Development and Application of Methods for Extraction and LC/MS/MS Analysis of Sex Steroids and Conjugates from Fish Feces

By

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Abstract

Non-lethal monitoring of animals using fecal steroid analysis is frequently employed to assess the health/reproductive status of individuals or populations, and may be applied to environmental monitoring of fish. Fecal steroid analysis requires both the non-polar parent and the polar glucuronide/sulfate conjugated forms to be considered. To address this challenge in fish, a reproductive steroid extraction method with HPLC/MS/MS analysis was first developed for Cortland's *in vitro* bioassay medium. A 2-step liquid:liquid extraction method successfully captured parent sex steroids and their conjugates. This method was then applied to investigate routes of endocrine disruption in trout gonads exposed to environmentally relevant concentrations of polybrominated diphenyl ether (PBDE) flame retardants, and selected metabolites. Of the two classes, hydroxylated metabolites of PBDE-47 had the greatest effect on steroidogenesis in both male and female trout gonadal tissues.

The extraction and LC/MS/MS analysis techniques were then applied to fish feces. The most effective matrix treatment for feces was a lipid removal agent, Cleanascite^{TM}. It removed lipid and pigmented compounds left by acid washing, and prolonged column life without affecting hormone recoveries. Using paired plasma and feces samples from rainbow trout it was determined that fecal estrogens could predict plasma concentrations. Changes in plasma estrogens were typically not reflected in feces, which varied less, until the subsequent week. Fecal concentrations of E_2-17 glucuronide best predicted plasma E_2 concentrations in female rainbow trout, while the strongest relationship was between plasma E_2-3 sulfate and fecal E_2-17 glucuronide.

Plasma clearance time and partitioning of estrogens into feces, urine and bile was then monitored in two experiments. The first involved *in vivo* administration of radiolabeled E_2 into the blood of rainbow trout, and in the second radiolabelled E_2 was introduced into the fish gut. Overall, E_2 was cleared from plasma in 72 hours, and estrogens in feces can be a composite of hormones metabolized over > 4 days. Approximately 68% of E_2-H^3 injected into the gut entered enterohepatic circulation. Understanding hormone metabolism and clearance, and the roles of dietary uptake and enterophepatic circulation, will facilitate further development of fecal steroid analysis as a non-lethal assessment tool for monitoring fish reproduction.

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Chapter 1 - General Introduction

Traditional Environmental Monitoring of Fish

Traditional programs to evaluate biological effects of point and non-point source contaminants often involve lethal sampling to evaluate the health of fish populations. For example, in Canada the Environmental Effects Monitoring (EEM) for pulp and paper mills and metal mines was developed to monitor effects for these industrial effluents, and to ensure that federal regulations are protective of the aquatic receiving environments (Environment Canada 2010, 2012). Early studies of fish downstream from bleached kraft mills showed that lower plasma sex steroid levels, decreased gonad size, increased age to maturity, and decreased fecundity were evident (McMaster et al. 1991; Munkittrick et al. 1992; Munkittrick et al. 1998). The purpose of the EEM fish survey is to evaluate resident fish health in terms of growth, energy storage, survival and reproductive potential. The sampling protocol involves 20 males and 20 females of two fish species to be sacrificed at regular intervals in order to gain this information (Munkittrick et al. 2002; Environment Canada 2010, 2012). The program requires a minimum of two sampling seasons at the exposed site, and at least one reference site, often in an upstream/downstream design. Considering that there are over 130 pulp and paper mills and approximately 100 active metal mines in Canada (Munkittrick et al. 2002; AQUAMIN 1996), almost 37000 fish are sacrificed per year to satisfy the monitoring requirements for EEM alone. This calls into question the impact that these environmental programs may be having on resources that they were intended to protect, particularly

when many metal mines are located in less productive headwater regions where fish populations tend to naturally be smaller (Vannote et al. 1980; Ribey et al. 2002).

Environmental monitoring programs have mainly focused on large-bodied fish species. The biggest challenge in using larger-bodied fish species as sentinels is their potential for movement over large distances. The white sucker (Catostomus commersoni) has been used as a monitoring species for some time (McMaster et al. 1991; Munkittrick et al. 1992; Gagnon et al. 1994; Servos et al. 1992; vanden Heuvel et al. 1996; Oakes et al. 2003). However, Doherty et al. (2010) found by tracking white suckers with implanted transmitters in the Saint John River that they would move on average 2.5 km or less during the winter months and up to 40 km at spring spawning. During spawning there was the potential for stock mixing as only 70% of the fish returned to their previous wintering grounds. This suggested that white suckers may not be a good choice for monitoring in a large river system.

Questions of residency and exposure make it difficult to associate potential effects with point source contaminants, especially when there are multiple discharges that occur in relatively close proximity. There has been a shift towards the use of small-bodied, non-migratory forage fish as sentinel species. This helps to address the issues of mobility and certainty of exposure (Munkittrick et al. 2002). Many small-bodied fish species are ideal sentinels because they have small home ranges (Minns 1995). Studies tracking individual slimy sculpin (Cottus cognatus) movements, a small-bodied fish, demonstrated during 10 months of monitoring that there was a median displacement of only 11.3 m (Gray et al. 2004). Small-bodied fish also tend to be more abundant, so that repeated sampling has a proportionally smaller impact on resident population numbers. Another

key advantage to small-bodied fish is that they have been shown to respond to environmental stressors at the population level more quickly than large-bodied fish (Kidd et al. 2007). Finally, small species are not typically harvested commercially or taken as sport fish, and they tend to be easily cultured in the lab (Gibbons et al. 1998).

Reproductive status in fish is most often evaluated by determining gonad size and measuring circulating levels of plasma reproductive steroids. Small-bodied fish are often too small to collect a sufficient volume of plasma for sex steroid analysis. However, an *in vitro* technique (McMaster et al. 1995) using gonadal tissue has emerged as a reliable alternative. Both techniques provide information on the reproductive status of fish, and are basic tools in the studies of fish endocrine function, but both also require the fish to be sacrificed. In situations where fish populations are small, where impacts of repeated sampling are a concern, or where Species at Risk are of interest, lethal sampling is not possible.

Non-Lethal Sampling of Fish

Non-lethal techniques have been developed for EEM fish survey programs that are able to provide the growth and population structure information that is usually derived with traditional lethal sampling programs. These survey methods compare fish length frequency data at impacted versus reference sites (Gray et al. 2002; Environment Canada 2010, 2012). However, the new techniques fall short because they do not provide information on fish reproduction. An example of how this shortcoming can be important is illustrated using data from a preliminary site assessment designed to assess the potential impacts of point source pesticide inputs (Figure 1.1, Peters et al. 2005). When

length-frequency distributions are compared for fish captured at a reference sites versus a pesticide exposed site, it is clear that the young-of-the-year (YOY) cohort are missing at the highest pesticide exposure site (Cherry Road), whereas they are present at the other unimpacted upstream or reference sites. While the non-lethally collected data are useful for showing a potential recruitment issue in fish at the Cherry Road site, they do not allow an evaluation of the cause of the reproductive impairment. It is not known if a one-time event (eg. a storm washing lethal concentrations of pesticide into the stream) might have been responsible for fewer larval fish, or if a chronic endocrine disruption might exist at the site that prevents adults from successfully reproducing.

Some non-lethal measures of reproductive assessment have been employed for environmental monitoring, but they are often only applicable to a limited number of species. For example, slimy sculpin and Johnny darters (*Etheostoma nigrum*) which lay eggs in nests guarded by males (Gray et al. 2002; Scott & Crossman 1998) can be assessed by counting eggs to compare fecundity between sites (Gray et al. 2002). However, longnose dace (*Rhinichthys cataractae*), fathead minnow (*Pimephales promelas*) and several stickleback species which have also been used as sentinel species in the EEM fish survey employ other reproductive strategies that do not allow this sort of assessment (Scott & Crossman 1998).
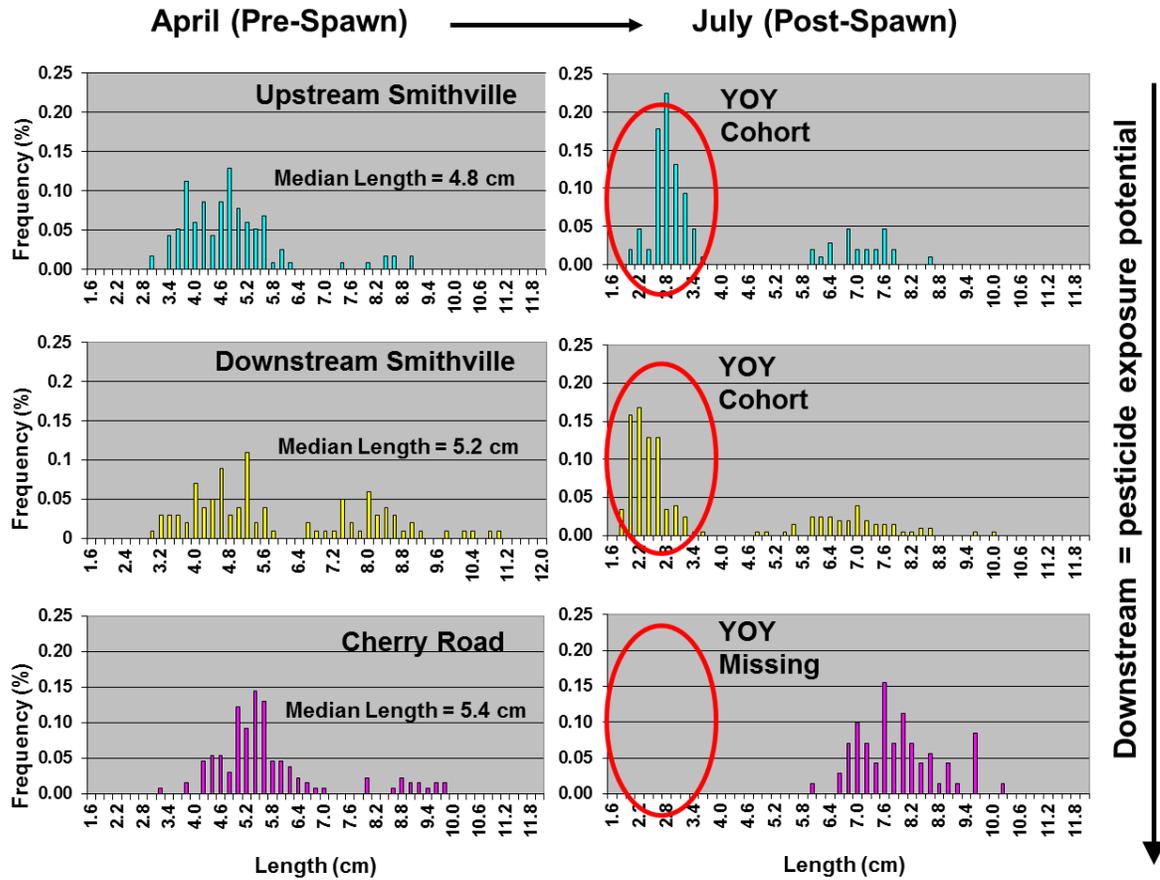
Figure 1.1 Golden Shiner length frequency distributions for collections at upstream or reference sites (Upstream and Downstream Smithville) and a pesticide impacted site (Cherry Road) where young of the year (YOY) were not present (taken from Peters et al. 2005).

To expand the ability to assess fish reproduction non-lethally, indicators that have comparable sensitivity and applicability need to be developed. The developed metrics need to provide similar information to that afforded by measuring gonad size and circulating levels of plasma sex hormones.

Reproductive Hormones in Feces

The analysis of steroids in excreta has been used by zoos to monitor animal health and reproductive state (Whitten et al. 1998). Measuring sex steroids in feces provides information on the reproductive status of animals without inducing capture or handling stress (Ziegler and Wittwer 2005). However, this requires recognition that there are several excretion forms found in feces. Reproductive steroids are excreted as parent steroids as well as glucuronide and sulfide conjugated forms. Conjugation as glucuronides or sulfates increases the polarity of lipid soluble steroids, allowing them to be excreted in either feces or urine (Pankhurst 2008; Whitten 1998). The proportions of these forms varies with species (Whitten et al. 1998), and therefore a variety of extraction methods have been developed for primates (Shideler et al. 1993; Ziegler et al. 1996), cats (Terio et al. 2002; Morato et al. 2004), birds (Jensen and Durrant 2006), rodents (Young et al. 2001) and elephants (Ganswindt et al. 2002).

Limited assessment of steroids has been performed in fish excreta. Dominance and courtship behaviours have been linked these with reproductive steroids in urine of male cichlid fish (Olivieri et al. 1996). Vermeirssen & Scott (1996) identified multiple routes of steroid excretion in rainbow trout including gill, urine and bile. However, relating circulating levels of hormones and reproductive status of fish with fecal analysis of the sex steroids as a non-invasive tool in fish has not been adequately explored.

The biologically active, free forms of the sex steroids, including estradiol and testosterone, have short half-lives in circulation because they are either bound to plasma carrier proteins or rapidly conjugated for excretion. In rainbow trout, the half-life of E_2 is less than 30 minutes (Pankhurst 2008). Steroid binding proteins (SBPs) serve to keep

"free" concentrations of hormones within a narrow range, as well as to protect them from being removed from circulation via conjugation (Pankhurst 2008; Felig & Frohman 2001; Bhagavan 1992). Another group of binding proteins, sex hormone binding globulins (SHBG), are high affinity sex hormone transporters that regulate delivery of the steroids molecules to target tissues (Hammond 2011, Mommsen & Korsgaard 2008).

Metabolic conjugation involves the addition of either glucuronic acid or a sulphate group to the sex steroid hormones. Conjugation increases their solubility to facilitate excretion through bile then into feces or through renal routes, and in most cases reduces or eliminates their biological activity (Pankhurst 2008; Gunderson et al. 2001; Parks & LeBlanc 1998; Zhu & Conney 1998; Arukwe et al. 1997; Strott 1996). Although this primarily takes place in the liver of both mammals and fish (Pankhurst 2008; Kirk et al. 2003; Zhu & Conney 1998; Stott 1996), it can also occur in extra-hepatic tissues including the gonads and gills of fish (Pankhurst 2008; Leguen et al. 2000). Conjugated reproductive steroids can serve biologically active roles in fish as sex pheromones. Sulfate and glucuronide conjugates of 17α, 20β-dihdroxyprogesterone are considered to be among the primary reproductive chemical signals released by fish (Vermeirssen & Scott 1996; Scott & Sorensen 1994; van den Hurk et al. 1987).

Glucuronidation begins with the activation of glucuronic acid to the high-energy intermediate, uridine diphosphate glucuronic acid (UDPGA).

UDPGA   +   Steroid-OH $\longrightarrow$ Steroid glucuronide

glucuronosyltransferase (UGT)
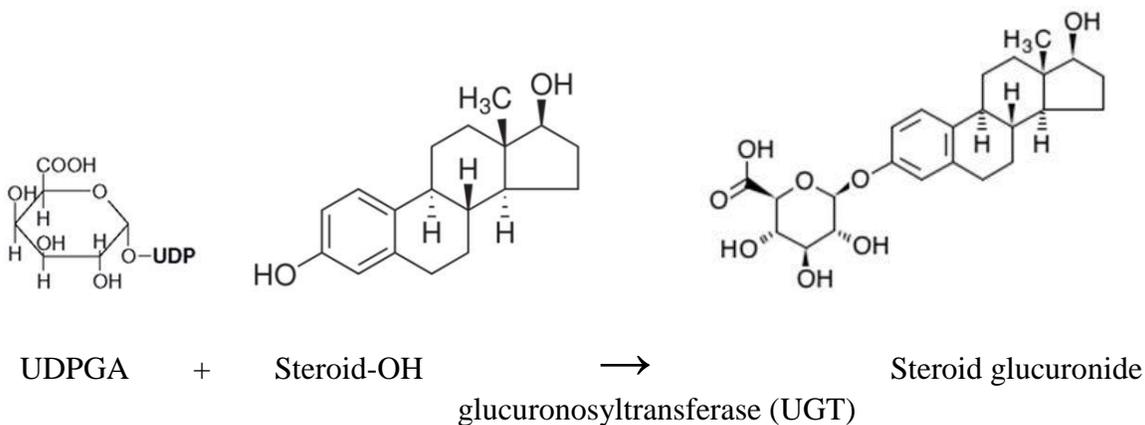
Figure 1.2   Formation of glucuronide conjugated steroids

UDP-glucuronosyltransferase (UDPGT or UGT) is the enzyme that catalyzes the transfer of glucuronic acid to the steroid functional hydroxyl group (Figure 1.2). Although not necessary for the reaction, compounds with hydroxyl and amino groups are good substrates for UGT (Lemke &Williams 2008; Zhu & Conney 1998; Di Guilio et al. 1995; Clarke et al. 1991).

Membrane-bound UGT is located in the endoplasmic reticulum (ER) of many tissues, particularly liver (Lemke & Williams 2008; Clarke et al 1992; Clarke et al. 1991). These enzymes have two domains: the N-terminus and the C-Terminus. The N-terminus contains the signal peptide on the cytoplasm side of the ER membrane that aids in transporting the aglycone (compound to be conjugated) into the lumen. This domain also contains the aglycone binding site. The C-terminus binds the UDPGA and contains the active site for catalyzing the reaction with the aglycone on the lumen side of the ER membrane. The newly formed glucuronide is then transported across the ER membrane to the cytosol and excreted from the cell (Lemke & Williams 2008; George & Taylor 2002; Clarke et al 1991).

The cellular position of UGT alongside the microsomal family of Cytochrome P450 monooxygenases is important because one of the primary functions of P450 enzymes is hydroxylation. After sex steroids have been hydroxylated by P450 enzymes, they are readily available for UGT glucuronidation. Although, this enzyme system provides sites at which the sex steroids may be conjugated, it is not necessary as the parent estradiol molecule can be conjugated at positions 3 and 17, and testosterone at position 17 (Strott 1996; Clarke et al. 1991). The conjugated steroid is then readily excreted into the urine or bile of the animal for elimination from the body (Lemke & Williams 2008; George & Taylor 2002; Clarke et al. 1992; Clarke et al. 1991). Steroids that are excreted in the bile are not necessarily destined for excretion because the glucuronide bond can be hydrolyzed in the gut by β-glucuronidase activity in the intestinal flora (Lemke & Williams 2008; MacDonald 1983) and mucosal membranes (MacDonald 1983; Whitten 1998; James et al 2001).

Sulfate conjugation is also an important biotransformation pathway for steroids (Lemke & Williams 2008; Zhu & Conney 1998; Strott 1996). There are two separate cellular locations for sulfotransferase activity; in the cytoplasma and membrane-bound in the Golgi apparatus. The site that conjugation takes place generally depends on the substrate that is being targeted, with steroid conjugation taking place in the cytosol (Lemke & Williams 2008; Strott 1996). As with glucuronidation, sulfation requires the sulfate group (SO_4^{2-}) to be activated before it is transferred to the steroid. The donor molecule of this active sulfate group is 3'-phosphoadenosine 5'-phosphosulfate, also known as PAPS. The synthesis of PAPS is a two-step process; first adenosine 5'-phosphosulfate (APS) is produced through the reaction of inorganic sulfate (SO_4^{2-}) with

adenosine triphosphate (ATP), catalyzed the ATP sulfurylase (Figure 1.3). The second

step is catalyzed by APS phosphokinase to form PAPS and ADP from APS and ATP. In

the case of sex steroids, the transfer of the sulfonate radical group (SO^{3-}) from PAPS to

the hydroxyl acceptor site is catalyzed by a sulfotransferase (SULT) enzyme (Lemke &

Williams 2008; Kirk et al. 2003; Zhang et al. 1998; Strott 1996). The rate limiting step of

this process is the availability of PAPS and inorganic sulfate from which it is derived

(Lemke & Williams 2008).

ATP-sulfurylase

1. SO_4^{2-} + ATP $\longrightarrow$ APS

2.   APS-phosphokinase

         APS + ATP $\longrightarrow$ PAPS + ADP

PAPS   +   OH-Steroid $\longrightarrow$ Sulfate Conjugated Steroid

Sulfotransferase (SULT)
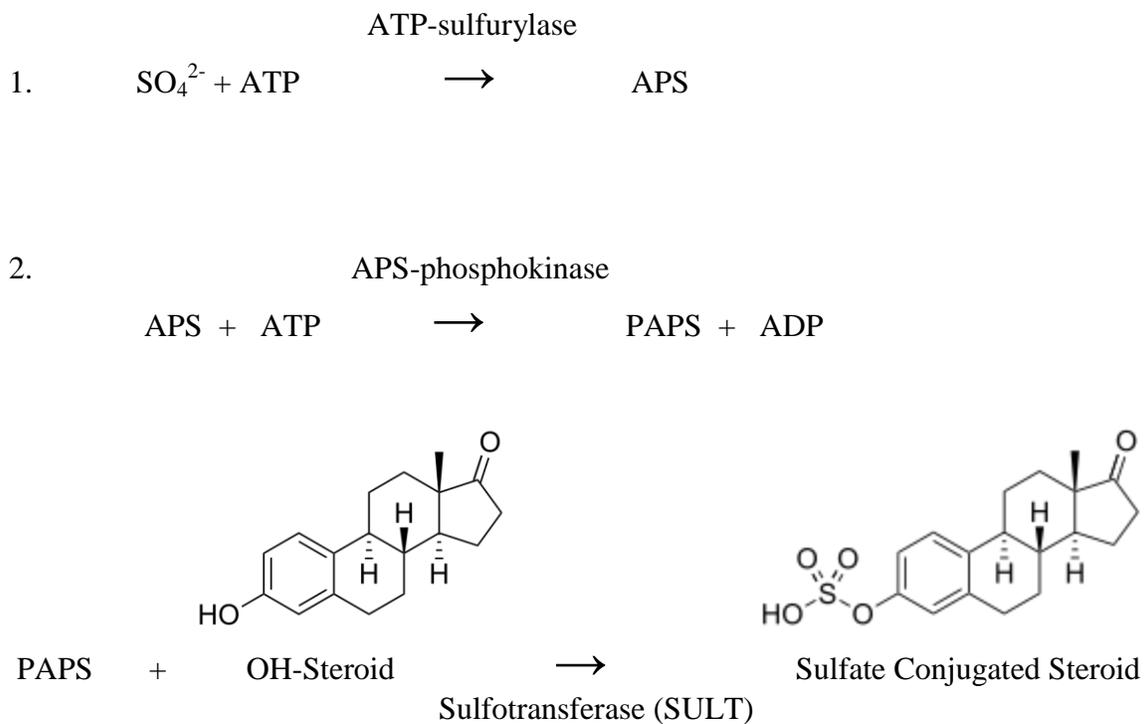
Figure 1.3   Formation of sulfate conjugated steroids

The UGT and SULT enzyme families each contain subfamilies with different isoforms

and substrate selectivity. Identification and isoform activity in fish tissues is an active

area of research (James 2011; Kurogi et al. 2011). In zebrafish (*Danio rerio*), there have

been 45 UGTs identified (Huang & Wu 2010) and 15 SULTs (Kurogi et al. 2011).

Problems with current hormone analysis

Radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA) are the most common methods used for steroid hormone analyses. Many of these are available in commercial kit form, and have become popular because they are quick, simple to use and relatively inexpensive. Immunoassay (IA) techniques are limited because only one hormone can be measured at a time. In situations where multiple hormone profiles are required, as in environmental monitoring for hormone disruption or clinical studies, the use of IAs can become very expensive. Conducting multiple hormone analyses with IA also requires larger sample volumes (Soldin & Soldin 2009; McMaster et al. 1992).

There are also concerns regarding IA antibodies cross-reacting with other hormones, conjugated metabolites or binding globulins, potentially overestimating hormone concentrations (Taieb et al. 2003; Moal et al. 2007; Ziegler & Wittwer 2005; Soldin & Soldin 2009). Immunoassays which include an organic solvent extraction step reduce the antibody cross-reactions by denaturing proteins while not accounting for the conjugated hydrophilic hormones (Stanczyk et al. 2003). This extra step, however, does not eliminate all cross-reactivity because unconjugated metabolites, including androstenedione, can interfere with testosterone antibodies (Stanczyk et al. 2003). Immunoassay kits have high detection limits, and show high variability at low hormone concentrations due to non-linear calibration curves with increased variability at tail ends (Taieb et al. 2003; Stanczyk et al. 2003; Moal et al. 2007; Kushnir et al. 2011). Studies comparing immunoassays consistently show that there is high variation between results from testosterone kits (Taieb et al. 2003; Moal et al. 2007) and estradiol (Hanselman et al. 2004; Lee et al. 2006) from several different manufacturers.

Chromatographic methods, including GC/MS and early LC/MS methods, have also been used to determine steroid hormones. These analyses were very labour intensive and required derivatization steps. The added extraction steps decreased precision of hormone analysis and there were reports that derivatization at extreme pH could cause the hydrolysis of steriod conjugates, resulting in falsely elevated hormone measurements. When results were compared between different laboratories, derivatization steps resulted in estradiol measurements that were 10 to 20% higher than for methods without this step (Soldin & Soldin 2009).

As research on endocrine disruptor mechanisms advance, the importance of hormone metabolites and the ability to measure them has increased. IA kits are currently not available for sex hormone conjugates, and preliminary work in our lab has shown that sulfate conjugates of estrogen and glucuronide conjugates of testosterone can make up 50% of hormone production by fish gonadal tissues *in vitro* (Peters et al. 2007). Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is the most sensitive and specific method of hormone analysis currently available. It allows the simultaneous analysis of hormones and their conjugates without the elaborate sample preparation procedures such as enzyme deconjugation (Soldin & Soldin 2009; Kushnir et al. 2011; Carvalho 2011).

Focus and Highlights of This Thesis

The steroidogenesis *in vitro* assay using fish gonadal tissue was developed to replace measurements of steroids in plasma of small bodied fish and to allow investigation of steroidogenesis pathways. The assay measures the capacity of the fish

gonadal tissue to produce reproductive steroids. Analysis of parent hormone production (estradiol, testosterone and 11-ketotestosterone) is typically measured using radioimmunoassay and sometimes EIAs. However, as mentioned previously, up to 50% of hormones produced by the tissue are conjugated metabolites, which are not accounted for by RIA or EIA measurements. The *in vitro* medium used for the in vitro assays is a semi-complex matrix containing salts and proteins. Developing a protocol to extract both parent and conjugated hormones from this semi complex medium for simultaneous analyses using HPLC/MS/MS was the focus of the second chapter of this thesis. Conjugation of steroid hormones increases their solubility in polar media, facilitating their excretion in urine or bile, but this increased solubility requires careful consideration of solvents that are capable of extracting both the parent and conjugated steroid molecules (Dorgan et al. 2002; Gemmill et al. 2011; McMaster et al. 1992).

Having established a viable method for steroid and conjugate extraction and analysis using LC/MS/MS, Chapter 3 demonstrates the use of the techniques to investigate the endocrine disrupting potential of a group of environmentally relevant brominated flame retardants polybrominated diphenyl ethers (PBDEs) and their hydroxylated metabolites. This chapter stresses the importance of also examining sex hormone metabolites in endocrine disruption studies, because effects cannot always be detected by simply assessing the production of the parent steroids.

In Chapter 4 the *in vitro* medium extraction methods were further developed to target feces as a more complex media. Whereas the *in vitro* media included salts and protein, feces also contains fats and pigments that can potentially interfere with extraction efficiencies. The biggest challenge was not in simply extracting hormones from the

feces, but in also removing co-extracted matrix interferences without compromising the detection of either parent or conjugated steroid hormones. The use of a 2-step extraction method, followed by sample treatment with a lipid removal agent, facilitated quantification of parent fecal estrogens and their conjugated metabolites at the pg/mg dry feces level. To determine the ability of fecal steroids to predict circulating plasma steroids concentrations, paired feces and plasma samples from female rainbow trout were compared weekly through three months leading up to spawning. The technique was validated to show that fecal E_2 metabolites are a good predictor of circulating E_2 concentrations in rainbow trout.

Having established a relationship between steroids in feces and plasma, studies in Chapter 5 examined what fecal sex steroid concentrations represent for the fish in terms of existing circulating plasma concentrations, together with rates and routes of excretion using radiolabelled estradiol. Uptake of dietary estrogen and enterohepatic circulation were also investigated. This information is relevant for monitoring programs where fish are collected near sewage treatment facilities, and there is the potential for synthetic and native hormone exposure and uptake. This research shows that gut uptake of hormones can significantly contribute to circulating plasma levels of estrogens, and that enterohepatic recirculation prolongs the availability of bioactive free forms of sex hormones. Understanding these factors will facilitate the development of fecal sex steroid analysis as a tool for non-lethal monitoring of fish reproductive health.

Research Objectives

1) Develop and optimize a method for the extraction and HPLC/MS/MS analyses of free and conjugated sex steroids from a tissue incubation medium used to investigate gonadal steroidogenesis in fish (Chapter 2).

2) Applied the extraction technique with HPLC/MS/MS analyses to investigate endocrine effects of polybrominated diphenyl ethers and their hydroxylated metabolites on steroidogenesis in fish gonadal tissue (Chapter 3).

3) Develop and optimize fecal extraction and analyses of free and conjugated estrogens in fish feces using HPLC/MS/MS. The estrogen and metabolite profiles in rainbow trout feces will be characterized and compared to hormone levels found in plasma circulation of the same fish through the seasonal reproductive cycle for method validation (Chapter 4).

4) Determine plasma estrogen clearance time and partitioning into feces, urine and bile using radiolabelled estradiol (Chapter 5).

5) Using radiolabelled estradiol, determine how the uptake of exogenous reproductive steroid hormones through consumption and enterohepatic circulation contributes to plasma hormone concentrations and hormone levels excreted in feces (Chapter 5).

Hypotheses

- Free and conjugated reproductive steroid hormones can be simultaneously extracted from *in vitro* medium and analyzed by HPLC/MS/MS with a simple liquid-liquid extraction method

- The *in vitro* extraction and analysis method can detect potential routes of endocrine disruption from exposure of fish gonadal tissue to polybrominated diphenyl ethers

- Conjugated and free forms of hormones extracted from fecal matter can be simultaneously measured and quantified by HPLC/MS/MS

- Fecal steroid measurements can estimate plasma hormone concentrations and reflect the reproductive status of fish

- Ingestion and enterohepatic circulation is an important route of exposure for natural and synthetic exogenous hormones in fish

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Chapter 2 - Extraction of multiple sex steroids and their conjugates from steroidogenesis assay medium followed by simultaneous HPLC/MS/MS analysis

Abstract

Emphasis on the environmental activity of endocrine disrupting chemicals has highlighted the need for techniques to measure endogenous hormones and their metabolites in fish, either in plasma or from a variety of *in vitro* test media. Existing immunoassay techniques limited to single hormone measurements are impaired by antibody cross reactivity and require time consuming extractions. Traditional methods to measure hormones that once relied on immunoassay (IA) techniques are being replaced with more sensitive, specific techniques based on liquid chromatography (LC) coupled with mass spectrometry (MS). Antibody cross-reactivity issues that are inherent to some IA techniques are now circumvented using LC/MS methods as multiple hormones and their metabolites can be measured simultaneously. However, some previous attempts to develop HPLC/MS/MS methods have been constrained by cumbersome extraction techniques. Measuring the *in vitro* production of sex steroids by fish gonadal tissue is an established technique for estimating the steroid biosynthetic potential in species for which the collection of sufficient volumes of plasma is not possible. This chapter discusses the development and optimization of a sex hormone extraction technique from Cortland's incubation medium in preparation for analysis by HPLC/MS/MS. Extraction efficiency of ten sex steroid hormones and their sulfate and glucuronide conjugates from

the incubation buffer were optimized using different extracting solvents, pH adjustment and by addition of an ion pair reagent. This *in vitro* technique allows the simultaneous investigation of several gonadal steroid biosynthetic pathways, as well their metabolism. This technique may also be used to identify modes of action by endocrine disrupting chemicals in the reproductive steroid pathways.

Introduction

Alterations in circulating levels of sex steroids can indicate exposure to compounds impacting the reproductive system of fish. Effects correlated with hormone changes include delayed maturation, reduced gonad size and fecundity, and the inhibited expression of secondary sex characteristics (Kime 1998; Munkittrick et al. 2002; McMaster et al. 2006). Assessing plasma sex steroid levels in small-bodied fish species is problematic due to insufficient plasma volumes. Therefore, the use of an *in vitro* incubation technique to assess the biosynthetic capacity of fish gonadal tissue to produce sex steroids has become a reliable alternative (McMaster et al. 1995), and is commonly used in environmental effects monitoring programs (McMaster et al. 2005). The assay can be used to compare steroidogenesis in fish from contaminated and non-contaminated sites, and can also be used to explore impacts of suspected EDCs in the steroid biosynthetic pathway. However, the assay is dependent on existing hormone analyses methods to determine endpoints.

Radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA) are the most commonly used methods for steroid hormone analyses. They are both competitive binding assays that require three basic components, the ligand, in this case the hormone to be measured, a known concentration of labeled ligand (this could be radiolabeled or colour-labeled), and an antibody that will specifically bind that ligand. The basic assumption in these assays is that the labeled ligand behaves in the same way as the natural ligand and competes for antibody binding sites. The amount of ligand in the sample is based on the ratio of labeled versus non-labeled ligand bound to the antibodies (McMaster et al. 1992; Hoofnagle & Wener 2009). Many of these assays are available as

commercial kits, and have become popular because they are rapid, simple to use and relatively inexpensive. They typically do not require extensive sample preparation and can be used on a variety of biological samples including feces (Jensen & Durrant 2006; Lupica & Turner 2009), plasma or serum (McMaster et al. 1992; Taves et al. 2010), saliva (Gozansky et al. 2005), hair (Karlen et al. 2011), water samples (Friesen et al. 2012) and *in vitro* medium (McMaster et al. 1995). They are limited, however, because only one hormone can be measured at a time. Where multiple hormones are of interest, as in environmental monitoring for hormone disruption or clinical studies, the use of IAs can become expensive, time consuming, and require large sample volumes that are difficult to collect. More problematic are concerns that antibodies used in IA can exhibit cross-reactivity with other hormones, conjugated metabolites or binding globulins, potentially leading to overestimated hormone concentrations (Taieb et al. 2003; Moal et al. 2007; Ziegler & Wittwer 2005; Soldin & Soldin 2009). Immunoassay kits are currently not available for conjugates of sex steroids (eg. excretion forms). Preliminary work in our lab has shown that sulfate conjugates of estrogen and glucuronide conjugates of testosterone can make up 50% of hormone production by fish gonadal tissues *in* vitro (Peters et al. 2007; Peters et al. 2001). Finally, linearity of IAs at high and low hormone concentrations is poor (Taieb et al. 2003; Moal et al. 2007; Hanselman et al. 2004; Lee et al. 2006; Stanczyk et al. 2003; Kushnir et al. 2011).

Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is currently the most sensitive and specific method of hormone analysis and allows the simultaneous analysis of hormones and their conjugates (Soldin & Soldin 2009; Kushnir et al. 2011; Carvalho 2012). However, because extraction techniques can be unreliable

and cumbersome, additional work is required to facilitate their use for environmental monitoring.

Here we describe the development of an extraction methodology to quantify, using LC/MS/MS, a suite of sex hormones and their conjugated metabolites produced by fish gonadal tissues in biological media. Extraction efficiency from Cortland's medium was evaluated using different solvents, pH, ion pairs, and solvent volumes. Cortland's medium was selected because *in vitro* incubation of trout gonadal tissue in Cortland's medium is a reliable and repeatable method for assessing the biosynthetic capacity of fish gonadal tissue to produce sex steroids (McMaster et al. 1995). It is commonly used in environmental effects monitoring programs (McMaster et al. 2005) to compare gonadal steroidogenesis in fish from contaminated and non-contaminated sites, and can also be used to explore impacts of suspected endocrine disrupting chemicals (EDCs) on the steroid biosynthetic pathway (Dube & MacLatchy 2000; Evanson & Van Der Kraak 2001). The extraction method was validated by comparing LC/MS/MS analyses of samples that were extracted with those that were directly analyzed without extraction.

Reliable extraction methodologies using HPLC/MS/MS would provide a powerful tool for screening potential EDCs, and aid in the investigation of the mechanisms by which they alter steroidogenic pathways.

Materials & Methods

Hormones, Solutions and Buffers

The sex steroid hormones targeted for extraction were the parent hormones 17β-estradiol (E2), estrone (E1), testosterone (T), and 11-ketotestosterone (11-KT), as well as

their conjugated forms 17β-estradiol 3-sulfate (E2-3S), 17β-estradiol 3-glucuronide (E2-3G), estrone 3-sulfate (E1-3S), estrone 3-glucuronide (E1-3G), testosterone sulfate (TS), and testosterone glucuronide (TG). Mass-labeled compounds tested for use as recovery internal standards (RIS) or internal precision standards (IPS) included 17β-estradiol 2,4,16,16-d_4 (d_4-E2), estrone 2,4,16,16-d_4 (d_4-E1), 17β-estradiol 2,4,16,16-d_4 3-sulfate (d_4-E2-3S), and progesterone 2,2,6,6,17,21,21,21-d_8 (d_8-P). The testosterone conjugates and 11KT were purchased from Steraloids (Newport, RI, USA). The d_4 mass-labeled estrogen conjugates were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Unlabelled conjugated estrogens were acquired from Sigma-Aldrich Chemical (St. Louis, MO, USA). All other mass-labeled and unlabelled parent hormones were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Cortland's saline is an incubation buffer that is routinely used in *in vitro* studies to maintain trout gonadal tissues (McMaster et al. 1995; Leatherland & Lin 2001; Pankhurst et al. 1995; Pankhurst et al. 1996). Human chorionic gonadotropin (hCG), was dissolved in Cortland's medium to a final concentration of 1 IU/mL. The composition of Cortland's medium used in this study was adapted from McMaster et al. (McMaster et al. 1995), with all ingredients purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

The ion pair, tetrabutylammonium hydrogen sulfate (TBAHS), (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in DDW to make a 1M solution. Distilled in glass grade hexane, diethyl ether, ethyl acetate (EA), ethanol (EtOH) and methanol (MeOH), and Optima grade methanol and water were purchased from Fisher Scientific (Napean, ON, Canada).

LC/MS/MS Optimization

Selection of the product ion transitions to be monitored for each hormone and multiple reaction monitoring (MRM) optimization were performed using a Sciex 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with electrospray ion source (ESI). The working parameters optimized for the ESI for both negative and positive ion modes can be found in Table 2.1. LC separations were performed on an Agilent 1100 series LC system (Agilent Technologies Canada, Inc., Mississauga, ON, Canada) equipped with a vacuum degasser, binary pump and autosampler. Two different C_{18} analytical columns were tested; the Genesis Lighten (50mm×2.1mm i.d., 4 μm particle size; Grace, Alltech Canada, Guelph, ON, Canada) and the Kinetex (50mm×2.1mm i.d., 2.6 μm particle size, Phenomenex, CA, USA).

Table 2.1: The LC/MS/MS working parameters and run time optimized for multiple reaction monitoring with electrospray ion source in positive and negative ion modes.

Ion Mode	Period	Run time (mins)	CUR	CAD	IS	GS1	GS2	Temperature (^{o}C)
negative	1	9.6	20.0	4	-4500	30	45	550
negative	2	11.4	20.0	4	-4400	30	45	550
positive	1	21.0	30.0	8	5000	30	55	550

A binary mobile phase of Optima grade water and MeOH was used at a flow rate of 300 μL/min. In negative ion mode, the initial composition of the mobile phase was 80:20 water/MeOH (v/v), held for 1 min, followed by a linear gradient to 100% MeOH in 8.5 min and held for 7.5 min. The column was allowed to equilibrate for 5 min between runs. Two monitoring periods were used in the method developed for negative ion mode; the first lasting for 9.6 minutes and the second for the remaining 11.4 minutes. The

method for hormones detected in positive ion mode was not divided into separate periods, and the same mobile phase was used as in negative ion mode. The proportion of MeOH was increased to 100% over 9 minutes, held for 5 minutes, with a final column equilibration of 7 minutes between samples.

Linearity was confirmed for each compound by injecting 1 μL of each hormone solution in MeOH in concentrations between 250 fg/μL and 25 ng/μL. The analytical detection limits (ADLs) of each hormone/conjugate was calculated by mathematically suppressing the signal to noise ratio for the known amount of hormone injected to a value of 5:1 using Analyst 1.5 software. The method detection limit (MDL) was determined by adding a known amount of each hormone to 6 extracted Cortland's medium blanks. The electronically determined signal to noise ratios were again suppressed to a value of 5:1, and the MDL was calculated as the mean $\pm$ 3x standard deviations for each hormone.

Solvent Tests

Four different solvents/solvent combinations were evaluated to determine extraction efficiencies for all of the sex hormones and conjugates; ethyl ether, ethyl acetate, 9:1 hexane:ethyl acetate (v/v), and 3:2 hexane:ethyl acetate (v/v). To test each solvent we modified a basic fish plasma method used to extract estradiol, testosterone, and 11-KT by McMaster et al. (McMaster et al. 1992).

Gonadal tissue *in vitro* assays contain 1 mL of incubation medium in each well of the 24 well plates. To emulate this, 975 μL of Cortland's medium was pipetted into each disposable glass test tube. The mass-labeled hormones d_4-E2, d_8-Prog, and the mass-labeled conjugate d_4-E2-3S were added in a 5 μL standard spike (500 pg/μL each in MeOH), to act as the RIS. A 20 μL spike of 500 pg/μL standard in MeOH containing ten

hormones and conjugates (E2, E2-3S, E2-3G, E1, E1-3S, E1-3G, T, TS, TG, 11-KT) was added to each tube for a final concentration of 10 ng/mL. The tubes were then vortexed for 20 seconds. Three mL of solvent was added to each tube, with 6 replicate tubes for each solvent tested. Tubes were then vortexed for 1 min, centrifuged for 5 minutes at $4000 \times g$ and then frozen for 10 minutes in a $-80^{\circ}C$ freezer. The top solvent layer was decanted from the bottom frozen aqueous layer into clean tubes. The extraction process was repeated with the second solvent fraction decanted into the same tube as the first. The solvent was evaporated to dryness under a gentle stream of N_2. Samples were reconstituted with 1 mL of MeOH, vortexed for 1 min and transferred to a 1.5 mL autosampler vial. Tubes were rinsed again with 0.5 mL of MeOH and this was added to the first mL. The samples were again evaporated to dryness under N_2, and the vials were vortexed twice with 100 µL of MeOH, then pipetted into 250 µL autosampler vial inserts. The final extracts were blown to dryness and reconstituted in 45 µL of MeOH. A 5 µL IPS solution (500 pg/µL in MeOH) was added, vials were capped and vortexed for 20 seconds.

Samples were analyzed by LC/MS/MS in both ion modes, separately, to include the full suite of spiked androgen and estrogen sex steroids. Peaks were electronically integrated, and the areas were calculated using the instrument software. Recovery for each hormone was calculated as a percentage of the peak area measured in the external standard of same concentration. Results are expressed as the mean of six replicates with standard error.

pH Tests

The pKa values of parent hormones and their conjugates can vary significantly; the pKa of estradiol is reported to be 10-10.5 (Van Der Kraak & Donaldson 1986; Kirdani & Burgett 1967) while the value for estradiol-3 glucuronide is between 3 and 4 (Janson et al. 2011). Therefore, the effect of altering pH of the Cortland's medium on extraction efficiency was also tested. The medium was adjusted to pH 12.0, 9.0, 7.6, 5.0, 4.0, 3.0, 2.0, and 1.0 using 2M HCl or NaOH prior to extraction. Medium was pipetted into test tubes and spiked as described above. Ethyl acetate and 3:2 hexane:ethyl acetate were evaluated in these studies, as they were the only two solvents that extracted both parent and conjugated sex hormones in the above test. The extraction and analysis proceeded in the same fashion as described above, but with 8 replicates for each test pH and solvent.

Addition of Ion Pair

Tetrabutylammonium hydrogen sulfate (TBAHS - 1M solution) was added to tubes containing hormone spiked Cortland's medium to improve hormone extraction efficiency by making the conjugates less polar. The volumes of TBAHS tested were 10, 50, 100 and 200 μL additions. The medium was extracted as previously described using one of ethyl acetate or 3:2 hexane:ethyl acetate. Each ion pair test group was replicated 8 times for both solvents.

Fraction Recoveries and Solvent Volume

The first series of tests examined the proportion of each hormone recovered in a 3 mL solvent extraction, repeated 3 times. The hormone spiked Cortland's medium was extracted at pH 7.6 with either ethyl acetate or 3:2 hexane:ethyl acetate. The extractions

were performed as previously described, however, in this test each fraction was analyzed separately. Cortland's medium at pH 1 was also tested with ethyl acetate tested as the extraction solvent.

Based on the results from the 3 mL extraction fractions, similar tests were repeated using 6 mL of ethyl acetate each time to increase the amount of estrogen sulfate conjugates extracted. Cortland's medium at pH 7.6 was extracted twice and the fractions were analyzed separately. The medium at pH 1 was only extracted once as the previous tests showed that a second extraction did not significantly increase the hormone yield. Each treatment was replicated 8 times. Analysis and calculations were performed as described above.

Final Protocol Tests and Validation of Method

Once the liquid:liquid protocol was optimized, the extraction efficiency from Cortland's medium was tested at lower spiked concentrations (1 ng/mL and 500 pg/mL) that are more representative of natural hormone production *in vitro*. These tests were conducted using the original 975 μL of Cortland's spiked medium with 5 μL RIS, as well as 875 μL of medium with the addition of 100 μL of 1 IU/μL hCG. Depending on the fish species of interest, hCG in commonly used to stimulate steroid production in gonadal tissue *in vitro* assays (McMaster et al. 1995). Extraction with and without the addition of these compounds was tested to ensure they did not interfere with the efficiency of the method.

