The anti-estrogenic and liver metabolic effects of DHAA in rainbow trout (*Oncorhynchus mykiss*).

By:

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ABSTRACT

Recent studies have shown that dehydroabietic acid (DHAA), a resin acid present in pulp and paper mills, may have anti-estrogenic effects in fish. A chronic-exposure toxicity experiment using immature rainbow trout (*Oncorhynchus mykiss*) was conducted in order to assess the endocrine disrupting and liver metabolic effects of the wood extractives DHAA and β-sitosterol (BS) regularly present in pulp and paper mills and the model estrogen 17β-estradiol (E2). It was found that exposure to 5 ppm of E2 significantly increased hepatosomatic index (HSI), vitellogenin (VTG) and plasma sorbitol dehydrogenase (SDH). This effect was reduced by mixing E2 with DHAA, indicating that DHAA does not cause its anti-estrogenic effects indirectly due to liver damage. Exposure to 5 ppm of DHAA caused a significant increase in liver citrate synthase (CS), and liver ethoxyresorufin-O-deethylase (EROD) activity after 7 days, however, the fish recovered by 28 days. This study also determined the effect of 14 different pulp and paper mill effluent extracts on liver enzyme metabolism through alterations in the activity of liver lactate dehydrogenase activity (LDH) and CS. This activity varied greatly between mills but most showed an induction of CS after 28 days exposure through i.p. injection. The results of the study indicate that DHAA may alter energy metabolism as well as cause anti-estrogenic effects in female juvenile rainbow trout.

Key words: Pulp mill effluent, VTG, EROD, anti-estrogenic, estrogenic, liver metabolic enzymes

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List of Abbreviations

E2	=	17β-estradiol
BS	=	β-sitosterol
DHAA	=	dehydroabietic acid
EROD	=	ethoxyresorufin-O-deethylase
VTG	=	vitellogenin
SDH	=	plasma sorbitol dehydrogenase
LDH	=	lactate dehydrogenase
CS	=	citrate synthase
HSI	=	hepatosomatic index
GSI	=	gonadosomatic index
K	=	condition factor
mg	=	milligrams
ppm	=	parts per million
mg/kg	=	milligrams per kilogram
mL	=	milliliter
mg/mL	=	milligrams per milliliter
h	=	hour
U	=	international units
ELISA	=	enzyme linked immunosorbent assay
i.p.	=	intra-peritoneal
CEPA	=	Canadian Environmental Protection Act

1.0 INTRODUCTION

Many studies have demonstrated that pulp and paper mills can affect fish, more specifically at the reproductive level (Hewitt *et al.,* 2008). These effects are thought to be caused by wood extractives such as phytosterols. However, effluent quality can vary substantially between mills using different manufacturing processes, mill furnish, operation conditions and effluent treatment, which contributes to a wide variety of effects between mills (Orrego *et al.,* 2011a, Kamaya *et al.,* 2005). A recent study by Orrego *et al.* (2011a) discovered a disruption of liver energy metabolism by enzymes such as LDH and CS following a single Intra-peritoneal (i.p.) of rainbow trout to Chilean pulp mill effluent. The effects of other mill effluents from other countries on these enzymes would be an interesting area to explore, as they are not as stringently regulated as North American mills.

While most studies on pulp and paper mill effluent have focused on the effects of plant phytosterols, but they are just one type of constituent. Resin acids are a class of wood extractives, naturally present in wood and in higher levels in conifers (Hernandez *et al.,* 2008). Like effluent quality they can vary greatly between mills. They represent some of the most acutely toxic chemicals in the waste water effluent (Oikari *et al.,* 1982). They are of concern as the ability of bacteria to cause biological detoxification of resin acids in secondary treated effluent is inconsistent (Wang *et al.,* 1995). While the modernization of mills has significantly reduced the amount of resin acids emitted, they are still found in

measurable levels in the receiving water and sediment downstream of mills (Wang *et al.,* 1995; Lahdelma and Oikari 2005; Leppanen *et al.,* 1998).

Dehydroabeitic acid (DHAA) is the most abundant resin acid and has been implicated in toxicological effects in fish (Oikari *et al.,* 1983). Although not a strong endocrine disruptor, recent studies have suggested that DHAA may act as an anti-estrogen (Chrisanson-Heiska *et al.* 2008; Orrego et al., 2011b). It has been shown that the anti-estrogenic activity of DHAA may not be mediated by the intracellular estrogen receptor (Teresaki *et al.,* 2009). It is suggested that since DHAA is known to be hepatotoxic, it may cause an indirect anti-estrogenic effect as a result of liver damage (Orrego *et al.,* 2010b). DHAA has also been implicated in metabolic energy disruption in trout hepatocytes (Rissanen *et al.,* 2003).

2.0 BACKGROUND LITERATURE

2.1 Pulp and paper mills

Paper is a versatile commodity essential to our society, commonly used for writing and printing. The advent of steam-driven paper making machines in the 19th century allowed paper to become easily accessible to the masses. Since then, the pulp and paper mill industry has grown worldwide. In Canada it is a billion dollar industry and today Canada ranks second to the US in pulp and paper manufacture, and first in pulp and paper exports (Mines, 2011). Canada has about 140 pulp and paper mills, with at least one in every province except Prince Edward Island (Mines, 2011).

The main objective of the pulping process is to separate cellulose fibres from lignin, thus freeing the fibres for paper making (CEPA, 1992). There are two main types of pulping processes: mechanical and chemical, with Kraft chemical pulping being the most predominant in Canada. There are also three different types of wood feeds used by mills: hardwood, softwood and mixed. An entire review article could be dedicated to describing the differences between the different mills and wood types, but what is most significant to the environment is that the pulp and paper mill process consumes large volumes of water and chlorine, and discharge considerable quantities of chlorinated organic matter into aquatic systems (CEPA, 1992). The pulp mill industry is the largest commercial user of water in Canada, and discharge large amounts of effluent (25,000-300,000 m^3/day) (CEPA, 1992). This waste has the potential to affect both humans and wildlife downstream.

2.2 Pulp and paper mill regulation

In the late 1980s, the world's attention was focused on the potential environmental impact of bleached pulp mill effluent after scientists in Sweden reported that fish collected near effluent discharges were adversely affected by the effluent (McMaster and Hewitt, 2010). Most of the early research (1980's) on pulp and paper mills was produced by Sweden and other European nations. These findings spurred the first conference on the environmental fate and effects of pulp and paper mill effluents held in Saltsjobaden, Sweden in 1991 (McMaster and Hewitt, 2010). This conference is held every 3 - 4 years, the most recent of which was held in Fredericton, New Brunswick in 2009.

From this 1991 conference in Sweden, Canada was quick to create regulations. A list and summary of regulations pertaining to the operation of pulp and paper mills in Canada can be found in Table 1. It should be noted that Canada has some of the most stringent monitoring strategies for mills, considering there are very little, if any, monitoring strategies in other developed countries, such as the USA, and Australia (Chiang *et al.,* 2010). The first of these regulations, the Pulp and Paper Effluent Regulations (PPER) created in 1992, put strict requirements on pulp and paper mills, such as creating discharge limits for total suspended solids and biochemical oxygen demand, and require effluent to be non-acutely lethal to fish (Lowell *et al.,* 2005). This requirement of effluent to be non-acutely lethal is common for most industries. While it is good to avoid acute lethality, this does not protect wildlife from chronic effects.

Thankfully, in 2004 an amendment to PPER or Regulations Amending the Pulp and Paper Effluent Regulations (RAPPER), was created which addresses this issue by requiring sub lethal toxicity testing. Due to these regulations, Canada is a world leader in research on pulp and paper mills.

As a result of the regulations of 1992, the effluents from pulping and bleaching operations are combined and, in most cases, treated prior to discharge. Primary treatment removes suspended solids through screening and settling, reducing the biological oxygen demand (BOD) of the effluents on the aquatic environment (CEPA, 1992). Secondary treatment involves contact with bacteria which decompose organic substances in the effluent. This process removes oxygen-consuming substances and many of the toxic compounds to fish (CEPA, 1992). However, pulp mill effluent still contains compounds that can adversely impact the environment.

Table 1: Timeline for creation of major acts/regulations pertaining to the pulp and paper mill industry.

Act/regulation	Year created	Main goals of act
Canadian fisheries act	1985	Management and monitoring of fisheries, Conservation and protection of fish and fish habitat
Pulp and Paper Effluent Regulations (PPER)	1992	Pollution prevention. Prescribes discharge limits for total suspended solids and biochemical oxygen demand. Requires effluent to be non-acutely lethal to fish
Environmental Effects Monitoring (EEM)	1992	Assess the uncertainty of the limits created in the PPER. Determine the effectiveness of these limits
Regulations Amending the Pulp and Paper Effluent Regulations (RAPPER)	2004	Canadian mills under RAPPER are required to conduct studies on: 1. A fish population survey to assess fish health. 2. A benthic invertebrate community survey to assess habitat. 3. The effects on the usability of fisheries resources, including a study of dioxins and furans in fish tissue

2.3 Canadian environmental effects

What the Environmental Effects Monitoring (EEM) has shown for Canada, is that since 1992, Canada has vastly improved the quality of our pulp and paper mill effluent (Lowell *et al.,* 2005). However, effluents have still been shown to affect fish health and habitat. The main effects observed in fish across Canada are: 1) nutrient enrichment or eutrophication (fatter, faster growing with larger livers); and 2) a reduction in fish gonad size that has remained constant for the past decade (Lowell *et al.,* 2005). As a result, most of the current research has focused on the effects of pulp and paper mills on the environment, with a focus on endocrine disruption. Compounds that cause endocrine disruption, known as endocrine disrupters are exogenous compounds which mimic hormones and can disrupt the function of endogenous hormones (Sonnenschein and Soto, 1998). They generally function by acting as mimics, agonists, antagonists for receptors and enzymes.