Validation was achieved by comparing hormone concentrations from samples that were extracted and analyzed with this newly developed method, to those from the same samples directly injected on the LC/MS/MS without extraction. *In vitro* incubations were

performed using six female rainbow trout (*Oncorhynchus mykiss*) obtained from Rainbow Springs Hatchery, Thanmesford, ON, Canada. The fish were held in an 800L tank at 13oC with a 12 hour light/dark cycle. Each fish was fed approximately 1.5% of their body weight six times per week. Upon sacrificing, each fish was weighed and measured (1.02 ± 0.12 kg and 37.8 ± 1.5 cm, gonad weight 31.0 ± 11.3 g), ovaries were removed and placed in Cortland's incubation medium (pH 7.6) on ice. Five ovarian follicles were placed in each well of the 24-well culture plates with 1 mL of Cortland's medium (basal) or Cortland's with 100 IU Human Chorionic Gonadotropin (hCG) for stimulation. There were triplicate wells prepared for both basal and stimulated conditions using each of the six fish ovaries. The plates were incubated at 12.0 ± 0.5 ^{o}C for 24 hours. The incubation medium from each well was collected by pipette and stored separately in 1.5 mL Eppendorf vials at -80 ^{o}C until analysis.

Samples were thawed, votexed and centrifuged for 5 minutes at 13000 rpm. Extraction of the samples was achieved using the final method outlined in the results and Figure 4. As the extraction method only uses 850 µL of a possible 1000 µL of assay medium, there was sufficient medium left for direct injection. Fifty microliters of sample was then transferred to 1.5 mL autosampler vials with 200 µL inserts. An internal precision standard (IPS) was added to each vial; 5 µL of this standard contained 2500 pg d4-estradiol and d4-estradiol 3-sulfate. Estradiol, estrone, estradiol 3-sulfate, estrone 3-sulfate and testosterone sulfate were quantified in the samples using LC/MS/MS. All data was log transformed and results of the directly injected samples were compared to the extraction method results using linear regression.

Results & Discussion

Analytical detection limits (ADLs) ranged from 2.8 pg/µL for E_2-3G to 0.20 pg/µL for T. Generally, the glucuronide conjugates had the highest detection limits of the 10 compounds tested. Hormone mass to charge (*m/z*) values and their transitions used for quantitation and confirmation, together with method detection limits (MDLs), ADLs and equations of the standard curve lines with R^2 values are shown in Table 2.2. Standard regressions constructed for all ten hormones and conjugates were linear from their ADLs up to injection amounts of 25 ng/µL. Overall, the chromatography was better using the Genesis analytical column and work described herein was completed with it.

Solvents and solvent mixtures were tested for their extraction efficiency of 10 parent and conjugated estrogens and androgens using our preliminary extraction method. Ethyl ether is commonly used in the literature for parent hormone extraction from plasma and other matrices (McMaster et al. 1992; Hanselman et al. 2004). A 9:1 solution of hexane:ethyl acetate (H:EA, v/v) (Gemmill et al. 2011; Dorgan et al. 2002) and 3:2 hexane:ethyl acetate (H:EA, v/v) (Harwood & Handelsman 2009) are used for E_2, T and 11-KT extraction. Finally, EA was also evaluated because it is the most polar solvent not miscible with water, and has been shown to be effective for glucuronide extraction (Pozo et al. 2008).

Overall, EA was the best solvent for extracting this suite of hormones (Figure 2.1). The extraction efficiency of parent estrogens was comparable to all the other solvent systems tested, however, recovery of the estrogen sulfate conjugates was 30% greater using EA than for the other solvents. The mean T recovery of 18% with EA was approximately half that of the two H:EA solvent systems tested,

40

Table 2.2: Mass to charge (m/z) values of the estrogens and androgens optimized on the LC/MS/MS in positive or negative ion mode, with transitions used for quantitation and confirmation. Analytical detection limits (ADL) and method detection limits (MDL) were calculated using a signal to noise ratio of 5:1, and the MDL is expressed as the mean + 3x standard deviations for each hormone.

Hormone	Abbreviation	Mass (m/z)	Transitions Monitored Quantitation	Transitions Monitored Confirmation	Ion Mode	MDL (pg/µL)	ADL (pg/µL)	Standard Curve Equation of the Line	R^2
Estradiol	E2	271.2	145.1	183.1	Negative	2.5	0.81	y = 174.35x + 151598.30	0.948
Estradiol - 3 Sulfate	E2-3S	351.2	271.0	80.0	Negative	1.3	0.53	y = 1325.61x + 173661.50	0.998
Estradiol - 3 Glucuronide	E2-3G	447.2	271.2	175.0	Negative	10.1	2.78	y = 433.77x + 29011.10	0.999
Estrone	E1	269.1	145.0	142.8	Negative	0.6	0.32	y = 525.49x + 406516.45	0.948
Estrone - 3 Sulfate	E1-3S	349.1	269.1	145.1	Negative	0.5	0.22	y = 2463.50x + 843884.86	0.989
Estrone - 3 Glucuronide	E1-3G	445.2	269.1	175.0	Negative	7.1	2.29	y = 443.52x + 31579.48	0.999
Testosterone	T	289.2	97.1	109.1	Positive	0.5	0.20	y = 337.85x + 295763.74	0.941
Testosterone Sulfate	TS	367.2	97.0	80.0	Negative	1.4	0.39	y = 1445.10x + 341362.02	0.993
Testosterone Glucuronide	TG	487.4	311.2	199.0	Positive	6.8	0.77	y = 98.03x + 20877.97	0.996
11-Ketotestosterone	11KT	303.3	121.1	259.2	Positive	3.1	1.88	y = 81.21x + 56475.22	0.967
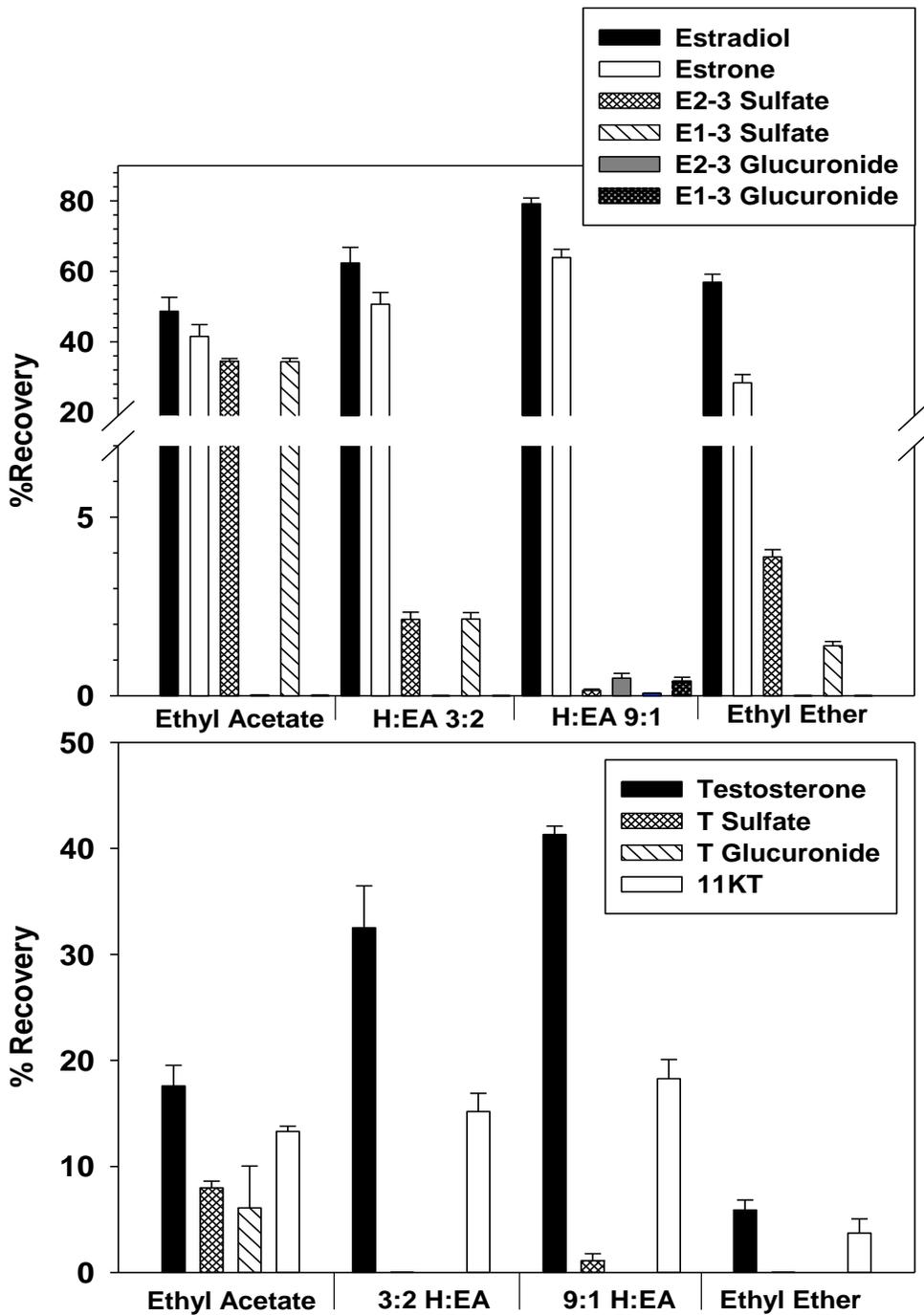
Figure 2.1: Spiked hormone recovery from Cortland's *in vitro* media extracted at assay pH of 7.6. From left to right the solvents/solvent mixes tested were 1) ethyl acetate 2) 3:2 hexane:ethyl actetate (v/v) 3) 9:1 hexane:ethyl actetate (v/v) 4) ethyl ether. Results are expressed as the mean $\pm$ SEM with n = 6.

but 11-KT was similar for the three groups containing EA. The 9:1 H:EA mixture demonstrated the highest extraction efficiency for E_2 (80%), E_1 (65%) and T (40%), but recovered less than 1% of the sulfate and glucuronide conjugates. Glucuronide conjugates proved to be the most difficult analytes to recover, and EA was the only solvent that showed detectable peaks, with a mean recovery of 6%. Overall, ethyl ether was the poorest solvent for extracting hormones from the Cortland's *in vitro* medium.

Metabolic conjugation involves the addition of either glucuronic acid or a sulfate group to the sex steroid hormones. Conjugation of steroid hormones increases their solubility to facilitate excretion into aqueous media (Kirk et al. 2003; Pankhurst 2008; Zhu & Conney 1998; Strott 1996). This increased solubility means that solvents such as hexane and ethyl ether, typically used to extract the parent hormones (McMaster et al. 1992; Gemmill et al. 2011; Dorgan et al 2002), were ineffective (Figure 2.1). Addition of ethyl acetate, a more polar solvent, to the hexane did improve the extraction efficiency, but not enough to deem it a viable option analytically. Based on these results, extraction optimization using 100% EA and 3:2 H:EA was pursued. These were the only solvent systems that extracted measurable amounts of the sulfate and glucuronide conjugates.

Adjusting pH had mixed effects on extraction efficiencies using H:EA. The Cortland's solution was adjusted to 8 different pH values between 12 and 1 before extraction with either 100% ethyl acetate or 3:2 H:EA (v/v). The H:EA solvent mixture was inconsistent in the extraction efficiency of parent hormones, with no clear effect of changing the pH (data not shown). Mean extraction values ranged from 40 to 80% for the E_2 and E_1, and less than 40% for T and 11-KT. None of the sulfate conjugates and TG

could be detected in any of the H:EA extracts. Estrogen glucuronide recoveries improved from less than detection limits at pH $\geq$ 4, up to 20% between pH 3 and 1 (data not shown).

Altering the pH of the Cortland's solution prior to extraction with 100% EA had the most significant effect on hormone recoveries of all tests explored. Estradiol and E_1 showed the best recoveries at low pH (Figure 2.2). At pH 12 and 9, mean E_2 and E_1 recoveries were about 85% and 70%, respectively. This dropped for both hormones to approximately 40% between pH 7.6 and 5, and then steadily increased to maximum mean extraction efficiency of 100+% at pH 1. The general increasing trend in recovery efficiencies of E_2 and E_1 at pH values less than 5 is consistent with their reported pKa (Kirdani and Burgett 1967; Hurwitz and Liu 1977). With respective pKa values of 10.3 and 10.5 for E_1 and E_2, negligible dissociation of these compounds is expected at pH of 8 or smaller, thereby favoring partitioning into EA. Recovery of the androgen parent hormones, T and 11KT, progressively improved from means less than 10% at pH 12, to 88% (T) and 80% (11KT) at pH 1.

Decreasing the pH of the sample prior to extraction increased the recovery of the glucuronide conjugated hormones (E_2-3G, E_1-3G, and TG), as they were the most affected by changes to pH (Figure 2.2). All three were not detectable in extracts from pH 12 to 7.6. Between pH 5 and 1, the extraction efficiencies greatly improved to means of 101, 102, and 94% for E_2-3G, E_1-3G and TG, respectively. Reported pKa values for E_2-3G and E_1-3G are *ca*. 3-4 (Janson et al. 2011). Reducing the pH to a value of 1 will drive these compounds into their neutral state thereby forcing them into the EA.
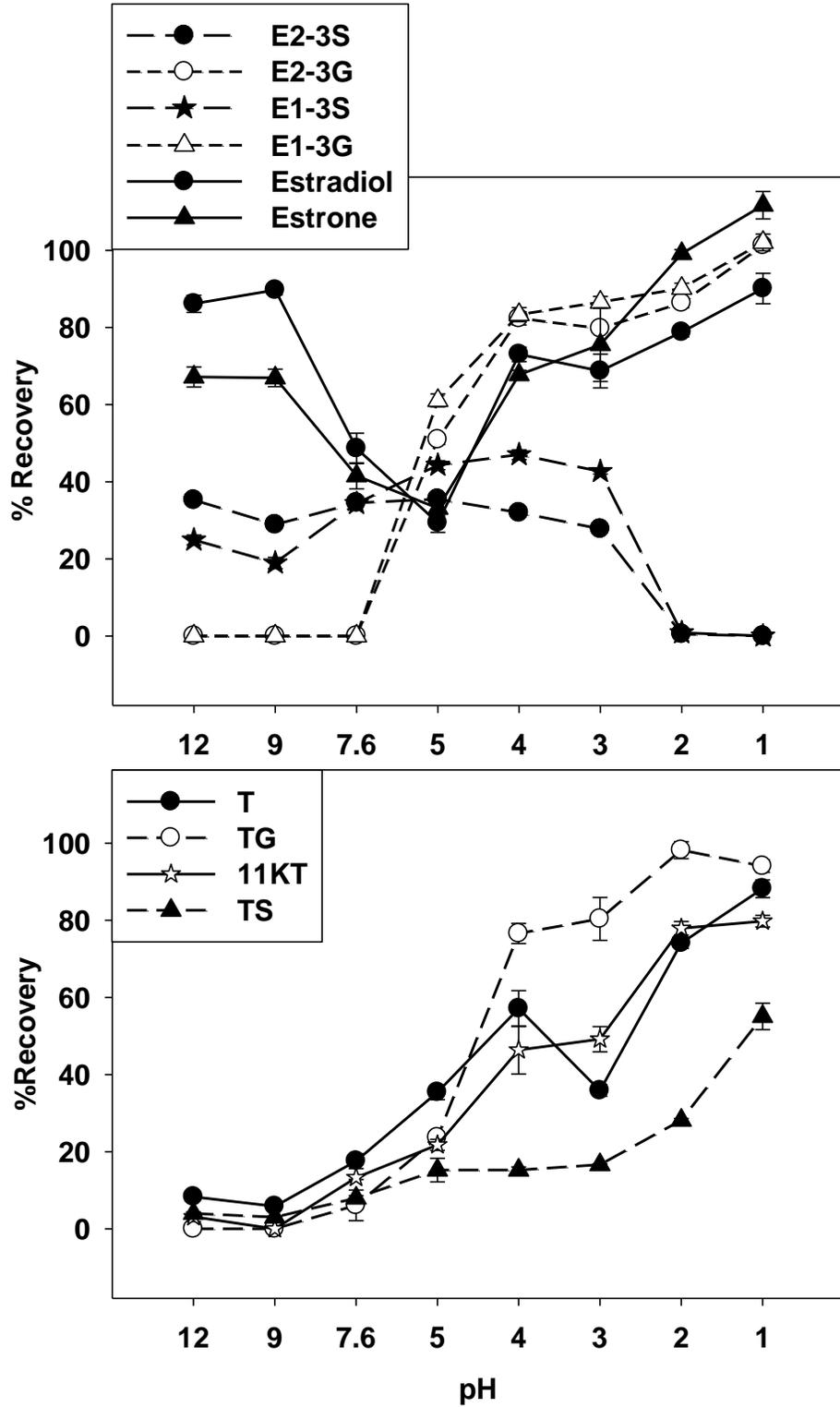
Figure 2.2: Recoveries of estrogens (A) and androgens (B) from spiked Cortland's medium by adjusting the pH between 12 and 1. Values are expressed as a mean ± SEM with n = 8.

Testosterone sulfate (TS) and the estrogen sulfate conjugates (E_2-3S and E_1-3S) did not show the same pattern in their extraction efficiencies with adjustments to pH. Testosterone sulfate results were more similar to the glucuronide conjugates with very low recoveries at pHs greater than 5, followed by increasing extraction efficiency with decreasing pH. Extraction efficiency did not improve, however, to the same degree as for the glucuronide conjugates; with maximum mean value of 55% recovery achieved for TS occurred at pH 1 (Figure 2.2).

Extraction efficiencies for the estrogen sulfate conjugates were consistent at pH values greater than 2. Mean recoveries for E_2-3S ranged between 27 and 35%, with E_1-3S being slightly more variable with mean ranges from 19 to 47%. The key difference in the estrogen sulfate results is that at the lowest pH values tested, these two compounds were non-detectable in the extracts (Figure 2.2); all other parent hormones and conjugate extraction efficiencies were best at pH 1. It is difficult to put the observed trends in recovery of E_2-3S and E_1-3S with changing pH into context as there are no reliable pKa values available.

Addition of the ion pair, TBAHS, was tested to improve the recoveries of the sulfate-conjugated hormones. By adding an ion pair with the opposite charge to the analyte of interest, the two compounds form an ionic bond, which neutralizes the charge of the analyte and increases hydrophobicity (Xu et al. 2009). TBAHS (1 M solution) was added in increasing volumes prior to the basic extraction method using 100% ethyl acetate as the extraction solvent. These experiments were conducted with the Cortland's medium at assay pH 7.6, and at pH 1 which had shown excellent recoveries of the other hormones.

The addition of TBAHS improved recovery of TS at both pHs tested, but not the estrogen sulfates (Figure 2.3).

There were other negative effects of the ion pair additions on parent hormone extraction efficiencies that precluded its routine use in a final assay. For example, at both pHs tested, extraction efficiencies of estradiol and estrone decreased 50-80% with increasing amounts of TBAHS. Testosterone, 11-KT and the glucuronide conjugate recoveries also significantly decreased at pH 1. Increased recovery at pH 7.6 with TBAHS additions between 50 and 200 µL, were variable and inconsistent (Figure 2.3). Given that the addition of TBAHS did not improve the extraction of the estrogen sulfate conjugates, and had negative effects on the recovery of the other hormones, it was not included in the final extraction protocol.

Because extraction fractions were combined for all previous tests, the proportion of the total recovery for each fraction was unknown. If the first extraction captured a sufficiently high percentage of the extractable hormones, then the method could be streamlined. On the other hand, if each fraction was closer to being equal, then total extraction efficiency could be improved simply by increasing the volume of solvent used each time. The preliminary extraction method using 3 mL of 100% ethyl acetate was performed on spiked Cortland's medium 3 times at pH 1 and 7.6. The fractions were analyzed separately to determine what hormone proportions were captured in each. Over 80% of the recovered parent and glucuronide conjugated hormones were extracted in the first fraction at both pHs (Figure 2.4). At pH 7.6, fractions 2 and 3 contained less than 3.3 and 0.9% of the parent and glucuronide conjugated hormones captured, respectively.

Fraction 2 captured more of the hormones at pH 1, with recoveries ranging between 2 and 13%. Fraction 3 contained less than 4% of the total at pH 1.
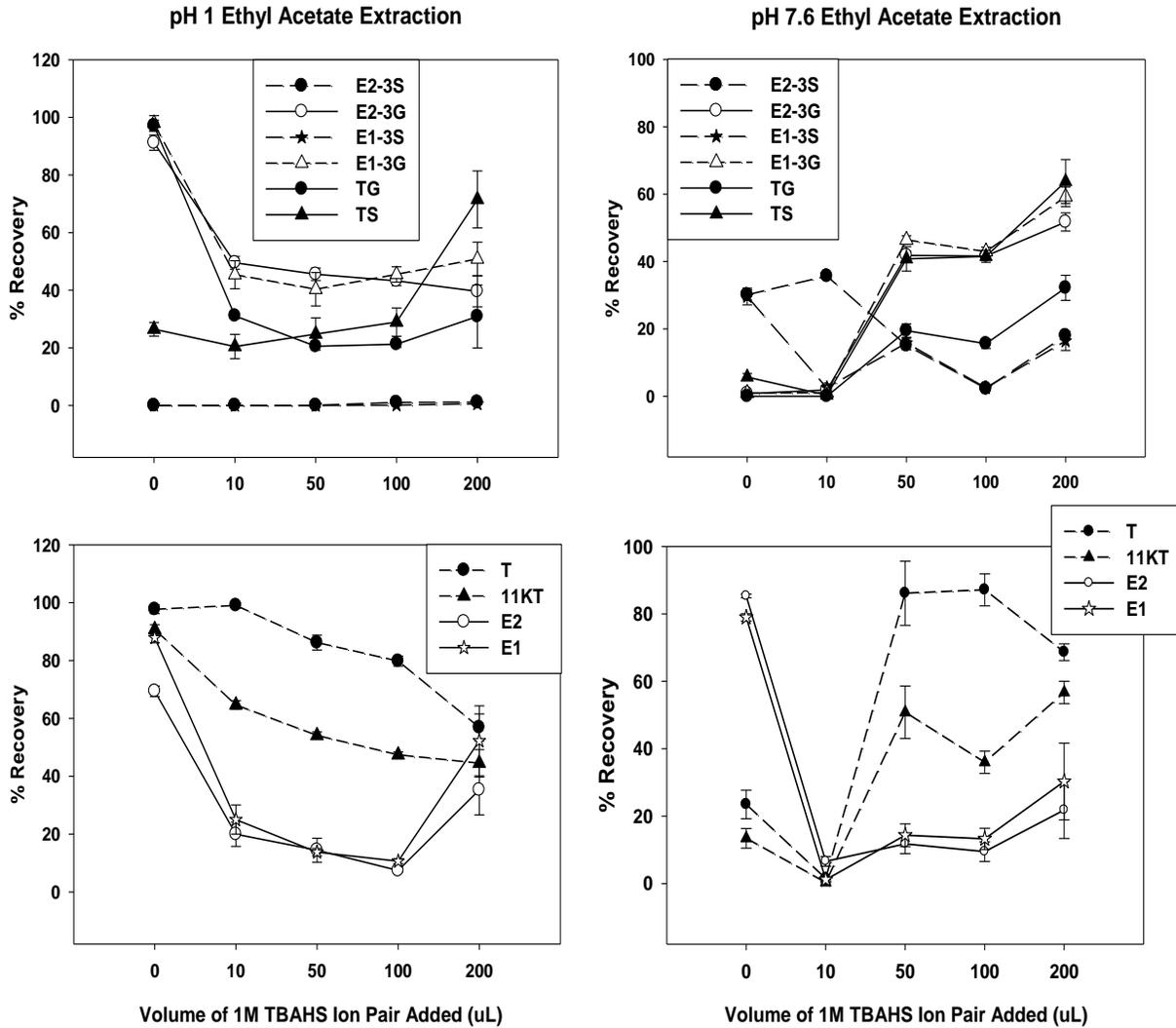
Figure 2.3: Changes in hormone recoveries with addition of increasing volumes of 1M tetrabutylammonium hydrogen sulfate (TBAHS) with the incubation medium at assay pH 7.6, and decreased to pH 1. Values are expressed as a mean ± standard error with n =8.
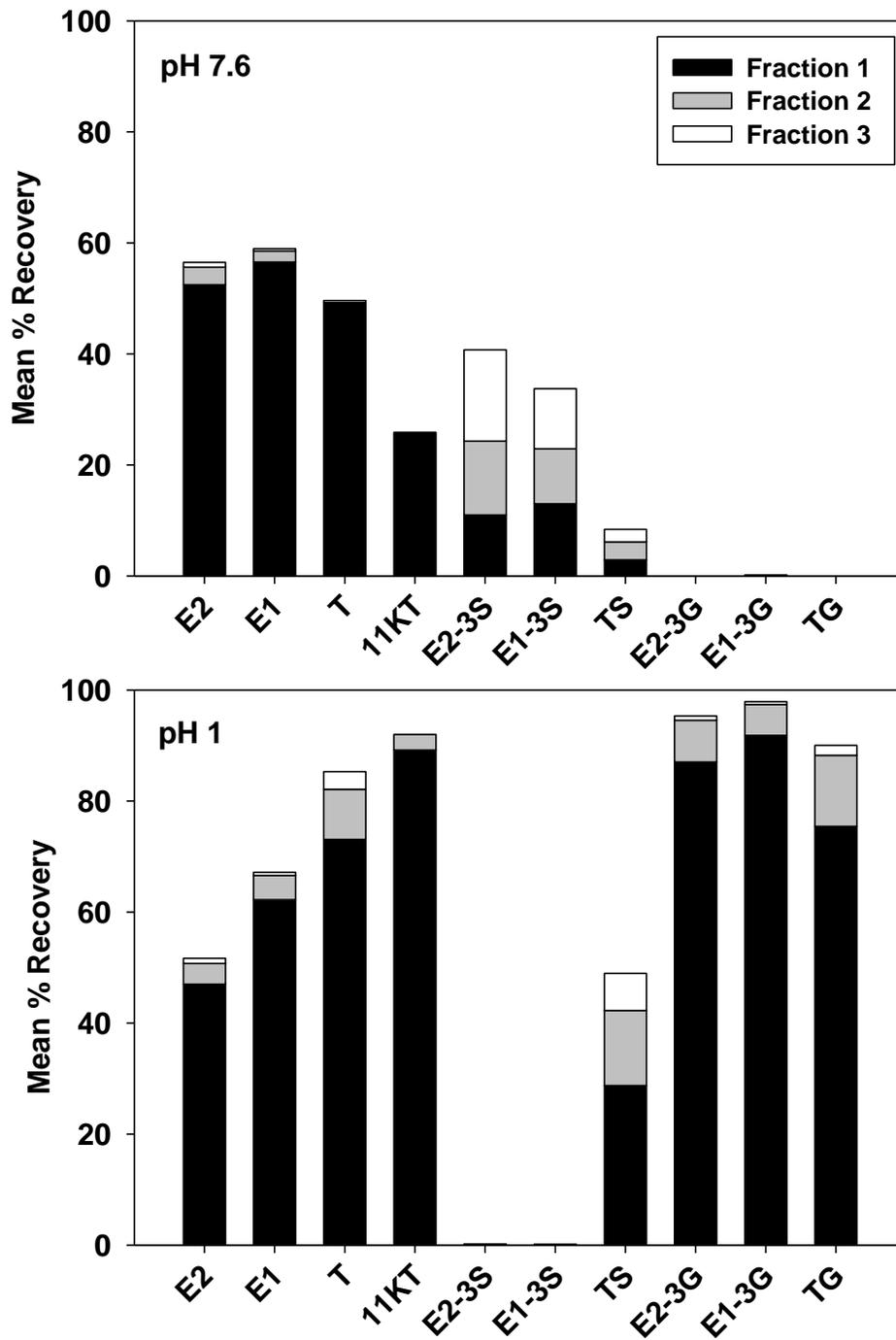
Figure 2.4: Hormone recoveries from Cortland's medium in three consecutive 3 mL extraction fractions of 100% ethyl acetate tested at pH 1 and 7.6.

Sulfate conjugates were extracted in similar proportions between the 3 fractions at pH 7.6 (Figure 2.4); about 12% in each fraction for the estrogens and 3% for TS. As observed in the pH experiment, estrogen sulfates were not recovered from any fraction at pH 1.   Testosterone sulfate was extracted from fractions 1 to 3 at 29, 14 and 7% efficiency at low pH. The test was repeated with 2 extractions of 6 mL each at pH 7.6 and once at pH 1. As expected, the sulfate conjugate recoveries improved proportionally to the increased volume of solvent used.

The final extraction protocol is actually two separate extractions steps that can be used independently or together, depending on the whether the gonadal tissues incubated were male or female, and/or the hormones being targeted. As illustrated in Figure 4, the androgen and glucuronide conjugate method requires the pH to be decreased to 1 prior to extraction with a single 6 mL volume of ethyl acetate. Recoveries achieved for the targeted hormones with this method were greater than 80% (Table 2.3).

Table 2.3: Recoveries (Mean + SEM) of testosterone (T), testosterone glucuronide (TG) and 11-ketotestosterone (11-KT) from Cortland's in vitro incubation medium spiked with 3 different quantities of hormones using the Androgen and Glucuronide Conjugate Extraction Method.

	Recoveries (%)		
Spikes	**T**	**TG**	**11-KT**
10 ng each hormone	79.8 ± 1.5	91.1 ± 2.0	76.5 ± 1.5
10 ng each hormone + 100 IU hCG	85.5 ± 1.3	98.1 ± 0.4	86.1 ± 0.8
1 ng each hormone	85.4 ± 1.2	93.5 ± 2.8	88.2 ± 2.6
1 ng each hormone + 100 IU hCG	96.4 ± 1.0	100.0 ± 3.0	90.2 ± 0.9
500 pg each hormone	95.0 ± 2.8	101.4 ± 3.5	101.4 ± 2.3
500 pg each hormone + IU hCG	88.7 ± 1.2	103.7 ± 2.6	91.1 ± 2.4

Recoveries appeared to increase with decreasing amounts of spiked analytes. However, this may be more a factor of increased variability of extraction efficiency at the lower concentrations. The method extracts the incubation medium at assay pH of 7.6 for estrogen and sulfate conjugates. By using 6 mL of EA, and extracting twice, approximately 50% of the estrogen sulfates, 65-80% of the parent estrogens, and about 20% of TS can be captured (Table 2.4).

Androgen and glucuronide conjugate extraction is then accomplished by decreasing the sample pH to 1, extracting once with 6 mL of EA, and then analyzing fractions separately on the LC/MS/MS (Figure 2.5). Following with the low pH extraction captures over 85% of the estrogen and testosterone glucuronides, as well as an extra 20-30% of the TS that can be added to the total from the pH 7.6 extraction (Table 2.4). The androgen/glucuronide conjugate extract must never be combined with the estrogen/sulfate conjugate extract, as this will result in the total loss of the sulfate conjugates due to a salting-out effect. The drawback of using the two extraction methods on the same sample is that the recoveries of parent testosterone and 11-KT suffer by up to 50% (Table 2.4).

Based on what we have observed with real gonadal tissue samples in our lab, the primary steroids produced by ovarian follicles are E_2, E_2-3 sulfate, T, and testosterone glucuronide (TG), while testicular tissues produce T, TG, and 11-KT. Therefore, in situations where hormones captured by each method are of interest, such as in estrogen and T production of oocytes, it is recommended that the samples be divided into two aliquots, with only one target extraction performed on each fraction.

Table 2.4: Recoveries (Mean + SEM) of 10 sex steroid hormone and their sulfate/glucuronide conjugates extracted from spiked Cortland's in vitro incubation medium using the optimized Estrogen and Sulfate Conjugates Extraction protocol (Fraction 1) followed by the Androgen and Glucuronide Conjugate Extraction protocol (Fraction 2). Medium was tested with and without 100 IU human chorionic gonadotropin (hCG).

Spikes	Fraction	Estrogen Recoveries (%)						Androgen Recoveries (%)			
		E2	E1	E2-3S	E1-3S	E2-3G	E1-3G	T	TG	11-KT	TS
10 ng each hormone	1	63.2	71.9	52.0	48.1	0.0	0.0	50.4	0.0	29.8	21.1
	2	8.5	9.3	0.0	0.0	91.5	95.3	23.2	97.0	0.0	15.3
	Total	73.8 + 3.6	81.1 + 4.3	52.0 + 0.9	48.1 + 1.8	91.5 + 2.1	95.3 + 2.0	73.6 + 1.5	97.0 + 1.7	29.8 + 2.1	36.4 + 2.3
10 ng each + 100 IU hCG	1	62.7	71.8	54.5	60.8	0.0	0.0	59.8	0.0	41.2	26.6
	2	9.0	10.1	0.0	0.0	97.8	97.7	28.6	108.7	0.0	8.6
	Total	74.2 + 2.1	81.9 + 3.2	54.5 + 1.4	60.8 + 2.5	97.8 + 2.9	97.7 + 2.5	88.5 + 2.6	108.7 + 3.6	41.2 + 1.7	36.6 + 1.8
1 ng each hormone	1	67.8	68.1	48.1	44.9	0.0	0.0	36.5	0	25.0	21.4
	2	7.9	5.3	0.0	0.0	97.5	97.7	7.6	84.3	0	34.9
	Total	75.7 + 2.7	73.3 + 1.3	48.1 + 3.1	44.9 + 3.4	91.5 + 1.4	97.7 + 2.0	44.1 + 2.2	84.3 + 2.1	25.0 + 1.9	56.3 + 1.4
1 ng each + 100 IU hCG	1	64.6	67.2	48.3	49.0	0.0	0.0	42.6	0.0	34.3	21.2
	2	7.2	4.7	0.0	0.0	99.6	100.9	6.7	95.3	0.0	38.4
	Total	71.0 + 0.9	71.9 + 1.0	48.3 + 1.2	49.0 + 3.1	99.6 + 1.3	100.9 + 3.1	49.3 + 4.0	95.3 + 2.8	34.3 + 0.9	59.6 + 3.1
500 pg each hormone	1	81.3	67.9	57.9	48.6	0.0	0.0	37.0	0	33.3	22.7
	2	11.0	2.9	2	0.0	95.2	92.1	18.2	91.7	0.0	21.1
	Total	92.3 + 2.1	70.9 + 2.9	57.9 + 3.8	48.6 + 4.3	95.2 + 3.1	92.1 + 3.0	55.2 + 3.2	91.7 + 3.2	33.3 + 1.9	43.8 + 3.7
500 pg each + 100 IU hCG	1	65.7	64.5	57.7	56.6	0.0	0.0	57.9	0.0	43.6	28.8
	2	10.3	3.4	0.0	0.0	98.8	97.1	14.7	95.2	0.0	28.1
	Total	76.0 + 3.0	67.4 + 1.4	57.7 + 2.0	56.6 + 2.9	98.8 + 1.8	97.1 + 1.9	72.6 + 1.7	95.2 + 10.2	43.6 + 2.0	56.9 + 2.5

**850 µL *in vitro* assay sample at pH 7.6
+ Recovery Internal Standard**

**Estrogens and
Sulfate Conjugates**

**Androgens and all
Glucuronide Conjugates**

**Extract with 6 mL
Ethyl Acetate**
Vortex for 1 minute
Centrifuge at 4000 rpm for 5 mins

**Adjust to pH 1 using
100 µL of 2M HCl**

**Snap freeze and
Decant solvent**

**Extract with 6 mL
Ethyl Acetate**
Vortex for 1 minute
Centrifuge at 4000 rpm for 5 mins

**Repeat and
Combine fractions**

**Snap freeze and
Decant solvent**

Evaporate to dryness

**Reconstitute in 50 µL of MeOH
Analyze using LC/MS/MS**

Figure 2.5: The final extraction protocol to facilitate targeting estrogens or androgens. If targeting sulfate and glucuronide conjugates, complete the "estrogen" extraction first, then decrease pH and conduct the "androgen" method. Analyze extracts separately on the LC/MS/MS as combining extracts of these two methods will result in loss of the sulfate conjugates.

Validation of Method

The direct injection of samples without extraction is not uncommon for LC/MS/MS analysis, particularly for drug testing using urine (Gustavsson et al. 2007). There are two important reasons to choose extraction of the incubation medium over straight injection analysis on the LC/MS/MS. The first is that the incubation medium has a high salt and protein content (McMaster et al. 1995) and some lipid from the testes and ovarian follicles (personal observations), that can quickly foul the column used to separate the compounds. The high volume of samples from *in vitro* studies greatly increases the mass spectrometer maintenance and cleaning operations which can prove to be costly.

Extraction also concentrates the sample allowing the quantification of analytes that would otherwise be below detection limits. In the 34 samples analyzed by straight injection, hormone concentrations were below detection limits in the following; 3% of samples for estradiol, 74% for estrone 3-sufate (E_1-3S) and 100% for testosterone sulfate (TS). In the extracted samples estradiol was quantified in all the samples also well as 100%, 97%, 74% and 59% were measured in E_2-3S, E_1-3S, E_1 and TS, respectively. Due to the unreliability in measuring estrone, E_1-3S and TS in straight injected samples, they were removed from the method comparison.

As there was no extraction conducted on the straight injection samples, the labeled hormones were added as internal precision standards (IPS) to determine suppression of the signal due to interfering components they may be present in the *in vitro* medium. Suppression in the non-extracted samples was calculated to be $38.3 \pm 3.2\%$ (SEM) for E_2-3S and $47.8 \pm 2.7\%$ for estradiol. Using d_4-Estrone (d_4-E1) as the IPS, suppression in the extracted group was calculated to be $61.6 \pm 2.0\%$ (SEM). The recovery internal standard

(RIS), which also takes into account signal suppression, was $60.5 \pm 1.4\%$ and $29.7 \pm 1.7\%$ for E_2-3S and E_2, respectively. It appears that recovery for E2 is very low, but this is masked by the fact that suppression in the *in vitro* samples is so high, as indicated by the IPS.

There was a significant relationship between hormone concentrations in the extracted and non-extracted samples for both E_2-3S ($R^2 = 0.970$, p<0.001) and E_2 ($R^2 = 0.791$, p<0.001). Figure 2.6 shows that the 1:1 plot of injected data falls within the 95% confidence interval of the regression of E_2-3S concentrations in extracted versus injected samples. It appears that the extraction method underestimates the E_2 concentrations in the samples when compared to the injection method (Figure 2.7). The 1:1 injected sample relationship is not within the 95% confidence interval for the extracted versus injected regression, however, it does fall well within the band that predicts the occurrence of 95% of the data based on the regression.

Underestimation of the E_2 concentrations in the samples may have more to do with the results of the direct injection than an inadequacy of the extraction method. Injected sample concentrations were low and sometimes close to the detection limit which could decrease the accuracy of the analysis (Zöllner et al. 2003). As the *in vitro* assay is run in triplicate, it was noted that there was more variability between the replicate samples when analyzed using direct injection compared to when the samples were extracted. This was particularly true in samples with low E_2 concentrations.
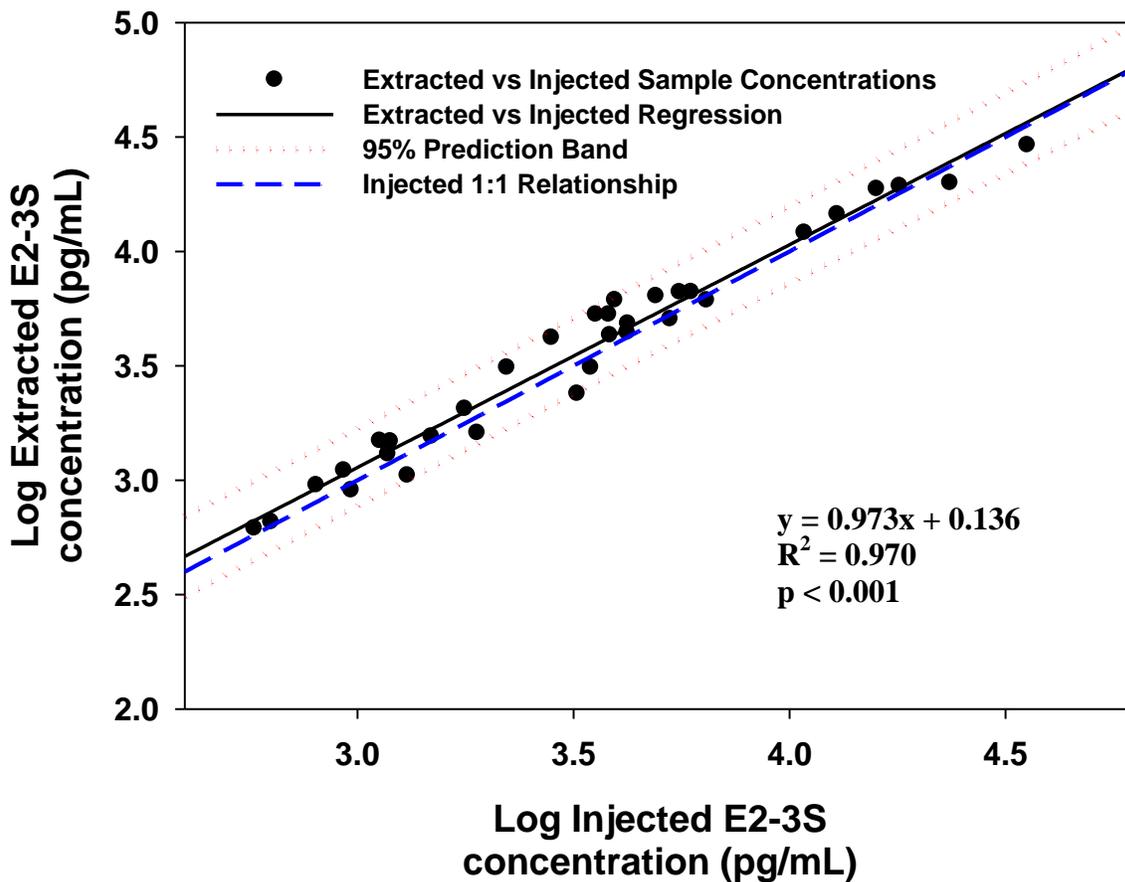
Figure 2.6: Regression of estradiol 3-sulfate (E2-3S) concentrations measured in the same samples using the developed extraction method prior to LC/MS/MS analysis versus directly injecting the samples without extraction (n = 34).

Two mass transitions were monitored for each analyte; one for quantification and one for confirmation. The ratio between these transitions is constant, and can be compared to those of a standard to ensure that the peak being measured is indeed the analyte of interest. It was observed that 1/3 of the E_2 transition ratios in the injected samples were different than the standard ratio by 30-45%. This exceeds what has been considered an acceptable difference for mass transition ratios in LC/MS/MS steroid analyses (Hauser et al. 2008; Diaz-Cruz et al. 2003). Transition ratios can be affected by co-eluting substances

with one or both of the same transition masses as the target analyte (Hauser et al. 2008), or when measuring peak areas close to detection limits (Zöllner et al. 2003). The E_2 mass transition ratios in the extracted samples did not show the same variation, nor did the E_2-3S ratios from either method. Since E_2 concentrations were low in the injected samples, and there was no extraction or clean-up method used to remove interfering compounds, it is possible that the direct injection method over-estimated the E_2 concentrations in the *in vitro* samples.
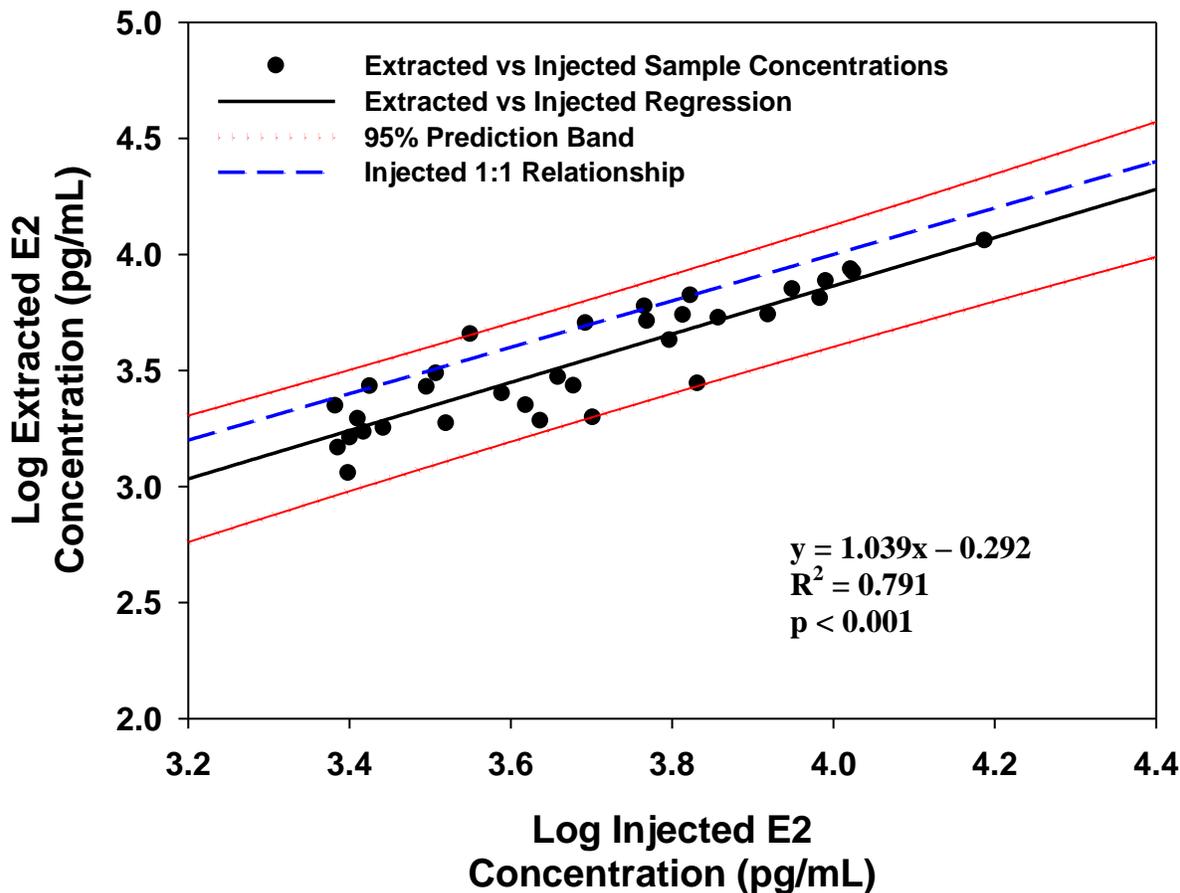
Figure 2.7: Regression of estradiol (E2) concentrations measured in the same samples using the developed extraction method prior to LC/MS/MS analysis versus directly injecting the samples without extraction (n = 33).

We have developed and validated a novel extraction technique and analysis using LC\MS\MS to simultaneously determine a full complement of sex hormones, and their equally important conjugated forms. The final protocol is simple and flexible enough to facilitate targeting specific hormone groups. Analysis of hormones and their metabolites from *in vitro* assay incubation medium will allow examinations of the regulatory mechanisms and the investigation of direct impacts of EDCs on steroidogenesis in fish gonadal tissues.

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Chapter 3: Investigating effects of polybominated diphenyl ethers (PBDEs) and their hydroxylated metabolites on *in vitro* gonadal steroidogenesis in rainbow and brown trout using LC/MS/MS analysis.

Abstract

Polybrominated diphenyl ether (PBDE) flame-retardants are lipophilic persistent organic compounds used in the manufacturing of plastics, electronic equipment, polyurethane foam and textile materials. PBDEs have become an important environmental problem due to their bioaccumulation and biomagnifion properties. Since the late 1970's, substantial increases of these compounds have been measured in wildlife and human adipose tissue, with 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) as the predominant PBDE congener found. The fully brominated, 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE-209), is the principal congener of the DecaBDE commercial formulation. Although these compounds are suspected endocrine disruptors from mammalian studies, little is known about the action of BDEs on fish reproductive systems. The potential effects of environmentally relevant concentrations of BDE-209, BDE-47, as well as the hydroxylated metabolites, 3OH, 5OH and 6OH-BDE-47, on steroidogenesis in brown trout (*Salmo trutta*) testicular tissue and BDE-47 and 6OH rainbow trout (*Oncorhynchus mykiss*) oocytes were investigated. Hydroxylated metabolites of BDE-47 had the greatest effect on steroidogenesis in both the male and female tissues. 3OH and 5OH-BDE-47 were anti-androgenic in males, the opposite effect to the 6OH metabolite. Incubation with 3OH also produced a stronger response than the 5OH. In the oocyte exposures to 6OH, there was increased E2-3S and 3G production with the simultaneous decrease in E2-17

glucuronidation. This suggests that more than one isoform of UGT exists in rainbow trout oocytes, or that SULT activity may be regulating UGT activity. These studies showed that measuring only the parent hormone does not provide a complete profile of the potential for PBDEs and their hydroxylated metabolites to affect fish reproductive steroid concentrations. If total estrogens, or E2 alone, had been analyzed significant effects would have gone undetected. Although this study was primarily focused on investigating the effects of xenobiotics on parent hormone production, it was also the first simultaneous investigation using LC/MS/MS of sulfate and glucuronide conjugate steroid production in fish gonadal tissues under both basal and hCG stimulated conditions. Under natural conditions, without PBDE exposure, conjugates were determined to make up over 80% of total testosterone produced by male brown trout testes, and 40-50% of estrogens produced by rainbow trout oocytes. Simultaneous determination of multiple steroid forms using the method developed in the previous chapter facilitated the discovery that the carbon at position 17 of E2 was the preferred site of glucuronidation over position 3 in rainbow trout oocytes.

Introduction

Polybrominated diphenyl ether (PBDE) flame-retardants are lipophilic persistent organic compounds used for manufacturing plastics, electronic equipment, polyurethane foam and textile materials. They have become an important environmental issue because some formulations are persistent, bioaccumulative and toxic. Since the late 1970's, concentrations measured in wildlife and human adipose tissue have increased, with 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) the predominant PBDE congener identified (Anderson & MacRae 2006, Staskal et al. 2006, Hites 2004, Boon et al. 2002). While the fully brominated, 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE-209) is the predominant congener of the DecaBDE commercial formulation, BDE-47 is a primary metabolite of BDE-209 (Roberts et al. 2011).

Recent studies have focused on the potential endocrine disrupting properties of PBDEs, which include effects on thyroid hormones and reproductive steroid production. Streroid hormone production has been examined exclusively in mammalian models, and Gregoraszczuk et al. (2008) showed that BDE-47, 99, 100 and 209 increased estradiol (E2) and testosterone (T) production in a porcine thecal cell in vitro assay; suggesting induction of aromatase (CYP19) and 17β-hydroxy steroid dehydrogenase (CYP17) activity, respectively. BDE-47 may have estrogenic activity via receptor mediated pathways (Dang et al. 2007, Hamers et al. 2006), with the 6OH form exhibiting significantly higher potency (Harju et al. 2007). BDE-47 may also act as an androgen receptor (AR) inhibitor by competing for binding sites (Stoker et al. 2005, Harju et al. 2007). Hydroxylated metabolites of BDE-47 (i.e.3OH, 5OH and 6OH) exhibit significant antiandrogenic activity by reducing the DHT-induced response in the yeast human androgen receptor (hAR) assay (Canton et al. 2007).

While little is known about the action of PBDEs on fish reproductive systems, maternal transfer from adult zebrafish (*Danio rerio*) to their offspring via oocyte yolk deposition can occur, emphasizing the potential environmental importance of these compounds to fish (Rattfelt et al. 2007). Moreover, a parallel study showed an increase in the number of atretic oocytes, and impaired hatching success in offspring of PBDE exposed fish, with no concurrent effect on fecundity and gonadosomatic index (Norman et al. 2007). Based on these limited results, additional examination regarding the effects of PBDEs on fish reproductive systems is warranted.

Using an *in vitro* technique, the potential effects of environmentally relevant concentrations of BDE209, BDE-47, as well as its 3OH, 5OH and 6OH hydroxylated metabolites, on steroidogenesis in brown trout (*Salmo trutta*) testicular tissue and rainbow trout (*Oncorhynchus mykiss*) oocytes was examined.   Testosterone, 11-ketotestosterone, estradiol, estrone and their conjugates were all analyzed simultaneously in the incubation medium using LC/MS/MS to examine effects on reproductive steroid metabolism.

Methods

Chemicals: PBDEs, Hormones and in vitro Medium

The PBDEs, 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (209), 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and 3OH-, 5OH-, OH6-BDE-47 were purchased from Wellington Laboratories (Guelph, ON Canada). The native and mass-labelled parent sex steroids 17β- estradiol (E2), estrone (E1), testosterone (T), 17β-estradiol 2,4,16,16-d_4 (d_4-E2), estrone 2,4,16,16-d_4 (d_4-E1), testosterone (d_2-T), and progesterone 2,2,6,6,17,21,21,21-d_8 (d_8-P), were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).   The

testosterone conjugates, testosterone sulfate (TS) and testosterone glucuronide (TG), as well 11-ketotestosterone (11-KT) were acquired from Steraloids (Newport, RI, USA). Unlabelled conjugated estrogens 17β-estradiol 3-sulfate (E2-3S), 17β-estradiol 3-glucuronide (E2-3G), 17β-estradiol 17-glucuronide (E2-17G), estrone 3-sulfate (E1-3S), estrone 3-glucuronide (E1-3G) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The d_4 mass-labeled estrogen conjugate 17β-estradiol 2,4,16,16-d_4 3-sulfate (d_4-E2-3S) was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada).

Cortland's saline incubation medium was adapted from McMaster et al. 1995 (see Appendix). The incubation buffer is routinely used in *in vitro* studies to maintain trout gonadal tissues (McMaster et al. 1995, Leatherland & Lin 2001, Pankhurst et al. 1995, Pankhurst et al. 1996). Human chorionic gonadotropin (hCG), dissolved in Cortland's medium to a final concentration of 100 IU/mL, was added to stimulate steroid production. All Cortland's medium ingredients were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

The solvents used in the extraction process, hexane and ethyl acetate, Optima grade methanol and water used as the mobile phase for LC/MS/MS analyses, and acetone as a PBDE carrier were purchased from Fisher Scientific (Ottawa, ON Canada).

Fish

Pre-spawning brown trout (*Salmo trutta*) were obtained from Westhawk Fish Hatchery (Westhawk Lake, MB Canada). Six males (1.69 ± 0.15 kg and 48.4 ± 1.4 cm, gonad weight 67.5 ± 4.2 g) and six females (1.61 ± 0.16 kg and 47.4 + 1.1cm, gonad weight 140.6 ± 17.2 g) were individually anesthetized in pH buffered MS 222 (0.5 g/L), sacrificed, weighed and

measured. Gonadal tissues were removed, weighed and immediately placed in Cortland's incubation medium (pH 7.6) on ice.

Additionally, six females rainbow trout (*Oncorhynchus mykiss*) obtained from Rainbow Springs Hatchery (Thamesford, ON Canada) were grown to pre-spawning condition at the Freshwater Institute, Winnipeg, MB. These fish were held in an 800L tank at $13^{o}C$ with a 12 hour light/dark cycle and fed approximately 1.5% of their body weight six times per week. Upon sacrificing, each fish was weighed and measured (1.02 ± 0.12 kg and 37.8 ± 1.5 cm, gonad weight 31.0 ± 11.3 g). Gonadal tissues were removed and treated as described above. Both groups of fish were held and utilized according to the DFO-Animal Use Protocol FWI-ACC-2010-014.

In vitro Incubations

In vitro incubations were adapted from McMaster et al. 1995, and described in the previous chapter. Briefly, 5 ovarian follicles or 20 mg of testicular tissue were placed in each well of the 24-well culture plates with 1 mL of Cortland's medium. BDE-47 and BDE-209 were added to the wells in 5 µL of acetone at the following final concentrations: 0, 100, 500, 1000, 3000, and 5000 pg/mL. The hydroxylated compounds were each added at concentrations of 10, 50, 100, 500 and 1000 pg, also in 5 µL of acetone to 1 mL of Cortland's medium. Each sample was run in triplicate under both basal and stimulated conditions using 100 IU human chorionic gonadotropin (hCG), and acetone only solvent controls were added for each individual fish. The plates were incubated at 12.0 ± 0.5 ^{o}C for 24 hours. The incubation medium from each well was collected by pipette and stored separately in 1.5 mL Eppendorf vials at -80 ^{o}C until analysis.

Extraction and LC/MS/MS analysis

The frozen media samples were first thawed, and vortexed to ensure homogeneity, and 850 μ L of sample was spiked with labeled recovery internal standards (RIS). Samples from male brown trout received d_8-P (positive ion mode), and d_4-E2 with d_4-E2-3S (negative ion mode). Labeled testosterone (d_2-T), was substituted for d_8-P as the positive ion mode RIS in the female rainbow trout samples. Hormones were extracted using a two-step liquid:liquid method developed in Chapter 2. An abbreviated outline is found in Figure 3.1. Final extracts were spiked with d4-E1 as the internal precision standard (IPS). Testicular tissue incubations were analyzed for T, T-S, T-G, and 11-KT. The oocyte incubation medium was analyzed for E2, E2-3S, E2-3G, E2-17G, E1, E1-3S and E1-3G concentrations.

Liquid chromatography (LC) was performed with an Agilent 1100 series system (Agilent Technologies Canada, Inc., Mississauga, ON, Canada) equipped with a vacuum degasser, binary pump and autosampler. A binary mobile phase of Optima grade water and MeOH was used at a flow rate of 300 μL/min. The composition and gradients of water/MeOH (v/v) mobile phase, and details of the two monitoring periods in negative ion mode and the single period in positive ion mode are described in the Methods section of Chapter 2.

Hormone separations were achieved using Genesis Lighten C_{18} analytical columns (50mm×2.1mm i.d., 4 μm particle size; Grace, Alltech Canada, Guelph, ON, Canada). The LC was coupled to a Sciex 2000 triple quadrupole mass spectrometer (MS/MS) (Applied Biosystems, Foster City, CA, USA) with electrospray ion source (ESI). Working parameters
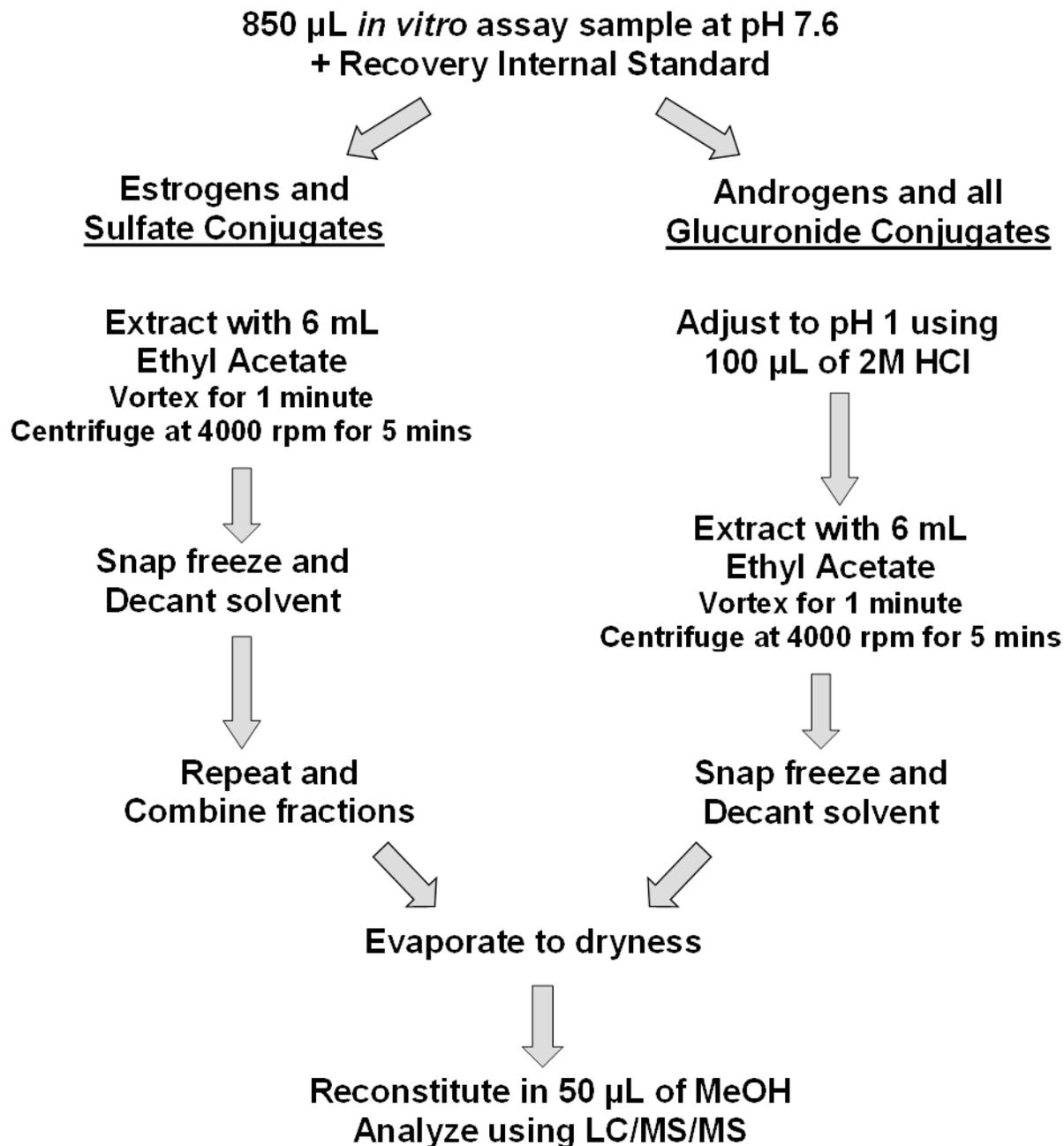
Figure 3.1: The final extraction protocol to facilitate targeting estrogens or androgens. If targeting sulfate and glucuronide conjugates, complete the "estrogen" extraction first, then decrease pH and conduct the "androgen" method. Analyze extracts separately on the LC/MS/MS as combining extracts of these two methods will result in loss of the sulfate conjugates.

Table 3.1: The LC/MS/MS working parameters and run time optimized for multiple reaction monitoring with electrospray ion source in positive and negative ion modes.

Ion Mode	Period	Run time (mins)	CUR	CAD	IS	GS1	GS2	Temperature (^{o}C)
negative	1	9.6	20.0	4	-4500	30	45	550
negative	2	11.4	20.0	4	-4400	30	45	550
positive	1	21.0	30.0	8	5000	30	55	550

optimized for the ESI for both negative and positive ion modes are shown in Table 3.1. The mass to charge (*m/z*) value and their product ion transitions used for quantitation and confirmation of each hormone screened for in the *in vitro* medium, together with their method detection limits (MDLs) and analytical detection limits (ADLs) are shown in Table 3.2A.

Table 3.2A: The mass to charge (*m/z*) value and product ion transitions used for quantitation and confirmation of each hormone with method detection limits (MDL) and analytical detection limits (ADL).

Hormone	Abbreviation	Mass (m/z)	Transitions Monitored Quantitation	Transitions Monitored Confirmation	Ion Mode	MDL (pg)	ADL (pg)
Estradiol	E2	271.2	145.1	183.1	Negative	2.5	0.81
Estradiol – 3 Sulfate	E2-3S	351.2	271.0	80.0	Negative	1.3	0.53
Estradiol – 3 Glucuronide	E2-3G	447.2	271.2	175.0	Negative	10.1	2.78
Estradiol – 17 Glucuronide	E2-17G	447.2	85.1	113.1	Negative		
Estrone	E1	269.1	145.0	142.8	Negative	0.6	0.32
Estrone – 3 Sulfate	E1-3S	349.1	269.1	145.1	Negative	0.5	0.22
Estrone – 3 Glucuronide	E1-3G	445.2	269.1	175.0	Negative	7.1	2.29
Testosterone	T	289.2	97.1	109.1	Positive	0.5	0.20
Testosterone Sulfate	TS	367.2	97.0	80.0	Negative	1.4	0.39
Testosterone Glucuronide	TG	486.8	310.9	198.9	Positive	6.8	0.77
11-Ketotestosterone	11KT	303.3	121.1	259.2	Positive	3.1	1.88

Methods for defining MDLs and ADLs are outlined in Methods from Chapter 2. The *m/z* values with product ion transitions for all mass-labeled hormones used as RIS and IPS are in Table 3.2B.

Table 3.2B: The mass to charge (*m/z*) value and product ion transitions used for quantitation and confirmation of each mass-labelled hormone.

Mass-Labelled Hormone	Abbreviation	Mass (m/z)	Transitions Monitored		Ion Mode
			Quantitation	Confirmation	
d4-Estradiol	d4-E2	275.2	147.1	187.1	Negative
d4-Estradiol - 3 Sulfate	d4-E2-3S	355.2	275.1	80	Negative
d3-Estradiol - 3 Glucuronide	d3-E2-3G	450.2	274.1	112.9	Negative
d4-Estrone	d4-E1	273.1	147.1	145	Negative
d2-Testosterone	d2-T	291.2	99.1	111.1	Positive
d8-Progesterone	d8-Prog	323.3	100.1	113.1	Positive

Statistical Analysis

Differences in individual hormone production by oocytes and testes were determined using ANOVA on log transformed data followed with Fisher's least significant difference pairwise analysis. The analysis of percent of total hormone production of glucuronides, sulfates and parent estrogens by fish oocytes was conducted using non-parametric Kruskal-Wallace 1-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results

Effects on Male Gonadal Tissue Steroidogenesis and Metabolism

Testosterone sulfate was not consistently measured in the Cortland's medium and was therefore excluded from further analysis.

i.   Parent Compounds - BDE-47 and BDE-209

The effect of BDE 47 and 209 were examined on androgens and conjugated testosterone. There was no clear trend of effects on T, TG, or 11-KT production with increasing concentrations of BDE47 (p>0.05) (Table 3.3). Results from BDE209 incubations revealed that the concentrations of androgens produced by Male 6 were more than 2 standard deviations away from the means of each hormone measured. Therefore, data from this individual was removed from the analyses. Androgen production was not significantly different between these control groups, except in the case of the TG where the acetone carrier group (AC) produced a significantly greater quantity of hormone under stimulated conditions (p = 0.034).

Under basal conditions, BDE209 had no effect on the production of T, TG or 11-KT from brown trout testicular tissues (p>0.05). Stimulated T and 11-KT production were not significantly different than the controls; p = 0.400 and p = 0.270 respectively (Table 3.3). At the 5000 pg/mL BDE209 incubation concentration, hCG stimulated brown trout testicular tissues produced significantly more TG than the control group (p = 0.040). There was no difference from the acetone carrier group (Table 3.3).

Table 3.3: Effects on select androgen production of Brown Trout testes incubated with increasing concentrations of BDE-47, BDE-209 and 6OH-BDE-47 under basal and hCG stimulated conditions (steroids values are means $\pm$ SEM).

[BDE] For Incubations		Testosterone (pg/mg tissue)		Testosterone Glucuronide (pg/mg tissue)		11-Ketotestosterone (pg/mg tissue)	
		Basal	hCG	Basal	hCG	Basal	hCG
BDE-47 (pg/mL)	5000	14.5 $\pm$ 3.7	29.2 $\pm$ 4.9	78.9 $\pm$ 11.8	197.8 $\pm$ 24.2	37.1 $\pm$ 8.5	94.1 $\pm$ 21.7
	3000	9.7 $\pm$ 1.3	29.8 $\pm$ 5.1	81.5 $\pm$ 11.4	145.1 $\pm$ 26.3	39.2 $\pm$ 6.5	102.7 $\pm$ 21.4
	1000	11.1 $\pm$ 1.7	34.8 $\pm$ 4.7	62.2 $\pm$ 9.4	167.7 $\pm$ 24.6	32.3 $\pm$ 4.6	110.6 $\pm$ 16.1
	500	10.5 $\pm$ 1.3	32.4 $\pm$ 4.2	81.5 $\pm$ 13.3	161.0 $\pm$ 24.3	36.4 $\pm$ 5.8	105.1 $\pm$ 22.1
	100	13.1 $\pm$ 2.0	38.3 $\pm$ 4.2	91.6 $\pm$ 12.3	212.1 $\pm$ 33.4	32.1 $\pm$ 3.6	114.4 $\pm$ 19.3
	Control	11.0 $\pm$ 1.5	31.1 $\pm$ 4.1	63.8 $\pm$ 7.3	169.5 $\pm$ 26.9	30.9 $\pm$ 3.5	85.0 $\pm$ 13.3
	AC	9.0 $\pm$ 0.8	35.2 $\pm$ 3.6	76.8 $\pm$ 8.2	171.7 $\pm$ 14.7	38.3 $\pm$ 4.2	88.2 $\pm$ 7.1
BDE-209 (pg/mL)	5000	9.8 $\pm$ 1.2	25.7 $\pm$ 3.0	83.6 $\pm$ 7.5	150.9 $\pm$ 19.6	32.8 $\pm$ 6.4	65.0 $\pm$ 13.0
	3000	9.8 $\pm$ 1.4	29.9 $\pm$ 3.8	71.7 $\pm$ 5.7	121.9 $\pm$ 15.2	29.2 $\pm$ 4.4	61.8 $\pm$ 15.0
	1000	9.3 $\pm$ 1.5	29.1 $\pm$ 3.9	58.5 $\pm$ 6.6	114.3 $\pm$ 10.7	25.7 $\pm$ 4.0	62.3 $\pm$ 9.5
	500	9.3 $\pm$ 1.3	25.2 $\pm$ 2.5	73.6 $\pm$ 12.1	107.4 $\pm$ 14.2	26.7 $\pm$ 5.1	57.3 $\pm$ 9.9
	100	9.1 $\pm$ 0.7	22.2 $\pm$ 2.3	69.9 $\pm$ 7.6	90.8 $\pm$ 12.0	32.9 $\pm$ 6.0	54.6 $\pm$ 9.9
	Control	7.9 $\pm$ 0.7	21.2 $\pm$ 1.7	58.0 $\pm$ 7.9	103.2 $\pm$ 10.7	21.5 $\pm$ 2.2	54.2 $\pm$ 6.6
	AC	8.1 $\pm$ 0.6	30.0 $\pm$ 3.0	75.6 $\pm$ 9.6	147.3 $\pm$ 10.2	28.2 $\pm$ 3.9	82.0 $\pm$ 7.2
6OH-BDE-47 (pg/mL)	1000	10.4 $\pm$ 0.8	43.8 $\pm$ 6.9	89.4 $\pm$ 6.1	326.8 $\pm$ 44.6	35.9 $\pm$ 2.5	160.6 $\pm$ 28.3
	500	12.0 $\pm$ 1.1	40.7 $\pm$ 4.3	92.4 $\pm$ 10.6	246.1 $\pm$ 26.5	38.4 $\pm$ 6.0	141.3 $\pm$ 16.1
	100	9.8 $\pm$ 0.8	29.2 $\pm$ 2.2	126.8 $\pm$ 10.8	189.0 $\pm$ 20.4	41.9 $\pm$ 5.7	95.7 $\pm$ 13.4
	50	9.9 $\pm$ 1.2	44.8 $\pm$ 6.8	119.6 $\pm$ 7.9	203.3 $\pm$ 26.2	35.6 $\pm$ 4.6	100.8 $\pm$ 13.1
	10	10.5 $\pm$ 0.8	37.8 $\pm$ 3.6	109.3 $\pm$ 5.7	233.8 $\pm$ 17.8	41.0 $\pm$ 5.0	131.5 $\pm$ 20.5
	Control	10.9 $\pm$ 1.5	31.1 $\pm$ 4.1	87.3 $\pm$ 7.6	192.0 $\pm$ 30.7	30.9 $\pm$ 3.5	84.9 $\pm$ 13.3
	AC	11.8 $\pm$ 1.4	32.9 $\pm$ 2.9	94.6 $\pm$ 9.9	162.5 $\pm$ 12.3	38.2 $\pm$ 4.2	88.2 $\pm$ 7.1

ii.   Metabolites – 3OH-, 5OH- and 6OH-BDE47

The metabolite, 6OH-BDE-47, exhibited a greater androgenic effect than the parent BDE47.   Tissues treated with 500 and 1000 pg/mL 6OH-BDE-47 under hCG stimulated conditions (Table 3.3) produced significantly more TG than the controls (p = 0.006). Differences in basal TG production appeared at lower concentrations of 6OH-BDE-47 (i.e. 50 and 100 pg/mL), but not at higher concentrations.   6OH-BDE-47 also induced a significant increase (p=0.006) in 11KT production in the hCG treatment group at 10, 500 and 1000 pg/mL, however, no effect was observed among unstimulated tissues.   There were no significant differences in T production by male testicular tissues incubated with 6OH-BDE-47 relative to the controls (Table 3.3).

Both 3OH- and 5OH-BDE47 affected androgen production differently than the 6OH-metabolite, exhibiting an inhibitory effect. Testicular tissues exposed to 5OH-BDE47 produced significantly less T, TG and 11KT than the controls in basal activity assays (p < 0.05).   The testes receiving lower concentrations were also more affected than the higher dose groups (Figure 3.2A-C). A similar pattern was evident among the hCG stimulated tissues, except that T was the only hormone significantly different than the controls at 10 pg/mL.

3OH-BDE-47 acted as a potent anti-androgen in brown trout testes in vitro incubations. The basal groups dosed with 3OH- at concentrations greater than 10 pg/mL had significantly lower T (p = 0.013) and TG (p = 0.009) production than the controls (Figures 3.3A-B). Basal production of 11KT was lower in all exposure groups, but not significantly (Figure 3.3C). Stimulated hormone production decreased concentration dependently with increasing 3OH-concentrations. Testosterone production was lower (p=0.024) than the controls at all of the exposure concentrations except 500 pg/mL. The mean control level of TG was significantly

higher than all 3OH- treatment groups. The acetone carrier (AC) group was marginally lower than the control (p = 0.597), therefore only the 50 and 1000 pg/mL exposure groups were significantly different from the AC (Figure 3.3A-B). 11-ketotestosterone was lower (p=0.026) at the two highest 3OH- concentrations (500 and 1000 pg/mL), as well as at 50 pg/mL (Figure 3.3C).
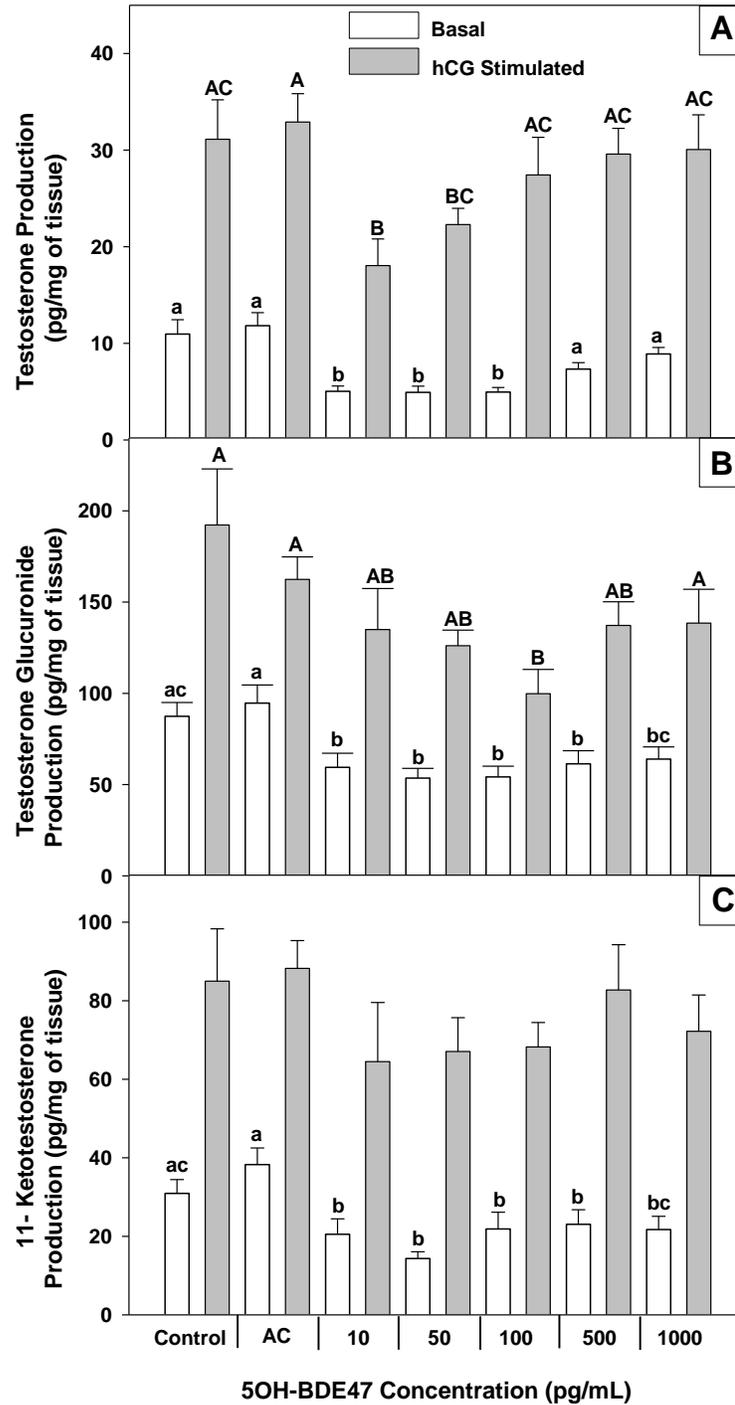
Figure 3.2 Changes in mean $\pm$ SEM testosterone (A), testosterone glucuronide (B) and 11-ketotestosterone (C) production *in vitro* by Brown trout testicular tissues exposed to increasing concentrations of 5OH-BDE-47 under basal (open bars) and hCG stimulated conditions (shaded bars). Letters denote treatment groups that are significantly different from each other determined by ANOVA ($p < 0.05$).
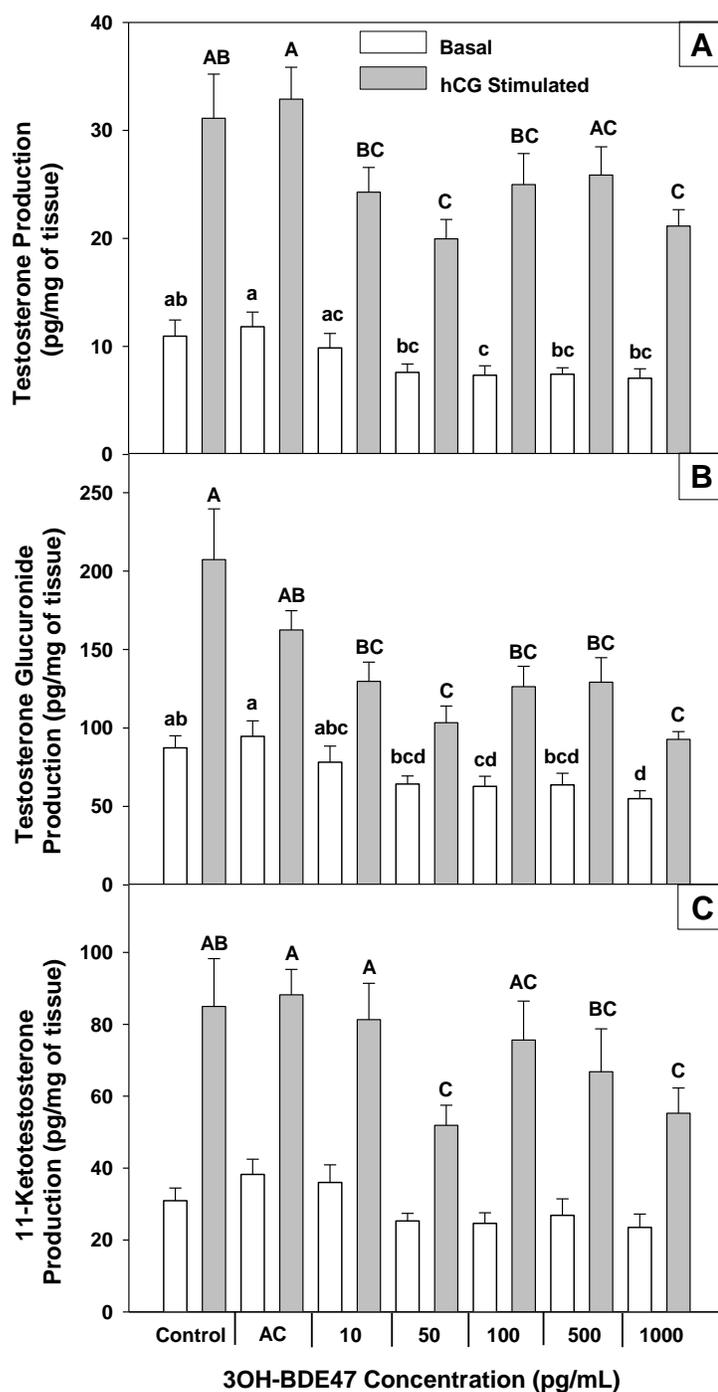
Figure 3.3 Decreases in mean $\pm$ SEM *in vitro* androgen production by Brown trout testicular tissues incubated with increasing concentrations of 3OH-BDE-47 under basal (open bars) and hCG stimulated conditions (shaded bars). Androgens measured were testosterone (A), testosterone glucuronide (B) and 11-ketotestosterone (C). Letters denote treatment groups that are significantly different from each other determined by ANOVA ($p < 0.05$).

iii.   Total Testosterone and the Proportion of Glucuronide Conjugate Produced

When T and TG hormone concentrations produced by the testicular tissues of brown trout were combined to determine "total testosterone" production, the results under basal conditions were similar to hormones analyzed individually. However, under hCG stimulated conditions, there were no differences in total testosterone for 5OH, 6OH or BDE-209 exposures even though significant differences were found if T and TG were analyzed separately (Table 3.4). BDE-47 and 3OH had the same results for total testosterone production as that observed in their T and TG individual hormones (Table 3.4).

TG was the predominant form of T produced by the brown trout testes *in vitro;* mean production ranged from 79.8 to 92.9% of total testosterone. The ratio of T to TG production was not consistent between basal and hCG stimulated conditions because the proportion of T produced significantly increased under hCG stimulated conditions ($p < 0.05$) for all BDE and metabolite exposures (Table 3.5).

The proportions of T and TG produced *in vitro* by the testicular tissues remained constant ($p > 0.05$) with increasing concentrations of BDE-47, BDE-209 and 3OH.   However, with exposure to 6OH- and 5OH0BDE, there were significant differences from the controls in the proportions of T and TG produced but only at 10 pg/mL 6OH and 50 pg/mL 5OH (Table 3.5). A general summary of all the male brown trout testicular tissue *in vitro* incubation results can be found in Table 3.6.

Table 3.4: Total testosterone (mean $\pm$ SEM) produced by brown trout testicular tissues *in vitro* during exposure to increasing concentrations of test BDEs.

[BDE] For Incubations		Total Testosterone	
		Basal	hCG
BDE-47 (pg/mL)	5000	93.4 $\pm$ 4.5	247.1 $\pm$ 36.9
	3000	96.6 $\pm$ 12.1	274.5 $\pm$ 82.7
	1000	73.2 $\pm$ 9.7	220.4 $\pm$ 40.8
	500	94.0 $\pm$ 13.7	193.4 $\pm$ 28.2
	100	109.5 $\pm$ 11.7	243.0 $\pm$ 38.1
	Control	69.8 $\pm$ 8.4	176.4 $\pm$ 24.5
	AC	85.8 $\pm$ 8.4	189.6 $\pm$ 11.4
3OH-BDE-47 (pg/mL)	1000	62.0 $\pm$ 5.1	113.9 $\pm$ 5.5
	500	74.1 $\pm$ 7.9	148.1 $\pm$ 17.1
	100	70.4 $\pm$ 6.1	151.4 $\pm$ 15.1
	50	71.9 $\pm$ 5.7	123.7 $\pm$ 11.5
	10	88.1 $\pm$ 11.0	150.9 $\pm$ 14.7
	Control	97.5 $\pm$ 9.5	236.6 $\pm$ 34.9
	AC	105.8 $\pm$ 10.6	189.6 $\pm$ 11.4
5OH-BDE-47 (pg/mL)	1000	75.8 $\pm$ 6.6	190.0 $\pm$ 29.9
	500	68.6 $\pm$ 7.8	160.9 $\pm$ 14.7
	100	59.1 $\pm$ 6.2	140.7 $\pm$ 12.8
	50	58.2 $\pm$ 5.5	147.3 $\pm$ 9.7
	10	64.5 $\pm$ 8.0	153.0 $\pm$ 24.6
	Control	78.2 $\pm$ 9.7	236.6 $\pm$ 34.9
	AC	105.8 $\pm$ 10.6	189.6 $\pm$ 11.4
6OH-BDE-47 (pg/mL)	1000	101.4 $\pm$ 6.3	352.6 $\pm$ 54.2
	500	104.4 $\pm$ 10.8	291.3 $\pm$ 29.0
	100	136.8 $\pm$ 10.4	220.4 $\pm$ 20.5
	50	133.8 $\pm$ 9.9	245.7 $\pm$ 32.1
	10	157.9 $\pm$ 19.7	293.3 $\pm$ 48.9
	Control	97.4 $\pm$ 9.5	236.3 $\pm$ 34.8
	AC	105.7 $\pm$ 10.6	189.6 $\pm$ 11.4
BDE-209 (pg/mL)	5000	93.4 $\pm$ 7.8	175.0 $\pm$ 21.5
	3000	81.5 $\pm$ 6.5	150.1 $\pm$ 18.4
	1000	67.9 $\pm$ 7.9	141.8 $\pm$ 13.3
	500	81.5 $\pm$ 13.5	132.6 $\pm$ 15.4
	100	79.0 $\pm$ 7.4	113.0 $\pm$ 13.6
	Control	67.8 $\pm$ 8.7	150.8 $\pm$ 20.0
	AC	83.7 $\pm$ 9.7	212.5 $\pm$ 18.3

Table 3.5: The proportions of testosterone (parent) and testosterone glucuronide produced as a percentage of total testosterone during *in vitro* incubation of testes with increasing concentrations of BDE compounds.

[BDE] For Incubations		% of Total Testosterone Produced			
		Parent		Glucuronide	
		Basal	hCG	Basal	hCG
BDE-47 (pg/mL)	5000	15.9	16.2	84.1	83.8
	3000	10.6	20.2	89.4	79.8
	1000	19.2	19.7	80.8	80.3
	500	11.3	17.3	88.7	82.3
	100	13.4	17.4	86.6	82.6
	Control	14.6	15.0	85.4	85.0
	AC	11.4	18.4	88.6	81.6
3OH-BDE-47 (pg/mL)	1000	12.0	18.7	88.0	81.3
	500	12.1	18.2	87.9	81.8
	100	13.0	17.2	87.0	82.8
	50	10.4	17.8	89.6	82.2
	10	12.0	15.9	88.0	84.1
	Control	14.7	14.3	85.3	85.7
	AC	11.1	17.7	88.9	82.3
5OH-BDE-47 (pg/mL)	1000	12.4	17.6	87.7	82.4
	500	11.8	18.3	88.2	81.7
	100	8.9	20.2	91.1	79.8
	50	8.1	14.9	91.9	85.1
	10	8.4	12.7	91.6	87.3
	Control	10.9	14.6	89.1	85.4
	AC	11.1	18.4	88.9	81.6
6OH-BDE-47 (pg/mL)	1000	11.2	14.6	88.8	85.4
	500	13.5	16.0	86.5	84.0
	100	8.2	15.7	91.8	84.3
	50	8.7	19.0	91.3	81.0
	10	7.1	15.5	92.9	84.5
	Control	10.9	14.6	89.1	85.4
	AC	11.1	18.4	88.9	81.6
BDE-209 (pg/mL)	5000	10.9	14.2	89.1	85.8
	3000	11.6	18.8	88.4	81.2
	1000	11.4	19.0	88.6	81.0
	500	13.0	20.3	87.0	79.7
	100	12.6	20.3	87.4	79.7
	Control	16.5	15.9	83.5	84.1
	AC	10.7	17.4	89.3	82.6

Table 3.6: Summary of androgen production in male brown trout testicular tissue incubations with PBDE congeners and metabolites. Arrows denote increases or decreases in mean hormone production compared to the controls. NSD is no significant difference (p > 0.05)

BDE / Metabolite	Testosterone	Testosterone Glucuronide	11-Keto testosterone	Total Testosterone
BDE-209	NSD	↑	NSD	NSD
BDE-47	-----	-----	-----	-----
6OH-BDE-47	NSD	↑	↑	NSD
5OH-BDE-47	↓	↓	↓	-----
3OH-BDE-47	↓	↓	↓	↓

Effects on Female Gonadal Tissue Steroidogenesis and Metabolism

Unforeseen method complications occurred with the original extraction protocol and the sulfated hormones were lost. A new extraction protocol was developed (see Chapter 2) to facilitate the isolation of these important conjugates. Once this was complete, female brown trout were no longer available, so the study was completed using female rainbow.

i. BDE-47

E1 and E1-3S levels were below detection in many basal samples of the BDE-47 incubations and E1-3G was not at measureable levels in any of the samples. Therefore all three compounds were removed from further analysis. There were no significant effects (p > 0.05) of BDE-47 on E2 or its conjugates under basal conditions (Table 3.7).

Production of E2 and E1 were not significantly altered by BDE-47 under stimulated hCG conditions (Table 3.7) (E2 p = 0.14; E1 p = 0.298). There were differences in sulfate conjugate production at the higher BDE-47 exposure concentrations. At the 3000-5000 pg/mL, E1-3sulfate production was lower than the controls, but not significantly (p = 0.133). There was a significant (p = 0.024) reduction in E2-3S production in oocytes exposed to BDE-47 at concentrations ≥ 1000 pg/mL compared to the acetone control. Incubating rainbow trout

oocytes with increasing concentrations of BDE-47 had no effect ($p > 0.05$) on individual glucuronide production under hCG stimulated conditions (Table 3.7).

ii.   6OH-BDE-47

The 6OH-BDE metabolite had a greater effect on rainbow trout oocyte estrogen production than the parent BDE-47 brominated flame retardant.   Increased exposure concentrations of 6OH- resulted in lower E2 and E1 production. However, only stimulated E2 production was significantly different ($p < 0.001$) than controls at 6OH concentrations greater than 10 pg/mL (Figure 3.4A).

Estrogen conjugate production was also affected by 6OH-BDE-47 exposure. E2-3S, E1-3S, and E2-3G all exhibited a trend of higher production with increasing 6OH- exposure concentrations, particularly under hCG stimulated conditions (Figure 3.4B-C). Mean E2-3S production increased significantly ($p = 0.042$) at the highest exposure concentration (1000 pg/mL) with hCG stimulation, compared to the control group, but not compared to the acetone carrier group (Figure 3.4B). 6OH-BDE-47 had the opposite effect on E2-17G production compared with the conjugates at the 3 carbon position. There were significant reductions in E2-17G production with increased exposure under both basal ($p < 0.001$) at 6OH concentrations over 10 pg/mL and hCG stimulated ($p = 0.038$) conditions at 50 and 500 pg/mL(Figure 3.4D).

Table 3.7: Estradiol and conjugate production (mean $\pm$ SEM) by rainbow trout oocytes incubated with increasing concentrations of BDE-47 and its 6OH- metabolite under basal and stimulated conditions.