It is thought that most of the effects from pulp and paper mill effluent arise from three main types of compounds present: resin acids (abietic acid), isoflavonoids (genistein) and phytosterols (sitosterol and stigmastanol) (Hewitt *et al.,* 2008). A very detailed review of the individual compounds and mill operating conditions was published by Hewitt *et al.,* (2008). Both isoflavonoids and phytosterols have the potential to act as endocrine disrupters; one cause of this may simply be their structure, which is similar to estradiol (E2) (Fig. 1). However, resin acids have generally been ignored by the literature. Many researchers have examined the effects of these three types of compounds individually, or on

an effluent basis. Very little research has been done to look at how these compounds interact. The main effects observed on fish in the environment are: changes in gonad size, changes in production of gonadal steroids (VTG), and induction of ethoxyresorufin-o-deethylase (EROD) activity.

As mentioned previously, one of the major findings of the EEM in Canada was that fish showed signs of nutrient enrichment (larger livers, bigger fish) and a reduction in gonad size (Lowell *et al.,* 2005). This is indicative of metabolic disruption, which is characterised by increased body condition (K) (length to weight ratio, fatter fish have a bigger K), increased liver size, and decreased gonad size (Hewitt *et al.,* 2008). It is known that there are some androgenic compounds in pulp mill effluent, such as testosterone, epitesterone and androstenedione (Van den Heuvel, 2010). While androgens are generally viewed as vertebrate steroids, these compounds are produced and play key physiological roles in plants (Van den Heuvel, 2010). These androgens have been conclusively shown to cause reduction in gonad size and masculinisation in female mosquito fish (Van den Heuvel, 2010). It is worth noting that it takes many years of exposure for these effects to occur. As a result, these effects are generally not seen *in vitro* and makes comparing *in-vitro* to *in-vivo* studies difficult.

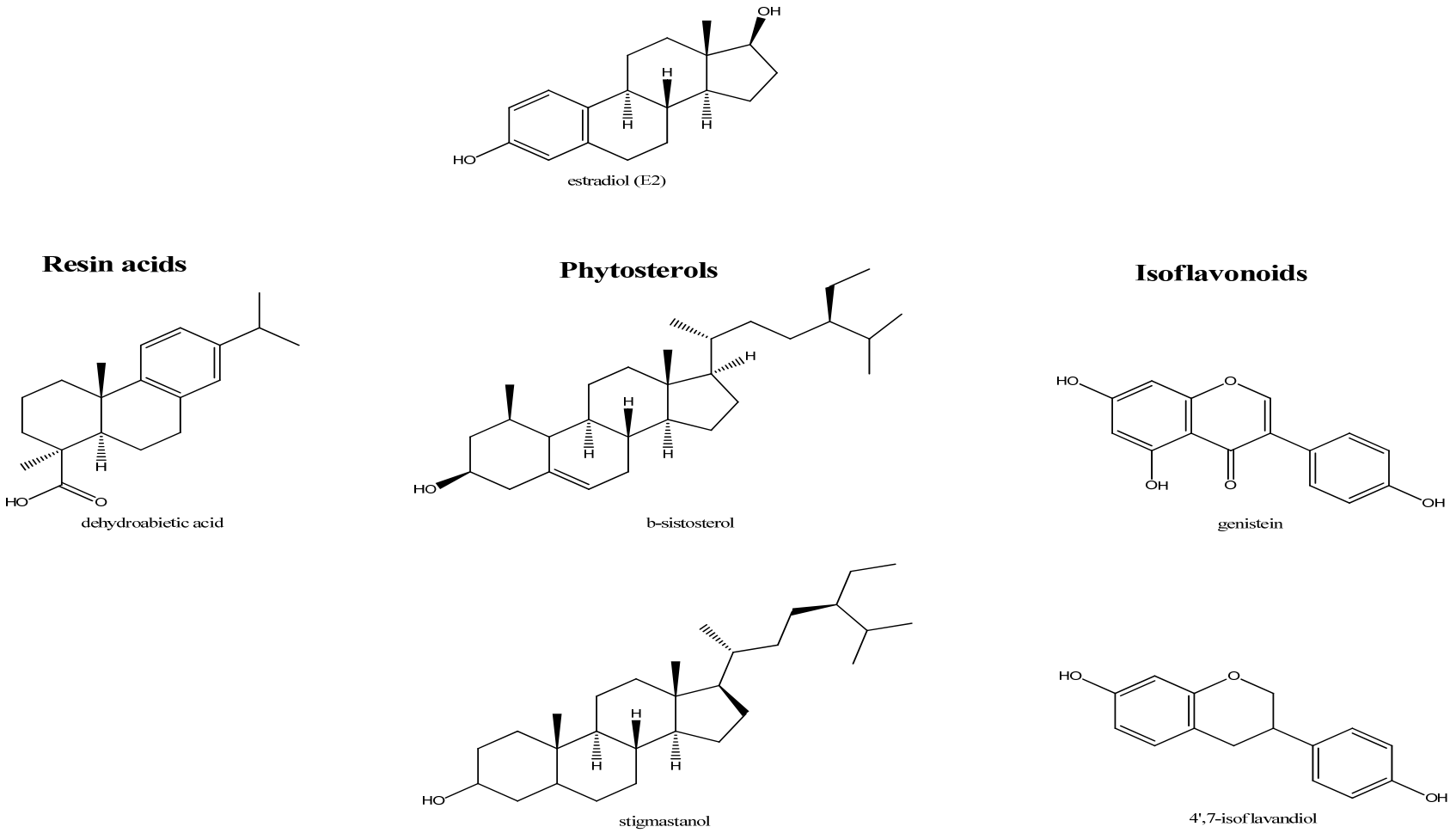


Figure 1: A comparison of the structures of resin acids, phytosterols and isoflavonoids with a natural hormone estradiol, created using ChemBioDraw Ultra 12.0.

2.4 Global environmental effects

While the environmental effects of pulp mill effluents are consistent throughout Canada, Europe and the USA this is not the case worldwide (Table 2). This variation in effects, along with the difficulty of assessing what are the causative components of the effluents makes mitigating them very challenging. In an attempt to help explain pulp mill effluent effects on fish, a recent study has linked changes in biomarker metabolic enzymes with pulp mill exposure (Orrego *et al.,* 2011a). Currently, there is no solution to reduce the current sublethal effects of pulp and paper mill effluents on the environment.

An increase in ethoxyresorufin-O-deethylase (EROD) activity has been regularly observed in fish exposed to pulp and paper mill effluents (Wartman *et al.,* 2009; Orrego *et al.,* 2008; Lindesjoo *et al,.* 2002). Oikari *et al.* (2010) showed a slight induction of EROD activity of 40% in caged brown trout exposed to diluted pulp mill effluent, which while thought to be a result of increased exposure to resin acids, was not felt to be sufficient enough to be considered a significant effect. Generally EROD is used as a biomarker for exposure to polycyclic aromatic hydrocarbons (PAHs) and organochlorines (Andersson and Forlin, 1992; Whyte *et al.,* 2000).

Another effect attributed to pulp and paper mill effluents is a change in the production of gonadal steroids such as vitellogenin (VTG) (Kovacs *et al.,* 2005; Orrego *et al.,* 2006; Sepulveda *et al.,* 2004). VTG is an egg yolk precursor protein. Usually an increase in natural VTG occurs in female fish prior to spawning/maturation. It is not usually expressed in males. An induction of VTG

is an indication of endocrine disruption (Jensen and Ankley, 2006, Tyler *et al.,* 2001). The effects of pulp mills on VTG are quite variable; some studies in Canada and Chile have shown that exposure to pulp mill effluent have increased VTG in fish (Kovacs *et al.,* 2005; Orrego *et al.,* 2006), while others in the USA have shown the opposite effect (Sepulveda *et al.,* 2004). Either way, pulp and paper mill effluents are clearly causing endocrine disruption.

Finally, most studies have focused on the exposure of pulp and paper mill effluent on fish. A recent study by Orrego *et al.* (2011b) demonstrated that all types of pulp and paper mill effluent (treated and untreated) have the potential to cause embryotoxicity (mainly delaying hatching time, and decreased hatchability) in embryos (post fertilization) of three different species (rainbow trout, American flagfish and Japanese medaka). Thus while most of these effects are discussed in terms of mature fish, it is important to note that these compounds can affect fish during many stages of their life.