[BDE] For Incubations		Estradiol (E2) (pg/mL)		E2-3 Sulfate (pg/mL)		E2-3 Glucuronide (pg/mL)		E2-17 Glucuronide (pg/mL)	
		Basal	hCG	Basal	hCG	Basal	hCG	Basal	hCG
BDE-47 (pg/mL)	5000	2936.0 $\pm$ 167.1	7827.8 $\pm$ 801.5	964.5 $\pm$ 91.7	4204.6 $\pm$ 282.8	290.5 $\pm$ 38.0	718.3 $\pm$ 122.5	654.9 $\pm$ 34.2	2579.8 $\pm$ 114.8
	3000	2808.4 $\pm$ 268.0	8067.8 $\pm$ 1021.1	1027.4 $\pm$ 84.7	4425.9 $\pm$ 417.6	312.9 $\pm$ 72.8	617.1 $\pm$ 59.4	660.3 $\pm$ 36.6	2541.8 $\pm$ 78.5
	1000	2903.7 $\pm$ 298.2	7031.5 $\pm$ 651.2	1018.1 $\pm$ 112.5	4228.3 $\pm$ 492.0	271.3 $\pm$ 37.0	723.3 $\pm$ 125.2	755.1 $\pm$ 40.9	2777.2 $\pm$ 166.2
	500	3234.1 $\pm$ 225.8	8452.3 $\pm$ 801.9	1088.3 $\pm$ 128.7	5233.9 $\pm$ 406.6	319.0 $\pm$ 56.4	808.8 $\pm$ 120.7	823.9 $\pm$ 79.2	2999.8 $\pm$ 213.9
	100	2464.9 $\pm$ 159.9	8554.1 $\pm$ 731.0	969.6 $\pm$ 74.1	5516.1 $\pm$ 590.3	270.4 $\pm$ 43.1	784.2 $\pm$ 97.6	794.6 $\pm$ 104.7	2798.4 $\pm$ 177.6
	Control	2419.7 $\pm$ 178.0	6496.3 $\pm$ 539.5	1110.9 $\pm$ 85.2	4985.5 $\pm$ 303.8	219.7 $\pm$ 22.7	636.2 $\pm$ 92.4	801.5 $\pm$ 59.1	2907.4 $\pm$ 306.3
	AC	2526.6 $\pm$ 222.0	7498.6 $\pm$ 576.4	1115.1 $\pm$ 87.7	5696.8 $\pm$ 292.0	240.7 $\pm$ 27.8	775.1 $\pm$ 79.4	774.3 $\pm$ 96.1	2889.2 $\pm$ 294.8
6OH-BDE-47 (pg/mL)	1000	2282.2 $\pm$ 233.7	5019.3 $\pm$ 297.3	1100.9 $\pm$ 183.3	7154.0 $\pm$ 979.2	284.8 $\pm$ 35.9	844.3 $\pm$ 89.8	491.9 $\pm$ 42.3	2280.9 $\pm$ 132.2
	500	2221.1 $\pm$ 202.7	5101.8 $\pm$ 503.9	1190.0 $\pm$ 145.4	5515.7 $\pm$ 519.0	237.0 $\pm$ 16.0	901.4 $\pm$ 82.8	560.3 $\pm$ 34.4	2082.1 $\pm$ 163.0
	100	2217.9 $\pm$ 243.7	5635.3 $\pm$ 454.7	1175.9 $\pm$ 161.1	4807.5 $\pm$ 539.2	226.3 $\pm$ 39.6	870.8 $\pm$ 121.9	565.4 $\pm$ 37.4	2519.9 $\pm$ 188.5
	50	2091.5 $\pm$ 212.4	4755.2 $\pm$ 287.5	1047.0 $\pm$ 141.4	4066.5 $\pm$ 521.2	215.1 $\pm$ 46.7	626.7 $\pm$ 96.3	495.3 $\pm$ 23.0	2020.3 $\pm$ 137.2
	10	2382.7 $\pm$ 257.0	6099.8 $\pm$ 328.2	1228.0 $\pm$ 151.2	4771.7 $\pm$ 481.0	231.8 $\pm$ 27.6	732.1 $\pm$ 83.0	675.4 $\pm$ 50.7	2383.0 $\pm$ 159.7
	Control	2722.0 $\pm$ 261.3	7028.4 $\pm$ 522.3	1110.9 $\pm$ 85.2	4625.7 $\pm$ 356.0	219.7 $\pm$ 22.7	713.2 $\pm$ 115.8	801.5 $\pm$ 59.1	2907.0 $\pm$ 306.3
	AC	2798.5 $\pm$ 273.2	7498.6 $\pm$ 576.4	1115.1 $\pm$ 87.7	8259.2 $\pm$ 233.9	240.7 $\pm$ 24.8	775.1 $\pm$ 79.4	705.0 $\pm$ 55.5	2889.2 $\pm$ 294.8
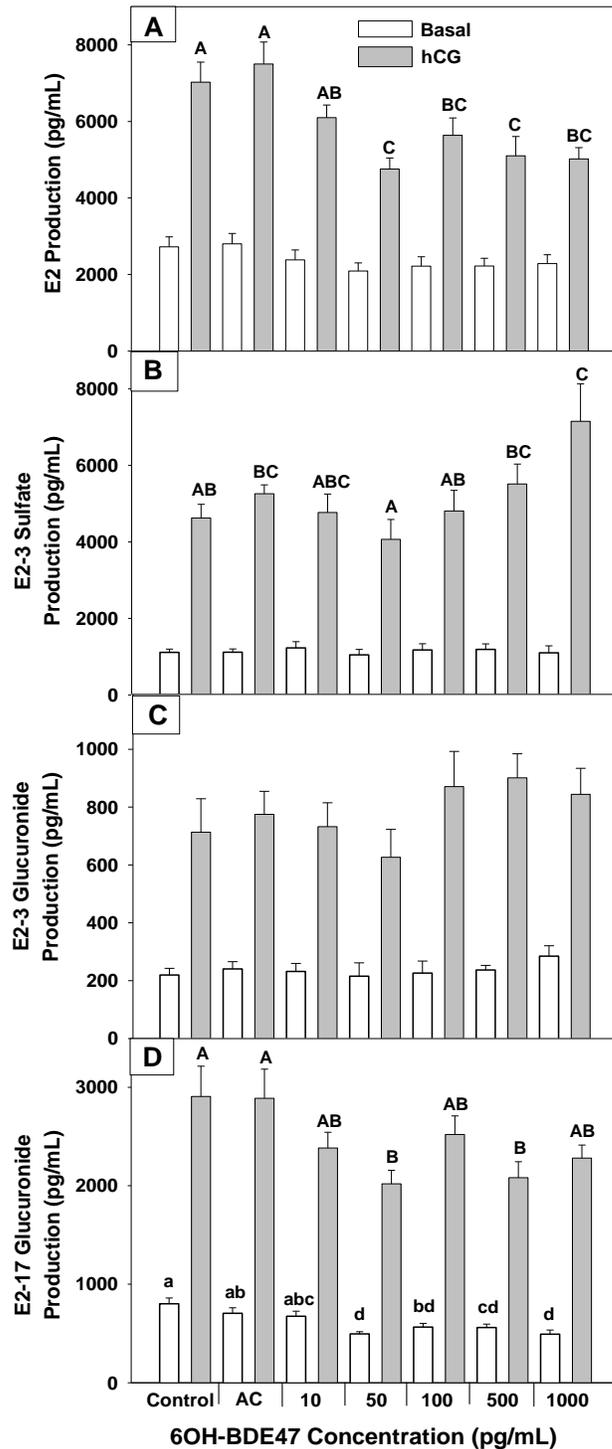
Figure 3.4 *In vitro* mean ± SEM estradiol and conjugate production by Rainbow trout oocytes exposed to increasing concentrations of 6OH-BDE-47 under basal (open bars) and hCG stimulated conditions (shaded bars). Letters denote treatment groups that are significantly different from each other determined by ANOVA (p < 0.05).

iii.   Total Estrogens and Proportions of Conjugates Produced

Because individual conjugates have not been quantified in this type of *in vitro* assay before, the sum of glucuronides, sulfates and parent estrogen hormones produced were compared among BDE-47 and 6OH-BDE-47 exposure concentrations. The three groups were also combined to determine total estrogens. There were no significant differences ($p > 0.05$) in total glucuronides, total sulfates, or total parent hormones produced by the rainbow trout oocytes under basal or hCG stimulated conditions at any BDE-47 or 6OH concentration (Table 3.8). When the three groups were summed as total estrogens, the three highest BDE-47 concentrations >1000 pg/mL produced lower total estrogens than the acetone carrier group and the 100 pg/mL groups ($p < 0.05$). There were no differences among the control group and the higher concentrations, and the 100 pg/mL group had elevated total estrogen production compared to the control ($p < 0.05$) (Table 3.8).

Regardless of the total concentrations of hormones produced, the proportions of glucuronide and sulfate conjugates, and parent estrogens were always constant ($p > 0.05$) across BDE-47 and 6OH treatments within basal or hCG conditions (Figure 3.5A-B).   Under basal conditions the mean sulfate production ranged from 24.7-35.0 %, and parent estrogens between 43.8-54.4 %. However, these proportions changed under hCG stimulated conditions. The mean percent sulfate production increased to 33.4-40.7% and the parent estrogens decreased to 36.3-43.4 % of total estrogens produced. This shift in parent and sulfate conjugate production was significant between basal and hCG conditions for both the BDE-47 (Figure 3.5A) and 6OH- exposures (Figure 3.5B).

Glucuronide production was consistent across treatments, and between basal and stimulated conditions. The mean glucuronide production for all groups ranged between 18.8 and 26.6 % of total estrogens. Although the proportion of glucuronides produced was only 3% higher under

Table 3.8: The contribution of glucuronides, sulfates and parent compounds to total estrogen production by rainbow trout oocytes incubated with increasing concentrations of BDE-47 and 6OH-BDE-47 under basal and hCG stimulated conditions. Hormone values are expressed as mean ± SEM.

[BDE] For Incubations		Total Glucuronides (pg/mL)		Total Sulfates (pg/mL)		Total Parents (pg/mL)		Total Estrogens (pg/mL)	
		Basal	hCG	Basal	hCG	Basal	hCG	Basal	hCG
BDE-47 (pg/mL)	5000	1185.5 ± 185.8	3298.2 ± 213.5	964.5 ± 91.7	4204.6 ± 282.8	2936.0 ± 167.1	7827.8 ± 801.5	1657.0 ± 141.6	14804.1 ± 644.5
	3000	1329.8 ± 245.8	2977.3 ± 160.6	1027.4 ± 84.7	4425.9 ± 417.6	2808.4 ± 268.0	8067.8 ± 1021.1	5190.7 ± 560.9	14389.2 ± 1050.4
	1000	1099.5 ± 107.7	3407.1 ± 226.6	1018.1 ± 112.5	4228.3 ± 492.0	2903.7 ± 298.2	7031.5 ± 651.2	5029.7 ± 510.8	14307.3 ± 944.1
	500	1454.9 ± 184.7	3808.6 ± 269.0	1088.3 ± 128.7	5233.9 ± 406.6	3234.1 ± 225.8	8452.3 ± 801.9	5950.1 ± 486.9	16355.7 ± 789.1
	100	1136.1 ± 159.9	3582.7 ± 224.9	969.6 ± 74.1	5516.1 ± 590.3	2464.9 ± 159.9	8554.1 ± 731.0	4592.3 ± 408.6	18066.9 ± 676.0
	Control	1184.7 ± 116.2	3620.5 ± 356.1	1110.9 ± 85.2	4985.5 ± 303.8	2419.7 ± 178.0	6496.3 ± 539.5	5279.0 ± 452.0	14922.7 ± 884.4
	AC	1180.4 ± 154.5	3664.3 ± 246.6	1115.1 ± 87.7	5696.8 ± 292.0	2526.6 ± 222.0	7498.6 ± 576.4	5308.1 ± 459.0	17246.0 ± 861.7
6OH-BDE-47 (pg/mL)	1000	855.6 ± 111.8	3125.1 ± 186.7	1137.9 ± 192.0	6574.5 ± 990.8	2282.2 ± 233.7	6043.6 ± 659.7	5056.2 ± 657.4	14591.0 ± 1105.9
	500	822.0 ± 42.6	3030.4 ± 200.9	1221.1 ± 148.2	4946.8 ± 573.1	2221.1 ± 202.7	5607.7 ± 588.2	5337.2 ± 715.0	13179.9 ± 897.8
	100	757.5 ± 43.6	3390.8 ± 254.6	1204.3 ± 162.3	5363.9 ± 660.8	2217.9 ± 243.7	5872.1 ± 485.5	4632.8 ± 655.2	14626.8 ± 941.0
	50	648.6 ± 31.8	2647.0 ± 214.7	1078.5 ± 142.3	5042.0 ± 740.4	2091.5 ± 212.4	4784.0 ± 323.4	4366.3 ± 549.7	13187.3 ± 1168.8
	10	955.1 ± 89.7	3115.1 ± 211.0	1275.5 ± 173.9	5866.7 ± 774.8	2382.7 ± 257.0	6068.7 ± 395.8	5339.5 ± 627.0	15197.6 ± 959.9
	Control	993.6 ± 56.9	3620.5 ± 356.1	1164.5 ± 100.0	4575.7 ± 389.3	2722.0 ± 261.3	7016.9 ± 569.4	5619.6 ± 515.5	14922.7 ± 884.4
	AC	917.6 ± 60.8	3664.3 ± 246.6	1137.8 ± 91.0	5875.5 ± 297.1	2798.5 ± 273.2	7781.5 ± 611.4	5706.8 ± 569.0	17246.0 ± 797.8
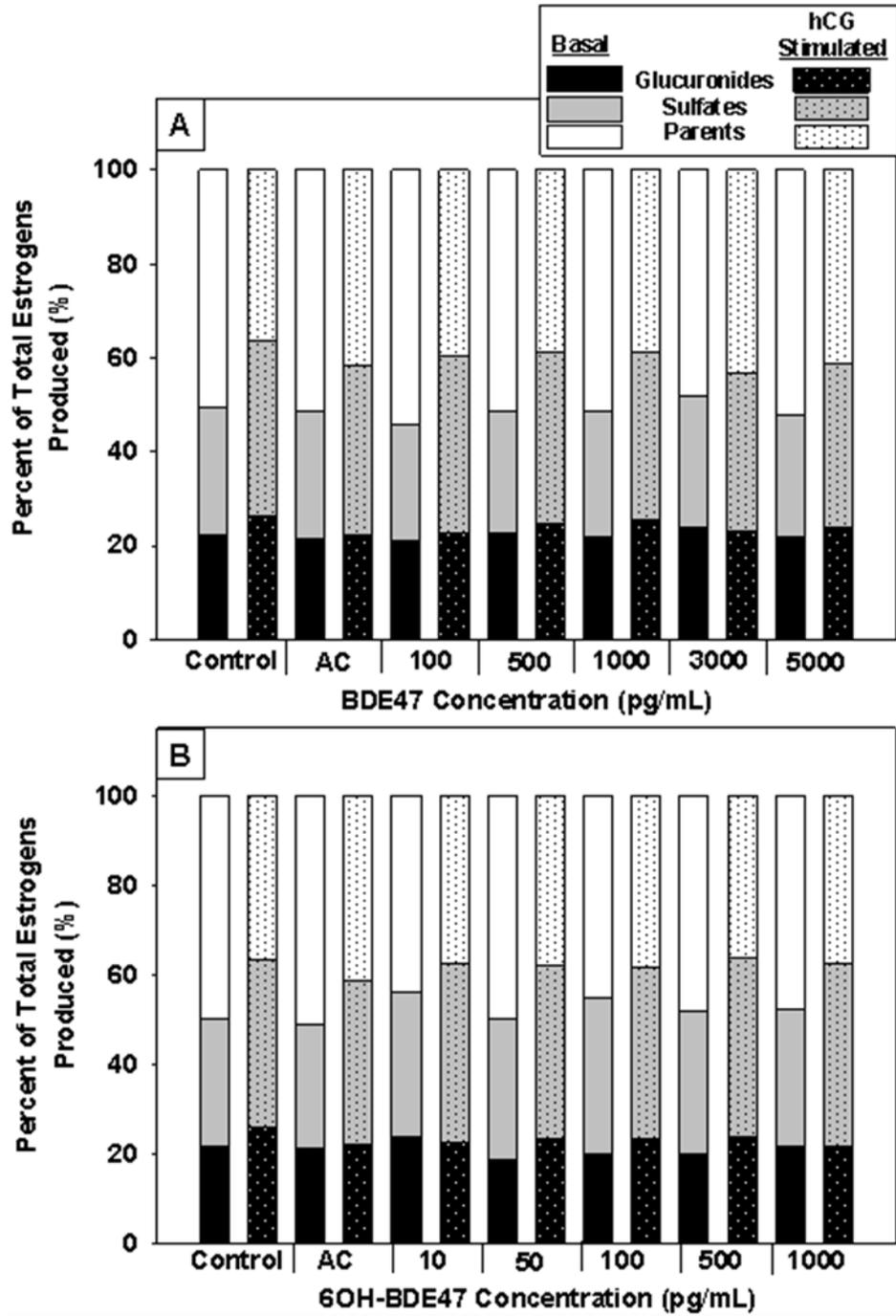
Figure 3.5: Changes in proportions of parent estrogens and sulfate conjugates produced by Rainbow trout oocytes under in vitro basal and hCG stimulated conditions. There were no differences (p > 0.05) in total glucuronide proportions produced, and increasing BDE-47 and 6OH-BDE-47 concentrations also had no effect determined by ANOVA (n=6).

stimulated conditions over basal, this difference was significant (p<0.05) (Figures 3.5A-B). This is the opposite of results observed in the male *in vitro* incubations where proportion of TG produced decreased under hCG stimulated conditions.

There were no significant effects of BDE-47 or 6OH on E2 glucuronides or total glucuronide production, but there appeared to be trends in the proportions of each glucuronide metabolite produced as a percentage of total glucuronides. The proportion of E2-3G produced by rainbow trout oocytes increased significantly as the exposure concentrations of 6OH-BDE47 increased in both basal (p < 0.001, Figure 3.6A) and hCG stimulated (p = 0.01, Figure 3.6B) conditions. Basal and hCG stimulated E2-3G production, as a percent of total glucuronides, were higher than the controls at 6OH- concentrations greater than 50 and 100 pg/mL, respectively.   The opposite was observed for the E2-17G metabolite, where production decreased (basal p = 0.001; hCG p = 0.003) at higher 6OH-BDE-47 concentrations. 6OH-BDE-47 concentrations greater than 100 pg/mL in both the basal and hCG incubated oocyte groups showed significantly reduced E2-17G production. It appears that there is a shift in the preferred site of E2 glucuronidation from position 17 to 3 under both basal and stimulated conditions. At its lowest, E2-17G production continued to account for approximately 75% of total E2-glucuronides produced (Figure 3.6).

BDE-47 did not have the same effect on the proportions of estradiol 3- and 17-glucuronides produced by oocytes. Basal production was different at low exposure, but not at higher concentrations (data not shown). There was no clear trend similar to the one observed with 6OH- exposures. The hCG stimulated group showed no significant changes in the proportions of the two glucuronide conjugates measured (data not shown). A results summary for the female rainbow trout in vitro oocyte incubations can be found in Table 3.9.
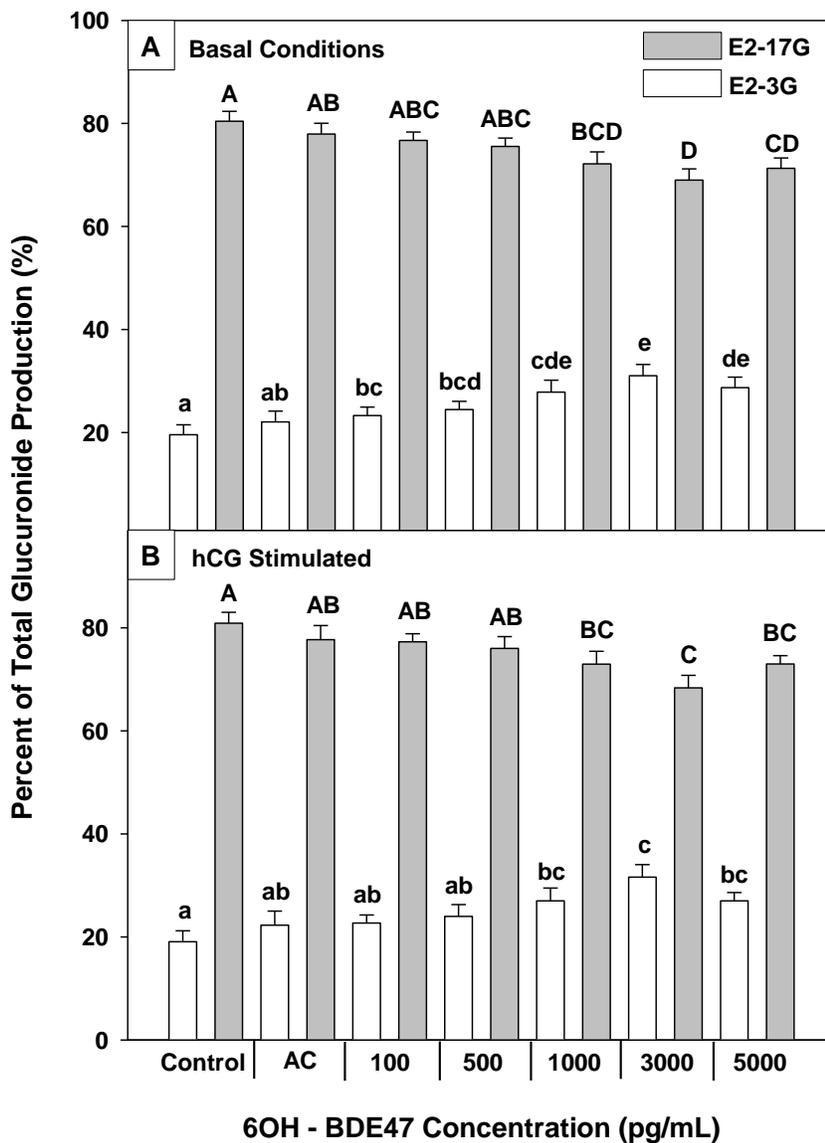
Figure 3.6: Changes in the favoured site of estradiol glucuronidation by Rainbow trout oocytes *in vitro* with increasing concentrations of 6OH-BDE-47 under basal (open bars) and hCG stimulated (shaded bars) conditions. Mean percent of total with SEM for E2 glucuronides are shown. Letters denote treatment groups that are significantly different from each other determined by ANOVA ($p < 0.05$).

Table 3.9: Results summary of rainbow trout oocyte in vitro incubation with BDE-47 and its 6OH hydroxylated metabolite. Arrows denote increases or decreases in mean hormone production compared to the controls. NSD is no significant difference from the controls (p > 0.05).

BDE / Metabolite	Estradiol (E2)	Estrone (E1)	E2-3 Sulfate	E1-3 Sulfate	E2-3 Glucuronide	E2-17 Glucuronide	Total Estrogens
BDE-47	NSD	NSD	↓	NSD	NSD	NSD	↓
6OH-BDE-47	↓	NSD	↑	NSD	NSD	↓	NSD

Discussion

Gonadal steroidogenesis to maintain optimal sex steroid concentration in the plasma is essential for fish reproductive success. Exposure to endocrine disrupting chemicals that interfere with the synthesis or metabolism of active sex steroids can result in altered circulating levels of sex steroids, which can then negatively impact reproduction in wild fish. Reduced gonad size and fecundity, inhibited expression of secondary sex characteristics and delayed maturity and feminization and masculinization have all been correlated with altered circulating levels of plasma reproductive steroids in fish (Kime 1998; Munkittrick et al. 2002; McMaster et al. 2006). However, there are inherent difficulties in assessing reproductive potential in wild fish by monitoring plasma sex steroids. Ascribing cause and effect for reproductive disturbance to specific environmental contaminants is also difficult (Diamanti-Kandarakis et al. 2009).

Laboratory studies can carefully control contaminant exposures and are useful for determining cause and effect relationships as well as the biochemical mechanism of actions for environmental contaminants. The studies described in this chapter used an *in vitro* incubation system to examine the mechanisms by which selected PBDE's and hydroxylated metabolites affect the production of sex steroids by fish gonadal tissues.

Conjugate Production

Maintaining optimal active reproductive hormone concentrations is a balance between steroid synthesis and catabolism. The two primary steroid excretion forms are sulfate and glucuronide conjugates. This is the first study to examine steroidogenesis in fish gonadal tissues including these two types of conjugates under both basal and hCG stimulated conditions. Conjugates were determined to make up over 80% of total testosterone produced by male brown trout testes, and 40-50% of estrogens produced by rainbow trout oocytes. Stimulation of the gonadal tissues by hCG significantly changed the ratios of reproductive steroid parent molecules and the conjugates that were produced. In females, the proportion of glucuronide and sulfate conjugates increased, while the parent hormone production decreased when the oocytes were stimulated with hCG. The opposite occurred in the testes, where the proportion of glucuronide conjugate significantly decreased with hCG stimulation. James (2011) postulated that concentrations of active hormones are partly regulated by changes in enzyme conjugation activity. The proportion of sulfate and glucuronide conjugation of E2 by rainbow trout oocytes could be enhanced due to the increased availability of the parent hormone substrate. Exposure to the polycyclic aromatic hydrocarbon benzo(a)pyrene increased the expression of glucuronide conjugating enzyme UGT1B in flounder (*Platichthys flesus*) 4 to 10 fold (Leaver et al. 2007) and induction of both UGT and SULT enzymes was reported in channel catfish (*Ictalurus punctatus*) exposed to 3-methylcholanthrene (3-MC) (Gaworecki et al. 2004). Although these studies did not determine conjugation of endogenous steroids, they do support the notion that once a threshold concentration of parent hormone is reached, some UGT or SULT isoform expression may be induced.

The proportional decrease of T glucuronidation observed in the brown trout testes exposures may indicate that UGT enzymes reached substrate saturation for that tissue. Species differences in UGT and SULT (Gaworecki et al. 2004) expression, as well as tissue differences (Daidoji et al. 2006, Sneitz et al. 2011) could also account for the observed dissimilarities between brown trout testes and rainbow trout oocytes in parent and conjugate production in response to hCG stimulation.

The presence of isoforms and the selectivity of UGTs in fish tissues is an active area of research (James 2011). This study has shown that the carbon at position 17 of E2 is the preferred site of glucuronidation over position 3 in rainbow trout oocytes. Human intestine and liver tissues contain different UGT isoforms which do not have the same affinities for 3OH and 17OH estrogens (Sneitz et al. 2011). The kinetics for the formation of these two conjugates is also different, with E2-3G forming more quickly than E2-17G in pooled human liver and intestine microsomes (Itaaho et al. 2008).

The position of E2 sulfonation can determine the function/fate of the conjugate in mammals. The conjugated 17β-E2-3S can be readily hydrolyzed back to the active parent hormone 17β-E2, while E2-17S is not, and therefore, is rapidly eliminated (Chetrite et al. 2000). The predominant pathway of 3OH or 17OH E2 conjugation depends on the isoform of SULT or UGT present, seasonal production of the hormone, and the tissue (James 2011). It is unknown which UGT and SULT isoforms are present in rainbow trout oocytes, but Huang and Wu (2010) have identified 45 UGT genes in zebrafish (*Danio rerio*) that can be divided into three families; UGT1, UGT2 and UGT5. Fifteen zebrafish SULT isoforms have also been identified and categorized into 4 different families; eight SULT1, three SULT2, three SULT3 and one SULT6

sulfotransferases (Kurogi et al. 2011). Ongoing work in this area is certain to provide additional information regarding the production of conjugated steroid hormones in other fish species.

Effects on Male Brown Trout Steroid Production and Metabolism

BDE-209 produced mildly androgenic effects on male brown trout steroidogenesis from this study. Both T and 11-KT production were elevated with increasing concentrations of BDE-209. Differences in the parent hormone (T) were not significant; however, the production of the TG metabolite was enhanced. The increased TG is likely due to higher levels of T substrate available for conjugation as the balance of total testosterone (T + TG) was not significantly higher than the controls. Several other studies have examined the effect of BFRs on androgen production in vertebrates. Earnest et al. (2012) found that feeding male rats a diet containing a BFR mixture composed of 44% BDE-209, did not affect their plasma T levels. BDE-47 also had no effect on plasma T and E2 concentrations in exposed juvenile turbot (Jenssen et al. 2004). An *in vitro* study using male tissues found that BDE-71 acted directly on the Leydig cells of rats to increase T production through the activation of the cAMP pathway to increase the expression of steroidognic acute regulatory (StAR) protein (Wang et al. 2011). Gregoraszczuk et al. (2008) suggested that increased T production with BDE-209 and BDE-47 exposure in porcine ovarian follicular cells was due to activation of CYP17 or 17β-hydroxy steroid dehydrogenase, enzymes responsible for the production of the T precursor, androstendione, or T itself, respectively.

BDE-47 can exhibit androgenic and anti-androgenic properties, at more than one point in the steroidogenic pathway. Zhao et al. (2011) reported stimulation in T production, together with an increase in StAR and cytochrome P450 side-chain cleavage (P450scc) activity, in rat

testes incubated with BDE-47. StAR facilitates the first step in steroidogenesis by moving cholesterol across the mitochondrial membrane where it is converted to pregnenolone by P450scc (Arukwe 2008). Johanning et al. (2007) also found no hepatic UGT enzyme activity in the offspring of male rainbow trout orally dosed with BDE-47, however they did observe a significant decrease in SULT activity in the F1 generation. In a follow-up to Gregoraszczuk's (2008) study, Karpeta et al. (2011) found that BDE-47 had no effect on CYP17 activity, which is important toward the end of the androgen synthesis pathway. The increase in T production was due to a stimulatory effect on 17β- hydroxysteroid dehydrogenase (17β-HSD), the enzyme involved in synthesizing T from androstenedione. Because BDE47 had no effect on androgen production at concentrations up to 5000 pg/mL, it may be that this compound does not exert an effect on fish testicular tissue at these concentrations. Exposure concentrations in Karpeta's study ranged from 5000 pg/mL to 50 ng/mL.

Incubation of brown trout testicular tissue with 6OH-BDE-47 resulted in slightly elevated 11-KT and T, and a significant increase in TG production. Increased glucuronidation of T by the testes indicates an activation of UDP-glucuronosyltransferase (UDPGT or UGT) Phase II enzymes. This may be partly due to increased availability of T substrate; however, exposure to OH-PBDEs can stimulate the activity of E2-UGTs (Lai et al. 2012). Any significant increases in parent T production were likely masked by its rapid conjugation. The marked surge in TG production also suggests that 6OH-BDE-47 does not compete with testosterone as a substrate for UGT. PBDEs can exert endocrine disrupting properties through competition with parent hormones as substrate for conjugation and through allosteric inhibition of the SULT enzyme family. More active parent hormone is left available to bind to receptors

resulting in enhanced estrogenic or androgenic activity (Kester et al. 2000, Kester et al. 2002, van Lipzig et al. 2005, Jurgella et al. 2006, Lai & Cai 2012).

Previous 6OH exposure studies, all using mammalian cells, have reported mixed results. Karpeta et al. (2013) reported no significant change in T production by porcine thecal cells incubated with 6OH, similar to He et al. (2008) with the H295R cell line. Anti-androgenic effects were reported by Canton et al. (2006, 2007) and Hamers et al. (2006) using the human androgen receptor (hAR) and AR Calux assays, respectively. An et al. (2011) found 6OH to be cytotoxic in the human hepatoma cell line HepG2, which could account for a decrease in T production.

This study determined that 3OH and 5OH-BDE-47 were anti-androgenic, the opposite effect to the 6OH metabolite. Incubation with 3OH also produced a stronger response than the 5OH. There are few studies available to compare the effects of these two metabolites. Similar to results for 6OH, there were no effects on T production observed in thecal cells exposed to 5OH-BDE-47 (Karpeta et al. 2012), and antagonist behavior in the yeast cell assay was observed for both 3OH and 5OH (Canton et al. 2007). Changes in glucuronidation activity can be due to less available active hormone for conjugation, and fluctuations in rates of catabolism in other pathways, including sulfonation (James 2011). However, UGT may be an important effector because the decline in TG production is greater than for T. Since we were not able to consistently measure the production of testosterone sulfate, activation of this pathway should not be ruled out.

PBDEs with the hydroxyl group in the ortho position are most reactive to UGT (Lai et al. 2012). More specifically, hydroxyl groups at carbon position 3 and 5 beside a bromine atom may open the PBDE structure, allowing it to be more readily conjugated (Lai & Cai

2012). Figure 3.7 shows that the 3OH- and 5OH-BDE-47 used in this study both have the reactive configuration. These compounds could be competing with testosterone as a UGT substrate.
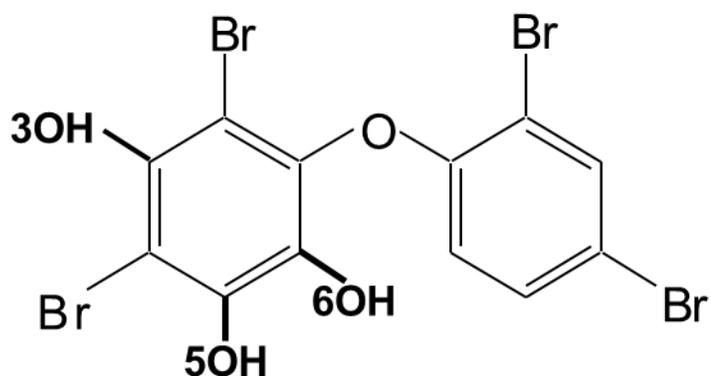
Figure 3.7: The structure of BDE-47 showing the 3 sites of hydroxylation and bromine (Br) position in the metabolites investigated.

Finally, cytotoxicity could also contribute to the observed decreases in androgen production by the brown trout testes, as both He et al. (2008) and Hamers et al. (2006) reported cell death in their assays with 3OH and 5OH exposure. Since T production was higher at 1000 pg/mL than 10 pg/mL in our 5OH exposure, cytotoxicity appears to be less of a factor for this compound.

Effects on Female Rainbow Trout Steroid Production and Metabolism

This study was the first to examine the balance of estrogen synthesis and metabolism, preferred conjugation routes, and effects of BDE-47 and its 6OH metabolite in rainbow trout ovarian follicles. Carbon position 17 was the preferred site of E2 glucuronidation, over the 3

position, in rainbow trout oocytes. Although 17G remains dominant, as the 6OH incubation concentration increases, there is a significant shift in this preference. The proportion of E2-3G formed increases and E2-17G decreases when expressed as proportions of total glucuronides produced. In rat liver microsomes exposure to 3OH-BDE100 also increased the production of the E2-3G, but had no effect on 17G (Lai et al. 2012). The predominant conjugation position depends on the UGT present (James 2011), due to their different affinities for 3OH or 17OH (Sneitz et al. 2011), suggesting that there is more than one form present in rainbow trout oocytes. Increasing exposure to BDE47 did not have a clear effect on the UGT conjugation site, however OH-BDEs are thought to be more potent in affecting steroid conjugation than the parent BDE compounds (Lai & Cai 2012).

Although 6OH-BDE-47 had no effect on total estrogens produced by rainbow trout oocytes, there was significantly less parent E2 and E2-17G present in the incubation medium. In the same samples, E2-3G production was elevated, and E2-3S was significantly induced, indicating induction of SULT and potentially one isoform of UGT by the hydroxylated BDE.

Altered conjugation activity has been examined in several other animal models using different environmental contaminants. The polyaromatic hydrocarbon (PAH), 3-methylcholanthrene, induces SULT and UGT activity in channel catfish (Gaworecki et al. 2004) and SULF and UGT were induced in the livers of mice fed octachlorostyrene, a synthesis by-product of chlorinated compounds (Yanagiba et al. 2009). Natural compounds that have been shown to induce SULT in mammalian tissues include caffeine (Zhou et al. 2012), and genistein, a potent phytoestrogen found in soy and soybean products (Chen et al. 2013). In contrast, an earlier study with zebrafish cystosolic sulfotransferases showed that genistein could inhibit E2

conjugation through direct competition (Ohkimoto et al. 2004). These contrary results highlight the diversity in species and tissue differences in SULT and UGT isoform activities.

The hydroxylated metabolites of polyhalogenated aromatic hydrocarbons (OH-PHAHs), including polychlorinated dibenzo-*p*-dioxins and dibenzofurans, and polybrominated diphenylethers are known to be estrogenic, although they have varying affinities for the estrogen receptor (ER) (Kester et al. 2000, Kester et al. 2002, van Lipzig et al. 2005). Hydroxylated halogen compounds are generally found to inhibit UGT and particularly SULT conjugation enzymes. Many exert their estrogenic action by competing with E2 as substrate for SULT conjugation, while others act on the enzymes allosterically (Kester et al. 2000, Kester et al. 2002, van Lipzig et al. 2005, Jurgella et al. 2006, Lai and Cai 2012). Either mechanism could increase concentrations of active E2, thereby resulting in estrogenic effects. The position of the OH and halogen groups are consistently the most important indicators of SULT and UGT inhibition. Compounds in which the OH and halogen groups are together, particularly co-planar molecules where there is an OH group at a para position with an ortho halogen substitution, had the greatest inhibitory effect on E2 metabolism (Kester et al. 2000, van Lipzig et al. 2005, Jurgella et al. 2006). The halogen may create a more open configuration, allowing the OH to be in a more reactive position (Lai & Cai 2012), or the halogen may increase dissociation of the OH, priming the xenobiotic for conjugation (Kester et al. 2000). Although these studies examined effects on E2 metabolism, the effects we observed for T conjugation in male brown trout testes may also be due to the hydroxylation position of the BDE47 metabolite. Both 3OH and 5OH-BDE-47 which inhibited TG production are positioned beside a bromine atom, whereas the 6OH that exhibited marginal stimulatory activity is not.

The stimulatory effect of 6OH-BDE-47 on E2-3S and E2-3G production we observed in rainbow trout oocytes, was similar to results from lake trout liver, but only at low concentrations of 4,4'-OH-TCB (Jurgella et al. 2006). In the case of 4,4'-OH-TCB, low doses may allosterically potentiate SULT and UGT, therefore increasing E2 metabolism, while higher concentrations could competitively inhibit the same enzyme (Jurgella et al. 2006). The simultaneous decrease in E2-17 glucuronidation, again suggests that more than one isoform of UGT exists in rainbow trout oocytes, or that SULT activity may be regulating UGT activity. In a recent study by Barrett et al. (2013), UGT2B4 expression was up-regulated in a human liver cell line when sulfotransferase activity was blocked. Further research is required to determine if SULT regulates UGT in fish, or if the presence of particular substrates alone induce or inhibit their activity.

Traditionally, reproductive assessments examined circulating steroid hormone profiles by determining the parent hormone alone. Similarly, in vitro steroidogenic assays have also determined the parent hormone exclusively. However, the studies described in this chapter have shown that measuring only the parent hormone does not provide a complete profile of the potential for PBDEs and their hydroxylated metabolites to affect fish reproductive steroid concentrations. If total estrogens or E2 alone had been analyzed, significant effects would have been masked. Future examinations of reproductive steroids in fish testes and oocytes, should consider parent hormone and metabolites, especially in light of the evidence presented here that xenobiotics may differentially affect the production of steroids and their predominant conjugate forms.

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Chapter 4: Extraction and clean-up of steroid hormones from fish feces and comparison with plasma concentrations

Abstract

Feces are a useful media for analysis of sex steorids and related metabolites and have been used in zoo and wild animal monitoring programs for some time, but to date, the techniques have largely not been applied to fish. An extraction and sample clean-up method for the simultaneous LC/MS/MS analysis of sex steroids and their conjugates in fish feces was developed. The pre-treatment of feces with acid (HCl to pH < 2) improved glucuronide extraction and lipid breakdown, but it also facilitated extraction of compounds that severely interfered with parent hormone analyses. Several techniques commonly used for tissue and lipid clean-up were tested to remove co-extracted matrix interferences without compromising the detection of either parent or conjugated steroid hormones. Various solid phase extraction (SPE) and gel permeation chromatography (GPC) methods were tested; however, none of these methods alleviated the matrix interferences while preserving the glucuronide conjugates. A 2-Step extraction method, with a series of acid washes, reduced the unknown interfering matrix peak to allow adequate detection (pg/mg dry feces) of the parent estrogens. To remove any remaining lipids and pigmented compounds a lipid removal agent, Cleanascite™ was effective for clearing the samples and prolonging the life of the column without affecting the recovery of the parent and conjugated estrogens. To demonstrate that parent and conjugated steroids can be used to estimate plasma hormone concentrations and reflect the reproductive status of fish, paired plasma and feces samples were collected weekly from 8 female rainbow trout for ten

weeks prior to spawning. Fecal samples were extracted and prepared for LC/MS/MS analysis of parent and conjugated reproductive steroids using the newly developed method. Changes in plasma hormone concentrations were typically not reflected in feces until the subsequent week, and estrogen concentrations were also more stable in feces than in plasma. Often a 10-fold change in plasma corresponded to only a 2-4 fold change in the feces; likely because steroid measurements in feces represent an integrated pool of free hormones metabolized from the plasma over a period of time. Regression analyses determined that E_2-17G measured in feces was the best predictor of both E_2 ($r^2 = 0.54$, $p < 0.01$) and E_2-3S ($r^2 = 0.64$, $p < 0.01$) in rainbow trout plasma. These results show that E_2 metabolites in feces can be used to estimate circulating E_2 concentrations through a reproductive cycle in rainbow trout.

Introduction

Concentrations of steroids in feces have been used to determine health and reproductive stages of mammals in zoos for their breeding programs (Asa 2001; Busso et al. 2005, 2007; Da Silva & Larson 2005; Dumonceaux et al. 2006; Graham et al. 2006; Hesterman et al. 2005; Shideler et al. 1993), and for reproductive assessments in wild animals (Beehner & Whitten 2004; Hunt et al. 2006; Mauget et al. 2007; Muehlenbein 2006; Strier & Ziegler 1997; Washburn et al. 2004). While some measurements of sex steroids have been made in fish urine, very little work has examined the utility of measuring them in fish feces. Steroids in feces are generally conjugated with larger water soluble molecules (eg. Glucuronic acid, sulphates) to make them more water soluble, and easily excreted in the bile. Conjugation occurs primarily in the liver of both mammals and fish (Lemke & Williams 2008; Pankhurst 2008; Kirk et al. 2003; Zhu & Conney 1998; Strott 1996), but extra-hepatic tissues including the gonads and gills of fish (Pankhurst 2008; Leguen et al. 2000) can also contribute to the metabolic pathway.

There is no universal method for preparing and analyzing steroids in feces (Couchman et al 2011), In fact, there are over a dozen different methods published for fecal reproductive steroid extraction from large game and primate feces (Schwarzenberger et al . 2013; Ziegler & Wittwer 2005). They vary in the extraction solvents and clean-up procedures employed, as well as the method used to quantify the targeted hormones. RIA and ELISA are the most common methods for sex hormone analyses because they are rapid and inexpensive. However, there are no kits or antibodies for determining conjugated sex steroids (Weltring et al. 2012). Therefore, the conjugates must first be hydrolyzed to derive the parent hormone, which is then analyzed using conventional tests. The two most common hydrolysis techniques are enzyme deconjugation and solvolysis using a strong acid (eg. sulfuric acid) (Weltring et al. 2012; Ziegler

& Wittwer 2005). These methods are labour intensive, and parent hormones must be analyzed after each separate hydrolysis procedure. Finally, previously developed protocols have been developed for relatively large samples (eg. several hundred milligrams to grams of dried feces). However for small bodied fish species (eg. Golden shiner), typically less than 30 mg (wet weight) of feces can be expressed, with a moisture content of 75% (Unpubl. observations).

Steroid analysis based on liquid chromatography-mass spectrometry detection have far lower detection limits than RIA and EIA (Ziegler & Wittwer 2005) and multiple hormones can be measured simultaneously, including conjugated metabolites (Couchman et al. 2011). A growing number of studies have employed LC/MS/MS analysis for fecal and urine hormones in mammals (Habumuremyi et al. 2014; Weltring et al. 2012; Walker et al. 2002; Young et al. 2001), and plasma in fish (Noyes et al. 2014), birds and mammals (Koren 2013). However, the technique has not been applied to the analysis of sex hormones in fish feces.

The development and optimization of a method to extract free estrogens and their conjugated metabolites from fish feces, with clean-up and preparation for LC/MS/MS analysis is described in this chapter. Several studies were performed to optimize the extraction procedures. Included among these were investigations using enzyme treatment to determine predominant conjugates in fish feces, different extraction solvents, matrix suppression assessments, solid phase and gas permeation chromatography and a clearing/lipid removal agent. Extracts were analyzed for free and conjugated estrogens. This new extraction and quantification technique was applied to fecal estrogen measurements with a goal to predict circulating hormone levels. Application of the techniques described for large bodied fish in this chapter could allow nonlethal and non-invasive measures of reproductive hormone profiles in fish. This strategy could be applied in future monitoring programs, perhaps even in small bodied fish.

Materials & Methods

Hormones, Solvents and Chemicals

All solvents (ethyl ether, methanol, ethanol, acetonitrile, acetone, chloroform, ethyl acetate, and hexane), Optima water and Scintiverse scintillation cocktail were purchased from Fisher Scientific (ON, Canada). Sephadex LH-20, heparin, B-glucuronidase (from E. coli) Type IX-A, sulfatase Type VIII and the ingredients for both the citrate and phosphate buffers (see appendix for buffer recipes) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The anaesthetic, tricaine methanesulfonate (MS-222), was purchased from Syndel (Nanaimo, BC, Canada). Supplies for obtaining whole blood samples and for preparing plasma (1.5 inch 22 gauge needles, 16 gauge needles, 1 mL and 3mL syringes, Vacutainers) were purchased from (BD Diagnostics, NJ, USA). Estradiol EIA coated plate assay kits were purchased from Cayman Chemical Company (MI, USA).

The sex steroid hormones targeted for extraction were the parent hormones 17β- estradiol (E_2) and estrone (E_1), as well as their conjugated forms 17β-estradiol 3-sulfate (E_2-3S), 17β-estradiol 3-glucuronide (E_2-3G), estrone 3-sulfate (E_1-3S), and estrone 3-glucuronide (E_1-3G). Mass-labeled compounds used as recovery internal standards (RIS) or internal precision standards (IPS) included 17β-estradiol 2,4,16,16-d_4 (d_4-E2), estrone 2,4,16,16-d_4 (d_4-E$_1$), 17β-estradiol 2,4,16,16-d_4 3-sulfate (d_4-E_2-3S), and d_3-17β-estradiol-3 glucuronide (d_3-E_2-3G). The d_4 and d_3 mass-labeled estrogen conjugates were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). H^3-Estradiol (H^3-E_2) and H^3-Estradiol-3 Glucuronide (H^3-E_2-3G) were purchased from Perkin Elmer (Woodbridge, ON, Canada). Unlabelled conjugated estrogens were acquired from Sigma-Aldrich Chemical (St. Louis, MO, USA). All other mass-labeled and

unlabelled parent hormones were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Stepwise Methodology

Developing a method to extract steroids from fish feces and refinement of the steps to clean-up and prepare the samples for LC/MS/MS analysis evolved through a series of experiments that are described below. While some of the methodology that was pursued eventually formed part of the final analysis, others that proved to be unsuitable are still described here to provide context for the final assay development protocol. Briefly, the series of experiments performed to develop and validate the final method included:

   i.   Collection of pooled fish feces

   ii.   Preliminary analysis of steroid conjugates in fish feces

  iii.   Establishing LC/MS/MS Parameters

   iv.   Extraction and Clean-up of samples for LC/MS/MS

          a. Liquid-liquid extraction

          b. Lipid analysis and the importance of acidifying samples

   v.   Solid phase extraction

   vi.   Gel Permeation Chromatography - GPC

  vii.   Liquid:Liquid Extraction with Lipid Removal Agent Cleanascite[TM]

          a. Effects of Cleanascite[TM] on glucuronide extraction

          b. Acid washing with LRA clean-up

          c. Determination of d3-E2-3G Suppression and Final Method

viii.   Method validation – Temporal comparison of hormone concentrations in plasma and

feces

i.   Collection of Pooled Fish Feces

Feces for the preliminary tests and first series of solvent extractions were obtained from female lake trout (*Salvelinus namaycush*) raised in the lab. Feces were collected from their intestinal track, pooled and frozen at -80ºC until they could be processed. The pooled feces was freeze-dried in Whirl Pak® bags, ground with a mortar and pestle and stored in sealed sample containers at -80ºC. Pooled feces contained 74% moisture based on comparisons of weight before and after freeze drying.

For the remaining sections of this study juvenile female rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Hatchery (Thamesford, ON Canada) and grown in our fish holding facility at the Freshwater Institute, Winnipeg, MB. The rainbow trout were held in an 800L tank in dechlorinated Winnipeg city tap water at 13ºC with a 12 hour light/12h dark light cycle. Fish were fed approximately 1.5% of their body weight six times per week. A second pool of feces used for method development was collected from 10 of the fish in this second group. To collect feces, fish were anaesthetized (80 mg/L, pH buffered tricaine methanesulfonate anesthetic (MS-222), placed on their side and the vent area was thoroughly dried with paper towel. Feces were expressed into a collection vessel using gentle abdominal pressure. The collection vessel was postioned with care to avoid collection of any urine which might be expressed from the urinary papillae. After collection of approximately 100g of pooled feces (wet weight), fish were allowed to recover in fresh aerated water in their original tanks. Samples were then frozen at -80ºC until freeze drying as described above.

Feces were collected as described above for the paired feces and plasma time series experiment for each individual fish. Whole blood samples were obtained from the same fish via the caudal artery using a heparinized 22G by 1.5" needle. Whole blood was centrifuged in heparinized Vacutainers for 7 minutes at 1000 rpm and the plasma was aspirated and stored at -80ºC until analysis.

ii.   *Preliminary analysis of steroid conjugates in fish feces*

To determine the predominant forms of estrogens excreted by fish in feces, five extraction methods commonly used in conjunction with deconjugation enzymes were tested. The effect of feces sample mass on extraction efficiency was also investigated.

Feces were collected and pooled from 10 female lake trout (*Salvelinus namaycush*) and prepared as described above. Samples weighing 5, 10, 20 and 40 mg, were weighed, in triplicate, into disposable glass test tubes. The 40 mg samples represented the low end of dried sample sizes used in the previously published studies, and the 5 mg represented the higher end of sample sizes that could be collected from small-bodied fishes (Unpublished Observ.). Five extraction methods were selected and modified from the literature (see Table 4.1). In all cases the initial step was to wet samples with one of the following solvents: 2 mL of MilliQ filtered (MQ) water, an aqueous buffer, or a polar solvent (see appendix for buffer descriptions). Each tube was vortexed to mix, and allowed to stand for 20 minutes before the solvent extraction step. The samples that were wetted with ethanol were then boiled in a test tube heating block for 20 min followed by vortexing for 5 min.

Five mL of a non-polar solvent, ethyl ether or 3:2 hexane:ethyl acetate, was added to extract the hormones from the wetted fecal samples and each tube was vortexed for 2 minutes.

In all cases, emulsions formed during vortexing with the non-polar solvent. Samples were centrifuged at 4000 rpm for 10 minutes to reduce the emulsion and separate the aqueous and solvent layers. The top solvent layer was aspirated to a clean test tube. The extraction and

Table 4.1: Wetting agents and solvents used in preliminary fecal hormone extraction tests.

Test Abbreviation	Wetting Agent (2 mL)	Solvent for Extraction (5 mL)	Reference
EE	MQ Water	Ethyl ether	McMaster et al. 1992
HEA	MQ Water	Hexane:Ethyl Acetate (3:2 vol)	Palace et al. 1993
CB	Citrate Buffer (with Brij 35)	Ethyl ether	Ziegler et al. 1996
PB	Phosphate Buffer (with Tween 20)	Ethyl ether	Jensen & Durrant 2006
BV	Ethanol (boil and vortex)	Ethyl ether	Dloniak et al. 2004 Wasser et al. 2000

centrifugation of the initial pellet was then repeated and the two solvent extracts were combined. The 10 mL of solvent phase containing the parent hormone, E_2, was dried down, and reconstituted in 500 uL of ELISA buffer. Estradiol concentrations were determined using commercial coated plate EIA assay kits (Cayman Biochemcial, Ann Arbor, MI, USA).

The water or buffer phase that was expected to contain the conjugated hormones was then treated first with 2 mL containing 2000 units of β-glucuronidase enzyme in 75mM phosphate buffer at pH 6.8. The samples were incubated for 21 h in a water bath at 37ºC, extracted and centrifuged twice with the non-polar solvent as describe above to capture the E_2 that had been liberated from the conjugated form by the β-glucuronidase enzyme.

The aqueous phase was then treated a second time with 2 mL of 75 mM phosphate buffer (pH 5.0) containing 15 units of sulfatase enzyme to liberate any sulfate conjugated estradiol in the samples. The samples were incubated in a 37°C water bath with the sulfatase for 21 h. Free

estradiol was then extracted twice from the samples using ethyl ether as described above. For both enzyme treatments, the 10 mL of extraction solvent was dried and samples were reconstituted in the EIA buffer. Concentrations of the free estradiol in both enzyme de-conjugated fractions were determined by EIA as described above. Concentrations were expressed as pg estradiol per mg of feces extracted.

Extraction efficiency of estradiol and its conjugate estradiol-3 glucuronide from the feces was tested using radiolabelled spikes, H^3-estradiol (H^3-E_2) and H^3-estradiol-3 glucuronide (H^3-E_2-3G). Second and third sets of test tubes with the same masses of feces (5, 10, 20 and 40 mg) were weighed out in duplicate. Each of these were spiked with the H^3-E_2 or H^3-E_2-3G and extracted with EE and PB methods previously tested. These two protocols were selected because EE produced the lowest, and PB produced the highest, emulsions during the extraction process and lower recoveries with increasing mass of feces were suspected to be related to greater emulsions. Samples spiked with the labelled conjugate were then treated with β-glucuronidase, incubated and extracted as described above. The samples were evaporated and reconstituted with Scintiverse cocktail and analyzed for 2 minutes per sample. Counts per minute (CPM) were measured per sample and compared to an external standard to calculate percent (%) recovery.

iii.   Establishing LC/MS/MS Parameters

While the preliminary analysis of the conjugates present in feces was performed using EIA to detect steroids, the ultimate goal was to develop a method that allowed simultaneous determination of parent and conjugated steroids, using LC/MS/MS. Selection of the product ion transitions to be monitored for each hormone and multiple reaction monitoring (MRM) optimization were performed using a Sciex 2000 triple quadrupole mass spectrometer (Applied

Biosystems, Foster City, CA, USA) with electrospray ion source (ESI). The working parameters optimized for the ESI in negative ion mode were CUR: 20, CAD: 8, IS: -4400, GS1: 20 and GS2 50 at 550 ℃ with a 20 minute run time. LC separations were performed on an Agilent 1100 series LC system (Agilent Technologies Canada, Inc., Mississauga, ON, Canada) equipped with a vacuum degasser, binary pump and autosampler. The C_{18} analytical columns used was the Genesis Lighten (50mm×2.1mm i.d., 4 μm particle size; Grace, Alltech Canada, Guelph, ON, Canada).

A binary mobile phase of Optima grade water and MeOH was used at a flow rate of 300 μL/min. In negative ion mode, the initial composition of the mobile phase was 80:20 water/MeOH (v/v), held for 1 min, ramped linearly to 100% MeOH in 8.5 min and held for 7.5 min. The column was allowed to equilibrate for 5 min between runs. Two monitoring periods were used in the method developed for negative ion mode; the first lasting for 9.6 minutes and the second for the remaining 11.4 minutes.

Samples were analyzed by LC/MS/MS and included the full suite of estrogens. Peaks were electronically integrated, and the areas were calculated using the instrument software. Recovery for each hormone was calculated as a percentage of the peak area measured in the external standard of same concentration.

iv.   Extraction and Clean-up of samples for LC/MS/MS analyses

a)   Initial liquid-liquid extraction using polar solvents

Five different solvents were assessed for their efficiency to extract parent and conjugated sex steroid hormones from a fixed mass of spiked feces: methanol, ethanol, propanol, acetone and acetonitrile. Pooled feces from the 10 female lake trout described above were used for these studies.

Five mg of ground and freeze-dried feces was spiked with 500 pg each deuterated estradiol 3-sulfate (d_4-E_2-3S), deuterated estrone 3-sulfate (d_4-E_1-3S), ^{13}C-estradiol (^{13}C-E_2), and ^{13}C-estrone (^{13}C-E_1). Three mL of solvent was added to the spiked feces and the samples were vortexed for 1 min, sonicated for 30 minutes and centrifuged at 3200 rpm for 10 min. The supernatant was dried to 1 mL under nitrogen, and centrifuged at 13500 rpm. The supernatant was again removed and dried to 200 µL and the samples were transferred to vials for injection on the LC/MS/MS. Two µL of sample was injected and analytes were separated using a HPLC column and system as described in section 3. The hormone peak areas of each sample were measured using Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA). Hormone recoveries were determined by analyzing the 500 pg spike standard externally, and calculating the percent recovered from the sample. To examine the potential for matrix effects, volumes of extract were injected at 1 µL increments from 1 to 4 µL. Suppression was estimated using the area counts of the 1 µL injections to predict area values of 2, 3, and 4 µL injections. Actual peak areas were expressed as percentages of estimated.

b) *Lipid analysis and the importance of acidifying samples*

To determine the lipid content and solvent extractables present in fish feces, 50 mg of freeze dried feces was weighed in triplicate and placed into boroscillicate glass test tubes. Two types of extractions were conducted; MeOH followed by DCM/Hexane (50:50 v:v) and DCM/Hexane alone. Five mL of MeOH or DCM/Hexane was added to the feces. Samples were vortexed for 2 minutes and centrifuged for 5 minutes at 4000 rpm. The upper solvent layer was removed and the extraction was repeated with the second extract added to the first. Samples extracted with MeOH were then extracted twice with DCM/Hexane (50:50 v:v) and the solvent

was placed in weighboats separate from the MeOH extracts. Weighboats were placed in a fumehood overnight to allow solvent evaporation, and were re-weighed the next day to determine the mass of the lipid (DCM/Hexane extract) and solvent extractable which could include simple carbohydrates, protein fractions and bile salts (MeOH extract).

v. Solid phase extraction

The solid phase extraction (SPE) systems tested for the clean-up of extracted feces are listed in Table 4.2. Two types of SPE systems were tested; pre-packed reverse-phase cartridges and silica hand-packed glass columns. A mixed hormone standard containing E_2, E_1, E_2-3S, E_1-3S, E_2-3G and E_1-3G in MeOH was used to add a 20 ng spike to water or solvents to test SPEs recoveries before fecal samples were tested. A recovery internal standard (RIS) was added to the fecal samples prior to loading them onto SPE cartridges. The RIS contained 20 ng each of d_3-E_2-3S and d_4-E_2. Before loading all samples on the LC/MS/MS, each were spiked with an internal precision standard (IPS) which contained 20 ng of d_4-E_1.

Pre-packed SPE cartridges were conditioned with 0.5-1 mL of MeOH followed with an equal volume of water, as per manufacturer's instructions. Chromabond cartridges (6cc with 500 mg packing - Macherey-Nagel GmbH & Co. KG, Düren, Germany) also had a neutral phosphate buffer added after MeOH and water for conditioning. Solvents were spiked with the mixed hormone standard and loaded onto the SPEs. Fecal extracts were prepared by wetting 50 mg of freeze-dried feces with 0.5 mL of 2M HCl, then 5 mL of MeOH was added, vortexed and centrifuged. The supernatant was loaded onto conditioned SepPak C18 (6cc with 500 mg packing – Waters, Milford, MA, USA), Bond Elut Plexa (3cc with 60 mg packing - Varian, Palo Alto, CA, USA), and Oasis HLB (6cc with 500 mg packing - Waters, Milford, MA, USA) cartridges.

Table 4.2: Gel Permeation Chromatography (GPC) and Solid Phase Extraction (SPE) methods tested for fecal sample clean-up

Method	Column/Stationary Phase Details	Sample Type	Solvents	Instrument
GPC	SX-3 Biobeads (60 g)	Solvent spiked with labelled hormones	1:3 EA:cyclohex	Knauer Smartline 6500 Shimadzu system
GPC	Sephadex^{TM} LH-20 packed glass column	Feces extract spiked with labelled hormones	3:2 EA:Hex	N/A
GPC	Sephadex^{TM} LH-20 packed in glass column	Feces extract spiked with labelled hormones	100% EA	N/A
GPC	Sephadex^{TM} LH-20 packed in glass column	Solvent spiked with labelled hormones	EA with 2.5% glacial acetic acid	N/A
GPC	Sephadex^{TM} LH-20 packed in glass column	Solvent spiked with labelled hormones and spiked fecal extract	100% MeOH	N/A
SPE (normal phase)	2 g of silica (0, 1, 5 and 10% deactivated) packed in glass mini columns	Solvent spiked with labelled hormones and spiked feces extract	1:1 chloroform:MeOH packed and wash 100% MeOH elusion	N/A
SPE (normal phase)	2 g of silica (0% deactivated) packed in glass mini columns	Feces extract spiked with labelled hormones	1:1 chloroform:MeOH or 100% EA packed with 100% MeOH elution 1 mL fractions collected	N/A
SPE (reverse phase)	Waters C^{18}, Varian Bond Elute Plexa, Oasis HLB	Neutral solvent and feces extracted with acid pre-treatment spiked with labelled hormones	MeOH, EA, EA with 2.5% glacial acetic acid	
SPE (reverse phase)	Oasis HLB	Spiked H$_2$O and MeOH:H$_2$O (4:1), and spiked feces extracts	EA, MeOH:H$_2$O (30:70 with 2% acetic acid, 30:70 with 2% ammonium hydroxide)	
SPE (reverse phase)	Chromabond HR-X	Spiked MeOH:H$_2$O (4:1), and spiked feces extracts	MeOH:H$_2$O (2:3), 100% MeOH, 100% EA	

Fecal extracts for Chromabond and HLB cartridges were prepared by adding 1 mL of water or MeOH:water (4:1) to 50 mg feces. Samples were then vortex for 1 minute and centrifuged for 5 minutes at 4000 rpm. Supernatant was removed and loaded on conditioned Chromabond and HLB cartridges. Cartridges were eluted with the corresponding solvents listed in Table 4.2. The elution of estrogens and their conjugates from the Oasis HLB SPE was adapted from Reddy et al (2005). All eluents were evaporated under N_2 and reconstituted in 50-100 uL MeOH for LC/MS/MS analysis.

Silica, 60-200 mesh (Fisher Scientific, Napean, ON) was baked overnight at 66 $^{\circ}$C and cooled in the oven. Optima water was added to deactivate 1, 5 and 10% of the silica active sites. This process masks the most energetic sites on the silica and improves the chromatography (McClure 1972).Two milligrams of silica was weighed out for each column, mixed with the appropriate solvent (see Table 4.2). Mini 10 mL glass columns fitted with Teflon septa were packed with silica and solvent. Five milliliters of packing solvent was used to rinse packed silica before use.

Solvent spikes for the silica tests were prepared as described above. Feces (50 mg) for the silica packed in chloroform:MeOH tests were extracted twice with 3 mL of the same solvent blend, and then evaporated under N_2 to a volume of 0.5 mL. The tube was rinsed with another 0.5 mL and loaded onto the column. Extractions for the ethyl ether packed silica used the same mass of feces. All samples were extracted twice with 3 mL of ethyl acetate, and the 6 mL was evaporated under N_2 to a final volume of 0.5 mL. Samples were transferred to the column and the tube was rinsed with 0.5 mL of solvent and added to the column. Columns were washed and eluted with solvent volumes listed in order in Table 4.2. Each solvent fraction was collected

separately, evaporated to dryness under N_2, and reconstituted in 50 µL MeOH for analysis by LC/MS/MS.

vi.   Gel Permeation Chromatography - GPC

Two types of GPC systems were tested: gravity-fed Sephadex^{TM} LH-20 packed columns and an automated Knauer Smartline 6500 Shimadzu system.

Sixty grams of SX-3 Biobeads were packed into the Knauer system.   Ethyl acetate:cyclohexane (1:3, v:v) as the mobile phase was pumped at a flow rate of 5.0 mL/min. A standard in 5 mL of EA:cyclohexane solvent mix containing 10 ng of each of the following estrogens and conjugates: E_2, E_1, E_2-3S, E_2-3G, E_1-3S and E_1-3G was injected into the GPC. Three fractions of 25 mL were collected and condensed in a rotovap to approximately a 1 mL volume and then transferred to a borosilicate test tube. The rotovap flask was rinsed with 1 mL of solvent and added to the sample. Samples, including the 1 mL fractions, were evaporated under N_2 to dryness and reconstituted in 50 µL MeOH for LC/MS/MS analysis.

Gravity-fed columns were prepared by soaking 10 g of Sephadex^{TM} LH-20 overnight for each column in the solvents selected in Table 4.2. Columns were packed and rinsed with 20 mL of fresh solvent before samples were loaded. Solvent spikes were prepared as previously described. Fecal samples for the ethyl acetate:hexane column were pretreated with 2M HCl as previously described, while all other fecal samples did not. Samples were extracted using 3 mL of the same solvent mixes that the Sephadex was soaked in, and treated as described above to a final volume of 0.5 mL followed with a 0.5 mL tube wash prior to loading the column.

As the fractions were collected, a solvent cap of 1 mL above the Sephadex was maintained. The first fraction collected was a volume of 5 mL, followed with 1mL fractions until

the band containing the yellow bile salts passed through. Subsequent fractions were 10, 20 and 25 mL volumes. The larger volumes of solvent were condensed and prepared in 50 µL of MeOH for LC/MS/MS analysis as described above.

vii.   Liquid:Liquid Extraction with Lipid Removal Agent Cleanascite[TM]

The SPE and GPC sample clean-up methods tested were unable to remove the interfering compounds thought to consist of lipids and bile acids. Preliminary tests found these compounds have the same mass transition as E_2-3G, but co-elute later with the parent estrogens (data not shown). The quantities were substantial enough to reduce the life of the HPLC column and would require more frequent cleaning and maintenance of the mass spectrometer.

Cleanascite[TM] is a lipid removal agent (LRA) that has been available for over 10 years. It is a non-ionic, solid-phase adsorbent suspended in a saline solution, and has been used for clarifying complex matrices such as bile (Guerrier et al. 2007). It has been reported to remove fats from serum (Castro et al. 2000) and feces (Shuber et al. 2002) without affecting hormones. Its use has also been promoted for prolonging the life of membrane and chromatographic columns particularly when analyzing complex matrices (Biotech Support Group 2011). In a preliminary test, 500uL of Cleanascite[TM] was added to three different fecal extracts. Each extract had been evaporated to dryness and reconstituted in 1.0 mL MeOH. The extracts varied in viscosity and ranged in colour from yellow to dark brown. Samples were refrigerated overnight and centrifuged at 13000 rpm for 10 minutes. The LRA successfully removed the viscosity and cleared the yellow/brown pigments, likely due to lipids and bile acids, sequestering them in a pellet at the bottom of the Eppendorf tube. The supernatant was clear to slightly opaque, with a very pale yellow hue in the sample that was originally dark brown (personal observations).

a) Effects of Cleanascite^{TM} on glucuronide extraction

Cleanascite^{TM} LRA performed as described by visually clearing the samples. It was expected that the parent steroid compounds would still be present in the supernatant as previously reported (Castro et al. 2000), but it was unknown if the glucuronide conjugates would also be unaffected by this treatment. To test this 50 µL of MeOH containing 500 pg/µL each d_3-E_2-3G and d_4-E_2 was added to 20 Eppendorf 1.5 mL microcentrifuge tubes. In replicates of 5, LRA was added in 25, 50, 75 and 100 µL volumes. Recovery internal standards (RIS) were made in triplicate with the same 50 µL of labeled hormone stock with one of 0, 25, 50, 75 or 100 µL MeOH added instead of LRA. RIS and LRA sample tubes were volume corrected with MeOH to the same final volume as the 100µL groups. All tubes were vortexed to mix, refrigerated overnight, and then centrifuged as above. Fifty microliters of supernatant from each sample was transferred to an autosampler vial with a 200 µL borosilicate insert. Each vial then received a 5 µL IPS of 5000 pg/µL d4-E_1. Samples were vortexed and analyzed as per the LC/MS/MS method described above. Mean recoveries from the LRA additions were calculated as the percent of the corresponding volume RIS means. Significant differences were determined by ANOVA.

b)   Acid washing with LRA clean-up

Treating biological samples with sulfuric acid (acid washing) as a lipid removal step prior to chromatographic analysis is a common practice in sample clean-up (Strid et al. 2013; Hovander et al. 2000). This strong acid treatment, however, has also been used to de-conjugate sex hormones preceding the extraction of the parent hormone in fecal analyses (Ziegler & Wittwer 2005). The use of 2M HCl was successful in the extraction of glucuronide conjugates

from Cortland's incubation medium, without altering the compounds (see Chapter 2). Acid washing the fecal samples up to 3 times was tested as a clean-up step before the addition of the LRA. One acid wash cycle consisted of adding 1 mL of 2M HCl to the 1 mL of extracted sample and vortexed vigorously for 1 minute. The samples were then extracted twice with EA and condensed to 1 mL as described above. This completed 1 acid wash cycle.

As with LC/MS/MS analysis of complex matrices, decreasing the sample injection volume or increasing sample dilution often decreases matrix interferences (Gosetti 2010; Van Eeckhaut et al. 2009). Preliminary tests showed that decreasing the injection volume of a fecal extract from 3 to 1 µL reduced IPS suppression 10-25%, with higher masses of feces extracted showing less improvement (data not shown). All subsequent testing used the 1 µL injection volume. Sample dilution alone, up to 10 times, decreased suppression up to 15%. However, the trade-off was that endogenous hormones present in small amounts, such as E_2, were no longer detectable (data not shown). The following section covers 7 experiments where sample dilution and volume of LRA were varied to optimize the clean-up of rainbow trout fecal extracts in preparation for estrogen and conjugate hormone analysis using LC/MS/MS.

Each fecal extract used for the optimization tests was prepared measuring 50 mg of freeze dried and ground rainbow trout feces, collected as previously described, into a borosilicate test tube. This mass was selected as it was found to be sufficient to detect endogenous estrogens and conjugates without adding too much matrix that interfered with the analyses (data not shown). The samples were then wetted with 1 mL of 2M HCl and set aside for 1 hour to break down lipids and aid in the extraction of the glucuronide conjugates by decreasing the pH. Hormones were extracted by adding 3 mL of ethyl acetate (EA), vortexing for 1 minute, and then centrifuging for 5 minutes at 4000 rpm. The top solvent layer was transferred to a clean test

tube, the extraction with EA was repeated and the two fractions were combined. The 6 mL of solvent was condensed to 1 mL under N_2.

Once the two acid washes were completed, the 1 mL sample was transferred to a 1.5 mL Eppendorf microcentrifuge tube. The sample was evaporated to dryness under N_2 and reconstituted in 100, 200,250, 320,450, 650, 1000 µL MeOH. Samples were vortexed until fully resuspended. Cleanascite™ was added to the sample in MeOH in one of the following volumes; 0, 25, 50, 80 or 100 µL, representing 0 - 33% of the final sample volume. The samples were gently mixed and refrigerated overnight. After centrifuging for 10 minutes at 13000 rpm, 50 µL of the supernatant was transferred to an autosampler vial with a 200 µL insert and 10 µL of 5000 pg/µL IPS was added. Samples were vortexed and estrogens and conjugates were analysed via LC/MS/MS as previously described.

c)   *Determination of d_3-E_2-3G Suppression and the Final Method*

The final method consisted of acid washing the sample twice, reconstituting the extract in 250 µL of MeOH, and clarifying with 50 µL of Cleanascite™ lipid removal agent. This method allowed good, consistent recoveries of the glucuronide conjugates while still being able to detect endogenous E_2 in our samples. As d_3-E_2-3G is used for the RIS, which incorporates recovery and suppression together, it was of interest to determine how much suppression was taking place with the final method. Fecal samples were measured, extracted and cleared using the final method, except 10 µL of 5000 pg/µL d_3-E_2-3G was added as an IPS just before LC/MS/MS analysis instead of as the RIS. Suppression was calculated as a percentage of the amount in the sample compared to that of an external standard.

As d_4-E_2 RIS and d_4-E_1 IPS suppression remained at approximately 80%, this would not be optimal for fish collected during seasonal lows in circulating hormones or juvenile fish. Acidification of the feces, necessary for the extraction of the glucuronide conjugates, was suspected to cause interfering compounds or matrix to be more readily extracted. Extraction of the feces with a less polar solvent mix to capture the parent estrogens, prior to acidification for the glucuronide extraction was explored. The less polar solvent mix would also need to be acid washed to remove the bulk of the lipids extracted. The following pre-acid extraction using a hexane/ethyl acetate blend was tested and compared to the results of the final method using acid first.

In five replicates, 50 mg of freeze-dried feces was weighed in glass test tubes. Three milliliters of 3:2 hexane:ethyl acetate was added for extraction. The sample was vortexed for 1 minute, centrifuged for 5 minutes at 4000 rpm, and then the supernatant was carefully aspirated as not to disturb the pellet. The extraction was repeated and the two fractions were combined. The samples were condensed, acid washed 2 times and prepared for LC/MS/MS as described above. The remaining pellet was resuspended in 1 mL of 2M HCL and set aside for 1 hour. The final extraction and hormone analysis method proceeded on the pellet samples as previously outlined. Recovery and suppression were calculated as percentages using external standards.

viii. *Method validation – Temporal comparison of hormone concentrations in plasma and feces*

To validate the final extraction and clean up method, hormone concentrations were determined in paired feces and plasma samples obtained from a group of fish sampled repeatedly over time. Female rainbow trout that where housed at the Freshwater Institute, Winnipeg, MB, were anaesthetized, weighed and measured prior to each fecal and plasma sample collection

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event. Sample collection methods are described above. On the first collection day, the dorsal fin rays of each fish were clipped each in a unique pattern for future identification. Fish were sampled biweekly in May and then weekly from June through to mid-August 2012. For any given sampling period, only those which had a successfully collected pair of plasma and feces samples were analyzed.

Fish plasma extractions were modified from (Gemmill et al. 2012). Briefly, in a glass test tube 300 µL of sample was spiked with a 10 µL RIS containing 500 pg/µL d_4-E_2 and d_4-E_2-3S, and vortexed to mix. Samples were extracted twice with 3 mL of 9:1 hexane:ethyl acetate (H:EA) to target parent estrogens, E_2 and E_1. Then samples were extracted twice with 3 mL acetonitrile (ACN) and the 4 fractions were combined. Samples were evaporated to dryness under N_2 and reconstituted in 1 mL MeOH, then transferred to autosampler vials. The test tubes were rinsed with 500 µL of MeOH and added to the vial. Samples were evaporated to dryness again and resuspended in 45 µL MeOH. A 5 µL IPS containing 5000 pg/µL d_4-E_1 was added to each vial and vortexed. LC/MS/MS analysis was as described above with 2 µL sample and standard injections.

Results & Discussion

Preliminary analysis of steroid conjugates in fish feces

All five methods extracted measureable amounts of free E_2 from the fecal samples (Figure 4.1A). The citrate buffer and BV in ethanol extracted marginally more E_2 than the other methods. Increasing fecal sample size resulted in lower hormone extraction efficiency with each method. Larger fecal samples also formed the greatest emulsions during extraction in both the aqueous and solvent phases.

Aqueous phases (i.e. water or buffer) that were treated with β-glucuronidase contained the highest proportion of E_2 extracted after the treatment, however the results were variable (Figure 4.1B). EE and HEA both showed decreased extractability with increasing sample size, similar to results for the unconjugated hormone. The two buffer groups (CV and PB) and the boil/vortex in ethanol group (BV) did not exhibit the same pattern. When 40 mg of feces was extracted, the same, or more E_2, was extracted as when 5 mg was extracted. The final treatment with sulfatase resulted in very little E_2 being liberated, (i.e. <1 pg/mg).

There was not one method that clearly outperformed the others in extracting E_2 or its conjugates from fish feces. All methods had varying degrees of emulsions that formed during the vortex phase of the extraction, and many had to be centrifuged to compact the mass before the extraction could continue. The volume of emulsion appeared to increase with increasing fecal mass that was extracted. Where emulsions were greatest, the lowest amounts of E_2 were quantified in the sample. None of the six published methods that were tested (Table 4.1) reported emulsion formation during the extraction process; however, it is known to be common during steroid extraction (Makin et al. 2010). The previously published methods generally employed the same volume of solvents, but the mass of feces extracted was greater than in the current studies.

Glucuronide conjugates were identified as the predominant form of E_2 present in rainbow trout feces, followed closely by free E_2. These two forms were, therefore, targeted for further development of the fish feces extraction protocol. Because low concentrations of E_2 were liberated by sulfatase (< 1pg/mg) this labour intensive step was eliminated. Jensen & Durrant (2006) also reported similar results when developing a fecal sex steroid extraction method for the brown kiwi (*Apteryx mantelli*).
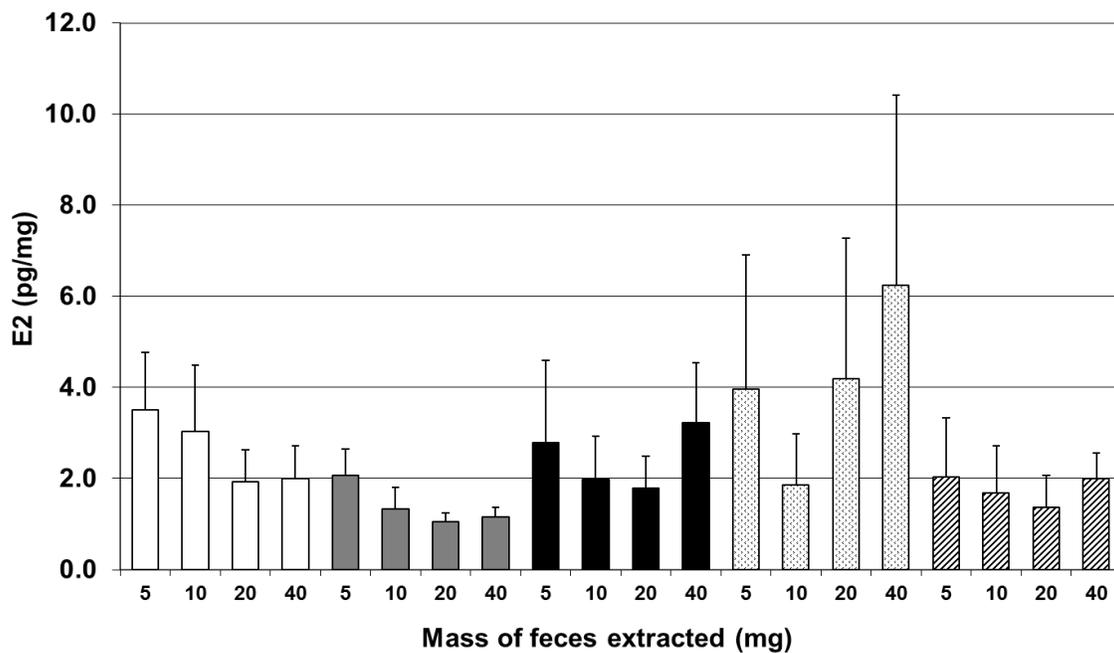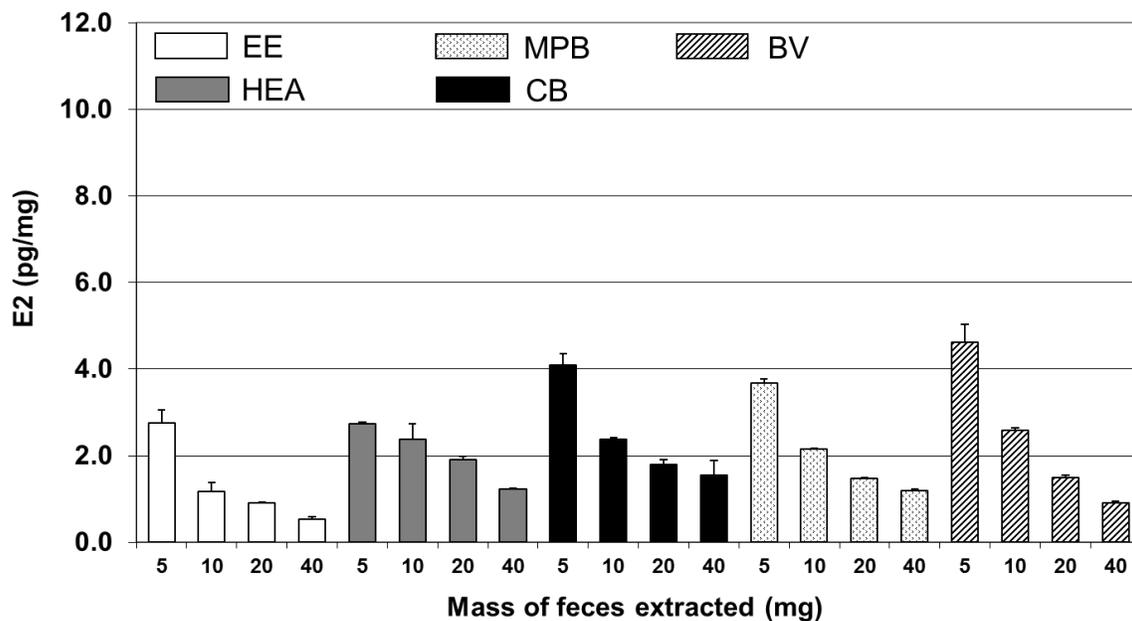
Figure 4.1: Estradiol (E$_2$) extracted from increasing masses of feces using five different extraction methods before (A) and after (B) treatment with β-glucuronidase enzyme.

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Extraction efficiency of the E_2-H^3 was above 80% for both EE and PB when 5 mg of feces was extracted. The extraction efficiency decreased as the amount of feces extracted increased, so that when 40 mg of feces was extracted, recoveries were just over 60%. McMaster et al (1992) reported that EE extraction of fish plasma captured >95% of parent sex hormones. From laboratory and field mice fecal samples, EE alone extracted 74% of spiked testosterone (T). Pre-wetting with MeOH increased T recoveries to 95% (Billitti et al. 1998). Before the addition of β-glucuronidase, the recovery of the H^3-E_2-3G was under 3% in all samples. After treatment with the enzyme, extraction efficiencies ranged from a mean of 93% for 5 mg feces to 78% for 40 mg using EE. Extraction with PB resulted in the formation of emulsions that increased with sample size. Spike recoveries with 5 mg were 50% and with 40 mg of feces recovery was only 38%.

Because only 3% of H^3-E2-3G was recovered using EE as the extraction solvent, the next steps in extraction method development focused on polar solvents to capture the water soluble conjugates. Buffers that contain surfactants were also eliminated from the method development due to the high production of emulsions and their observed negative affect on extraction efficiency.

Extraction and Clean-up of samples for LC/MS/MS

i. *Initial liquid-liquid extraction using polar solvents*

Five polar solvents were tested for their capability to simultaneously extract mass-labeled parent and conjugated sex hormones from fish feces. Of the solvents tested (methanol, ethanol, propanol, acetone and acetonitrile) for the liquid-liquid extractions, MeOH showed the best overall spike recovery; approximately 60% for free estrogens and 80% for d_4-E_2-3S. Ethanol

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and acetonitrile had the lowest recoveries, with means under 55% for all hormones spiked (Figure 4.2). Extractions employing MeOH or EtOH alone have shown good recoveries when one parent hormone at a time has been examined using RIA or EIA. Morato et al. (2004) reported >80% recovery of testosterone when using EtOH to extract captive jaguar (*Panthera onca*) feces. Recoveries from macaque feces were greater than 90% for H[3]-spiked testosterone,
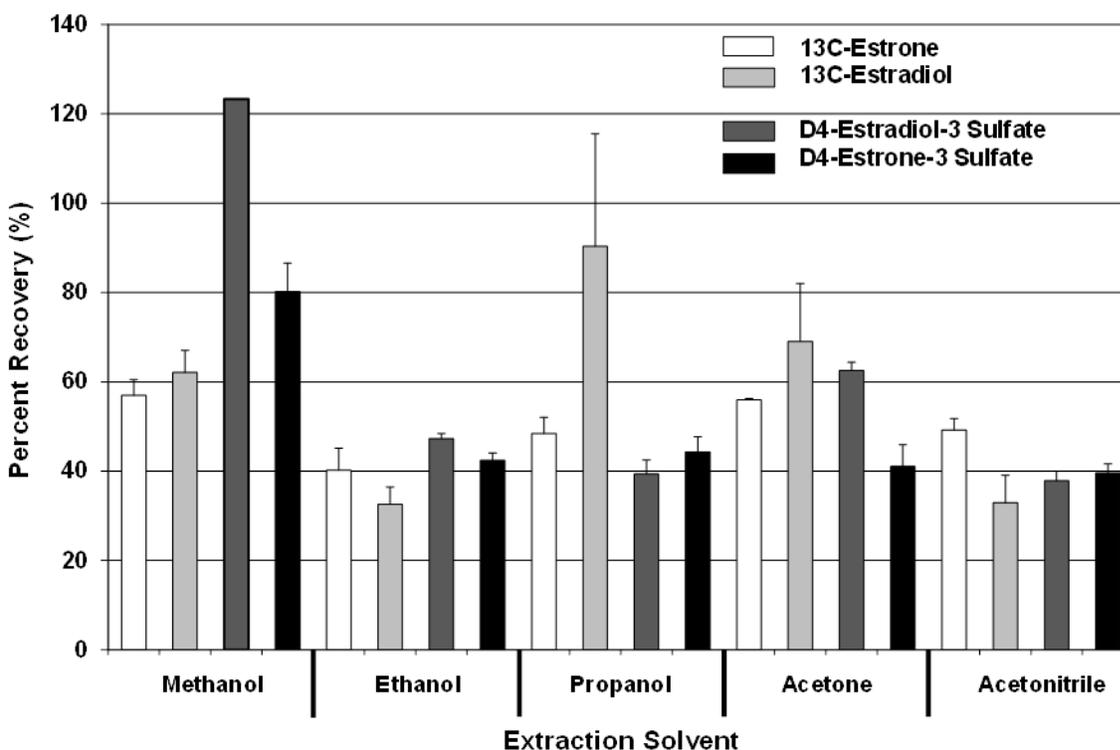
Figure 4.2: Mean percent recovery with SEM of labeled estrogens and sulfated conjugates from feces using five different solvents with 6 replicates for each extraction.

progesterone and cortisol using a MeOH extraction method with vortexing (Wasser et al. 2000). Da Silva et al. (2005) showed similar results for E_2 in sea otter feces (>90%) with MeOH.

When a complex material, such a feces, is extracted, there are other components that are removed at the same time which can interfere with the LC/MS/MS analysis (Couchman et al.

2011; Taylor 2005). These compounds can be similar in structure and/or size to the analyte of interest, but this is not necessary for matrix effects to exist. The electrospray ion source (ESI) used in this study works by highly charging ions of the analyte of interest. This is achieved by creating a fine spray of solvent droplets in the presence of a strong electric field, heat and compressed gas. The ions are transferred to the gas phase that ultimately reaches a detector (Taylor 2005). Co-eluting molecules can interfere with the ionization efficiency and transfer of the analyte of interest to the gas phase, or they can compete for charge and detection. This competition can diminish the signal or peak height of the analyte to be measured. This is referred to as matrix effect (Couchman et al. 2011; Cappiello et al. 2010; Taylor 2005, Van Eeckhaut 2009; Annesley 2003).

Matrix effect can often be diminished by decreasing the volume of sample injected by the autosampler for analysis; less sample injected means less matrix present to interfere in the analysis (Van Eeckhaut et al. 2009). By injecting smaller volumes, the peak area can quite often become larger and easier to quantify. A simple test to determine if there is a matrix effect in a sample is to inject increasing volumes of the same sample. If there is no matrix effect, the peak area should increase proportionally in a linear fashion. This technique was used to assess if there was a matrix effect in the samples, or if there was simply poor extraction efficiency.

Matrix effect tests indicated that the amount of detected hormone did not increase linearly with increasing injection volumes. The significant matrix effect was observed with all solvents tested and was most prevalent for the parent hormone, estrogen. When comparing the detected amount of E_2 using 2, 3 and 4 µL injections as a percentage predicted from the 1 µL injection counts, parent hormone suppression was 25.5 ± 6.8, 37.2 ± 7.4 and $46.1 \pm 6.7\%$ respectively. Conjugate suppression was generally less than for parent hormones, and did not

increase with higher injection volumes. Mean conjugate suppression was 16.7 ± 2.9, 22.9 ± 3.6 and $23.4 \pm 3.0\%$ for 2, 3 and 4 µL injection, respectively. These results are different than those reported by Bonfiglio et al. 1999. They compared ion suppression of four compounds with different polarities under the same mass spectrometric conditions and found that the most polar compounds had the greatest ion suppression. This however, may only be the case if the masses are similar as higher mass molecules have been shown to suppress the signal of smaller mass molecules (Gosetti et al. 2010).

When conducting LC/MS/MS analyses on biological matrices, particularly a very complex one such as feces, there are several ways in which co-extracted compounds can modify the signal of the analyte of interest. Interfering compounds can compete for available charges and/or for access to the droplet surface where the charges are transferred (Cappiello 2010). When interfering molecules compete and are ionized, they deplete the available charge, potentially causing the formation of neutral species of the analyte of interest, rendering them undetectable (Gosetti et al. 2010). Samples with complex matrices can also significantly increase the liquid phase viscosity. This can directly affect the formation and evaporation of the spray droplets by changing the surface tension, or affecting the ability of the droplet to reach the critical size required for efficient charge transfer. This would directly decrease the number of charged ions capable of reaching the gas phase and subsequently the detector (Cappiello 2010; Annesley 2003; Gosetti 2010). Once in the gas phase, the analyte is not safe from matrix effects, particularly if the interfering compound is highly basic. Analytes can lose or transfer their charge through neutralization reactions that occur in the gas phase (King et al. 2000; Cole 2000).

Matrix effects can be reduced by diluting the sample or decreasing the HPLC injection volume, however, this may not be desirable when target compound concentrations are low. Sample purification to remove or reduce the interfering components is the preferred choice because it prolongs column life, reduces instrument maintenance and typically requires/produces smaller sample volumes (Gosetti 2010; Van Eeckhaut et al. 2009). Solid phase extraction (SPE) and gel permeation chromatography (GPC) are two types of sample cleanup that were explored for this reason. Results from these experiments are described below.

ii.   *Lipid analysis and the importance of acidifying samples*

Using DCM:Hexane (v:v) trout feces were determined to contain 2.35 + 0.06% lipid by weight. MeOH extractables, which could include carbohydrates, protein fragments and bile acids, accounted for 20.1 + 1.8% of the fecal mass. Non-conjugated estrogens are lipophilic compounds that can be incorporated into lipid micelles (Malkin et al. 2010). Many of the parent sex steroid fecal extractions found in the literature contain a step to break down the lipid; these techniques include saponification (the use of a strong acid to break down the lipid), or the addition of a surfactant such as Tween or Brij (Ziegler & Wittwer 2005; Zielger et al. 1996; Jensen & Durrant 2006). Addition of such surfactants resulted in the formation of emulsions which were suspected to decrease hormone extraction efficiency. During the development of the Cortland's in vitro medium extractions (Chapter 2) the extraction of hydrophilic glucuronide conjugated sex steroids were significantly greater at pH <3 due to their estimated pKa of 3-4 (Janson et al. 2011). Therefore, it was expected that the addition of acid to the freeze dried fecal material would improve the extraction of both parent and glucuronide conjugated sex hormones.

Solid Phase Extraction

Because lipids and other solvent soluble fecal components can interfere with mass spectrometry analyses and foul HPLC column, removing these compounds prior to LC/MS/MS analysis was necessary. Ideally, clean-up steps should conserve both the parent and conjugated hormones for simultaneous analysis. Two types of SPEs were tested: pre-packed reverse-phase cartridges and silica hand-packed glass columns. Waters C^{18}, Bond Elux Plexa, Oasis HLB and Chromabond HR-X were the pre-packed commercial cartridges that were evaluated. Initial tests with hormones suspended in solvents returned >50 and 90% of the spiked hormones from the Waters and Bond Elute Plexa cartridges, respectively. Final recoveries were both over 100%, indicating signal enhancement. When acid extracted fecal samples were eluted on the Waters C^{18} columns the eluent was very cloudy. A solid mass formed when the eluent was evaporated under N_2 evaporation and the samples were not appropriate for injection on the LC/MS/MS instrument. The Varian cartridges did not appear to retain any of the pigments from the fecal extracts. Suppression in these samples was over 95% and there were no endogenous glucuronides detected (Table 4.3).