However, the biggest question is why is there such variation? Why do some mills cause increases in female sex hormones while others do not or even cause the opposite? Is this because of different study design/species used? Because of increases in hormones such as VTG, most of the pulp and paper mill research has been focused on phytosterols and isoflavonoids. This research has conclusively shown that phytosterols, such as sitosterol can cause increased levels of VTG while isoflavonoids such as genistein have been shown to cause feminisation in males (Hewitt *et al.,* 2008). Resin acids have been largely ignored as a source of endocrine disruption.

Table 2: The effects of pulp mill effluents on wild fish (modified from Hewitt *et al.,* 2008)

Country and year	Species of fish	Main effects	References
USA 1980-2006	Mosquito fish	Masculinisation of females (based on secondary sex characteristics)	Howell *et al.,* 1980, McMaster *et al.,* 2006
Sweden 1980's	Perch	Reduced gonad size	Sodergren *et al.,*1988
Canada 1992-2005	Many species	Reduced gonad size, circulating sex hormones, changes in secondary sex characteristics	Lowel *et al.,* 2005,
New Zealand 2006	Brown bullhead	Lower serum levels of steroid hormones, no change in gonad size	West *et al.,* 2006
Chile 2006	Rainbow trout	Increased gonad size, increased circulating sex hormones	Orrego *et al.,* 2006; 2010b

2.5 Resin acids

Most of the early research on resin acids was conducted in the 1980's. Amos Oikari published many studies that conclusively showed that resin acids such as dehydroabietic acid (DHAA) were hepatotoxic to fish at very low concentrations (Oikari *et al.,* 1983). DHAA is a common pollutant of softwood pulp and paper mills, and 90% of it is removed through secondary treatment (Hewitt *et al.,* 2006). Because of this, many researchers deemed it unlikely to cause effects. However, DHAA has a bioconcentration factor of up to 200 times and has been found to bioaccumulate in fish (Oikari *et al.,* 1985). The highest concentrations are accumulated in the bile, blood and liver of trout (Oikari *et al.,* 1982). In fact, the presence of resin acids in the bile of fish has been shown to be a good indicator (biomarker) of exposure to pulp and paper mill effluent (*Lindesjoo et al.,* 2002).

Consequently, despite only being released from pulp and paper mills in small amounts, DHAA has the potential to biomagnify up food webs. Most of the recent research by Oikari *et al.,* 2010 has been focused on showing the ability of DHAA to accumulate in sediment and impact the environment. Despite not being a very strong endocrine disrupter itself, DHAA has been recently shown to cause anti-estrogenic effects in rainbow trout (Orrego *et al.,* 2010). However, the mechanism by which DHAA acts as an anti-estrogen remains poorly understood. It has been shown that the anti-estrogenic activity of DHAA may not be mediated by the intracellular estrogen receptor (Teresaki *et al.,* 2009). It is suggested that since DHAA is known to be hepatotoxic, it may cause an indirect anti-estrogenic

effect as a result of liver damage (Orrego *et al.,* 2010). As a result, resin acids such as DHAA may be a factor in the masculinisation and the variance in endocrine disrupting effects. However, the exact mechanism by which DHAA affects the endocrine system is unknown, and should be investigated. Compounds like DHAA illustrate the complexity of studying whole mill effluents, but also illustrate the need for studies that examine the effects of combinations of compounds, rather than just considering the effects of single chemicals on their own.

2.6 General knowledge gaps

Pulp and paper mill effluents have been heavily researched over the past three decades. As previously mentioned, the exact mechanisms by which androgenic/estrogenic effects occur due to pulp and paper mill effluent exposure are still unknown. Some compounds are thought to play a role, but there has not yet been a direct link established between compounds and their effects. Research questions have changed and evolved over time (Fig. 2). One such question is: Why is there such variation in the chronic toxicity of effluents between mills and countries?

Due to recent changes to the global industry, and a number of mill closures in North America, there has been a significant shift in pulp and paper production to South America (McMaster and Hewitt, 2010). Mills have recently moved from the Northern hemisphere (Europe, Canada, USA) to the Southern (South America, New Zealand). Most of the modern and largest mills in the

world are operating in South America. These are of particular interest since they are operating in countries with few environmental regulations. They also pose interesting questions in that they utilize cultivated crops that are continuously replanted (McMaster and Hewitt, 2010). There is very little information on these effluent effects on receiving aquatic environments. As a result, it is likely that the bulk of the research on pulp and paper mills will migrate from a focus on impacts to the aquatic ecosystems of the Northern hemisphere to those of the Southern hemisphere.

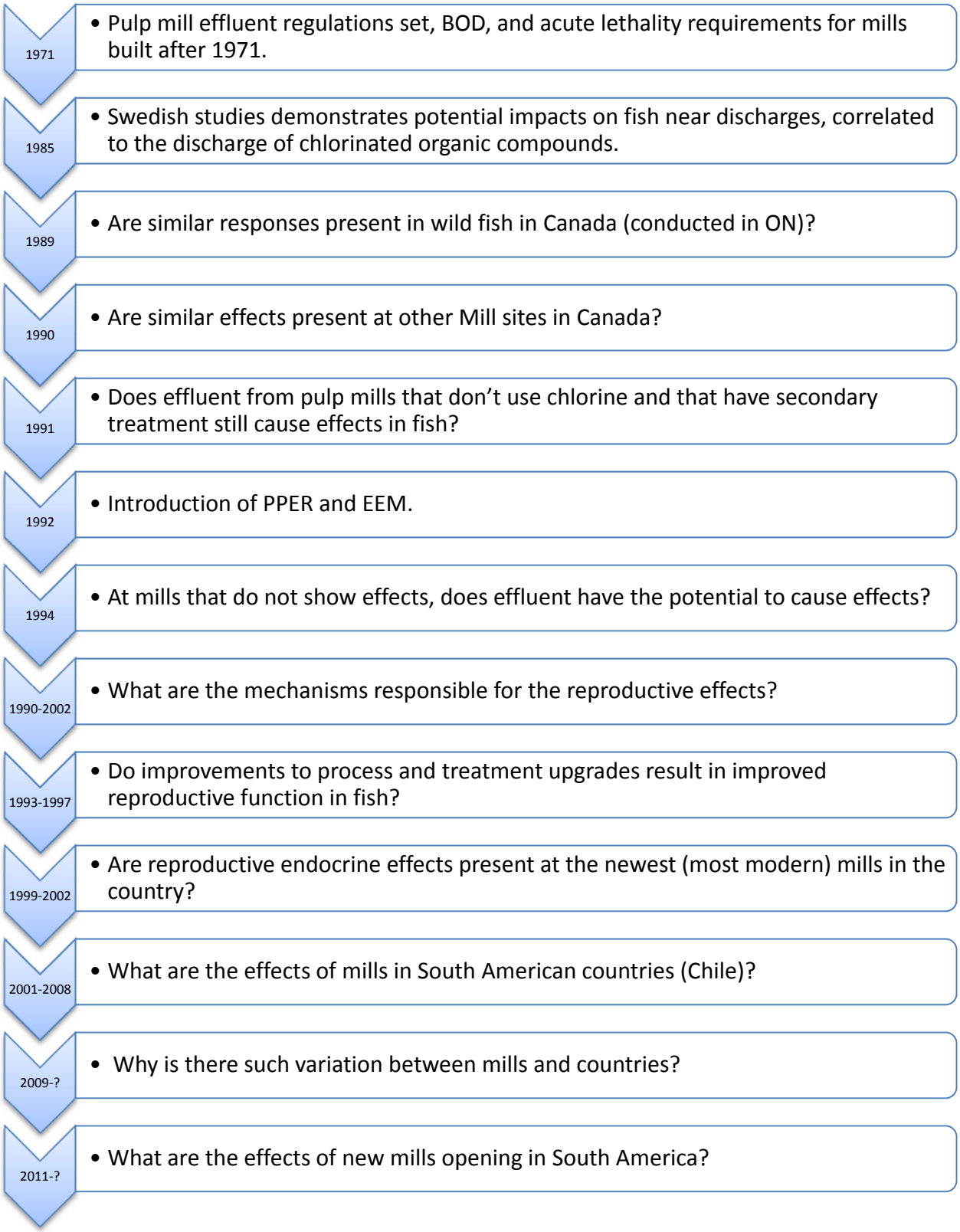


Figure 2: Timeline and evolution of research questions related to the study of the environmental impacts of pulp and paper mill effluents in Canada, modified from Hewitt *et al.*, (2008).

2.7 Biomonitoring

Risk assessment is the determination of the probability that a specific agent or chemical will give rise to an adverse effect (Watson and Mutti, 2004). Exposure to a chemical is extremely important in determining risk; sufficiently low exposure to even an extremely toxic chemical may not be a significant risk (Watson and Mutti, 2004).

Due to advances in analytical chemistry and the recognition of the substantial uncertainties involved in estimating environmental exposures, there has been a shift to assessing human exposure through biomonitoring (Hays *et al.,* 2007). Biomonitoring is an indicator of internal dose, and as such it can provide exposure estimates that are related to the concentration at the active site or organ (Hays *et al.,* 2007). For this reason, biomonitoring is becoming the preferred standard of environmental exposure assessment (Needham *et al.,* 1999).