Recovery of parent hormones from spiked solvent was very good (113.2 $\pm$ 8.8 %) for the Chromabond HR-X cartridge, with some signal enhancement. However, glucuronide recovery was poor (37.7 $\pm$ 1.4 %). Increasing the elution volume did not improve recovery when fecal extracts in H_2O or MeOH:H_2O were applied to the Chromabond HR-X cartridge. Mean glucuronide recoveries remained low and the suppression in the 100% ethyl acetate final elution fraction was too high to detect parent hormones (Table 4.3).

The Oasis Hydrophilic-Lipophilic Balanced (HLB) is a commonly used SPE cartridge for the purification of similar environmental matrices. It has been used for the cleanup of samples

containing free estrogens and some conjugates before LC/MS/MS analysis (Gadd et al. 2010) including, dairy shed effluent (Reddy et al. 2005), sewage influent and effluent (Labadie & Budzinski 2005), sewage effluent and river water, and sewage effluent and lake water (Isobe et al. 2003). The method in this study was adapted from Reddy et al. (2005). Mean recoveries were 87 % for both parent and conjugate hormones from spiked solvents. When feces extracted in H_2O were tested, most of the glucuronides were eluted in the 2% ammonium hydroxide, however, recoveries were poor (45%). Once corrected for suppression, mean parent recovery was approximately 75%. Extracts in MeOH:H_2O passed through the Oasis HLB with very little retention of pigments. Glucuronide recovery was 39.8 $\pm$ 1.3 % in this fraction, 45% less than the solvent spike, suggesting that the pigments are also interfering with the analysis of the glucuronides. The IPS suppression was over 90%, indicating that the IPS was not a good predictor of suppression for the glucuronides. The ethyl ether elution recovered approximately 75 % of the parent hormones and suppression had been reduced to 57.4 $\pm$ 4.4 % (Table 4.3).

Reddy et al. (2005) reported 62.9-74.5% (SD<8%) recovery of labeled glucuronide spikes, almost double what we observed. The stepwise washing of the cartridge, which was also performed in these studies, reduced suppression from 90-70% in Reddy's samples, and was similar to results from this study. Reddy et al. (2005) continued with cleanup using anion exchange chromatography and reduced suppression to 35%. It was estimate that 20-40% of the targeted analytes were lost during this cleanup process. Because conjugate concentrations are often quite low in feces, loss of this magnitude was deemed an unacceptable cost for reduced suppression for our purposes.

Table 4.3 Recovery and suppression tests of estradiol and its glucuronide metabolite in fecal samples using different solid phase extraction (SPE) cartridges for clean-up.

SPE Cartridge	Sample Type	Glucuronide Recovery (%)	Parent Estrogen Recovery (%)	Suppression (%)	Notes
Waters C^{18}	Unlabeled hormone spike in MeOH	83.7	121.3		Over 50% of hormone spike passed through cartridge
Waters C^{18}	Feces pretreated with acid – labelled hormone spike	------	------	------	Acidic conditions looked like cartridge had melted There was a solid mass left after evaporation Did not analyze samples
Varian Bond Elute Plexa	Unlabeled hormone spike in MeOH	102.0	108.8	------	Over 90% of hormone spike passed through cartridge
Varian Bond Elute Plexa	Feces pretreated with acid – labelled hormone spike	-----	0	>95	Pigments and lipids were not retained No endogenous glucuronides measured
Oasis HLB	Unlabeled hormone spike in MeOH	92.5	65.9		70% of Glucuronides passed through cartridge, parents were retained until eluted
Oasis HLB	Feces pretreated with acid – labelled hormone spike	N/A	13.7	88.8-100	The most endogenous glucuronides were eluted with MeOH, but pigments were in this fraction too
Oasis HLB	Labeled hormone spike in MeOH:H20 (4:1)	86.7 $\pm$ 0.3	86.9 $\pm$ 7.6	18.2 $\pm$ 4.2	Recoveries were good Parents and glucuronides eluted separately
Oasis HLB	Feces extracted with MeOH:H2O (4:1)	39.8 $\pm$ 1.3	74.7 $\pm$ 13.8	57.4 $\pm$ 4.4	Over 90% IPS suppression in glucuronide fraction Suppression less in ether elution containing parent hormones
Oasis HLB	Feces extracted with H2O	45.2 $\pm$ 1.0	83.4 $\pm$ 1.5	36.0 $\pm$ 2.0	78% Glucs eluted in 2% Ammonium Hydroxide mix All parents eluted in Ethyl Ether fraction
Chromabond HR-X	Labeled hormone spike in MeOH:H20 (80:20)	37.7 $\pm$ 1.4	113.2 $\pm$ 8.8	6.4 $\pm$ 3.5 35.8 $\pm$ 5.2	Good parent recoveries, signal enhancement Parents and glucuronides eluted separately 97% of glucuronides recovered eluted in 1st wash High suppression/low recovery in glucuronide fraction
Chromabond HR-X	Feces extracted with H2O	31.8 $\pm$ 0.64	4.2 $\pm$ 2.2	40.6 $\pm$ 3.9 98.9 $\pm$ 1.3	First wash, no parents Elution suppression too high to detect parents
Chromabond HR-X	Feces extracted with MeOH:H20 (80:20)	35.7 $\pm$ 1.5	13.0 $\pm$ 1.4	60.9 $\pm$ 10.9 96.6 $\pm$ 1.5	Suppression in washes Glucuronides passed through, it is thought parents were retained Sample and elution suppression too high to detect parents

During method development Gadd (2009) reported estrogen sulfate recoveries that ranged from 71-176%, with significant signal enhancement in some samples. Their corrected free estrogen recovery ranged between 60 and 83% using the Oasis HLB for sample clean-up. Similar results with free estrogen recoveries exceeding 80% were reported by Labadie & Buzinski (2005). Isobe et al. (2003) reported 71, 82, 91% recovery from spiked lake water samples for E_2-3G, E_1-3G, E_2-17G, respectively. Those authors used a second lengthy clean-up procedure followed the SPE extraction to separate free and conjugated estrogens into two sets of samples. The complete method was applied to sewage treatment effluent samples, however, recovery and suppression of free and conjugate hormones was not reported, and conjugates were simply reported as non-detectable. Using SPE cartridges to remove matrix elements, may add different interfering matrix components. Buffers (particularly phosphate buffers) often used to wet/rinse SPE cartridges can cause significant suppression during LC/MS/MS analysis (Lindegardh et al. 2008). Buffer salts significantly suppressed the signal of the analytes of interest. It has also been suggested that impurities from the SPEs themselves could cause suppression or enhancement of analyte signal during LC/MS/MS analysis (Cappiello et al. 2010).

Because none of the commercially available pre-packed SPEs adequately removed matrix interferences from the trout feces prior to LC/MS/MS analysis, three separate tests were conducted with hand-packed silica based columns. Hormone spiked solvent recoveries with various levels of deactivated silica (0, 1, 5 and 10%) packed in chloroform:MeOH 1:1 were not significantly different among different percentages of silica deactivation (p = 0.59). Recoveries ranged between 59.3-80.5% for d_3-E_2-3G and 84.0-101.2% for d_4-E_2. The only difference that the percentage of deactivation appeared to have was in the fraction that the glucuronides eluted. At 0 and 1% deactivation, one third to one half of the conjugates eluted in the first

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chloroform:MeOH wash, while at 5 and 10% approximately 50-65% eluted in the final 100% MeOH rinse. All d_4-E_2 eluted in the chloroform:MeOH wash, regardless of silica deactivation (Table 4.4).

When 0 and 10% deactivated silica columns were tested using fecal extracts, glucuronide recoveries using 10% silica were higher; likely due to the differences in elution time. Mean suppression for all columns in the first wash was 93.9 ± 0.6 %. This affected all parent hormone recoveries as well as glucuronides in 0% silica. Suppression in the third fraction, containing conjugates, was only $13.0 \pm 2.0\%$ when 10% silica was used (Table 4.5).

This experiment was repeated with silica packed in chloroform:MeOH or 100% ethyl acetate. Previous tests had collected fractions in 5 or 10 mL volumes, and it was hypothesized that by collecting smaller 1 mL fractions, target hormones may be collected separately from those causing the suppression. As expected, fractions with the greatest pigmentation also had the highest IPS suppression (Table 4.6). The chloroform:MeOH packed columns were successful in separating the conjugates from the pigments, but the parent hormones were still collected in the darkest fractions where suppression was as high as 92%. The EA packed column with 0% deactivated silica provided the best conjugate recovery at 71% and the least amount of suppression in the first fractions containing the parent hormones (24-26%). Gadd (2009) conducted a similar silica test with columns packed in chloroform:MeOH 1:1 to clean up samples from dairy shed effluent. E_2-3G recoveries were between 43 and 52%. E_2-17G and E_1-3G recoveries in the same test were significantly higher, as both means were over 80%. Gadd (2009) also tested a 5:1 chloroform:MeOH solvent ratio and successfully improved sulfate recoveries to a range of 87-94%. However, glucuronide recoveries dropped to unacceptable levels and no further testing was performed. Although suppression was improved by some of the

method adjustments pursued in our study, pigmented fractions were still dark and viscous. Therefore, other methods were tested to improve sample quality, prolong the life of the column, and reduce instrument cleaning events.

Table 4.4: Labeled hormones in solvent recoveries using chloroform:methanol (Chl:MeOH 1:1) hand-packed columns with 0, 1, 5 and 10% deactiviated silica.

IPS Corrected		0% Deactivated Silica				1% Deactivated Silica				5% Deactivated Silica				10% Deactivated Silica			
		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
10 mL Chl:MeOH	Wash	45.2	68.4	101.2	100.9	56.5	61.7	97.4	95.7	29.5	32.4	93.5	84.0	27.9	28.5	95.2	96.4
5 mL Chl:MeOH	Rinse 1	22.4	19.8	0.0	0.0	23.8	16.7	0.0	0.0	17.0	14.6	0.0	0.0	7.9	8.2	0.0	0.0
10 mL MeOH	Rinse 2	30.7	34.7	0.0	0.0	35.2	30.3	0.0	0.0	40.8	39.8	0.0	0.0	51.9	49.5	0.0	0.0
Total Recovery (%)		98.3	122.9	101.2	100.9	115.5	108.6	97.4	95.7	87.4	86.7	93.5	84.0	87.7	86.2	95.2	96.4

Only E2 is IPS Corrected		0% Deactivated Silica				1% Deactivated Silica				5% Deactivated Silica				10% Deactivated Silica			
		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2		d3-E2-3G		d4-E2	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
10 mL Chl:MeOH	Wash	25.8	42.9	101.2	100.9	35.7	44.5	97.4	95.7	19.0	21.5	93.5	84.0	15.4	15.5	95.2	96.4
5 mL Chl:MeOH	Rinse 1	10.3	9.4	0.0	0.0	11.6	8.9	0.0	0.0	10.5	8.9	0.0	0.0	5.0	5.5	0.0	0.0
10 mL MeOH	Rinse 2	27.1	27.1	0.0	0.0	28.5	27.1	0.0	0.0	32.3	32.3	0.0	0.0	43.9	38.3	0.0	0.0
Total Recovery (%)		63.1	79.3	101.2	100.9	75.7	80.5	97.4	95.7	61.8	64.8	93.5	84.0	64.3	59.3	95.2	96.4

Table 4.5: Labeled hormone recovery and suppression from fecal samples using deactivated silica hand-packed columns

		0% Deactivated Silica						10% Deactivated Silica						Mean IPS Suppression
		d3-E2-3G			d4-E2			d3-E2-3G			d4-E2			
		A	B	C	A	B	C	A	B	C	A	B	C	
5 mL Chlor:MeOH	Rinse 1	24.0	26.1	32.2	104.7	105.9	118.8	22.6	19.8	28.5	106.0	113.9	109.1	93.9 $\pm$ 0.6
5 mL Chlor:MeOH	Rinse 2	19.6	24.3	19.8	16.0	14.6	12.9	14.5	14.7	16.1	9.5	13.7	11.9	53.2 $\pm$ 1.1
5 ml MeOH	Rinse 3	20.2	21.6	20.2	0.0	0.0	0.0	36.0	37.7	30.7	0.0	0.0	0.0	13.0 $\pm$ 2.0
5 ml MeOH	Rinse 4	3.1	3.7	4.0	0.0	0.0	0.0	11.3	9.2	7.6	0.0	0.0	0.0	30.0 $\pm$ 1.7
Total Recovery (%)		66.9	75.6	76.3	120.7	120.6	131.6	84.3	81.4	82.9	115.5	127.6	120.9	

Table 4.6: Recovery and suppression results using silica packed in 100% ethyl acetate or chloroform:methanol (Chloro:MeOH) to separate labeled hormones from interfering fecal matrix.

Ethyl Acetate Packed	Rinse Solvent	Fraction	Mean IPS Suppression (%)	Mean Recovery		Mean Corrected Recovery		Fraction Appearance
				d3-E2-3G	d4-E2	d3-E2-3G	d4-E2	
0% Deactivated Silica	1 mL EA	1		0.0	0.0	0.0	0.0	Clear
	1 mL EA	2	24.5	0.6	0.0	0.9	0.0	Light yellow
	1 mL EA	3	26.2	0.9	37.5	1.2	50.8	Dark brown
	1 mL EA	4	24.7	0.1	22.0	0.2	29.2	Very light yellow
	1 mL EA	5	8.4	0.0	7.3	0.0	8.2	
	1 mL EA	6	12.6	0.0	0.0	0.0	0.0	
	EA:MeOH	7	56.9	18.1	0.0	43.5	0.0	
	100% MeOH	8	30.1	51.6	0.0	83.4	0.0	
			Totals	**71.3**	**66.8**	**129.1**	**88.2**	
10% Deactivated Silica	1 mL EA	1	13.9	0.0	0.0	0.0	0.0	Clear
	1 mL EA	2	19.2	0.7	8.7	0.8	10.7	Dark brown
	1 mL EA	3	22.6	0.5	42.7	0.6	53.0	Yellow
	1 mL EA	4	19.5	0.0	22.9	0.0	29.6	Very light yellow
	1 mL EA	5	17.6	0.0	0.0	0.0	0.0	
	1 mL EA	6	11.3	0.0	0.0	0.0	0.0	
	EA:MeOH	7	35.7	10.3	0.0	16.4	0.0	
	100% MeOH	8	16.4	54.9	0.0	65.8	0.0	
			Totals	**66.4**	**74.3**	**83.6**	**93.4**	

Table 4.6: (Continued)

Chloroform: MeOH	Rinse Solvent	Fraction	Mean IPS Suppression (%)	Mean Recovery		Mean Corrected Recovery		Fraction Appearance
				d3-E2-3G	d4-E2	d3-E2-3G	d4-E2	
0% Deactivated Silica	1 mL EA	1	28.2	0.0	0.0	0.0	0.0	Clear
	1 mL EA	2	77.0	1.8	2.8	8.3	12.5	Yellow
	1 mL EA	3	92.1	15.2	6.3	200.0	80.8	Dark brown
	1 mL EA	4	75.3	10.4	5.2	42.4	21.2	Very light yellow
	1 mL EA	5	40.2	8.1	0.0	13.6	0.0	
	1 mL EA	6	32.8	10.0	0.0	15.0	0.0	
			Totals	45.6	14.2	279.5	114.6	
10% Deactivated Silica	1 mL EA	1	29.2	0.0	0.0	0.0	0.0	Clear
	1 mL EA	2	80.4	2.8	7.8	15.8	41.1	Dark brown
	1 mL EA	3	81.5	9.4	10.9	50.1	59.0	Dark yellow
	1 mL EA	4	64.0	6.7	5.0	18.7	14.0	Very light yellow
	1 mL EA	5	44.2	3.9	0.0	7.1	0.0	
	1 mL EA	6	26.8	4.5	0.0	6.2	0.0	
			Totals	27.4	23.7	98.0	114.2	

Gel Permeation Chromatography (GPC)

The automated Knauer GPC system using S-X3 Bio beads was the first to be tested. Of the 10 ng of E_2 and E_1 loaded into the system, only 42 and 38% were recovered, respectively. No glucuronides were recovered in any of the three fractions collected.

GPC has been used successfully to remove lipids from fish tissue prior to analysis of estrogens and phenolic endocrine disrupting compounds. Liu et al. (2012) also used a column packed with Bio Beads S-X3 and cyclohexane:Ethyl acetate (1:1 v:v). Recovery of estrogens (E_2, E_1, EE_2 and E_3) were reported to range between 83.3 and 100.6%. Glucuronide conjugates were not targeted in their study. There was no recovery of the glucuronides in our study even when additional volumes of solvent were used to elute the column produced nothing. This GPC system was therefore not pursued.

A second type of GPC system using gravity-fed columns packed with 10 g of Sephadex^{TM} LH-20 beads was evaluated. Recovery of d_3-E_2-3G was 1% and 46% of d_4-E_2 from the EA:HEA column in 3 fractions of 10 mL each. All of the pigmented material passed through the column in the 2nd fraction, and the glucuronides were thought to be bound to the Sephadex such that EA:HEA was too non-polar to elute them. The second trial with 100% EA soaked Sephadex^{TM} proved to be the same, with no d_3-E_2-3G detected after passing 140 mL through the column. This GPC system was also deemed inappropriate.

In a third trial, Sephadex was packed in columns with either EA + 2.5% glacial acetic acid or 100% MeOH. Hormone spikes were added in solvent only and 7 fractions of 5-10 mL each for a total of 50 mL were collected and analyzed. Once again, no d_3-E_2-3G was detected in any of the fractions. Most of the d_4-E_2 recovered was collected in fractions 4-6, and recovery exceeded 130%, suggesting signal enhancement. Results from the MeOH packed

column were considerably better, with over 75% of the d_3-E_2-3G spike being recovered in the first 2 fractions (first 15 mL), and 59% of d_4-E_2 measured in fractions 3 and 4 (15-25 mL).

For the last GPC trial, MeOH extracted feces spiked with labelled hormones was added to a 100% MeOH and Sephadex^{TM} packed column. After the first 5 mL fraction, 15 fractions of 1 mL each were collected and analyzed for hormone spike recovery. Two extra fractions of 5 mL each were collected in an attempt to capture d_4-E_2, because results from the previous test suggested that more than 20 mL of solvent was required for complete elution. Contrary to the first MeOH test, d_4-E_2 eluted first in fractions 11 and 12, which correspond to 15-16 mL of eluted MeOH, and most of d_3-E_2-3G was detected in fractions 13 to 15 (17-19 mL MeOH). Two distinct yellow pigmented bands formed as the sample passed through the column. Each band eluted in the same fractions as the respective spiked hormones. Low recoveries of d_3-E_2-3G (33.5%) were determined, likely due to interfering compounds in the pigmented band. Although the d_4-E_2 recovery was acceptable at 80% without IPS correction, the glucuronide recovery was poor and the sample quality was not improved. The top centimeter of the packing material retained some yellow pigments, even after additional solvent was diverted through the column. Gadd (2009) also observed matrix compounds irreversibly bound to the LH-20 material and suggested that column performance could be affected. Some of the unrecovered glucuronides may also have been bound like the pigments. Changes in viscosity when MeOH was used as the solvent were also reported (Gadd 2009), although this was not encountered in our study. Overall, GPC results were not acceptable for both parent and glucuronides and the technique was deemed inappropriate for LC/MS/MS cleanup of fish feces samples.

Liquid:Liquid Extraction with Lipid Removal Agent Cleanascite[TM]

i. Will Cleanascite[TM] effect glucuronide extraction?

The lipid removal agent, Cleanascite[TM], successfully cleared fecal extracts and removed pigments in preliminary tests. However, it was unknown if the glucuronide conjugated hormones would also be removed. d_3-E_2-3G and d_4-E_2 spiked MeOH samples were treated with LRA volumes of 25, 50, 75 and 100 µL as per the manufacturer's instructions. The addition of Cleanascite[TM] at volumes up to 66% of total sample volume did not affect hormone recovery, particularly glucuronides, by more than 10% (Table 4.7). Spiked MeOH treated with 50 µL of LRA (33% of final sample volume) had the best d_3-E_2-3G and d_4-E_2 recoveries, and total hormone recovery was significantly greater than the other volumes tested ($p < 0.05$). This method was utilized in the next step of testing with fecal samples with acid washing to improve sample clean-up for LC/MS/MS analysis and hormone recovery.

Table 4.7: Mean percent (%) recovery with SEM for labelled hormones in MeOH with the addition of increasing volumes of Cleanascite[TM] Lipid Removal Agent (LRA).

LRA Volume (µL)	d_3-E_2-3G	d_4-E_2	d_4-E_1
25	95.6 ± 0.7	98.6 ± 1.0	90.9 ± 1.7
50	101.9 ± 1.7	111.7 ± 1.6	95.9 ± 4.0
75	98.4 ± 1.1	90.4 ± 1.8	73.9 ± 2.9
100	90.7 ± 1.5	93.9 ± 0.8	101.6 ± 1.8

ii. Acid washing with LRA clean-up

Acid washing of the extracted samples was tested to determine if decreased IPS suppression was caused by matrix interference. A single acid washing cycle did not improve the appearance of the samples, which remained dark amber in colour, indicating the presence of

lipid and/or bile derived compounds. The colouration of the samples was significantly reduced to pale yellow in those treated with 3 acid wash cycles. After the single acid wash, IPS suppression was 96%. Two and three acid washes decreased suppression by 15 and 18%, respectively. Interfering compounds have the same mass transition as E_2-3G, but elute very closely to the parent hormones, E_1, E_2 and their d_4-labelled counterparts used as the RIS and IPS (Figure 4.3). Not only did increasing the number of acid washing cycles reduce the peak height of the interfering compounds, but it also shifted it to the left, further decreasing its potential to mask the IPS signal. Because acid wash is time consuming, and because three acid washes improved the IPS suppression by only 3% over two washes, two washes were included in the final method.

Following the acid wash step, sample dilution and the volume of LRA were adjusted to optimize sample clean-up for LC/MS/MS analysis. When the final sample volume exceeded 350 µL, the ability to detect native E_2 in the samples was negatively affected. Decreases in d_3-E_2-3G recoveries were also observed when the LRA volumes accounted for over 20% of the sample's final volume. Therefore, a final volume of 300 µL with 50 µL of that contributed by LRA was selected for the final protocol because it conserved d_3-E_2-3G recovery and allowed good detection of native hormones. Although the addition of LRA had less of an effect on IPS recovery than simple dilution, its ability to clarify the samples was significant. Removal of the lipids and pigmented compounds that increased viscosity of the samples certainly prolonged the life of the HPLC columns and reduced time loss due to instrument cleaning and maintenance. Details of the tests can be found in Table 4.8.
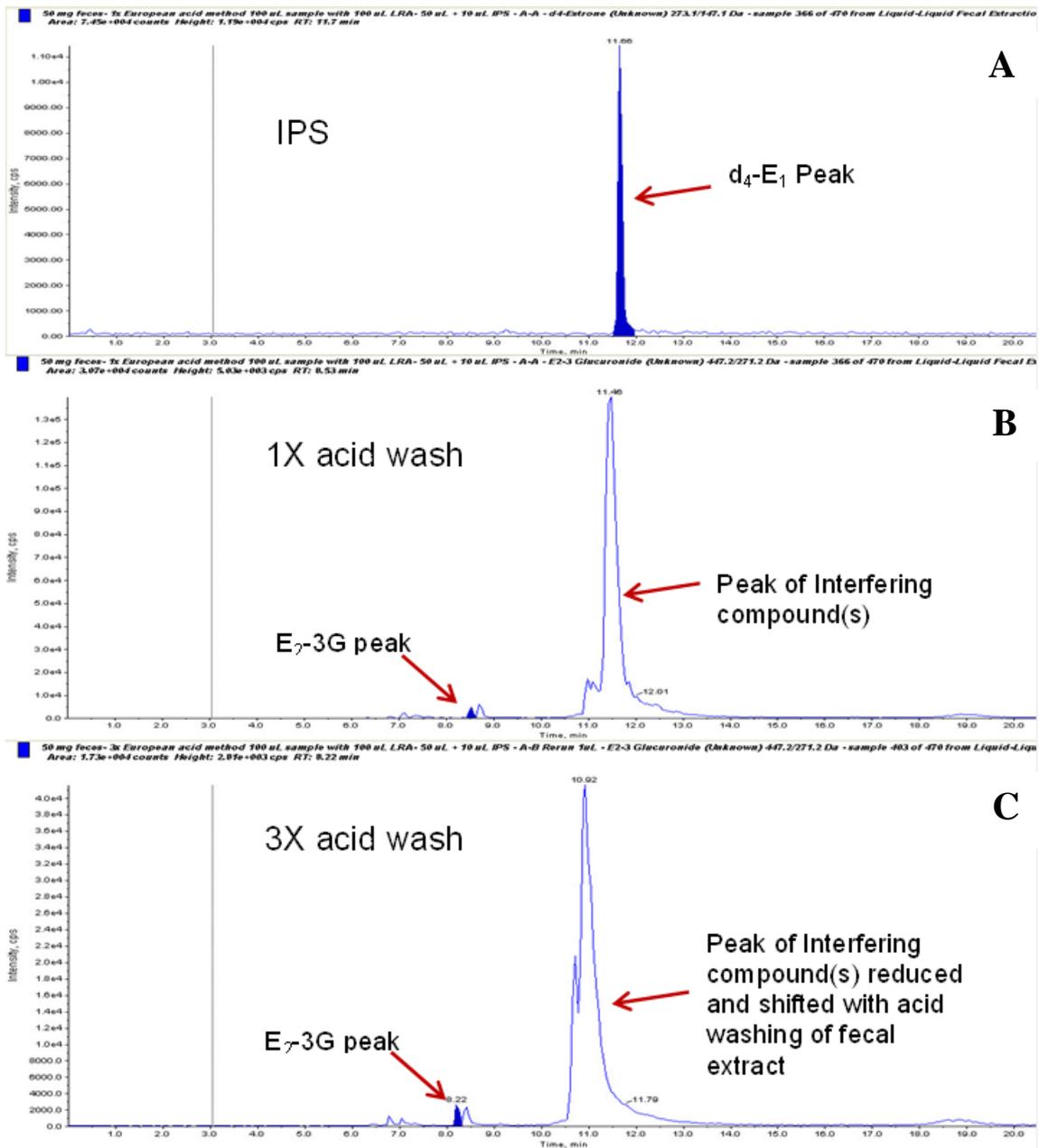
Figure 4.3: Peak of interfering compound(s) with the same mass transitions as E_2-3 glucuronide (B) and elution time as native and labeled E_2 and E_1(A). As acid washes are increased from 1 to 3 times the unknown peak is reduced and shifts left, decreasing suppression effects on parent estrogens.

Table 4.8: Mean percent suppression and recovery with SEM for sample and liquid removal agent (LRA) volumes tested. Ability to measure native E_2 in the samples is indicated. Sample and LRA combination used in final method is highlighted.

Final Sample Volume (µL)	LRA Volume (µL)	LRA % of Final Volume	Mean IPS Suppression (%)	d_3-E_2-3G Recovery (%)	Measurable Native E_2	n
100	0	0	98.7 ± 0.04	41.0 ± 1.6	No	n = 3
250	50	20	84.5 ± 1.7	81.4 ± 2.9	Yes	n = 3
300	0	0	74.5 ± 0.6	77.3 ± 2.0	Yes	n = 5
300	50	17	76.6 ± 0.8	84.0 ± 3.6	Yes	n = 4
300	100	33	77.7 ± 0.8	70.7 ± 1.4	Yes	n = 4
350	100	29	69.8	59.4	Yes	n = 1
400	80	20	73.7 ± 0.4	74.4 ± 0.8	No	n = 6
500	50	10	69.0 ± 1.2	70.1 ± 2.4	No	n = 5
700	50	7	53.6 ± 1.0	69.1 ± 2.1	No	n = 5
1000	0	0	55.2	70.8	No	n = 1

iii.   Determination of d_3-E_2-3G Suppression and the Final Method

To determine suppression of d_3-E_2-3G and native glucuronide conjugated hormones, d_3-E_2-3G was added as an IPS just prior to analysis instead of as the RIS. Signal suppression accounted for $12.8 \pm 1.9\%$ of the d_3-E_2-3G loss when used as a RIS. This indicates that the method consistently extracts $\geq 85\%$ of glucuronides from rainbow trout feces. Before signal suppression was factored in, d_3-E_2-3G recoveries ranged from just over 70% (Table 4.9) up to the mid 84% (Table 4.8). The results also emphasizes the importance of using multiple labeled internal standards when specific groups of hormones are being analyzed, particularly when those compounds have different polarity (Taylor 2005). Correcting the glucuronide results from fecal extracts with d_4-E_2 and the IPS (d_4-E_1) would have grossly over-estimated recovery and suppression, respectively.

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Table 4.9: Recovery comparison of the 2-Step versus Acid Pre-treatment Final Methods (n=6)

	IPS (d_4-E_1) Recovery (%)	d_3-E_2-3G Recovery (%)	d_4-E_2 Recovery (%)	d_4-E_2 IPS Corrected (%)
Step 1: Hex:EA 3:2 no acid pre-treatment, extract 2x acid wash	86.3 ± 1.1	0.0	56.3 ± 2.7	65.5 ± 3.7
Step 2: Add acid, extract with EA, extract 2x acid wash	18.6 ± 1.3	72.6 ± 2.0	5.3 ± 0.3	28.7 ± 1.5
12.8% d_3-E_2-3G Suppression		**83.3 ± 2.3**		**94.1 ± 2.6**
Acid pre-treatment method, Extract 2x acid wash	18.9 ± 0.3	76.8 ± 1.8	18.2 ± 0.5	96.2 ± 1.3
12.8 % Suppression		**88.1 ± 2.0**		**96.2 ± 1.3**

Wetting the fecal sample with 2M HCl to improve glucuronide conjugate extraction facilitated the extraction of compounds which co-eluted with the parent estrogens, and suppressed their signal, during LC/MS/MS analysis. The use of a hexane:ethyl acetate 3:2 (v:v) solvent extraction step before acidification for glucuronide extraction was explored to improve recovery of the parent hormones. As expected, extracting the fecal samples first with the polar solvent blend significantly decreased IPS suppression from approximately 80% to under 15% (Table 4.9). Once the 2M HCl was added for Step 2 of the extraction, the IPS suppression was returned to >80%. The recovery of d_4-E_2 was >50% before IPS suppression correction, enabling detection of endogenous parent estrogens in situations where lower concentrations are encountered. Once corrections were made for IPS suppression and previously calculated d_3-E_2-3G suppression, the results were very similar between the 2-Step and Acid pre-treatment methods. There was less than 5% difference between both d_4-E_2 and d_3-E_2-3G recovery when comparing methods. The added pre-acid extraction step doubles the sample preparation time

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and LC/MS/MS analysis time, but this trade-off reduces suppression, which could improve the ability to detect lower endogenous E_2. Lower E_2 concentrations would be expected in fish during post-spawning or juvenile life stages. Preliminary tests should always be conducted to determine the appropriate method for a given set of samples. The final fish feces extraction protcol can be found in Figure 4.4.

Having established a workable method for feces extraction and LC/MS/MS analysis of parent and conjugates hormones, the technique was applied to examine relationships between hormone concentrations in feces and the corresponding values in plasma. These studies are described in the following section.

50 mg freeze-dried fecal material ground to powder
+ Recovery Internal Standard (RIS)

Non-Polar Parent Hormones

Extract with 3 mL 3:2 Hexane:Ethyl Acetate
Vortex for 1 minute
Centrifuge at 4000 rpm for 5 minutes
Aspirate, Repeat, Combine Fractions

Polar Glucuronide Conjugates

Wet sample with 1 mL 2M HCl
Vortex and set aside for 1 hour

Extract with 3 mL Ethyl Acetate
Vortex for 1 minute
Centrifuge at 4000 rpm for 5 minutes
Aspirate, Repeat, Combine Fractions

Evaporate to 1 mL under N2 (Do not combine Polar and Non-Polar Fractions)

Acid Washing

Add 1 mL 2M HCl
Vortex for 1 minute
Extract with 3 mL Ethyl Acetate
Centrifuge at 4000 rpm for 5 minutes
Aspirate, Evaporate to 1 mL under N2
Repeat

Clarifying Sample with Cleanascite™ Lipid Removal Agent

Transfer sample to 1.5 mL microcentrifuge tube
Evaporate to Dryness under N2
Reconstitute with 250 µL MeOH
Vortex
Add 50 µL Cleanascite™
Gently Vortex
Refrigerate Overnight
Centrifuge 10 minutes at 13000 rpm

LC/MS/MS Analysis

Transfer 50 µL of supernatant to autosampler vial with 200 µL insert
+ Internal Precision Standard (IPS)

Figure 4.4: The final feces extraction protocol to facilitate targeting non-polar parent steroids or polar glucuronide conjugates. If parent hormone concentrations are expected to be low, as in juveniles or post-spawning fish, they are targeted with the non-polar extraction first. The pH can then be decreased and conjugates extracted. Analyze extracts separately on the LC/MS/MS because the polar extract contains matrix components that interfere with detection of low concentration parent hormones. If parent hormone concentrations are sufficiently high then the polar conjugate method will adequately capture both polar and non-polar hormones.

Comparison of fecal and plasma hormone concentrations through time for method validation

Plasma and feces were collected from 8 female rainbow trout to determine if parent and conjugated estrogen levels in the fecal samples could predict circulating plasma estrogen concentrations. E_2 was the predominant estrogen measured in the plasma of these fish, with concentrations ranging from 1000 to 82000 pg/mL. The 2nd highest estrogen concentration was either E_1 or E_2-3S: both typically ranged from 300 to 6000 pg/mL. E_1-3S was the lowest concentration of estrogens in plasma: 50 to 330 pg/mL. In contrast, in the feces, parent hormones were lowest; E_2 ranged from 16 to 769 pg/mg. E_1 concentrations were between 2 and 80 pg/mg and 48% of the 107 samples had non-detectable levels. The predominant estrogen glucuronide found in the rainbow trout feces was E_2-17G at 24 to 2925 pg/mg. E_2-3G and E_1-3G in trout feces were very similar ranging from 4 to 390 pg/mg.

Changing plasma concentrations of hormones were typically not reflected in feces until the following week (Figure 4.5). Estrogen concentrations were also more stable in feces than in plasma, often with a 10-fold change in plasma corresponding to a 2-4 fold change in the feces (Figure 4.6). This is likely because steroid measurements in feces represent an integrated pool of free hormones metabolized from the plasma over a period of time. In contrast, plasma samples are snap-shots of an individual's hormone levels at that moment (Goymann 2005). The length of the metabolite integration period in feces depends on defecation rates, which can vary between species. For example this period ranges from a few minutes to hours in birds (Goymann 2005), 1 or 2 days in large carnivores (Palme et al. 2005), and up to 2 weeks in some lizard species (Alexander et al. 2001). Many factors can affect defecation rates: diet (Klasing 2005), temperature (Alexander et al. 2001), feeding rates (Goymann et al. 2006),

season (Rogers et al. 1987), age of the animal (Seraphin et al. 2008), circadian rhythms (Sousa & Ziegler 1998) and sex of the animal (Cavigelli et al. 2005). In many cases, these factors are interrelated (Palme 2005, Goymann 2012).

The ability to correlate hormone measurements in feces with increases or decreases in plasma concentrations also depends on the pattern of the hormone released into circulation. If hormones are released as a single pulse, (eg.cortisol during an acute stress event) it is unlikely that the peak would contribute much to the overall pool of metabolized cortisol in the feces. It would also be difficult in a field situation to collect a plasma sample without causing additional handling stress.
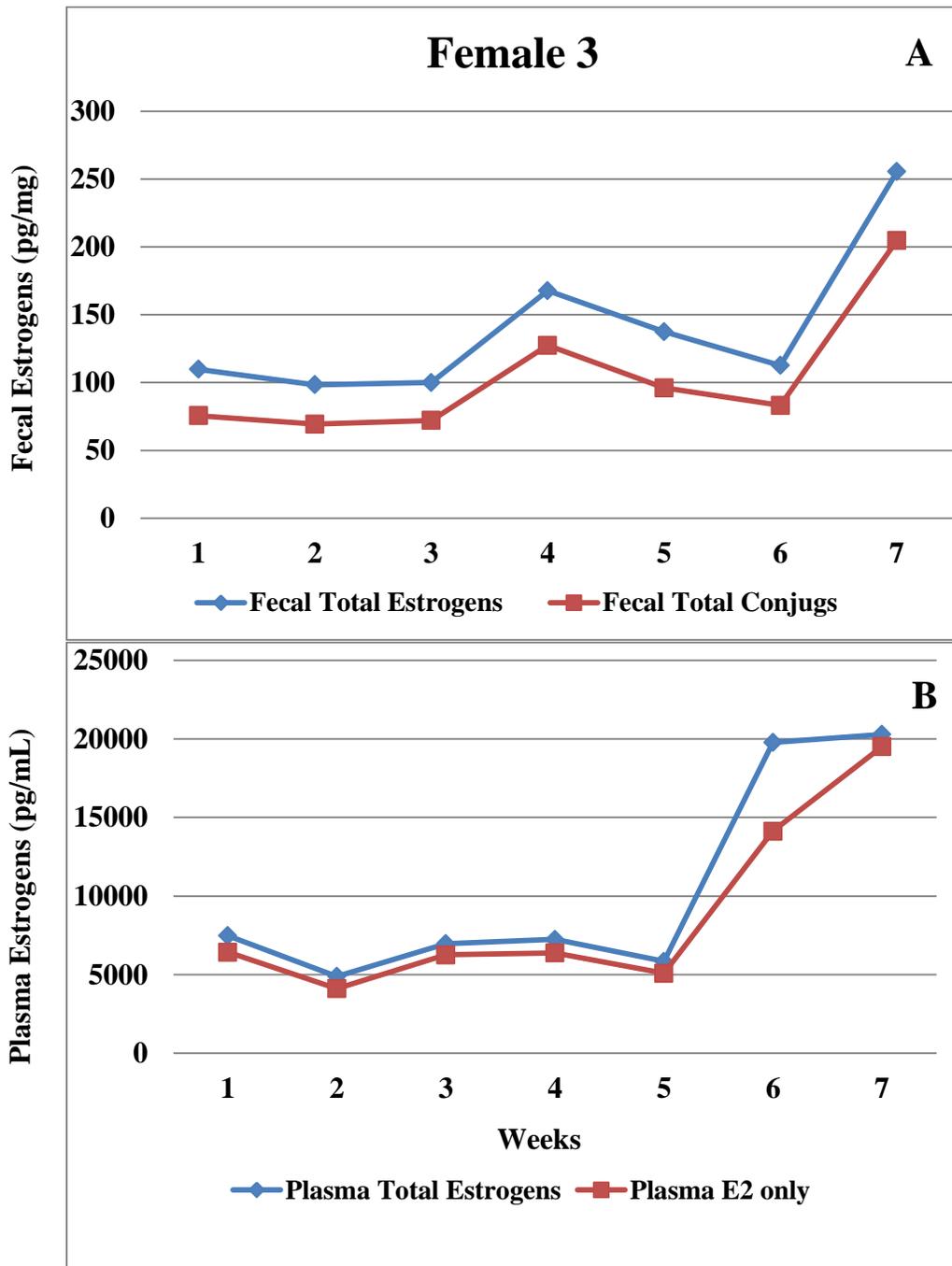
Figure 4.5: Paired plasma and fecal estrogen analyses demonstrating how an increase in plasma hormone concentrations at week 6 (B) is not reflected in the fecal concentrations until week 7 (A).
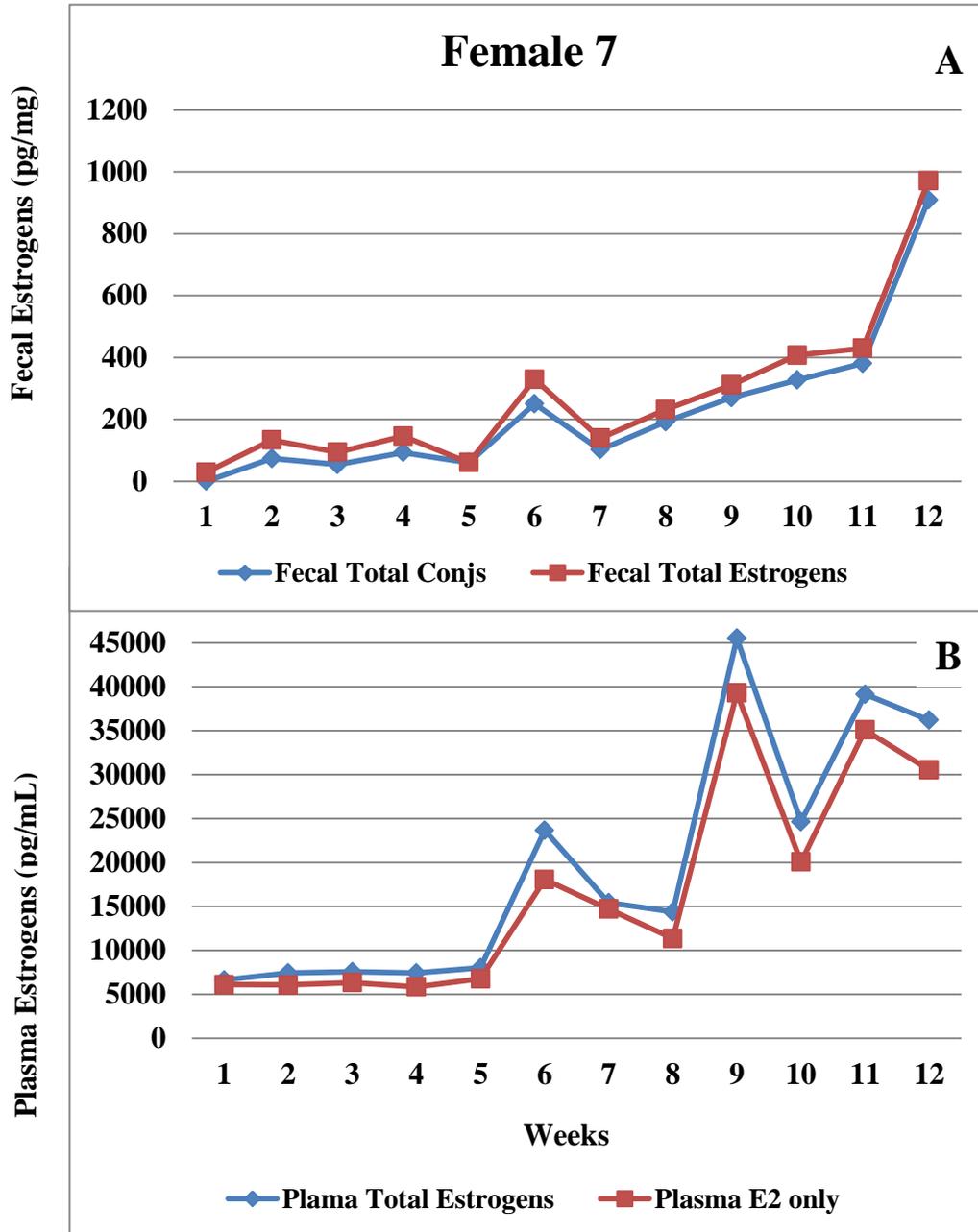
Figure 4.6: Paired plasma and fecal estrogen analyses demonstrating how a 10-fold increase in plasma hormone concentration (B) results in only 2-4 fold change in fecal concentrations (A).

Longer-term increases in circulating hormone concentrations (eg. during chronic stress, or a spawning event or the estrus cycle) would be more likely reflected in the feces hormone concentrations (Goymann 2005). Figure 4.7 adapted from Goymann (2005) illustrates the difference in how a pulse or prolonged hormone release could affect concentrations measured in both plasma and feces.
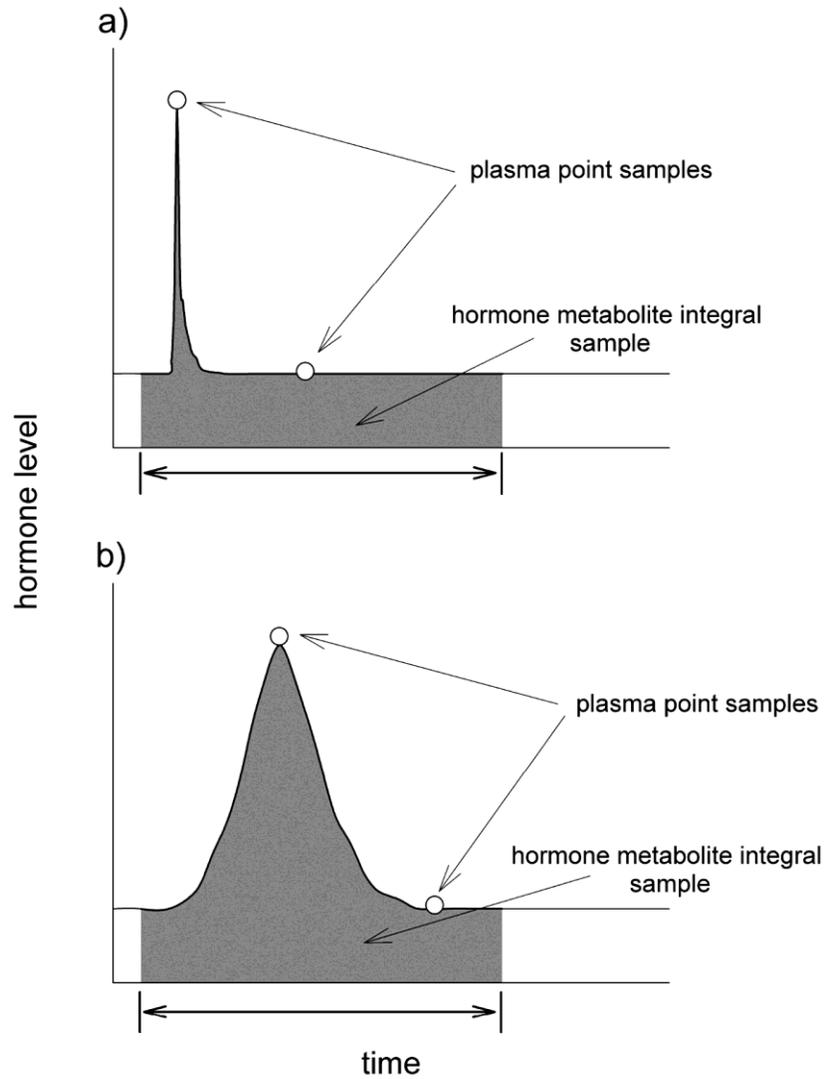
Figure 4.7: This illustrates how plasma hormone measurements are "snap shot" point samples (open dots) while fecal hormone measurements are an integrated pool of metabolites over a longer period of time (gray shading). A brief spike in hormone concentrations (a) would be difficult to detect in feces, but a long term peak (b) contributes more to the total pool represented by the area under the peak (Goymann 2005).

Plasma concentrations of E_2 are most reported for females. Therefore, determining which hormones, or groups of hormones and conjugates, in feces would best predict circulating E_2 was of interest, particularly for the development of a non-invasive hormone sampling protocol. Regression analyses determined that fecal concentrations of E_2-17G best predicted plasma E_2 concentrations in female rainbow trout. This relationship was significant (p < 0.01), with plasma levels accounting for 54% of the variation in feces concentrations (Figure 4.8). The strongest relationship was between plasma E_2-3S and fecal E_2-17G ($r^2 = 0.64$, p < 0.01) (Figure 4.9).
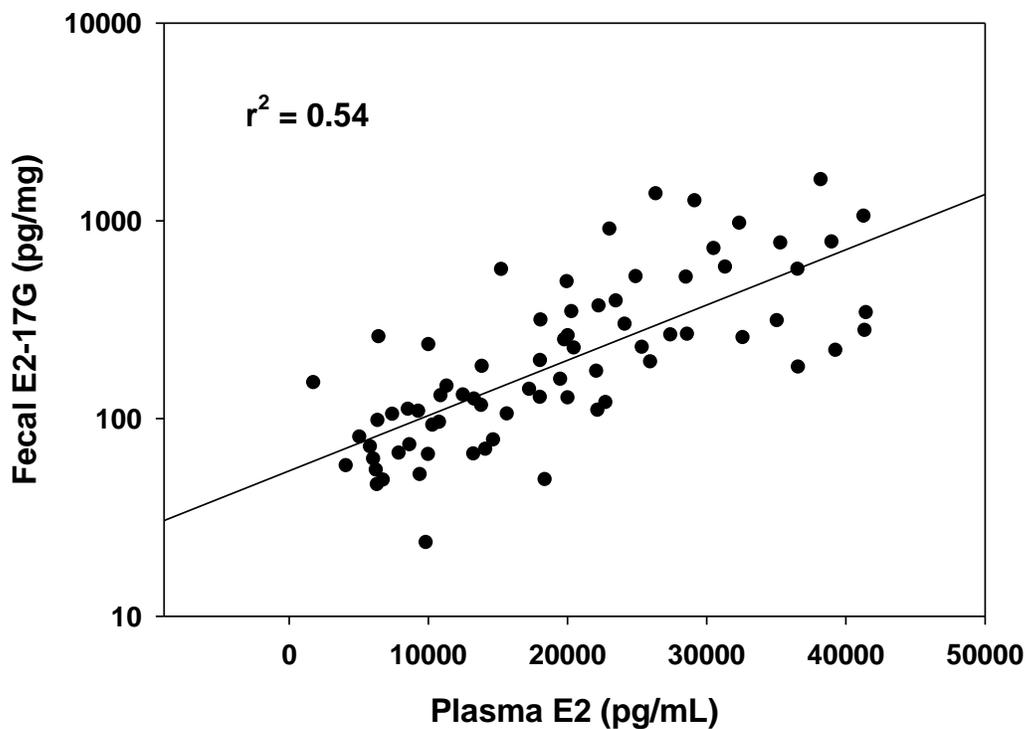
Figure 4.8: Fecal measurements of E_2-17 glurcuronide (E_2-17G) best predicted ($r^2 = 0.54$) plasma E_2 concentrations in female rainbow trout (p<0.01).
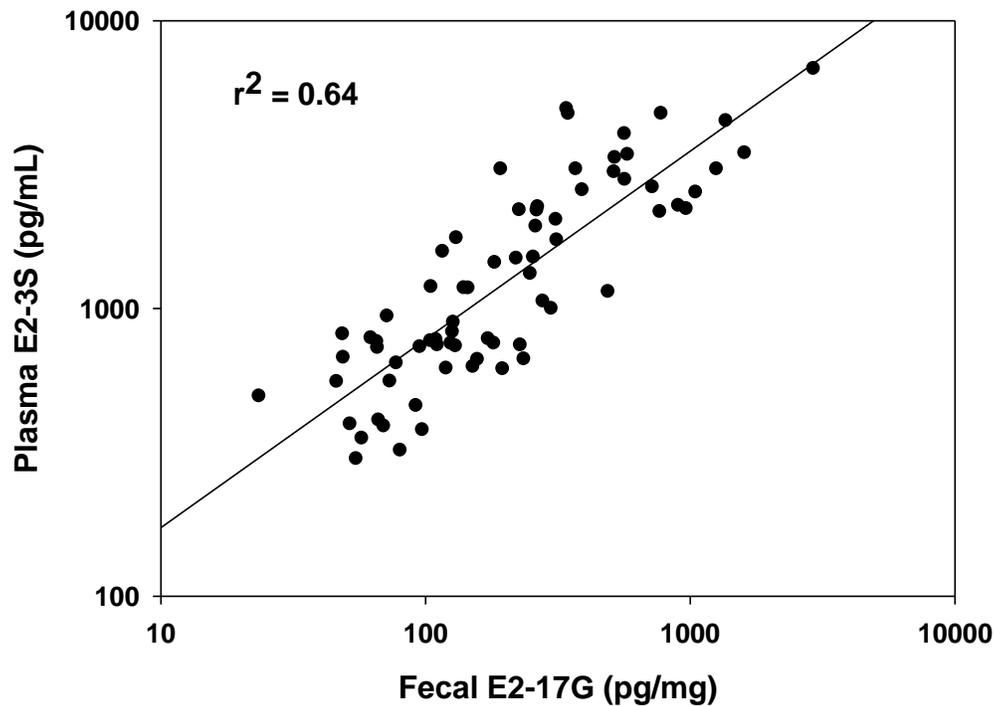
Figure 4.9: The strongest relationship ($r^2 = 0.64$) between plasma and fecal estrogens and metabolites measured was found between plasma E_2-3 sulfate (E_2-3S) and fecal E_2-17 glucuronide (E_2-17G) ($p<0.01$).

Macchi et al. (2010) reported similar results ($r^2 = 0.58$, $p < 0.01$) between plasma and fecal E_2 levels in wild boar (*Sus scrofa*). The correlation coefficient (r^2) between free fecal estrogen and plasma estrogen conjugates throughout a non-conception ovarian cycle in Goeldi's monkeys (*Callimico goeldii*) was 0.65 (Pryce et al. 1964). Free plasma cortisol and fecal cortisol metabolites were also correlated ($r^2 = 0.59$, $p < 0.05$) in free-ranging snowshoe hares (*Lepus americanus*) (Sheriff et al. 2010).

Using a series of experiments, several facets of parent and conjugated steroid extraction and analysis from fish feces have been examined. Ultimately, a method was developed and optimized that allows feces to be extracted and then analyzed using

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LC/MS/MS. Acceptable detection of both parent and conjugated estrogens was achieved and optimized. Finally, the technique was validated to show that fecal E_2 metabolites are a good predictor of circulating E_2 concentrations in rainbow trout. Additional work is required to test this method as a non-lethal measure of reproductive status and health in small-bodied fish, as they are more frequently targeted in environmental monitoring programs.

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Appendix

Phosphate Buffer Recipe

0.1M sodium phosphate monobasic
0.1M sodium phosphate dibasic
0.1% bovine serum albumen
0.5% Tween 20
20% Methanol
0.9% NaCl

Add above to 800 mL of MilliQ distilled water and mix for 15 minutes or until dissolved. Fill to 1 litre with MilliQ water.

Citrate Buffer Receipe

0.05M citrate
0.15M 0.1% sodium azide
0.1% gelatin
0.1% Brij
20% methanol

Add above to 800 mL of MilliQ distilled water. Heat to 45-50 ^{o}C while mixing for 15 minutes or until gelatin has dissolved. Fill to 1 litre with MilliQ water.

Chapter 5: Uptake of Radiolabelled E_2 from Gut and Clearance from Plasma in Rainbow Trout

Abstract

In order for fecal hormones to effectively be used as an environmental monitoring tool for determining reproductive health in fish, a thorough understanding of the endpoint is essential. Fecal steroid measurements represent an integrated pool of metabolized parent hormones cleared from circulation over a period of time. Because there is more than one route for hormones to enter the intestine, and enterohepatic circulation can result in steroids reentering the plasma pool, the assessment of steroid metabolism and excretion is complicated. Using radiolabeled E_2 injected directly to the circulation of Rainbow trout via the dorsal aorta, plasma clearance time and partitioning of the hormone into feces, urine and bile was monitored. A second experiment was conducted where E2-H3 was introduced directly into the gut of rainbow trout using gelatin as a vehicle to determine the role enterohepatic circulation plays in plasma hormone balance and excretion. In the plasma injected fish, E_2-H^3 was measured in the 24 hour fecal sample and peaked at the 48 hour sample. After 72 hours post-injection, over 35% of the labeled hormone was collected in feces and bile, approximately 10% in urine, and only 0.4% had been taken up into the muscle, kidney, gonad and liver. The labeled estradiol injected into the gut appeared in the plasma within 30 minutes, peaked after 4 hours, and was finally cleared within 96 hours. Routes of excretion and tissue distribution results were similar to the plasma injection experiment. Approximately 68% of the E_2-H^3 injected into the gut entered the enterohepatic circulation. These studies demonstrated that

enterohepatic circulation can be an important route of exposure to natural and synthetic exogenous hormones in fish living in effluent receiving environments, particularly sewage effluent. Not only may particles be ingested, but teleosts may drink seawater containing effluent to maintain osmoregulatory balance, allowing compounds to enter via the gut. Understanding plasma hormone clearance, and the role of dietary uptake and enterophepatic recirculation in hormone excretion, will facilitate the development of fecal sex steroid analysis as a tool for non-lethal monitoring of fish reproductive health.

Introduction

Environmental monitoring programs have traditionally used lethal sampling and tissue contaminant analysis of wild animals to examine potential anthropogenic impacts. However, increasing public and regulatory pressure to reduce lethal sampling has driven the development of new techniques that do not require lethal sampling. For wild fish populations, these have included muscle biopsy and fin clip contaminant analysis (Červenka et al. 2011; Rolfhus et al. 2008), minimally invasive assessments of population structure (Arciszewski et al. 2010), and ultrasound analysis of gonad size (Petochi et al. 2011; Evans et al. 2004). While these methods represent an important step forward for environmental toxicology, they provide limited information regarding the reproductive status of wild fish.

In the previous chapter of this thesis I reported that reproductive steroid hormones measured in fish feces are closely correlated with concentrations in circulation (see Chapter 4). However, fecal concentrations are integrated measures, representing a sum total of steroids cleared from the circulation over time. Free or unbound lipophilic sex steroids, including estradiol (E_2), are cleared from the circulation by conjugation enzymes. Conjugation involves the addition of glucuronic acid or sulfate moieties to increase the polarity and solubility of the hormones. This activity facilitates excretion of the parent lipid soluble steroid in the polar urine or feces. Conjugation primarily takes place in the liver of fish, but may also take place in extra-hepatic tissues including the gonads, gills and intestine (Pankhurst 2008; Leguen et al. 2000; Kime & Ebrahimi 1997, Clarke et al. 1991; James et al. 2001).

Depending on their structures, conjugates can have different eventual excretion pathways, with only some finding their way into feces. For example, sulfate conjugates derived from the liver enter circulation where they are eventually filtered by the kidneys and excreted in urine. Glucuronide conjugated hormones also enter the circulation, but they are typically excreted from the liver into the gall bladder as a component of bile (Whitten et al. 1998; Clark et al. 1991; Clarke et al. 1992). After a meal, bile is secreted into the intestine to aid in digestion and absorption of lipids. Bile is also the excretion route for endogenous substances, including conjugated steroids and xenobiotics (Grosell 2000; Clarke et al. 1991).

To be excreted in feces, steroid hormones can enter the intestine via bile or through the bloodstream. However, the presence of a conjugated steroid molecule in the intestine does not necessarily mean that it will ultimately be excreted in feces. Conjugated steroid molecules can be deconjugated by bacterial glucuronidase or mucosal cell glucuronidase (Whitten et al. 1998; James et al. 2001), releasing the parent steroid molecule so that it can be reabsorbed into the bloodstream. This cyclical deposition from the circulation into the gut and re-release back to the circulation is referred to as enterohepatic circulation, and can prolong the in vivo residence time of steroids in the fish. With more than one route to the intestine, and the phenomenon of enterohepatic circulation, it becomes clear that fecal steroid measurements represent an integrated pool of metabolized parent hormones over a period of time (Figure 5.1). This is in contrast to plasma samples which are snap-shots of circulating hormone levels at that moment (Goymann 2005).
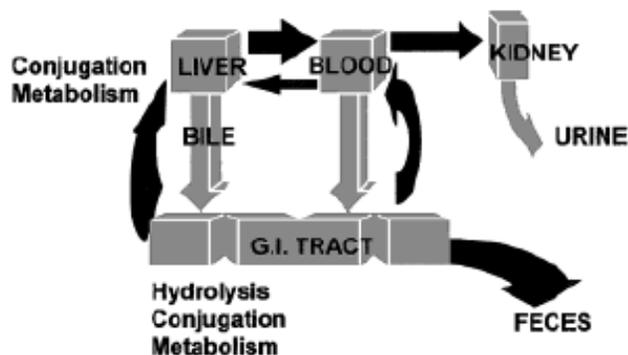
Figure 5.1 Pathways of metabolism, reabsorption and excretion of steroid hormones (Whitten et al. 1998).

For the purposes of environmental monitoring, the analysis of steroid hormones in fecal samples becomes further complicated because fish living in contaminated waterways can absorb hormones or hormone active substances from the environment, either across the gills or from the diet (Runnalls et al. 2010; Maunder et al. 2007). Lipophilic, unconjugated sex hormones can be ingested bound to various particle sizes. Many coastal towns still discharge directly into marine and estuarine environments (Blewett et al. 2013a) and particles from these sources are known to be ingested by fish. Since euryhaline and marine teleosts must also drink the seawater to maintain osmoregulatory balance, the gut and enterohepatic circulation can be other routes of steroid uptake (Blewett et al. 2013b).

In order to effectively use fecal hormones as a method to determine reproductive health in fish, a thorough understanding of the endpoint is required. The study described in this chapter addressed this objective by injecting radiolabelled estradiol (E_2-H^3) directly to the circulation of adult rainbow trout and determining the clearance time and partitioning of E_2-H^3 into feces, urine and bile. The deposition of E_2-H^3 in tissues was also analyzed to estimate tissue uptake and loss through the gills. In a follow up

experiment, E_2-H^3 was introduced directly to the gut of fish in gelatin vehicle (i.e. gavage exposure) to simulate a meal laden with exogenous hormone. Appearance of the labelled E_2 in the plasma, as well as the percentage of labelled hormone that simply passed through the gut was monitored. Distribution into various tissues and bile were again determined. Together this data increased understanding of the role of enterohepatic circulation in plasma hormone concentrations, especially for fish living in sewage treatment plumes. The data provide additional information for the continued development of fecal sex steroid analysis as a tool for non-lethal monitoring of fish reproductive health.

Methods & Materials

Chemicals and Surgical Supplies

All solvents (ethyl ether, methanol, ethanol, acetonitrile, acetone, chloroform, ethyl acetate, and hexane), Optima water and Scintiverse scintillation cocktail were purchased from Fisher Scientific (ON, Canada). The anaesthetic, tricaine methanesulfonate (MS-222), was purchased from Syndel (Nanaimo, BC, Canada). Supplies for taking fish blood samples, plasma collection (1.5 inch 22 gauge needles, 16 gauge needles, 1 mL and 3mL syringes, Vacutainers) and urinary catheter tubing were purchased from (BD Diagnostics, NJ, USA). Tygon tubing for the fecal catheters was purchased from Norton Performance Plastics (Akron, Ohio). H^3-Estradiol (H^3-E_2) was purchased from Perkin Elmer (Woodbridge, ON, Canada)

Fish

Juvenile female rainbow trout (50 g) (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Hatchery (Thamesford, ON Canada) and held in an 800L tank at 13°C with a 12 hour light/dark cycle at the Freshwater Institute, Winnipeg, MB until they were approximately 500 to 800 g in mass. Fish were fed approximately 1.5% of their body weight six times per week. Fish were held and utilized as per DFO Animal Use Protocol FWI-ACC-2011-014.

Radiolabelled E_2-H^3 plasma injections

The experiment required that the fish be both catheterized and cannulated to collect blood, urine and feces at each time period. The E_2-H^3 labeled plasma was prepared from a pool of plasma collected from fish obtained from the same stock. To remove endogenous hormones, 5 mg of activated charcoal was added to 10 mL of plasma in a borosilicate test tube and vortexed to mix. The plasma mixture was refrigerated over-night then centrifuged at 4000 rpm for 30 minutes (Palace et al. 1997). The steroid-free plasma supernatant was removed and a 1 mL solution was made with 39 μL of E_2-H^3 (1.0mCi/mL), for a labeled hormone concentration of 1.5 ng/mL.

Eight fish underwent procedures to surgically insert an aortic cannula as well as anal and urinary catheters as described by Palace et al. (1997), Black et al. (1995) and Scott & Liley (1994), respectively (Figure 5.2).
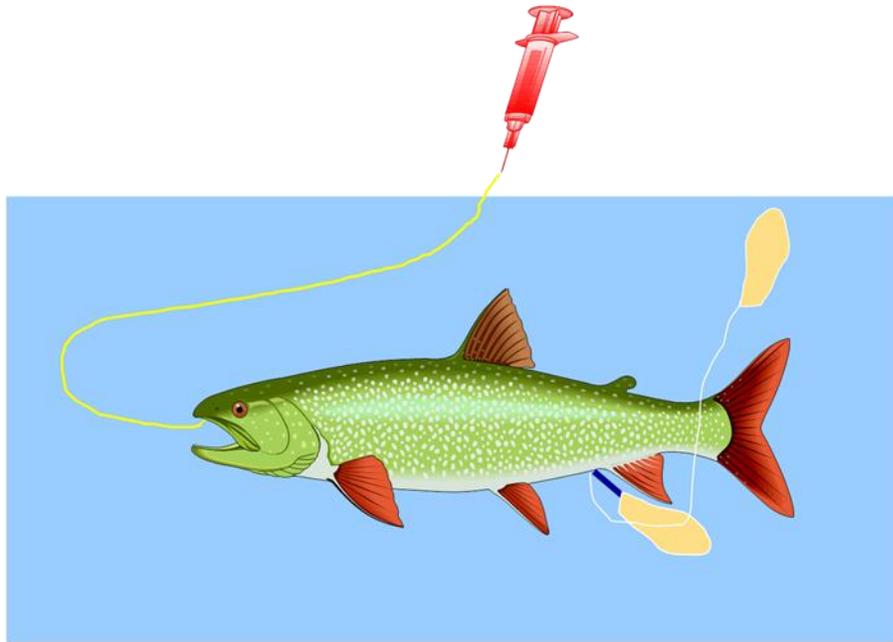
Figure 5.2: Dorsal aortic cannula, and urinary and fecal catheters to collect excreta from rainbow trout.

Briefly, each fish was lightly anesthetized and secured on a surgical table. Aerated physiological saline (0.9% NaCl) and saline containing 0.2 g MS222/L were alternately directed through the buccal cavity as required to maintain anesthesia so that the fish exhibited only slight fin movement. Physiological saline was used to irrigate the fish during surgery to reduce osmotic stress during the procedure, as outlined by Brown et al. (1986). Approximately 15 Litres of saline/aesthetic was required for the surgery.

An 18 gauge catheter filled with physiological saline containing 25 U heparin per ml was inserted into the aorta through the palate using a 20 gauge needle as described by Palace et al. (1997). A polyethylene tubing cannula (ID 0.58 mm, OD 0.965 mm, total length approximately 100 cm) (Clay Adams, NJ) also filled with 25 U heparin per ml in saline was fed through the catheter and into the aorta. The cannula was then sutured in place on the palate in three places and the catheter was removed. The insertion area was

treated with Polysporin antibacterial ointment (Pfizer Canada, Markham, ON) post operatively. Unobstructed blood flow from the aorta through the cannula was confirmed by pulsatory blood flow at the end of the cannula. Using a syringe, the cannula was refilled with saline and heparin, and the end of the cannula was sealed with Critoseal plastic putty (Monject Scientific, St. Louis, Mo).

The urinary catheter and collection vessel consisted of PE205 polyethylene tubing which had a latex balloon secured to the end to collect the urine. Holes were cut along the sides of the tubing for the top 1.5 cm of the insertion end of the tubing to facilitate urine entry into the tube. A 5 mm anchor collar was fitted onto the tubing 2 cm from the end and secured with surgical glue. The saline filled tubing was then inserted into the urinary papillae until the anchoring collar was not visible, approximately one quarter to half-way into the fish bladder. The tubing was tied to the urinary papillae and sutured to the anal fin of the fish. The latex balloon floated freely at the side of the fish and did not appear to impede swimming.

For anal catheterization, polyethylene tubing (ID 6mm, OD 7mm, total length approximately 4 cm) was fitted with a 5 mm wide anchoring collar of tubing approximately 5 mm from the end of the catheter. A purse-string suture was stitched around the anus of the fish. The end of the tubing without the collar was inserted about 1.5-2cm into the anus of the fish and the purse string was tightened and stitched to the anchoring collar. The catheter was anchored to the anal fin. A latex balloon was fastened to the free end of the anal catheter with the anchoring collar for the collection of feces.

The complete surgery (1 cannula and 2 catheters) took approximately 15 minutes after anaesthetization. After surgery, each fish recovered and was housed individually in a 185 liter aerated fiberglass tank receiving 200 ml/min dechlorinated water (14 ± 1ºC). An automatic lighting system provided the required 12 hour light/dark cycle. Additional aeration was supplied by air stones to maintain oxygen saturation at >90%. Tank volume, water depth and cannula length were such that blood could be withdrawn, and heparin-saline solution administered through the cannula above the surface without handling or disturbing the fish. The tubing length allowed it to hang outside of the tank and provided ample slack for the fish to swim during sampling.

Once the fish began to recover, the end of the aortic cannula was cut and a 16 gauge blunt needle with syringe was attached. This syringe was used to withdraw the heparinized saline in the cannula and blood at the distal end of the cannula. The syringe was removed and replaced with a multi-way valve. Two syringes were attached to the multi-way valve, one containing the spiked plasma and the other with heparinized saline. Each fish was injected with 100 µL of spiked plasma via the cannula followed by 300 µL of heparinized saline. The internal volume of the 100 cm of tubing was calculated to be 264 µL, therefore 300 µL was sufficient to deliver the spiked plasma through the cannula and refill it with heparinized saline. The tanks were covered to reduce stress of the fish and allowed blood sampling to occur without the fish further stressing the fish.

Sample collection

Blood samples were collected at time 0, 10 minutes, and 1, 2, 4, 8, 16, 24, 36, and 48 hours. For each sampling, the cannula was cut, and the saline with 250 u/mL heparin was withdrawn with a syringe fitted with a 16 gauge blunt needle. A fresh syringe was used to collect a 200 µL blood sample. A third syringe filled with saline and 250 U heparin/mL was then attached to the needle and the residual blood in the tube was returned into the fish, followed by an extra 30 µL saline with heparin to prevent clotting in the tubing prior to the next sampling time. The total volume of blood in a 500g fish is approximately 20.5 mL (Gingerich & Pityer 1989). Therefore, at any given sampling only 200 µL was removed from the fish (approximately 1 % of the total volume). Blood samples were centrifuged, and 100 µL of plasma was pipetted into a 20 mL scintillation vial with 10 mL Scintiverse, scintillation cocktail and vortexed. Counts per minute (CPM) were determined using a Packard Tri-Carb 2100TR liquid scintillation counter. Any extra plasma was frozen at -80ºC.

The fecal and urinary bags were changed every 24 hours. Flow to the tanks was stopped, and water was slowly drained until approximately 12 inches remained in the tank. Water with 80 mg/L MS 222 was added to the tanks to anaesthetize the fish. The fish was removed from the water and held on a dissecting table while the bags were changed (total handling time < 1 minute). The fish was returned to the water, and any feces that had not been collected in the bag was removed from the tank and stored in 20 mL scintillation vials. Flow was returned to the tank and the fish was monitored until fully recovered. The contents of the fecal and urinary bags were stored in 20 mL scintillation vials at -80ºC until analyzed.

After the last sampling time, each fish was individually anaesthetized with 0.5g/L of pH buffered MS-222 until operculum and fin movements ceased. Blood was collected from the caudal artery using heparinized 22G needles on 5cc syringes. Urine bags were removed and sample was pipetted into 20 mL scintillation vials. The spinal cord was severed and the predominant tissues that could contain estrogen receptors (liver, gonad, muscle) and estrogen metabolites (bile, kidney and intestinal contents) were sampled for E_2-H^3 content. Organs and muscle samples were weighed and frozen at -80°C. The fish was carefully dissected to expose the gall bladder, and bile was also collected in a syringe by puncturing gall bladder with a 22 gauge needle. The sample was stored in 20 mL scintillation vials until analyses.

Gavage

Six fish were anaesthetized and aortic cannulas surgically inserted as described above. Gelatin containing radiolabeled estradiol, E_2-H^3, was injected into the fish as described by Palace et al. (1996). To determine the individual dose, each fish was weighed and the required volume of warmed ethanol/gelatin containing the hormone (concentration = 50 ng/ml, corresponding to an activity of 13.2 μCi activity per 1 kg of body weight) was administered. Spiked gelatin was prepared by dissolving 1 gram of 60 bloom gelatin (Sigma Chem. Co., St. Louis, MO.) in 15 mL of MilliQ water in a 32ºC water bath. E_2-H^3 (1.0 mCi/mL) was added and the solution was delivered directly to the stomach through polyethylene tubing (ID 1.57 mm) attached to a 16 gauge needle mounted on a 3 ml syringe (Figure 5.3). The warmed gelatin quickly cools and sets in the gut of fish, reducing regurgitation and facilitating a full dose delivery to the gut.

Following oral dosing, the fish recovered in the experiment holding tanks. Each surgery was approximately 10 minutes after anaesthesia. Fish were housed in individual 15 liter glass aquaria receiving 0.25 L/min dechlorinated water (12 ± 1ºC). Aeration was supplied by air stones and an automatic light system will provide the required 12 hour light/dark cycle.
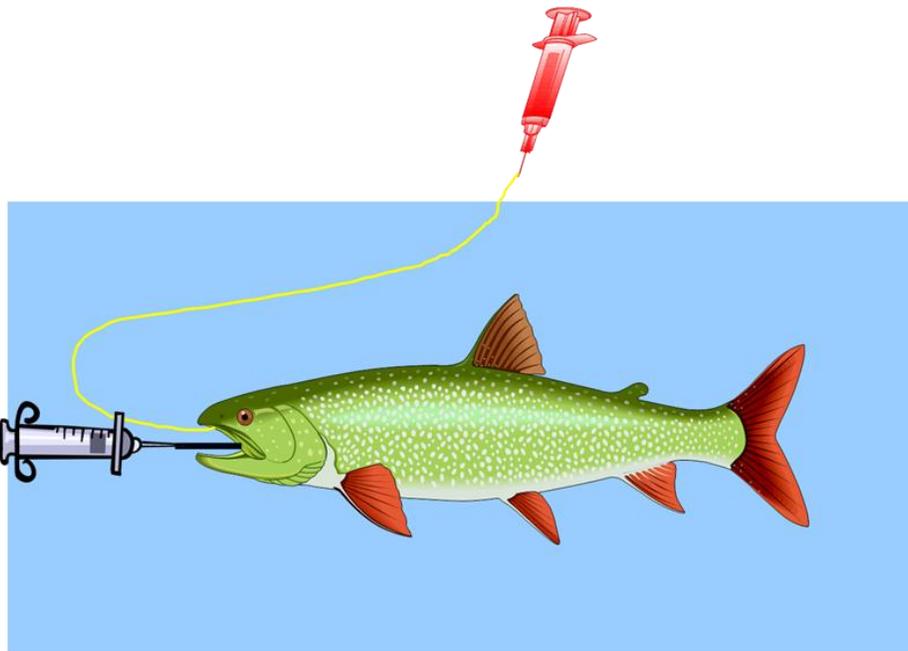
Figure 5.3: Dorsal aortic cannula and gavage method to inject radiolabeled gelatin into the fish gut to determine enterohepatic uptake of hormones in rainbow trout.

At selected times in the experiment (0, 30 minutes, and 1, 2, 4, 8, 16, 24, 48, 72 and 96 hours) serial sampling of blood was conducted as described above to analyze gastric uptake of the radiolabeled hormones. Fecal cannulas and collection bags were not employed for the gavage experiment. Instead, fecal pellets were collected from the tank bottom and samples were also manually expressed from the fish. Expelled feces were collected from the tank and frozen at -80ºC until analysis. At the 24, 48, 72 and 96 hour

samplings, the fish were lightly anaesthetized and fecal samples were collected manually as described in Chapter 4. Briefly, while holding the fish with one hand, the thumb and forefinger apply pressure on each side of the fish abdomen while moving towards the vent. Feces was collected in labeled sample cups and frozen at -80ºC until analysis. At the conclusion of the experiment, fish were euthanized and necropsied as described above.

Sample Analyses

All blood samples were treated as described above. The samples were centrifuged, and plasma was obtained. A 100 µL subsample was pipetted into a 20 mL scintillation vial with 10 mL Scintiverse, scintillation cocktail and vortexed. The quantity of E_2-H^3 in the 100 µL plasma sample, determined by counts per minute (CPM), was analyzed by a beta counter. Any extra plasma was frozen at -80ºC. Based on the mass of the trout, the total blood volume in each fish was estimated using the formula of 5.06 mL/100g (Gingerich & Pityer 1989). This was then used to calculate an estimate of E_2-H^3 in circulation at that time point to monitor clearance and/or uptake from the gut, depending on the experiment.

Once the collection bags had been changed and the fish returned to the tank, the urine and fecal samples could be processed. Urine samples were transferred from the collection bags to scintillation vials using disposable droppers. Samples were frozen at -80ºC until analyzed. When there was enough sample collected, increasing volumes (from 25 µL up to 1000 µL) were added to scintillation vials with 10 mL of Scintiverse. This facilitated an estimation of the volume that would allow E_2-H^3 to be detected while minimizing the effect of colour quenching (See Colour Quenching Section below).

Using the smallest volume with E_2-H^3 detected over background levels, the radioactivity was calculated for the total volume of each urine sample. This was used to estimate the total proportion of circulating E_2 that is excreted via urine.

During processing of the fecal samples, the solids were kept separate from mixed liquid samples. The fecal collection bags typically contained fecal slurry as they were found to not seal well. The slurry was pipetted into 20 mL scintillation vials and then the bags were rinsed with MQ water. The rinse was also collected and stored separately. All samples were stored at -80ºC until analyzed.

For analyses, mixed solid and liquid slurry samples were centrifuged at 4000 rpm for 10 minutes, and the top liquid was pipetted into clean vials and the solids remained in the original vials. The liquid was analyzed as the urine samples were above; with serial increases in volume and then corrected for total volume of the sample. Solid samples were oven dried at 26ºC for 48 hours. The dried feces were weighed and ground with a mortar and pestle, and then acidified with 2M HCl to form a slurry. The acidified samples were vortexed and sat overnight. Samples were extracted with 3 mL ethyl acetate and analyzed in serial increases as described above to a final volume of 1 mL in 10 mL of Scintiverse. Counts per minute (CPM) were determined using a beta counter and samples were corrected for extraction volume.

The gonad, liver, muscle and kidney samples were cut into smaller 0.5 cm^3 pieces and oven dried at 26ºC for 72 hours. Tissues were ground, extracted and analyzed for H^3-E_2 as described above for the solid feces samples.

iv.   *Colour Quenching in sample analysis*

A test was conducted to determine if colour quenching in the urine and bile samples would affect the analysis of the E_2-H^3 in this study. Colour quenching in radiolabelled analyses of biological samples occurs when samples contain pigmented substances. The photons that are emitted by scintillant molecules are absorbed by the coloured solution, reducing the efficiency of the liquid scintillation counting. In order to determine what the maximum volume of sample could be before significant colour quenching effected results, the following test was conducted:

A solution was made using 5 µL of stock E_2-H^3 in 995 µL EtOH, to which 10 µL was added to each 20 mL scintillation vial with 10 mL of scintillation cocktail. Counts per minute (CPM) for each vial with only labelled material was recorded. Increasing volumes (10, 25, 50, 75, 100, 250, 500, 1000 µL) of either urine or bile from the surgery test fish were added to the vials, and then they were reanalyzed. The quenched CPMs were determined as a percentage of the original before the urine or bile was added. The lowest mean percent quenched values were compared to means of increasing volumes of bile or urine by ANOVA.

Results

Colour Quenching

Color quenching increased in plasma from $1.6 \pm 0.2\%$ with 10 µL of sample to 6.3 ± 1.0 with 100 µL of sample (Figure 5.4). Quenching was significant in sample volumes greater than 250 µL, however results were also more variable in volumes greater than 50 µL.
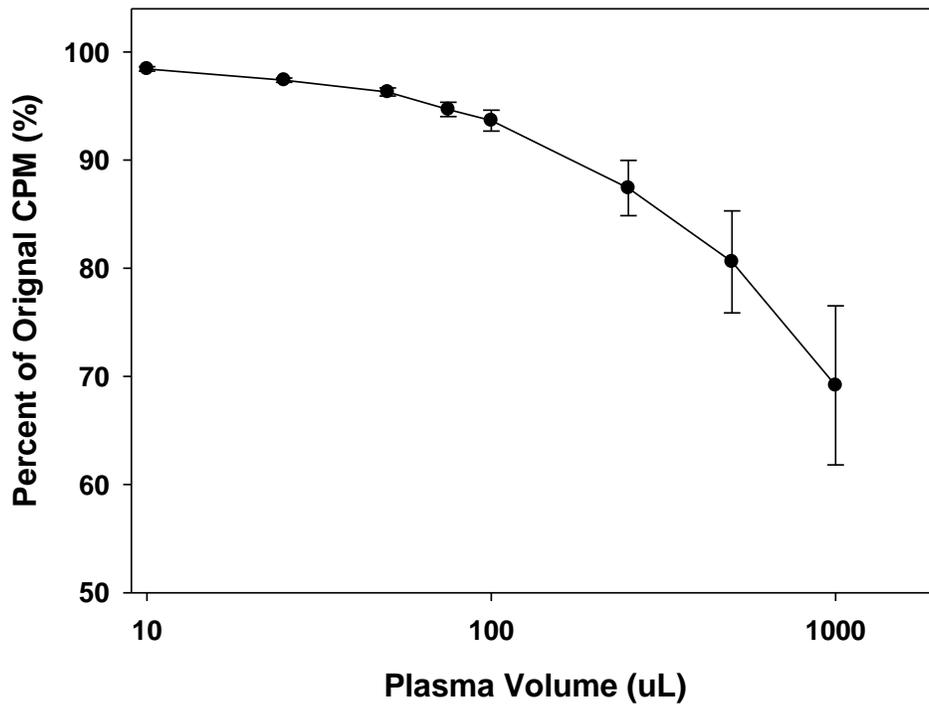
Figure 5.4 Mean percent of original CPM from E_2-H^3 with increasing volumes of fish plasma, indicative of color quenching (n=3).

The colour quenching in the bile samples affected counting efficiency more than for plasma samples, and bile results were more variable between fish (Figure 5.5). Because bile sample volumes required individual assessment based on color, each individual sample was analyzed using several volumes to account for colour quenching.
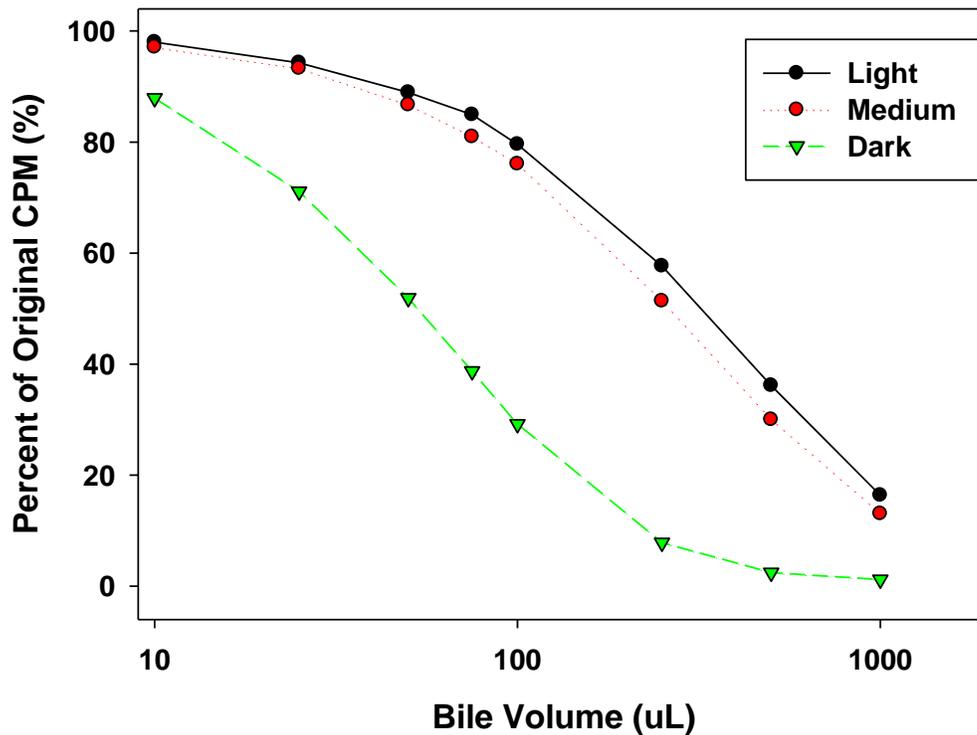
Figure 5.5: Percent of original CPMs from E_2-H^3 in increasing volumes of bile from three different fish representing light, medium and dark coloured bile samples.

Plasma results

Unobstructed flow was maintained in the dorsal aorta (DA) cannula in five of the seven fish for the full 72 hours. The two remaining fish were lightly anaesthetized and blood samples were collected via the caudal vein. The 10 minute sampling showed that the amount of radiolabelled hormone delivered into each fish was similar, expect for Fish F which had approximately 6-times more in circulation than the other fish. It is possible that there was an insufficient volume of saline injected into the cannula after the labelled hormone to adequately rinse the cannula before the next sampling time. By the 2 hour sample time the plasma radiolabelled counts were similar between fish with mean CPM of 9020 ± 565 (SEM). The labeled E_2 was cleared quickly from circulation so that by 72

hours the CPMs were only 0.2% of the original counts measured at the 10 minute post injection sampling (Figure 5.6).
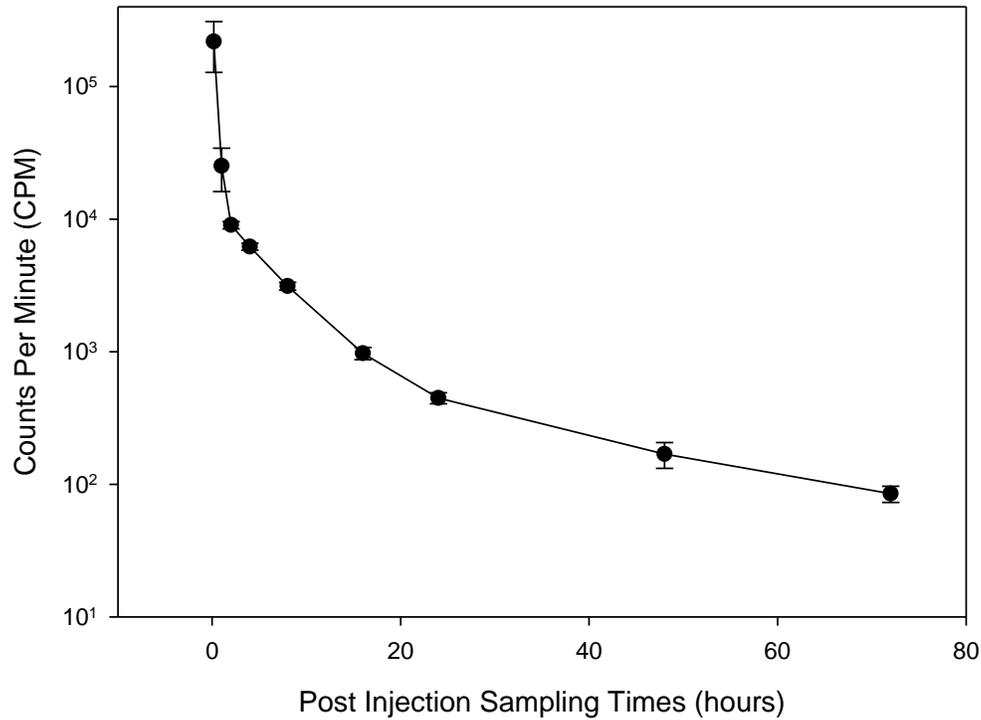
Figure 5.6: Activity of H^3 labeled estradiol (E$_2$) in circulation of mature female rainbow trout (n = 5).

Fecal results

Collection of feces using the fecal tubes and collection bags had two primary problems. Large volumes of fecal material tended to by-pass the tube, forming a pocket around the tube. Often samples were collected from the tank bottom. Water also entered the sample bags, creating a slurry. To overcome this, when the fecal bags were changed the contents were centrifuged at 4000 rpm for 20 minutes. The supernatant was removed and stored separately from the solids. It was assumed that water soluble conjugates were

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present in the liquid portion of the sample and unconjugated E_2-H^3 was in the solids. The labeled hormone in the bile would ultimately have been included in the feces, and therefore was included in the total fecal excretion analysis. Radiolabel hormone measured in the liquid portion of the fecal samples did not change significantly over the 3 days it was monitored. However, there was greater variation between individuals at the 72 hour sampling. Labelled hormone in the solid feces increased every 24 hrs throughout the experiment, and was significantly higher at the 72 hr sampling (Figure 5.7).
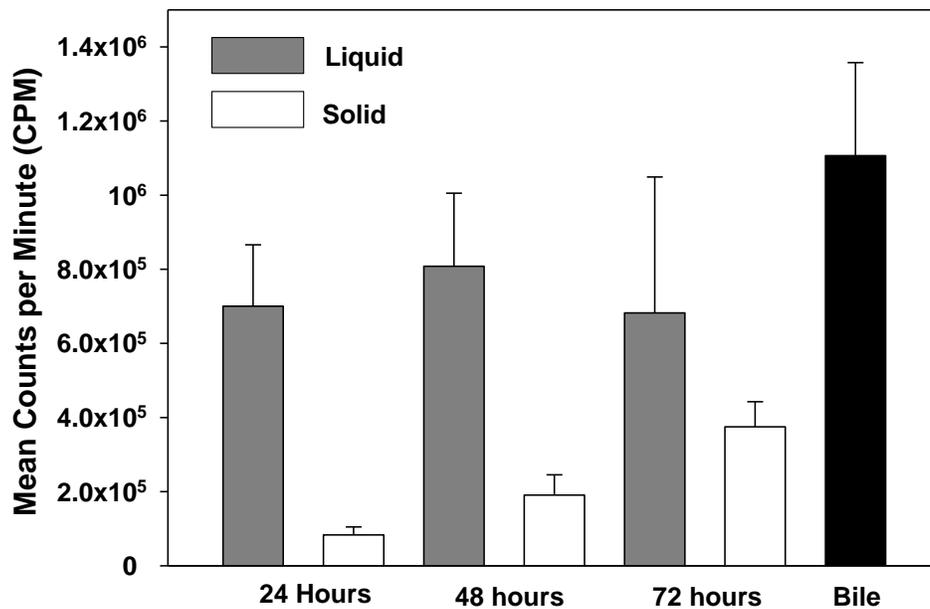
**Figure 5.7:** Activity of radiolabelled estradiol (CPM, expressed as Mean ± SEM) in solid and liquid feces of female rainbow trout, 24-72 hours post injection with E_2-H^3. Bile analysis occurred at 72 hours when fish were lethally sampled.

Of the total E2-H3 excreted in feces 53.9 ± 5.6% was in the form of glucuronides in the liquid, and 18.8 ± 3.6 % was the parent hormone form in the solids. The bile contained

27.3 $\pm$ 4.5% of the labelled hormone in the fecal component (Figure 5.8). The contribution of feces and bile to the overall distribution of the injected labelled plasma were 24.9 and 10.4% respectively (Figure 5.9).
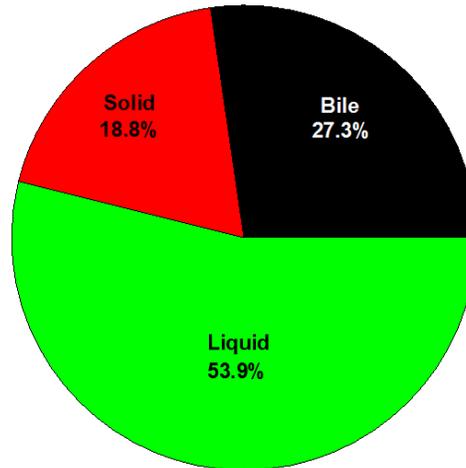
Figure 5.8 Distribution of E_2-H^3 in the three fecal components. The solid and liquid results are a composite over three days of collection, while the bile was sampled at 72 hours.

Urine results

After 24 hours urinary catheters in two fish became dislodged. Fish G and M were the only fish in which the urinary catheters remained in place for the duration of the experiment. However, the catheter in fish G appeared to be obstructed based on low radioactivity in samples from this fish. The volume of urine collected within the first 24 hrs ranged from 0.76 – 74.4 mL. Fish M was the only fish to maintain a flowing cannula for the duration of the experiment. Volumes of urine collected from this fish at 24, 48 and 72 hours were 74.4, 52.8 and 59.3 mL, respectively. The flow rate for this fish (4-5.6 mL/kg/hour) was similar to the 3-5 mL/kg/hour previously reported for pre-spawn adult female rainbow (Scott & Liley 1994). Because Fish M was the only subject that samples

were successfully collected from for the duration of the experiment, results from it were

used to predict urine production and E_2-H^3 excretion for the other fish. Corrected mean

excretion of the radiolabeled hormone via urine calculated to be was 9.7 ± 0.3 % of the

activity of the injected plasma (Figure 5.10).