However, there are significant limitations to biomonitoring. For example, genetic differences, seasonal cycles, acclimation, type of tissue measured, gender, nutritional status and other factors all may influence correlations between chemical exposure and biomarker responses (Forbes *et al.,* 2006). While these factors are controllable in experimental studies, they may act as confounding factors in field studies (Forbes *et al.,* 2006). Despite these limitations, biomonitoring can potentially decrease the uncertainty inherent in estimating exposures by conventional exposure assessment methods as well as provide a more biologically relevant measure of true exposure (Hays *et al.,* 2007). The

development and study of metabonomic, or metabolic techniques for biomonitoring is a relatively new field and is viewed as very important for the advancement of this field (Watson and Mutti, 2004).

2.8 Energy metabolism as a biomarker

Aquatic organisms exposed to xenobiotics may exhibit biochemical, physiological and/or behavioral responses. In field studies, biomarkers of effects are preferred to biomarkers of exposure, because individual biological responses can be extrapolated to impacts at a population level (Cohen *et al.,* 2001).

Metabolic enzymes such as citrate synthase (CS), lactate dehydrogenase (LDH), and cytochrome C oxidase (CCO) are components of the respiratory system and the production of adenosine triphosphate (ATP). These enzymes can be used to reflect the metabolic capacity of tissues (Pelletier *et al.,* 1993; Gagnon and Holdway, 1998). Studies have shown that changes in respiratory enzyme activity may be used as a biomarker of xenobiotic exposure in marine organisms (Table 3).

Table 3: Effects of various compounds on metabolic enzymes

Compound	Species	Main effects noted	Location	Reference
Endosulfan	*Mus Musculus*	Increased LDH activity	Liver	Kurutas *et al.,* 2006
Distillery effluent	*Cyprinus carpio*	Increased LDH activity	Liver	Ramakritinan *et al.,* 2005
Hydrocarbons	*Macquaria novemaculeata*	Increased LDH and CCO activity	Liver	Cohen *et al.,* 2001
water accommodated fraction of crude oil	*Salmo salar*	Decreased LDH and CS activity, no change in CCO	Gill	Gagnon and Holdway, 1998
NaPCP	*Pagrus auratus*	Increased LDH and CCO activity, no change in CS	Liver	Tugiyono and Gagnon, 2002
PCP-126	*Pagrus auratus*	Increased CCO activity, no change in CS or LDH	Liver	Tugiyono and Gagnon, 2002
Heavy metals and organochlorines	*Cyprinus carpio and Capoeta tinca*	Correlation between LDH and heavy metals and organochlorines	Liver	Ozmen *et al.,* 2008
Pulp and paper mill effluent	*Oncorhynchus mykiss*	Increased LDH activtity	Liver	Orrego *et al.,* 2011a

Aquatic organisms may reduce their metabolic rates when aerobically stressed (Long *et al.,* 2003). A key adaptation supporting long-term survival in the absence of oxygen is a sharp reduction of metabolic rate (DeZwaan *et al.,* 1995). When contaminants induce alterations in the carbohydrate metabolism of fish, intolerant species will suffer higher mortalities and reduced reproduction, causing replacement of underexploited resources by other species which are better adapted for performing more effectively in a polluted environment (Gagnon and Holdway, 1998). Shifts in the distribution of energy may alter carbohydrate metabolism and may consequently affect the commercial value and quality of fish (Gagnon and Holdway, 1998). Thus maintaining a normal metabolic rate is essential to the health of aquatic organisms.

A current approach for assessing cytotoxicity is monitoring respiratory activity of the mitochondrion, a sensitive non-specific subcellular target site (Khan and Cutkomp, 1982). This organelle is common to most eukaryotic cells and is involved in a number of vital cellular processes (Haubenstricker *et al.,* 1990). By detecting changes in mitochondrial function after exposure to a xenobiotic, one can correlate toxic effects with exposure concentration (Haubenstricker *et al.,* 1990). Biological membranes consist of a lipid matrix in which proteins are embedded (Bretscher, 1973). Consequently, xenobiotics that are likely to affect these membranes are generally lipophilic.

The synthesis of ATP by mitochondria can be influenced by a variety of compounds, which typically act as either uncouplers or inhibitors (Haubenstricker *et al.,* 1990). Uncoupling agents prevent ATP synthesis by promoting the

dissipation of energy gained through electron transfer (Haubenstricker *et al.,* 1990). Inhibitors tend to act in one of two ways: either inhibiting oxidative phosphorylation to prevent the ATP-forming mechanism from utilizing the high energy compounds generated from electron transfer, or inhibiting electron transfer which blocks the utilization of oxygen (Haubenstricker *et al.,* 1990). For example, organic compounds which can affect the translocation of protons across the mitochondrial membrane will affect cellular respiration, resulting in impaired levels of energetic metabolism (Gagnon and Holdway, 1998).

Aerobic enzymes such as CS and CCO generate ATP for use in normal cellular processes (Gagnon and Holdway, 1998). In contrast, anaerobic enzymes such as LDH provide energy mainly during high rates of swimming (Gagnon and Holdway, 1998). Stressed fish may increase their anaerobic metabolism while experiencing a decrease in aerobic metabolism.

Metabolic enzymes can vary depending on fish size and growth rate (Pelletier *et al.,* 1993), feeding status (Gagnon and Holdway, 1998; Polakof *et al.,* 2007; Yang and Somero, 1993), reproductive state (spawning compared to non-spawning) (Kiessling *et al.,* 1995), temperature (Seddon, 1997), season (Pelletier *et al.,* 1993; St-Pierre *et al.,* 1998; Thibault *et al.,* 1996; Ozmen *et al.,* 2006), and injury (Kuznetsov and Gnaiger, 2006). These variables can be controlled in the laboratory, but are modifying factors for wild caught fish. However, even in a laboratory setting under optimal conditions, CS, LDH and CCO activities have been measured to vary up to 18, 18 and 10% in control fish, respectively

(Gagnon and Holdway, 1998). Consequently, adequate sample sizes must be used in order to ensure that significant results can be obtained.

2.9 Anaerobic metabolism

LDH is a soluble enzyme located in the cytosol of all cells in the body, providing information on cellular glycolytic capacity (Kuznetsove and Gnaiger, 2006). LDH catalyses the last step in glycolysis; the reversible oxidation of lactate ion to pyruvate ion:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \longleftrightarrow \text{L-Lactate} + \text{NAD}^+$$

This regenerates NAD+ from NADH for glycolysis, which leads to the production of ATP. The activity of LDH is a measure of the anaerobic capacity of the cell (Gagnon and Holdway, 1998; Kuznetsov and Gnaiger, 2006).

When exposed to a xenobiotic which causes stress, an increase in LDH activity would be expected following a decrease in CS, to compensate for reduction in ATP production (Long *et al*., 2003). This has been seen in mussels following water-borne exposure to hydrocarbons (Long *et al.,* 2003).

The Pasteur effect refers to an enhanced rate of glycolysis when oxygen is limiting as a compensation for the decline in ATP generation by oxidative metabolism (Heath, 1988). The rate of ATP generation from anaerobic glycolysis never approaches that of oxidative metabolism (Heath, 1988). Essentially, when an aquatic organism is stressed a general increase in anaerobic metabolism is expected.

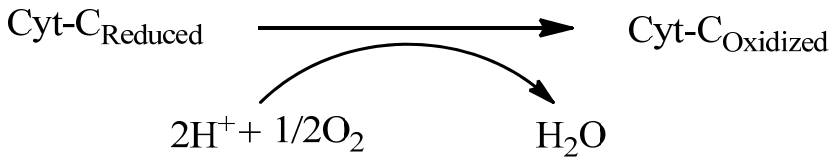
2.10 Aerobic metabolism

CS catalyses the first step of the citric acid cycle (Krebs cycle), the conversion of acetyl coenzyme A (Acetyl Co A) and oxaloacetate to produce carbon dioxide, citrate, and NADH:

$$\text{Acetyl-CoA} + \text{Oxalacetate} + H_2O \longrightarrow \text{Citrate} + \text{CoA-SH}$$

Much like with LDH, the NADH produced leads to the production ATP. CS is the first 'pacemaker' enzyme of the cycle and is located within the cytoplasm of mitochondria (Dickson *et al.,* 1993). Citrate synthase activity is thus a useful measure of the aerobic capacity of cells (Gagnon and Holdway, 1998; Tugiyono and Gagnon, 2002).

Another key enzyme in aerobic metabolism is cytochrome C oxidase (CCO) which is the terminal step in the electron transport system. It catalyses the oxidation of a reduced form of cytochrome C:



CCO is located in the inner membrane of the mitochondria (Gagnon and Holdway, 1998). As CCO and CS are both mitochondrial enzymes it would be expected that the two enzymes would vary similarly (Tugiyono and Gagnon, 2002). However, one study by Tugiyono and Gagnon (2002) found that following exposure to PCB-126 CS was unaffected while CCO activity increased. The stability in the CS activity compared to the variability in the CCO activity suggests that metabolic perturbations related to PCB exposure occur within the electron

transport system involving CS but before or during the reactions involving CCO (Tugiyono and Gagnon, 2002).