Tissues

The distribution of labeled hormone after 3 days was measured in four tissues;

muscle, gonad, liver and kidney. Hormones were extracted from dried tissue subsamples

and then expressed as original wet-weight tissue. The percentage of muscle in rainbow

trout was estimated at 51% (Salem 2013). Muscle and kidney mass as well as liver and

gonadosomatic indices are shown in Table 5.1. These four tissues comprised 54% of the

mean fish total mass but accounted for only 0.4% of total injected labelled hormone after

three days (Figures 5.9 and 5.10).

Table 5.1 Length, weight and tissue sample masses for the E_2-H^3 labeled plasma injected fish.

Fish	Body Weight (g)	Length (cm)	Estimated Muscle Mass (g)	Estimated Kidney Mass (g)	Gonad Weight (g)	Liver Weight (g)	GSI	LSI	Total Mass of 4 Tissues Sampled (g)
F	675	35.0	345.6	4.51	2.70	8.75	0.40	1.30	361.6
G	595	34.0	304.6	5.12	1.35	7.61	0.23	1.30	318.7
I	655	35.0	335.4	4.57	3.45	8.00	0.53	1.20	351.4
K	530	34.0	271.4	4.84	7.26	8.38	1.37	1.60	291.9
L	695	35.2	355.8	6.58	4.86	9.80	0.70	1.40	377.0
M	555	32.0	284.2	5.68	2.10	7.66	0.38	1.40	299.6
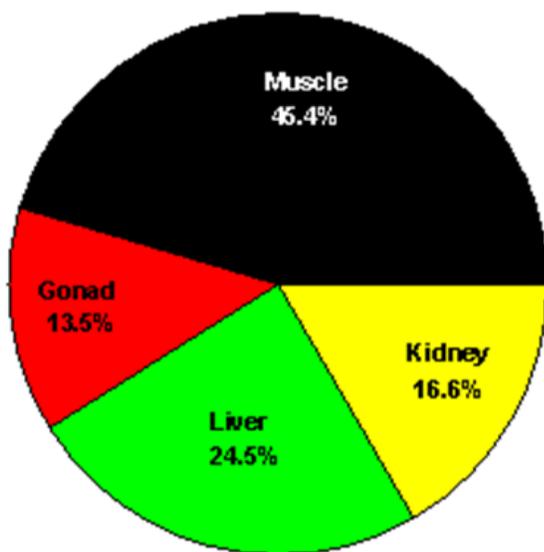
Figure 5.9: Tissue distribution of E_2-H^3 in rainbow trout 72 hours after plasma injection of E_2-H^3.

Totals

The two excretion media, feces and urine, accounted for 24.9 $\pm$ 4.3 and 9.7 $\pm$ 0.3% of the total originally injected E_2-H^3. Bile, also considered part of fecal excretion, contained 10.4 $\pm$ 2.2% of the E_2-H^3 three days post injection. Seventy two hours after injection there was still 0.2 $\pm$ 0.03% in plasma and 0.4 $\pm$ 0.05% bound in tissues (Figure 5.10). The majority of the E_2-H^3 (i.e.54.4 $\pm$ 5.1%) was unaccounted for, and likely lost via gills. Water samples were collected 1 hour post injection, but radioactivity was not detected. The large tank volume, high water flow necessary for trout and the speed at which the labelled E2 was cleared from the plasma can account for the lack of E2-H^3 detection in the water.
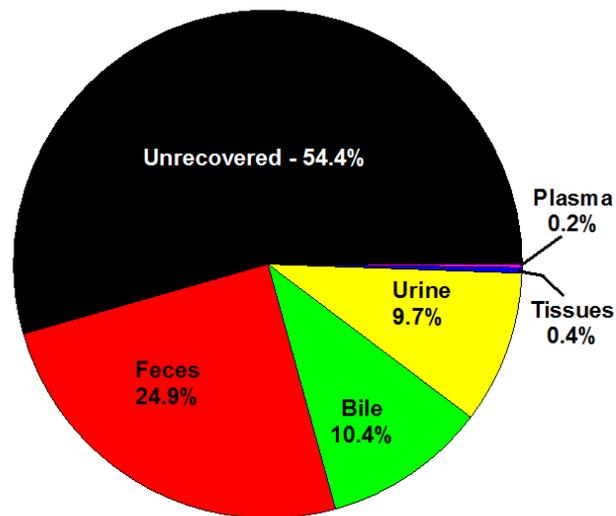
Figure 5.10: Percent distribution of E_2-H^3 in tissues of rainbow trout 72 hours post E_2-H^3 injection to the plasma.

Gavage Results

Delivery of the E_2-H^3 in gelatin to the stomach via gavage was successful, as were aortic cannula surgeries. However, after 24 hours, fish 6G appeared to have regurgitated some of the administered dose. Analysis of the gelatin type substance confirmed H^3 activity, necessitating the removal of this fish from further analysis.

Within 30 minutes E_2-H^3 appeared in the plasma of the remaining 5 fish, with activity, peaking at the 4 hour sample time. Five hundred (500) CPM/100 μL corresponded to 20 pg/mL in the plasma and at peak activities mean plasma CPM was 506.9 ± 91.5, or 20.3 pg/mL E2-H^3. Beginning at 8 hours, plasma levels declined slowly until background levels of radioactivity were reached between 72 and 96 hours (Figure 5.11). After 96 hours, E_2-H^3 remaining in the plasma accounted for 0.1% of the total

delivered dose.   This is similar to the 72 hour sample period from the previous experiment (i.e. 0.2%) where E_2-H^3 was delivered directly to the plasma.
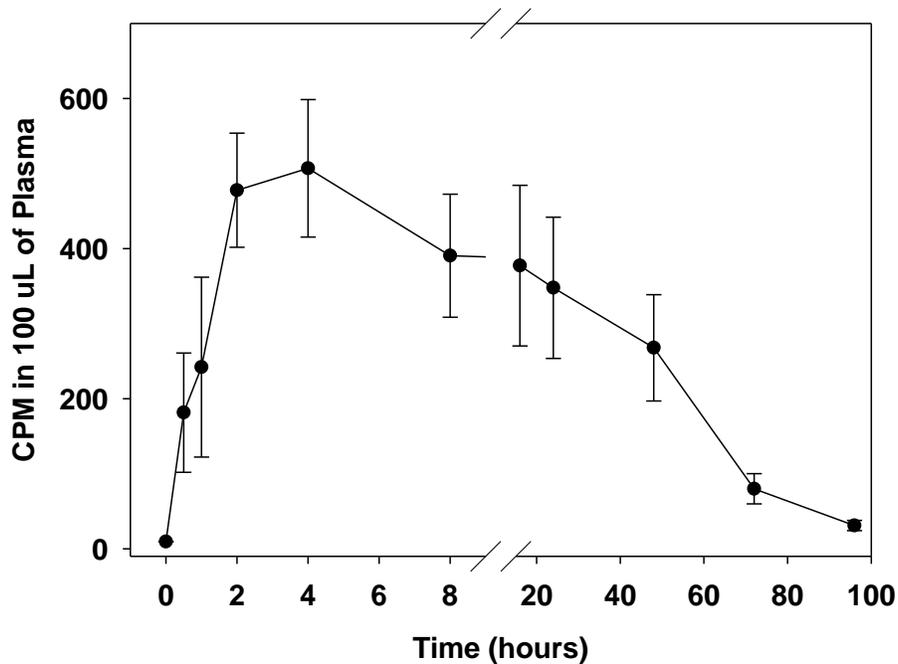
Figure 5.11: E_2-H^3 in plasma of rainbow trout after gavage delivery of gelatin bolus containing the hormone.

Muscle and kidney accounted for 46.3% and 14.1% of the original dose of E_2-H3, respectively. These proportions were similar to those from the previous experiment in which the E_2-H^3 was injected directly to the plasma of fish. Mean GSI was 0.70 ± 0.25, and the gonad contained 2.7% of the total E_2-H^3 in tissues. Liver somatic index (LSI) was1.14 ± 0.12 (Table 5.2) and the liver contained 36.9% of the E_2-H^3 in tissues (Figure 5.12). The percentage of labelled hormone found in the tissues was 0.5% of total injected (Figure 5.14).

Table 5.2 Length, weight and tissue masses of the E_2-H^3 gavage experiment fish.

Fish	Body Weight (g)	Length (cm)	Estimated Muscle Mass (g)	Estimated Kidney Mass (g)	Gonad Weight (g)	Liver Weight (g)	GSI	LSI	Total Mass of 4 Tissues Sampled (g)
1G	760.0	37.0	389.1	4.3	1.0	7.2	0.1	0.9	401.5
3G	905.0	37.2	463.4	3.7	7.0	10.2	0.8	1.1	486.2
4G	650.0	34.7	332.8	4.3	1.3	6.1	0.2	0.9	344.5
8G	670.0	34.0	343.0	4.2	9.9	10.2	1.5	1.5	367.3
9G	875.0	39.0	448.0	6.6	8.3	11.2	0.9	1.3	474.0
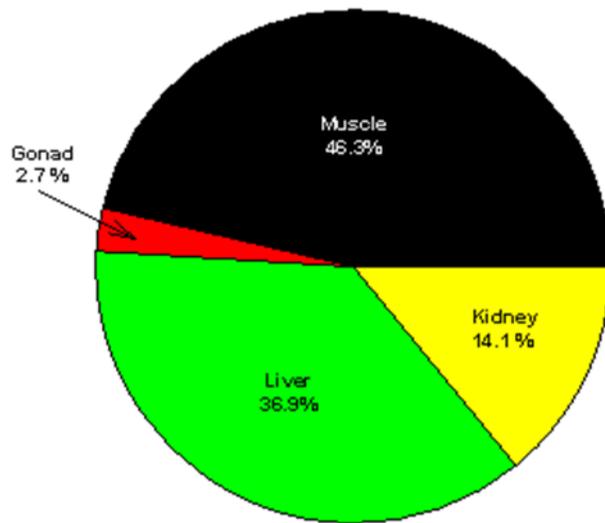
Figure 5.12: Distribution of E_2-H^3 in the tissues 96 hours after the female rainbow trout received the labelled hormone via gavage.

Because fecal cannulas and collection bags were not used in the gavage experiment, there were only solid fecal samples to extract, no liquid component. Fecal analyses were conducted for every 24 hour period. Fecal E_2-H^3 was highest in the 48 hour sample and significantly decreased in the 72 hour sample. The 96 hour fecal samples were the most variable (Figure 5.13).
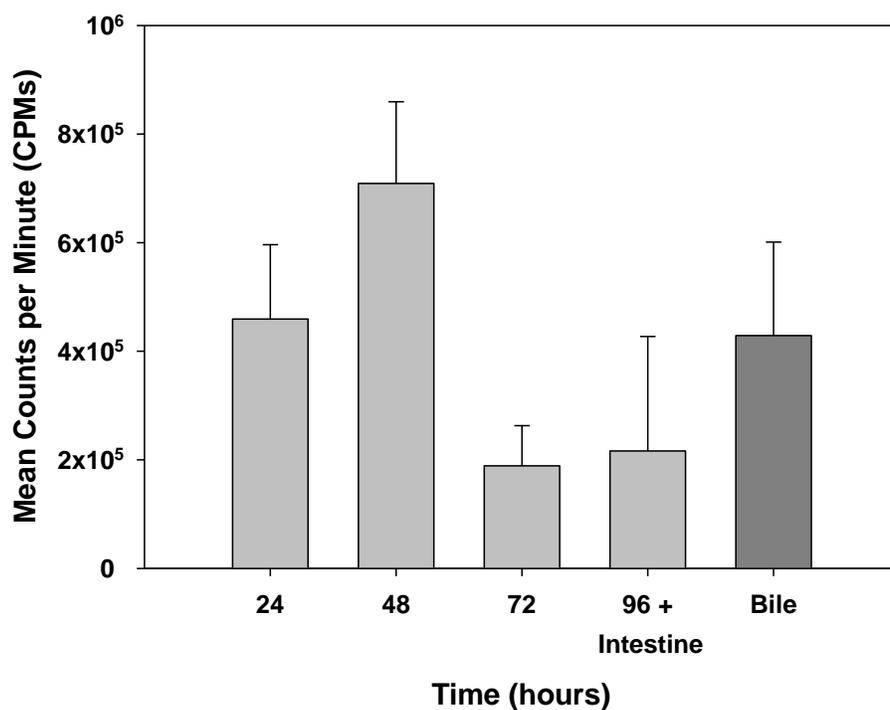
Figure 5.13: Labelled estradiol in fish feces collected every 24 hours post gavage.

In total, feces collected over the four days contained 15.5% of the total administered dose of E_2-H^3. Radiolabel recovered in the bile was 415.0 ± 101.8 pg and accounted for 4.6% of the total dose. Seventeen percent of the E_2-H^3 dose was unaccounted for after 96 hours (Figure 5.14).
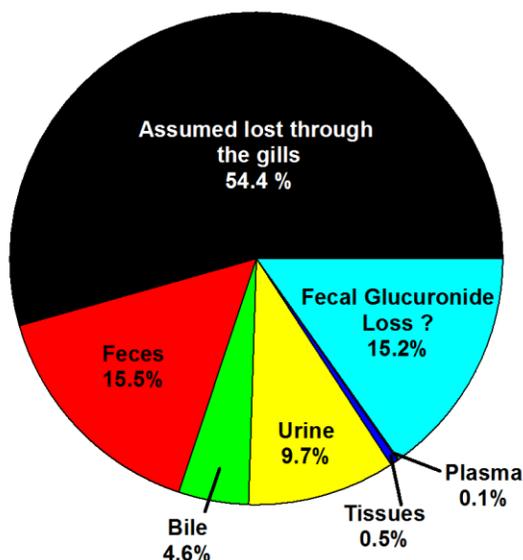
Figure 5.14: Tissue distribution and excretion of E_2-H^3 96 hours after rainbow trout received the labelled hormone via gavage.

Discussion

Radiolabeled estrogen, injected either directly to the plasma or delivered in via gavage provided a means to examine plasma kinetics, tissue uptake and excretion pathways of the hormone in rainbow trout. When the hormone was injected to the plasma, only 6.5% of the E_2-H^3 remained in circulation after 2 hrs. This rapid elimination was likely accomplished via the gills. A similar study by Vermeirssen & Scott (1996) also showed 20 to 45% of injected [^{3}H]17,20β-P in male or maturing rainbow trout, and 40% of the hormone in spermiating fish was rapidly excreted through the gills. Most of the hormone in both cases was still in free, unbound/unmetabolized form. The gills in trout can metabolize reproductive steroids via sulfate conjugation to facilitate release into water (Kime & Ebrahimi 1997). While the lipophilic nature of E_2 (log K_{ow} = 3.94; Ying et al. 2002) would appear to prohibit diffusion into water without first being conjugated,

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Vermeirssen & Scott, reported only [3H]17,20β-P found in urine and bile had been conjugated and that the hormone found in water was free (unconjugated). It has been suggested that free steroid hormones measured in fish holding water have diffused from the gills due to the concentration gradient between the water and fish plasma (Scott & Ellis 2007).

After an initial rapid elimination, E_2-H^3 in the plasma decreased more slowly and was still detectable after 72 hours. E_2 plasma clearance times can range between species, and similar concentrations to those cleared in fish from this study have been shown after 5 hours in trout (Baroiller et al. 1987), after more than 24 hours in Sturgeon (Pelissero et al. 1991) and after 96 hours in immature eels (Querat et al. 1985). There are two primary mechanisms by which circulating hormone levels may be conserved; enterohepatic circulation which will be discussed in greater detail below, and binding to steroid binding proteins (SBP) or sex hormone binding globulins (SHBG). SBPs are non-specific albumins that bind all groups of steroids with low affinity. These proteins act to enhance solubility of lipophilic steroids in plasma and serve as a reservoir by increasing their half-lives. SHBGs, in contrast, specifically bind estrogens and androgens with affinity four to five orders of magnitude higher than SBPs. They function as the primary transporters of biologically active sex steroids and regulate their access to target tissues (Hammond 2011; Mommsen & Korsgaard 2008). SHBGs are produced in the liver, and play a major role in the uptake of steroids and xenobiotics (eg. Ethynyestradiol) from water across the gill surface (Scott et al. 2005; Maunder et al. 2007; Hammond 2011; Blewett et al. 2013b). Salmonids also contain an extra SHBG gene denoted as *shbgb*. The protein product of this gene is expressed mostly in the ovary, gill and muscle, but not the liver

(Hammond 2011). Concentrations of SHBG protein in fish plasma varies based on reproductive status (Pottinger 1988; Foucher et al. 1992; Laidley & Thomas 1997) and the affinity of SHBG for testosterone and estradiol also differs between species (Miguel-Queralt & Hammond 2008; Tyler et al. 2005; Hobby et al. 2000a), sex (Scott et al. 2005) and reproductive status. Specifically there is a marked increase in SHBG affinity for E_2 in vitellogenic females compared to non-vitellogenic (Hobby et al. 2000a). This would facilitate E_2 delivery of estrogen to receptors in the liver to increase vitellogenin production (Hammond 2011, Mommsen & Korsgaard 2008). In vitro studies with human hepatocytes suggest that this process could be part of a feedback mechanism, because E_2 binding stimulates SHBG production (Bobe et al. 2010). The rapid elimination of E_2-H^3 in fish from this study suggests that SHBG may quickly reach an E_2-H^3 binding saturation, facilitating excretion of the excess hormone across the gill epithelium. It has been suggested that stress might affect SHBG binding of E_2 because increased cortisol could potentially compete for sites. However, recent results suggest that only SBP binding is altered with stress (Hobby et al. 2000b; Hammond 2011).

Labelled E_2-H^3 that was injected into the circulatory system appeared in the feces at the 24 hr sampling. The two possible routes for hormones to enter gastro-intestinal track are from the circulation and bile (Whitten et al. 1998; MacDonald 1983; James et al. 2001). The mean proportion of E_2-H^3 in the liquid fractions of the fecal samples (assumed to be conjugated) was seven times greater than levels in the solid portion (assumed to be free hormone). This was expected for rainbow trout based on results presented in Chapter 4. After the peak in fecal conjugate excretion at 48 hours, the mean levels were lower and more variable. E_2-H^3 from the solid fecal portions, however, was

highest at 72 hours. De-conjugation in the intestine can occur through the activity of sulfatase and glucuronidase enzymes. Glucuronidase activity in the intestine is either from gut fauna bacteria or in the mucosal lining cells. Sulfatases are strictly bacterially derived. Deconjugated hormones can be reabsorbed into circulation, serving as another mechanism to conserve plasma steroid concentrations (MacDonald 1983). Zhu et al. (1996) have demonstrated that cellular estrogen glucuronidase activity can be induced with increased estrogen exposure in hamster kidney. If increased E_2-^{3}H-glucuronide substrate in the intestine stimulates mucosal glucuronidase excretion in fish from this study, it may help to explain the increase in free hormone in the feces. Enterohepatic circulation would also have contributed to the persistence of radiolabelled E_2 in the plasma of fish from these studies. It appears that hormones do not have to be in their free forms to be taken up from the gut, because conjugates can readily pass through catfish intestine and enter the bloodstream (James et al. 2001).

The gavage experiment examined uptake and tissue distribution of E_2-H^3 delivered to the rainbow trout gut. The data suggests that the labelled hormone gelatin bolus was digested and cleared from the gastrointestinal track of the fish within 48 hours of gavage. The percentage of radioactivity found in the solid feces accounted for just over 14% of the injected total. If we assume that loss through urine (9.7%) and gills (54.4%) was similar to the proportions determined in the labelled plasma injected fish, then only17% of the administered dose was unaccounted for (Figure 5.14). This would likely have been E_2-H^3 glucuronide conjugate loss to the water. Fecal pellets retrieved from the tank could have been in contact with the ambient water for up to 24 hrs, during which losses of conjugated hormones may have occurred in the flow through fish holding

tanks. Approximately 14% of the radiolabel was found in the feces, and an estimated 17% was lost as water soluble conjugate, meaning almost 68% of the gavage dose of E_2-H^3 was potentially taken up from the gut. Common synthetic steroids have a higher rate of excretion in feces than similar natural hormones (MacDonald 1983). About 7% of both circulating native estradiol (E_2) and estrone (E_1) are excreted in feces, compared to 30% of the synthetic ethynyl estradiol (EE_2) (Sandberg & Slaunwhite 1951; Reed et al. 1972). Stable isotope studies in marine and freshwater systems confirm that fish prefer to forage in sewage outfall areas (Connelly et al. 2013; de Bruyn et al. 2003), increasing the potential to consume synthetic hormones. As EE_2 is 20 times more potent than E_2 for inducing vitellogenin production in fish (Caldwell et al. 2012), and is half as water soluble with a K_{ow} of 4.15 facilitating particle adsorption (Ying et al. 2002), the gut could be a significant route of synthetic hormone uptake.

It took approximately four hours for the labelled E_2 to be taken up from the gut and achieve peak concentrations in the plasma of the rainbow trout. This is almost 3 times faster than rates reported for sturgeon (Pelissero et al.1991). Many of the factors that can affect gastric uptake of hormones are the same as those that influence their plasma clearance, particularly changes in concentration and hormone affinity for SHBG. Temperature can affect ovarian and hepatic metabolic processes (Kime & Saksena 1980; Manning & Kime 1984), and feeding which simulates release of bile into the gut (Scott et al. 2005) can also affect gastric uptake rates. Residence time in the gut, also called defecation rate or rate of clearance, is also critical. Longer residence time allows for greater opportunity for the hormone to be reabsorbed into circulation (Goyman 2012; Palme 2005). Clearance rates can vary between species from a few minutes to hours in

birds (Goymann 2005), 1 or 2 days in large carnivores (Hulsman et al. 2011; Palme et al. 2005), and up to 2 weeks in some lizard species (Alexander et al. 2001). It has been estimated in marine tropical fish that gut clearance can take place over several hours to more than a day (Polunin et al. 1995).

There are several factors that affect clearance rates, and in many cases they are interrelated (Palme 2005; Goymann 2012). The diet of an organism can have a significant effect on gut passage time, and on hormone metabolism and uptake (Goymann 2012; Klasing 2005). Carnivorous fish have been shown to consume 3 to 4% of their body weight, compared to approximately 20% observed in herbivorous fish (Horn 1989), and have a longer gut retention time (Mill et al. 2007; Choat et al. 2004). Coral reef fish species which ate a higher quality mixed diet of algae and zooplankton were found to have slower ingestion rates and longer gut processing times than species whose diet consisted of detritus and sediment (Choat et al. 2004). Even within herbivorous fish there is variation; with lower ingestion and excretion rates indicating a higher energy diet (Polunin et al. 1995). The fish assemblage and competition between species can also affect foraging rate and diet (Paszkowski 1985). Likewise, spatial and temporal foraging segregation of the sexes within a species is well documented (Griffiths et al. 2014). These different foraging strategies can result in different gut clearance times between male and female fish. Finally, the diet of a fish can change at different life stages. For example, the number of taxa consumed usually increasing with size (Sandheinrich & Hubert 1984), and this can affect the rate of clearance significantly among different life stages.

Effects of digestive anatomy and diet on the gut clearance and fecal hormone concentrations in birds have been well described (Klasing 2005; Goymann 2005).

Species with high energy diets such as nectar and seeds or frugivorous birds possess simple digestive tracts, but differ in the mass of droppings produced, with the high fiber fruit-eaters excreting large amounts. Herbivorous birds have ceca and gizzards and often require symbiotic microflora to aid in digestion (Klasing 2005). Similarly, there are fish species that have gizzard-like stomachs (*Acanthuridae* - surgeonfishes) or a pharyngeal mill (*Scaridea* -parrotfishes) to increase mechanical digestion or house fermentative microbes, which can increase clearance time (Polunin et al. 1995).

The mass of feces produced can significantly affect concentrations of hormones measured (Goymann 2012). Increased dietary fiber intake in humans (Pusateri et al. 1990), baboons (Wasser et al. 1993) and European stonechats (Goymann 2005) resulted in decreased estrogen, progesterone and testosterone fecal concentrations, respectively. Essentially, the higher fiber mass acts to dilute hormone concentrations. The same affect would be observed where metabolic rate and/or feeding rates were increased. The opposite would potentially occur (higher concentrations of fecal hormones) when feeding and metabolic rates are low (Goymann 2012) (Figure 5.15). However, lower fecal mass and increased retention time may off-set higher fecal hormone concentrations by allowing more time for reabsorption.
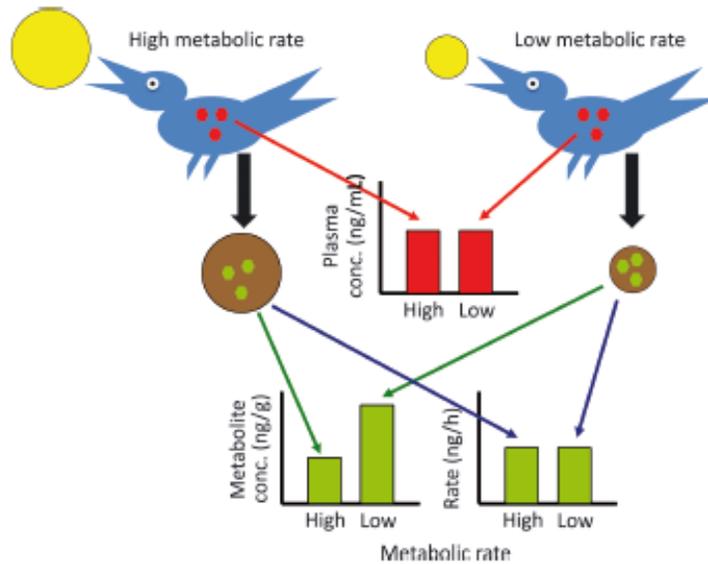
Figure 5.15: The influence of metabolic rate, food consumption and fecal mass on hormone metabolite measurements in feces. The left bird has a high metabolic rate and consumes a large amount of food (large yellow circle). The right bird has a low metabolic rate and consumes a small amount of food (small yellow circle). The birds have the same plasma hormone concentration (red symbols and central bar graph), and metabolites produced (green symbols inside the brown feces, and lower right bar graph). The left bird puts the same amount of hormone metabolites into a larger fecal mass (brown circles) due to its higher metabolic rate and food intake. The concentration of hormone metabolites appears higher in the right bird because the fecal mass is smaller (taken from Goymann 2012).

Temperature can affect metabolic rate and hormone excretion rates, particularly in fish. Higher temperatures accelerate fish defecation rates (Horn & Gibson 1990; Biette & Geene 1980; Afeworki et al. 2013) independently of increased feeding rate (Fauconneau et al. 1983). Diel changes in feeding rates also affect gut passage time (Afeworki et al. 2013), with changes in defecation rates lagging by as little as a few minutes (Afeworki et al. 2013) to 5 hours (Polunin & Koike 1987).

There were differences in E_2-H^3 tissue distribution, particularly between gonad and liver, when the plasma injection and gavage experiments were compared. There was no significant difference in gonad size ($p = 0.86$) between injected and gavage groups,

however, injected fish contained 13.5% of E_2-H^3 found in the tissues, while gonads in gavage fish contained only 2.7%. The difference may be due to changes in the livers. Although the gavage fish livers were not significantly larger (p = 0.14), the fish were a few weeks older and getting ready to spawn. Gavage fish livers contained 12.4% more of the E_2-H^3 total in the tissues than the plasma injected fish. This could be due to increasing numbers of estrogen receptors in the liver that occurs in during vitellogenesis (Smith & Thomas 1991).

The studies described above have shown that estrogen levels in a fecal samples can be a composite of more than 4 days of hormone metabolized from the plasma or diet in rainbow trout. Estrogens taken up directly into circulation via the gills from the environment would be cleared from the plasma in 72 hours. In contrast, estrogen from the diet peaks in circulation after 4 hours and is cleared from plasma within 96 hours. Tissue distribution within the tissues is consistent except for liver and gonad, which appear to change with reproductive status. Finally, fecal elimination accounts for approximately 35% of estrogen excretion. However, because there are many factors that can affect fecal hormone concentrations, careful study design and sample collection standardization are imperative, particularly when using feces to infer concentrations of hormones in the plasma among fish from different locations.

To reduce handling stress of fecal collections on fish, particularly small-bodied species, it is advantageous to collect naturally eliminated fecal casts. Conjugated steroids are likely lost more quickly than parent hormones from the fecal cast into the surrounding water. Future studies could examine parameters that affect the rate of hormone dispersal in naturally eliminated fecal pellets such as temperature, time in water and surface area of

fecal pellet. Optimizing sample collection would bring the analysis of fecal hormones another step towards being included in standard non-lethal practices of accessing fish reproductive health.

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Chapter 6 – Summary and General Discussion

Reproductive health is essential for maintaining sustainable fish populations, and assessment of this is an integral part of our national Environmental Effects Monitoring (EEM) program. However, almost 37000 fish are sacrificed every year to fulfill the EEM monitoring requirements to assess reproduction in fish. Fish are sacrificed in this program to evaluate gonad size as the primary measure of reproductive fitness. Concerns that lethal environmental sampling could impact naturally small populations in the less productive headwaters where many assessments take place (Ribey et al. 2002), or where Species at Risk are present, have led to increased emphasis on nonlethal sampling protocols (Gray et al. 2002). Current non-lethal techniques examine fish growth and population structure, but are limited in their ability to examine reproductive health of fish. Blood can be easily collected from large fish to analyze sex steroids; however, collecting sufficient plasma volumes from small-bodied fish non-lethally is not possible. Because small-bodied, non-migratory forage fish are commonly used as sentinels (Munkittrick et al. 2002), development of a viable non-lethal alternative was warranted. This thesis took on the challenge of developing an extraction for reproductive steroids in feces and an HPLC/MS/MS analysis method to augment non-lethal techniques for fish reproductive health assessments.

Reproductive steroids are conjugated with either glucuronide acid or sulfate moieties. This conjugation increases the polarity of steroids and facilitates their excretion. As a result, conjugates are the predominant form of steroids in feces. Traditional use of RIA and EIA analyses are not applicable without lengthy deconjugation steps because the

antibodies used for detection in these assays are only available for parent (non-conjugated) hormones. HPLC/MS/MS allows the simultaneous analysis of multiple analytes, including conjugated steroids, with substantially lower detection limits (Soldin & Soldin 2009; Kushnir et al. 2011; Carvalho 2012). Using a step-wise progression of experiments that each tested a separate hypothesis, an extraction and LC/MS/MS analysis protocol for reproductive steroids and their conjugated metabolites was developed for the complex matrix of fish feces.

The first step began in Chapter 2 with the development of an HPLC/MS/MS method for determining steroids and conjugates in Cortland's *in vitro* bioassay medium. Cortland's is a complex medium that contains salts and proteins. It is a predictable matrix because it does not contain lipids, bile acids and other unknown matrices found in feces. Preliminary work showed that sulfate conjugates of estrogen and glucuronide conjugates of testosterone comprised up to 50% of hormones produced by fish gonadal tissues *in vitro* (Peters et al. 2001; Peters et al. 2007). Developing an extraction method for the free hormones and conjugates from *in vitro* medium provided a good starting point for hormone extraction from more complex matrices.

The first hypothesis tested in this thesis was "Free and conjugated reproductive steroid hormones can be simultaneously extracted from *in vitro* medium and analyzed by HPLC/MS/MS with a simple liquid-liquid extraction method". Optimizing the extraction efficiencies of the sex steroid hormones and their conjugates from Cortland's incubation medium was explored using different extracting solvents, using an ion pair reagent, and by adjusting pH. A total of ten parent estrogens, androgens and their conjugated metabolites were initially targeted: testosterone (T), 11-ketotestosterone (11-KT),

testosterone glucuronide (TG), testosterone sulfate (TS), estradiol (E_2), E_2-3 sulfate (E_2-3S), E_2-3 glucuronide (E_2-3G), estrone (E_1), E_1-3sulfate (E_1-3S), and E_1-3 glucuronide (E_1-3S). The biggest challenge was to develop a method that simultaneously extracted the sulfate conjugates along with the glucuronides. The most important variables for optimizing the extraction of these two steroids from Cortland's were pH, followed by the use of ethyl acetate (EA) as the extracting solvent. Low pH reduced sulfate extraction efficiencies, but increased those for glucuronides.

Ultimately, it was not possible to simultaneously extract sulfate and glucuronide conjugates from the medium. However, a 2-step liquid:liquid extraction method was developed. Sulfate extraction was solvent volume dependent and step 1 included two 6 mL EA extractions resulting in >60% recovery. Step 2 involved reducing the medium pH to 1 using 2M HCl to target the glucuronides followed by extraction with EA. The final protocol facilitated targeting specific hormone groups and only required evaporation, with no further clean-up prior to LC/MS/MS analysis.

The extraction technique was validated by comparing hormone concentrations measured in samples that were extracted against samples that were directly injected on the LC/MS/MS without extraction. The relationship between extracted and non-extracted samples was highly significant for both E_2-3S ($r^2 = 0.970$, p<0.001) and E_2 ($r^2 = 0.791$, p<0.001), indicating that the extraction method was operating as intended. The development of an extraction method and HPLC/MS/MS analysis suitable for detecting free and conjugated reproductive steroid hormones from an *in vitro* medium, successfully addressed the first hypothesis of this thesis.

In Chapter 3, the utility of the developed HPLC/MS/MS method was applied to an experiment situation to address a second hypothesis: "The in vitro extraction and analysis method can be used to detect potential routes of endocrine disruption from exposure of fish gonadal tissue to polybrominated diphenyl ethers". Using an established *in vitro* steroidogenesis assessment method, and the extraction with LC/MS/MS analysis developed in Chapter 2, the potential effects of environmentally relevant concentrations of BDE-209, BDE-47, as well as its 3OH, 5OH and 6OH hydroxylated metabolites, on steroidogenesis in brown trout (*Salmo trutta*) testicular tissue and BDE-47 and 6OH rainbow trout (*Oncorhynchus mykiss*) oocytes were investigated. While the *in vitro* technique had been used to examine the effects of xenobiotics on parent hormone production, this was the first study to examine sulfate and glucuronide conjugate steroid production in fish gonadal tissues under both basal and hCG stimulated conditions. Under natural conditions, without PBDE exposure, conjugates were determined to make up over 80% of total testosterone produced by male brown trout testes, and 40-50% of estrogens produced by rainbow trout oocytes. Simultaneous determination of multiple steroid forms using the method developed in chapter 2 facilitated the discovery that the carbon at position 17 of E_2 was the preferred site of glucuronidation over position 3 in rainbow trout oocytes. Furthermore, stimulation of the gonadal tissues by hCG significantly changed the ratios of reproductive steroid parent molecules and the conjugates that were produced. In females, the proportion of glucuronide and sulfate conjugates increased, while the parent hormone production decreased when the oocytes were stimulated with hCG. The opposite occurred in the testes, where the proportion of glucuronide conjugate significantly decreased with hCG stimulation.

Hydroxylated metabolites of BDE-47 had the greatest effect on steroidogenesis in both the male and female tissues. 3OH and 5OH-BDE-47 were anti-androgenic in males, the opposite effect to the 6OH metabolite. Incubation with 3OH also produced a stronger response than the 5OH. PBDEs with the hydroxyl group in the ortho position are known to be most reactive with the uridine glucuronosyltransferase (UGT) enzyme (Lai et al. 2012). More specifically, hydroxyl groups at carbon position 3 and 5 beside a bromine atom may open the PBDE structure, allowing it to be more readily conjugated (Lai & Cai 2012). 3OH- and 5OH-BDE-47 used in this study both have the reactive configuration, and could be competing with testosterone as a UGT substrate. In the oocyte exposures to 6OH, there was increased E_2-3S and 3G production with the simultaneous decrease in E_2-17 glucuronidation. This suggests that more than one isoform of UGT exists in rainbow trout oocytes, or that SULT activity may be regulating UGT activity. The studies in Chapter 3 addressed the intended hypothesis. Specifically, the *in vitro* extraction and analysis method was used to detect potential routes of endocrine disruption in fish gonadal tissue exposed to polybrominated diphenyl ethers. Most importantly, these studies showed that measuring only the parent hormone does not provide a complete profile of the potential for PBDEs and their hydroxylated metabolites to affect fish reproductive steroid concentrations. If total estrogens or E_2 alone had been analyzed, significant effects would have gone undetected.

In Chapter 4, the extraction and analysis techniques that were developed in Chapter 2 for an *in vitro* medium were applied to develop a method for extraction and LC/MS/MS analysis of steroids and their conjugates in the far more complex medium of fish feces. This addressed the third hypothesis: "HPLC/MS/MS can be used to

simultaneously measure conjugated and free forms of hormones extracted from fecal matter". Acid treatment prior to extraction was particularly important in order to achieve optimal glucuronide recovery. The pre-treatment of feces with acid improved glucuronide extraction and lipid breakdown, but it also facilitated extraction of compounds that severely interfered with parent hormone analyses. These highly pigmented compounds, likely bile acids, had the same mass transitions during LC/MS/MS analysis as the estrogen glucuronides, but were eluted at the same time as the parent hormones. The greatest challenge for addressing the third hypothesis was developing a method to clean up and concentrate the samples, without losing the target hormones, in preparation for LC/MS/MS analysis. Several techniques commonly used for tissue and lipid clean-up were tested, including various types of solid phase extraction (SPE) and gel permeation chromatography (GPC). None of these methods alleviated the matrix interferences while preserving the glucuronide conjugates. Treating the extracted sample with a series of acid washes, followed by ethyl acetate (EA) extraction after each wash, reduced the unknown interfering matrix peak to allow adequate detection of the parent estrogens. To remove any remaining lipids and pigmented compounds a lipid removal agent, Cleanascite^{TM} proved to be effective for clearing the sample, and prolonging the life of the column without affecting the recovery of the parent and conjugated estrogens. As with the Cortland's medium extraction developed in Chapter 2, the final method for feces extraction was two separate extractions; the first used a hexane:ethyl acetate (H:EA) (3:2, v:v) prior to the addition of acid. This targeted the parent estrogens without extracting the glucuronides or the interfering compounds. Next the feces sample was acidified and extracted with EA to target the glucuronides. Both

sets of extracts were acid washed and treated with Cleanascite^{TM} before LC/MS/MS analyses. By extracting first with the H:EA, the labeled d_4-E_2 internal precision standard (IPS) suppression was reduced from a mean of 81.4 to 13.7%. In the second feces extraction with acid pre-treatment, the d_3-E_2-3G labeled glucuronide IPS was suppressed on average by 12.8%. After correcting for suppression, when the 2-step method was compared to the 1-step acid-pretreatment method, the recoveries were less than 5% different. This affords flexibility in selecting the method; if the parent estrogen levels are anticipated to be low, then the 2-step method with H:EA first would be most appropriate. If E_2 concentrations are expected to be higher, then the 1-step method could be used to stream-line extraction time and instrumentation use by reducing the number of samples by half. The development of this protocol addressed the hypothesis that HPLC/MS/MS can be used to simultaneously measure conjugated and free forms of hormones extracted from fecal matter.

Once a method for extracting and analyzing steroids and conjugates in feces was developed, the next experiment provided the biological context for feces steroid measurements and addressed the fourth hypothesis: "Fecal steroid measurements can be used to estimate plasma hormone concentrations and reflect the reproductive status of fish". Paired plasma and feces samples were collected weekly from 8 female rainbow trout for ten weeks prior to spawning to determine if parent and conjugated estrogen levels in feces could predict circulating plasma hormone concentrations.

Changes in plasma hormone concentrations were typically not reflected in feces until the subsequent week, and estrogen concentrations were also more stable in feces than in plasma. Often a 10-fold change in plasma corresponded to only a 2-4 fold change

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in the feces; likely because steroid measurements in feces represent an integrated pool of free hormones metabolized from the plasma over a period of time. In contrast, plasma samples are snap-shots of an individual's hormone levels at that moment (Goymann 2005). The length of the metabolite integration period in feces depends on many factors including diet (Klasing 2005), temperature (Alexander et al. 2001), feeding rates (Goymann et al. 2006), season (Rogers 1987), age of the animal (Seraphin et al. 2008), circadian rhythms (Sousa & Ziegler 1998) and sex of the animal (Cavigelli et al. 2005).

There was a vast range of E_2 plasma hormone concentrations from 1000 to 82000 pg/mL, and E_2-3S concentrations from 300 to 6000 pg/mL over time. Regression analyses determined that E_2-17G measured in feces was the best predictor of both E_2 (r^2 = 0.54, p < 0.01) and E_2-3S (r^2 = 0.64, p < 0.01) in rainbow trout plasma. Based on these results, the hypothesis that fecal E_2 metabolites could be used to estimate circulating E_2 concentrations through a reproductive cycle in rainbow trout was supported.

In order to effectively use fecal hormones as a method to determine reproductive health in fish, a thorough understanding of the endpoint is essential. Fecal steroid measurements represent an integrated pool of metabolized parent hormones cleared from circulation over a period of time. As there is more than one route for hormones to enter the intestine, and enterohepatic circulation can result in steroids reentering the plasma pool, the assessment of steroid metabolism and excretion is complicated. In Chapter 5, using a radiolabeled E_2 injection into the fish circulation, plasma clearance time and partitioning into feces, urine and bile was monitored. E_2-H^3 was measured in the 24 hour sample and peaked at the 48 hour sample. After 72 hours post-injection, over 35% of the labeled hormone was collected in feces and bile, approximately 10% in urine, and only

0.4% had been taken up into the muscle, kidney, gonad and liver. Estimating how much E_2-H^3 was lost through the gills and urine was the greatest challenge of this study because maintaining urine catheter insertion and flow was problematic. The large rainbow trout used in this study required larger holding tanks (greater volume) and a substantial water flow, such that measuring E_2-H^3 in the water was not possible because of the great dilution that would have occurred. However, using injection recovery balance, hormone loss via gills was estimated to be approximately 54%.

The most common route of hormone disrupting chemical (EDC) and exogenous hormone uptake discussed for fish living in effluent receiving environments, particularly sewage effluent, is via the gills (Scott et al. 2005; Blewett et al. 2013a). Understanding the role enterohepatic circulation plays in plasma hormone balance is particularly important for these fish but is often overlooked. Many coastal towns directly discharge untreated sewage into the marine and estuarine environments (Blewett et al. 2013b). Not only may particles be ingested, but teleosts drink this seawater/effluent mix to maintain osmoregulatory balance, allowing compounds to enter via the gut (Blewett et al. 2013a). The last hypothesis addressed in this thesis was: "Ingestion and enterohepatic circulation can be an important route of exposure for natural and synthetic exogenous hormones in fish". To simulate exogenous hormone uptake via the diet or from drinking, E_2-H^3 was introduced directly into the gut of fish using gelatin as a vehicle (i.e. gavage exposure). Appearance of the labeled E_2 in the plasma, as well as the percentage of labelled hormone that simply passed through the gut was monitored. The labeled estradiol appeared in the plasma within 30 minutes, peaked after 4 hours, and was finally cleared within 96 hours. Routes of excretion and tissue distribution results were similar to the plasma injection

experiment. Approximately 68% of the E_2-H^3 injected into the gut entered the enterohepatic circulation, supporting the hypothesis that ingestion and uptake through enterohepatic circulation can be an important route of exposure for natural and synthetic exogenous hormones in fish.

By performing the studies and addressing the hypotheses in this thesis, several advances have been realized. These include:

1) An extraction and analysis method for simultaneously measuring conjugates and parent reproductive steroids from *in vitro* medium using LC/MS/MS was developed.

2) Using exposures of BDE-209, BDE-47 and 3 hydroxylated metabolites, I showed that carbon 17 is the preferred position for glucuronidation of estradiol in rainbow trout oocytes. Furthermore, exposure of oocytes and testicular tissue to the hydroxylated metabolites of BDE-47 effects steroidogenesis by either potentiating the SULT and UGT enzyme systems or competing with parent steroids as substrates these Phase II enzyme systems.

3) An extraction and clean-up procedure for the LC/MS/MS based measurement of estrogens and their conjugated metabolites in the complex medium of fish feces was developed. .

4) I determined that fecal E_2 metabolites are a good predictor of circulating E_2 concentrations in rainbow trout.

5) Using radiolabelled hormone injection to the circulation of the gut, I increased the understanding of the integrative nature of fecal hormone excretion and the role of enterohepatic circulation in plasma hormone balance in fish. The results provide

essential information for the continued development of fecal sex steroid analysis as a tool for non-lethal monitoring of fish reproductive health.

Future Work

Future examinations of reproductive steroids in fish testes and oocytes, should consider parent hormone and metabolites, especially in light of the evidence presented here that xenobiotics may differentially affect the production of steroids and their predominant conjugate forms. Because E_2-17G is the primary glucuronide formed, the suite of conjugates analyzed should also include E_2-17S. Identification of the SULT and UGT isoforms and their substrate preferences in fish gonadal tissues could be used to predict effects of hydroxylated brominated flame retardant and other structurally similar xenobiotics on steroidogenesis.

Finally, this thesis demonstrated that fecal E_2 metabolites are a good predictor of circulating E_2 concentrations in rainbow trout. Additional work to test this method as a non-lethal measure of reproductive status and health in small-bodied fish is necessary, because they are more frequently targeted in environmental monitoring programs. To reduce handling stress for collecting feces from fish, particularly small-bodied species, it is advantageous to collect naturally eliminated fecal casts. Due to their water-soluble nature, conjugated steroids are likely lost from the fecal cast to the surrounding water more quickly than the lipophilic parent hormones. Future studies are needed to examine parameters that affect the rate of hormone dispersal in naturally eliminated fecal pellets such as temperature, time in water and surface area of fecal pellet. Optimizing sample collection would bring the analysis of fecal hormones another step towards being

included in standard non-lethal practices of accessing fish reproductive health. And finally, the method would require testing in a laboratory or mesocosm setting before being applied to a field situation.

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