CS does not catalyze a limiting step in mitochondrial ATP production (Cooney *et al.,* 1981). Conversely, CCO catalyzes a rate limiting step in mitochondrial ATP production (Balaban and Heinman, 1989; Tager *et al.,* 1983). CCO and CS are positively correlated and their activities are affected by growth rate, size, physical condition and season (Pelletier *et al.,* 1993). Consequently, in order to obtain a full understanding of an organs aerobic metabolism, both CS and CCO should be studied.

2.11 Sorbitol dehydrogenase

Sorbitol dehydrogenase catalyzes the reversible interconversion of fructose and polyhydric alcohol sorbitol (Dixon *et al.,* 1987; Tugiyono and Gagnon, 2002):

$$\text{D-fructose} + \text{NADH} \rightleftharpoons \text{D-Sorbitol} + \text{NAD}^+$$

This oxidation-reduction reaction occurs predominantly in the liver (Dixon *et al.,* 1987). With the exception of small quantities in the kidneys and testes, SDH is not found in other tissues (Wiesner *et al.,* 1965). Serum/plasma SDH (SDH) is frequently used to indicate mammalian liver damage (Raghavendra and Rao, 2000; Wiesner *et al.,* 1965); it has also been used commonly for fish (Dixon *et al.,* 1987; Holdway *et al.,* 1994; Mukherjee and Jana, 2006; Ramakritinan *et al.,* 2005; Tugiyono and Gagnon, 2002). Its use is preferred to utilizing other liver enzymes such as LDH, glutamate oxaloacetate transaminase or glutamate

pyruvate transaminase, since SDH is not elevated by other organ diseases but rather is specific to liver damage (Dixon *et al.,* 1987; Wiesner *et al.,* 1965). Under normal conditions SDH concentration is negligible in the bloodstream (Ozretic and Krajnovic-Ozretic, 1993). Its presence in blood serum/plasma indicates that hepatocytes and other liver cells have been damaged. In fact, biochemical lesions caused by single i.p. injection of toxicants have been shown to release SDH to the blood, preceding histological damage (Dixon *et al.,* 1987).

Sorbitol dehydrogenase activity decreases by up to 79% of initial activity if stored at room temperature but samples can be successfully stored for up to one week at -20º C (Tornquist *et al.,* 2000). However, SDH activity in rainbow trout can be maintained indefinitely without loss when samples are stored in liquid nitrogen (Dixon *et al.,* 1987). It has been also shown that the activity of SDH in rainbow trout is unaffected by fasting, sex or fish weight and that the activity clearly reflects the exposure of fish to hepatotoxic chemicals in a dose-dependent fashion (Dixon *et al.,* 1987). Gender has been shown to be a factor when interpreting results from mature fish; consequently gender must be considered or sexually immature juvenile fish used to avoid this confounding factor (Dixon *et al.,* 1987).

To summarize, changes in serum/plasma SDH activity act as a sensitive biochemical indicator of liver damage in salmonids (Dixon *et al.,* 1987). SDH has been proven to be more responsive at lower levels of toxicant exposure than other common measures of liver health such as LSI or histopathology (Dixon *et*

al., 1987). Since compounds like DHAA are known to be hepatotoxic, SDH should be a good biomarker of such exposure and effects.

2.12 Ethoxyresorufin-O-deethylase (EROD) activity

The measurement of EROD activity is a well established *in vivo* biomarker of exposure, most notably to halogenated/polycyclic aromatic hydrocarbons (PHH/PAH) and structurally related compounds (Whyte *et al.* 2000). Although EROD activity is best viewed as a biomarker of exposure, it has been also suggested to be a good biomarker of effects (Whyte *et al.,* 2000). To measure the activity of EROD in the liver one generally measures the activity of Cytochrome P4501A1.

Cytochrome P450 is the terminal oxidase component of an electron transfer system located within the microsomal fraction of the cell, and is involved in the metabolism of many xenobiotics (Andersson and Forlin, 1992). Cytochrome P450 is the substrate binding component that determines the specificity of the reaction (Andersson and Forlin, 1992). EROD is essentially an indirect biomarker of exposure for mixed-function oxygenases (MFO) which degrade xenobiotics.

The biotransformation of xenobiotics occurs in two phases. Phase I involves metabolism of the compound by the cytochrome P450 enzyme system, which introduces an oxygen atom into the molecule. It is thought that when a chemical binds to the cytosolic aryl hydrocarbon receptor (AhR), it stimulates the rate of P4501A production (Whyte *et al.,* 2000). Phase II detoxification involves

the conjugation of the now more reactive compound with an endogenous molecule such as sulphate, creating a conjugated product that is generally less toxic, more water soluble, and thus more easily excreted (Andersson and Forlin, 1992). While there are many ways to measure the activity of CYP1A, most have focused on determining it by measuring the ability of this protein to convert 7-ethoxyresorufin to a fluorescent product (resorufin) (Whyte *et al.,* 2000).

Ethoxyresorufin-O-deethylase activity has been extensively studied in rainbow trout (Andersson and Forlin (1992), Whyte *et al.,* 2000). It should be noted that EROD activity can be influenced by a large number of abiotic and biotic factors including water temperature, fish age, and reproductive phase (Andersson and Forlin, 1992).

EROD induction can also vary from compound to compound. In order to better quantify and understand effects on EROD, Whyte *et al.,* (2000) described three ranges of EROD induction: weak, moderate and strong. Weak contaminants induce EROD < 10-fold above control levels, moderate contaminants induce EROD 10-100 fold above control fish, and strong contaminants induce EROD >100 fold above controls.

While CYP1A is actively involved in Phase 1 detoxification processes, it is important to note that this enzyme (the p450 subfamily) is also actively involved in regulation of the endocrine system (Fig. 3). As many of the compounds present in pulp mill effluent are known endocrine disruptors, it is possible that potential effects caused by the induction of EROD activity are a result of endocrine disruption rather than a result of detoxification. While most studies

generally treat EROD induction as an indicator of the activation of detoxification pathways, it is important to note that this might not always be the case.

2.13 Vitellogenin

Vitellogenin (VTG) is a phospholipoprotein produced in the liver of oviparous animals that is a yolk precursor protein (Jensen and Ankley, 2006). Normally, VTG is produced in mature females through the activation of estrogen receptors by 17β-estradiol as part of the normal reproductive cycle controlled through the hypothalamic-pituitary-gonadal (HPG) axis (Jensen and Ankley, 2006). VTG is produced in the liver; it then travels through the blood to reach target organs such as the gonads. As a result, measuring the presence of VTG is plasma is utilized as a reliable biomarker for endocrine disruption.

2.14 Condition factor

Condition factor (K) is generally used as an indicator of fish health. It is usually expressed as a percentage of bodyweight compared to fish length. It can vary from species to species, but with rainbow trout it is expressed as a function of total weight compared to the cubed fish length. Generally, increased condition factor is indicative of healthier fish as they are bigger and fatter, while a lower condition factor indicates decreased health, smaller, leaner fish with reduced fat stores. A reduced K can result from stress due to contaminant exposure in the field, but is generally not expected during short lab exposures. With rainbow trout a normal baseline K should be close to 1.

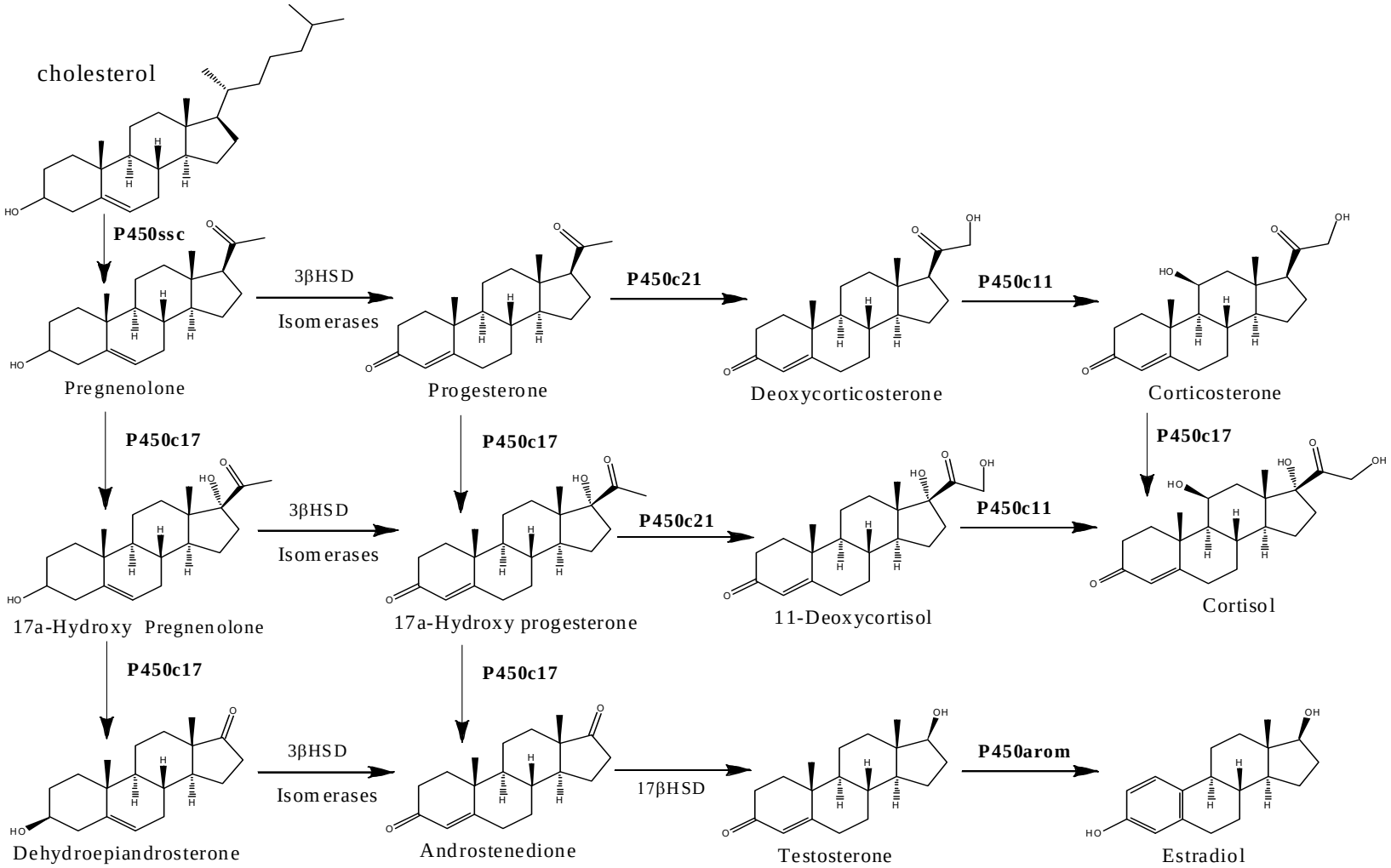


Figure 3: Steroidogenic pathway depicting both steroids and enzymes; the P450 family is in bold.

2.15 Hepatosomatic index

Hepatosomatic index (HSI) can also be used as an indicator of fish health. It is calculated by expressing the liver weight as a percent of total body weight. It generally is a crude indicator of liver activity, with a larger HSI indicating increased liver activity, possibly a result of toxicant removal.

2.16 Gonadosomatic index

Gonadosomatic index (GSI) like HSI is measured by measuring the gonad weight and expressing it as a percent of total bodyweight. It is generally used as an indicator of fish sexual maturation. Increased GSI indicates an increase in gonad size which is usually a result of sexual maturation.

2.17 Rainbow trout as a test species

One model bioindicator species is the rainbow trout (*Oncorhynchus mykiss*); it is well studied, easy to handle, cultured in lab, gives good responses for biomarkers, and is not easily stressed from handling (Battiprolu *et al.,* 2006; Clark and Rodnick, 1998; Sturve *et al.,* 2007; Vijayan *et al.,* 1997).

The life-stage of rainbow trout that is most often used and investigated in the laboratory is the juvenile stage (Capkin *et al.,* 2006; Clack and Rodnick, 1998; Polakof *et al.,* 2007; Tintos *et al.,* 2007). This life stage is preferable since it is well documented, as well as it allows for the assessment of endocrine disruption. Feeding has been found to affect energy metabolism in liver (Polakof *et al.,* 2007). Thus, rainbow trout are typically fasted 24-hours prior to dissection

in order to eliminate enzyme rhythms which are dependant upon feeding (Polakof *et al.,* 2007).

Temperature also affects the tolerance of rainbow trout to certain chemicals (Capkin *et al.,* 2006). Thus, temperature in laboratory studies should be controlled and maintained. Size has also been found to influence the ability of rainbow trout to handle toxicants; trout generally become more tolerant of contaminants as they grow in size (Capkin *et al.,* 2006). Thus size and weight should be controlled in any experiment that attempts to determine the effects of contaminants on rainbow trout. Finally gender, as previously stated, may affect energy metabolism in rainbow trout, and should consequently be considered (Battiprolu *et al.,* 2006).

2.18 Intra-peritoneal injection

Intra-peritoneal (i.p.) injections are a common method used for administering fish hormones and vaccines (Rottmann *et al.,* 2001). I.p. injection has more recently been used as a method to experimentally expose fish to compounds of interest (Orrego *et al.,* 2008). I.p. injection involves first diluting the compound one wants to inject in a carrier (a non-toxic fluid, ex. corn oil). One then injects a volume based on the weight of the organism in order to achieve the dose desired (usually µl per g (wet weight)). The benefit of using the carrier is that it allows for a slower absorption of the chemical by the liver (Mills *et al.,* 2001). One problem with water/food borne exposures is the inherent uncertainty regarding how much toxicant is taken up by the organism. Using i.p. injections

allows for the delivery of known doses to the organisms of interest. This is particularly useful as it is possible to achieve constant specific dose, regardless of the size of the individual. However, i.p. injection has its limitations which include: increased stress to fish, reduced environmental relevance and increased potential hazard to researchers.

2.19 Conclusion

The study of the reproductive effects of pulp and paper mill effluents on fish is among the largest single body of research on endocrine disrupting substances in the environment (Van den Heuvel, 2010). It is evident that the occurrence of reproductive effects in different fish species may be more complex than previously thought, with multiple causes and mechanisms. It is also clear that estrogens and other endocrine disrupting substances are native to the wood being pulped. Understanding the effects of the large number of compounds and their interactions poses a particular challenge for researchers. The development of biorefinery science and technology is evolving at a rapid pace without consideration of the biological effects (Van den Heuvel, 2010). Every plant material processing facility will have a resultant effluent, and each of those effluents will have the potential of containing some of the rich diversity of bioactive natural products present in plants (Van den Heuvel, 2010). Thus, an understanding of the mechanisms and causes of reproductive dysfunction in fish related to pulp and paper effluents will be essential to prevent further environmental problems.

3.0 RESEARCH OBJECTIVES

3.1 Overall objective

The main objective of the International Comparative Experiment is to determine the effects of pulp and paper mill effluent and exposure on liver metabolic capacity. The objective of the DHAA experiment is to determine the effects of exposure to DHAA on both liver metabolism and endocrine disruption, both alone and in combination with the hormones BS and E2. Both of these experiments will be conducted by a multiple intraperitoneal injection using rainbow trout (*Oncorhynchus mykiss*) as an experimental model.

3.2 Specific objectives

1) To assess anaerobic and aerobic capacity of the liver, following exposure to DHAA and pulp mill effluent using LDH and CS activity.
2) To determine the effects of BS, DHAA and E2 on hepatoxicity by SDH activity.
3) Determine the estrogenic effects of BS, DHAA and E2 through VTG production
4) To determine if the anti-estrogenic effects of DHAA are caused indirectly by liver damage.

3.3 Major null hypotheses

1) DHAA and pulp mill effluent do not affect liver metabolic enzymes
2) DHAA does not cause estrogenic, anti-estrogenic effects or affect VTG
3) DHAA does not cause liver cell damage

4.0 EXPERIMENTAL METHODS

4.1 Chemicals

Chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and Fisher Scientific (Ottawa, Ontario Canada) unless otherwise indicated.

4.2 Experimental animals

For the International survey project (ISP) experiment a total of 420 juvenile laboratory hatched and reared rainbow trout *Oncorhynchus mykiss* (41.8 ± 13.8 g), were used.

For the DHAA exposure experiment a total of 184 female juvenile rainbow trout *Oncorhynchus mykiss* (92.4 ± 18.8 g), were used. Fish were obtained from the Linwood Acres Trout Farms LTD (Campbellcroft, Ontario).

Fish were acclimated for two weeks to 12.1 ± 1º C flowing, filtered, dechlorinated water in 1500 L tanks (7.5 kg/m^3 of density and 3 L/min tank flow) and exposed to a constant 16 h light- 8 h dark photoperiod with ½ h of simulated dawn and dusk included in the light phase. Fish were fed once a day (5PT pellets, Martin Mills Inc. Elmira, ON, Canada) until satiated.

4.3 ISP Effluent samples

This study examined the effects of 11 different pulp and paper mill Solid Phase Extracted (SPE) effluent samples. Four of the mills were Canadian, two New Zealand and five were Brazilian. Secondary treated pulp mill effluent

samples were obtained from mills which used a variety of pulping processes such as Kraft or thermo mechanical pumping (TMP), and pulp type such as hardwood (HW) or softwood (SW). For a general summary of the mills used in this experiment see Table 4.

4.4 ISP Mill sample preparation

Samples were previously prepared by a Soxhlet Extraction followed by a Silica Column work-up, and the final extractives were obtained by Solid Phase Extraction according with Milestone *et al.,* 2011 (fall submission).

4.5 DHAA sample preparation

Samples were prepared bi-weekly prior to injection. Test compounds were first dissolved in acetone, allowing the solution to be then mixed into corn oil. The solutions were then left in the fume hood to allow the acetone to evaporate. It should be noted that all samples were dissolved in acetone, in order to maintain a suitable control; ie acetone was also added to the corn oil control. The test compounds included a steroid standard the estrogen 17β-estradiol of 5 ppm (E2), one pulp mill phytosterol standard β-sitosterol of 5 ppm (BS), 3 different DHAA concentrations (5.0, 0.5 and 0.05 ppm), 3 mixed DHAA and E2 concentrations (5 ppm E2 + 5.0, 0.5 and 0.05 ppm DHAA respectively), a DHAA + BS mixed (5ppm), and a carrier control (intraperitoneal injection of corn oil and tagged fish, CO).

For example, to achieve a dosing solution of 5 ppm DHAA, 20 mg of DHAA was weighed out in a 1.5 mL micro-centrifuge tube on a micro-balance. In the fume hood 200 µL acetone was added to dissolve the DHAA, it was then added to 10 mL of corn oil in a 10 mL amber serum injection bottle. Contents were vortexed then the acetone was allowed to evaporate.

It should be noted that 500 µL of acetone was required to dissolve the 5ppm E2 and 700 µL for the 5ppm BS; ~500 µL of acetone was added to the corn oil control in order to ensure it is a suitable carrier control. After the acetone had evaporated the bottle was then capped with a rubber septum and sealed shut by crimping an aluminum ring around the rubber septum and the top of the bottle. All injection solutions were stored in the -20º C freezer. All solutions were thawed 1 hour prior to use, and vortexed.

Table 4: Summary table of pulp and paper mills participating in this study. (Orrego et al., 2009)

Mill	Mill Type	Pulp Type	Wood Type	Waste system
Canada A	Pulp	ECF Kraft	HW	AS
Canada B	Integrated	TMP	SW & HW	AS
Canada C	Integrated	TMP	SW	AS
Canada D	Pulp	ECF Kraft	SW	Novel
Brazil A	Pulp	ECF Kraft	HW	ASB
Brazil B	Pulp	ECF Kraft	SW	AS
Brazil C	Pulp	ECF Kraft	SW & HW	AS
Brazil D	Pulp	ECF Kraft	HW	AS
Brazil E	Pulp	ECF Kraft	HW	AS
New Zealand A	Integrated	ECF Kraft & TMP	SW & HW	ASB
New Zealand B	Integrated	ECF Kraft	SW	ASB

4.6 Dose calculation and intraperitoneal injection

ISP

A preliminary 96h acute toxicity test was performed to estimate non-lethal intraperitoneal doses of the steroid standards (17β-estradiol and testosterone) and pulp mill extracts. The doses were determined immediately prior to injection based on individual fish weight (Rottmann *et al.,* 2001). Fish were injected with 25 µl of mill extract, 5 µl of steroid standard, 1 µl corn oil per 1 g of body weight.

A total of 420 juvenile laboratory hatched and reared rainbow trout *Oncorhynchus mykiss* (41.8 ± 13.8 g), were used in a 28 day pulse exposure toxicity experiment. The test compounds included two steroid standards including testosterone (T) and the estrogen 17β-estradiol (E2), eleven pulp and paper mill extracts: four Canadian mills (CAN A, CAN B, CAN C, and CAN D), two New Zealand mills (NZ A, and NZ B), five Brazilian mills (BR A, BR B, BR C, BR D, and BR E), and a control consisting of a carrier control (intraperitoneal injection of corn oil and tagged fish, CO). Fish were anesthetized in tricaine methanesulfonate (MS-222, 100 mg/L buffered with sodium bicarbonate) and then weighed, placed in a tray with flow through 12º C oxygenated water and given an intraperitoneal injection using a precision animal health syringe 187 (SOCOREX, Swiss) and 22G1 disposable hypodermic needles (B-D 305155). After injection fish were tagged with color plastic t-bar anchor tags (Orrego *et al.,* 2011 fall submission), in front of the dorsal fin, and moved into a flowing recovery tank system. Fish were then placed in 70 L glass tanks (30 fish per treatment 15

fish per tank, 8.96 kg/m^3) under the same experimental conditions used during the acclimatization period.

DHAA

As mentioned previously, a total of total of 184 juvenile rainbow trout *Oncorhynchus mykiss* (92.4 ± 18.8 g), were used in a 28 day pulse exposure toxicity experiment. The test compounds were mentioned in section 2.4. Injections commenced on the 12th and 13th of July 2010. Fish were injected with 100 µl/100 g or of 1 µl of treatment per 1 g of body weight. Fish were anesthetized in tricaine methanesulfonate (MS-222, 100 mg/L buffered with sodium bicarbonate) and then weighed, placed in a flowing water board holder system and given an intraperitoneal injection using a precision animal health syringe 187 (SOCOREX, Swiss) and 2R2 hypodermic needles (Unimed, CH-12002 Lausanne, Switzerland). After injection fish were tagged with sequentially numbered floy T-Bar anchortags (FD-68B FF), one colour and numbered sequence for each treatment. Fish were tagged in front of the dorsal fin, and moved into a flowing recovery tank system. Fish were then separated into replicate groups and placed in 2 1500 L tanks (3 sampling periods and 2 replicates per treatment (18 fish per treatment) 90 fish per tank, 8.0 kg/m^3) under the same experimental conditions used during the acclimatization period with the exception that water temperature consisted of 12.98 ± 1.06º C and fish were fed twice per day (5PT pellets, Martin Mills Inc. Elmira, ON, Canada) until satiety.

4.7 Fish sampling

ISP

Six fish were sacrificed before the experiment to evaluate their initial state of health and to get time zero measurements of enzyme activity, condition factor (K), liver somatic index (LSI) and gonadosomatic index (GSI). Groups of six fish per treatment were serially sampled after 4, 7, 14, 21 and 28 days after injection. Fish were anaesthetized by 100 mg/L MS-222 buffered with sodium bicarbonate in 10 L of water. They were then placed ventral side up on a dissection tray and euthanized by exsanguination (In accordance with Canadian Council on Animal Care Guidelines CCAC). Blood was removed from the caudal vein using a 22G1 disposable needle and a 4 mL vaccutainer. The trout were then opened longitudinally by inserting scissors in the vent and cutting towards the head, ending just after the operculum. The liver and gonads were extracted, placed in 2 mL cryo-tubes, flash frozen in liquid nitrogen and stored at -80º C.

The blood containing vaccutainers were spun at 4000 rpm at 4º C for 10 minutes, supernatant was removed and placed in 1.5 mL micro-centrifuge tubes and stored -80º C. The remaining trout which were not sampled were re-injected with their respective doses weekly, on days 7 (252 remaining trout) 14 (168 remaining) and 21 (84 remaining), using a multiple intraperitoneal injection protocol (Orrego *et al.,* 2011 fall submission). For a visual representation of the injection and sampling procedures see Fig 4.

DHAA

Four fish were sacrificed before the experiment to evaluate their initial state of health and to get time zero measurements of enzyme activity, condition factor (K), liver somatic index (LSI) and gonado somatic index (GSI). Groups of six fish per treatment were serially sampled after 7, 14, and 28 days after injection. Fish were anesthetized as above and both liver and plasma samples were obtained in the same manner as the ISP above and stored at -80º C in cryovials. The remaining trout which were not sampled were re-injected with their respective doses weekly, on days 7 (120 remaining), 14 (60 remaining), and 21 (60 remaining), using a multiple intraperitoneal injection protocol. For a visual representation see Fig. 5.

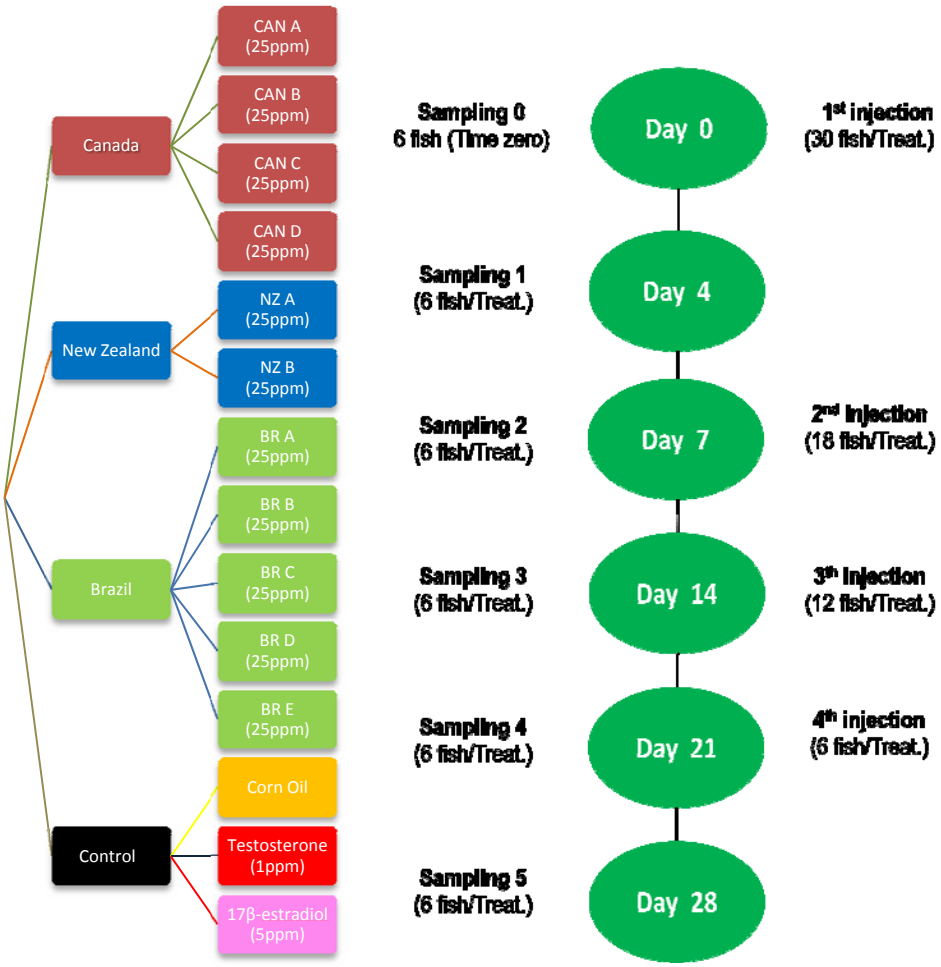


Figure 4: ISP treatments (left) and sampling/injection procedure (right)

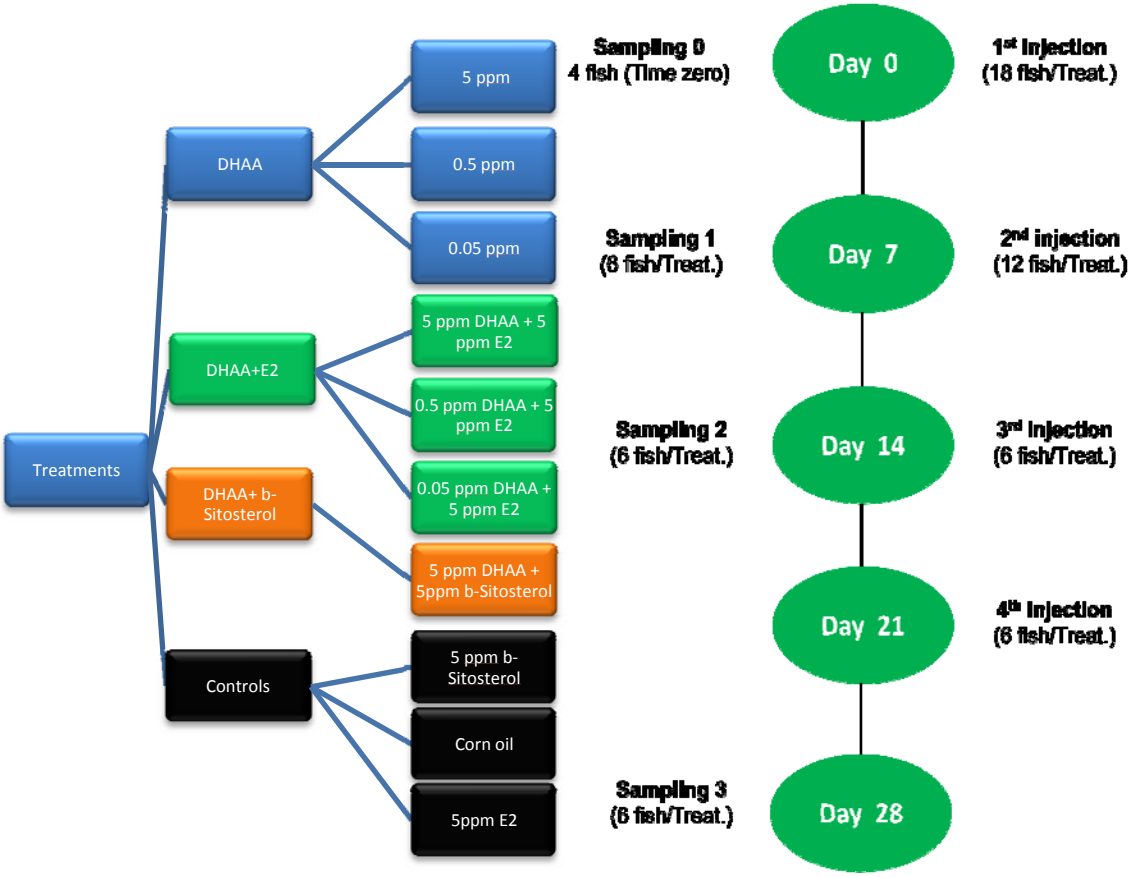


Figure 5: DHAA treatments (left) and injection procedure (right)

4.8 Bradford protein analysis

Bradford protein analysis was performed on liver and blood plasma. The technique used was modified from the cuvette technique by Bradford (1976). Samples were diluted in Mili-Q water (1/10 liver, 1/100 plasma) and plated in triplicate on a 96 well UV mircro-plate. It should be noted that EROD protein and metabolic protein were determined separately due to their different homogenization techniques. A standard curve was prepared by diluting a BSA standard in Mili-Q water (0 – 0.8 mg/mL). After adding 10 µL of samples/standards to each well 225 µL of Bradford reagent (Bioshop Inc.) was added to each well and incubated for 45 minutes. Absorbance was read in a plate reader (Bio-Tek Synergy HT micro plate reader) at 595 nm. Concentration of protein was determined by the standard protein calibration curve.

4.9 Physiological indices

Physiological condition indices were calculated on the basis of morphometric information, including the condition factor (K) (100 x weight/length^3), gonadosomatic index (GSI) (100 x gonad weight/total organism weight), and hepatosomatic index (HSI) (100 x liver weight/total organism weight).

4.10 Liver metabolic enzyme analyses

Anaerobic capacity of liver was measured by lactate dehydrogenase (LDH) activity. Aerobic capacity of liver was measured by citrate synthase (CS) activity. Both liver enzymatic activities were measured in liver homogenized

using an Ika: T-25 basic Ultra-Turrax homogenizer on level 3 for 30 s in imidazole buffer (9 volumes of 50 mM buffer to tissue, pH 7.6) and centrifuged at 2300 g for 10 minutes at 4º C. The supernatants were collected and stored at -80º C.

To determine LDH activity samples were thawed on ice and 25 µL was transferred in triplicate to a 96 well UV micro-plate and incubated at room temperature for 10 minutes. During the reaction a dosing solution consisting of 0.2 mM NADH and 1 mM sodium pyruvate in imidazole buffer was prepared (pH 7.6 at 25º C). After the incubation 225 µL of dosing solution was added to each well to start the reaction. LDH activity was measured by following the rate of oxidation of NADH to NAD^+ at 340 nm for 3 minutes (Bio-Tek Synergy HT microplate reader), in a 96 well (nonbinding clear) plate (Costar, distributed by Fisher scientific, Ottawa, ON, Canada), following a protocol based on the Kuznetsov *et al.,* (2006) and Georgiades and Holdway (2007) protocols.

To determine CS activity samples were thawed on ice, and 25 µL was transferred to control and sample wells in duplicate. Afterward, 150 µL Tris buffer (100 mM, 8.1 pH) was added to each sample well, while 175 µL was added to the control wells. The plate was incubated for 5 minutes at room temperature. Then 25 µL of 0.1 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB) was added and the plate was incubated for another 5 minutes. After the incubation 25 µL of 0.1mM acetyl-CoA was added to each well and the plate was incubated for 10 minutes at 25º C in the microplate reader. After this 25 µL of 0.15 mM oxaloacetate was added to the sample wells to start the reaction. CS activity was

followed at 412 nm for 5 minutes (Bio-Tek Synergy HT microplate reader) in a 96 well (nonbinding clear) plate (Costar, distributed by Fisher scientific, Ottawa, ON, Canada), following a protocol based on the Kuznetsov *et al.,* (2006) and Georgiades and Holdway (2007) protocols.

4.11 SDH analysis

Plasma was thawed on ice and 50 µL was transferred to a micro UV-cuvette (Plastibrand®, Germany) with 450 µL of 1.28 µM ß-Nicotinamide Adenine Dinucleotide (β-NADH), reduced form diluted in 0.1 M Tris buffer (pH 7.5 at 25º C). This was then incubated for 10 minutes at room temperature to allow the keto acids in the plasma to react. Following incubation, 100 µL of 4 M D-fructose solution was added to commence the reaction. SDH activity was determined by following the rate of oxidation of NADH to NAD^+ at 340 nm for 1 minute (Varian Cary 50 BIO UV Visible Spectrophotometer), based on the protocol used by Tugiyono and Gagnon (2002). A control consisting of a known concentration of SDH (sorbitol dehydrogenase from sheep liver lyophilized powder, ≥ 20 units/mg protein) was used. This assay was run in duplicate. Enzymatic activities were measured in mili-international units (mU). It should be noted that any samples which read under the detection limit were assigned a value of 1 for statistical analysis purposes.

4.12 EROD analysis

Cytochrome p450 enzyme (CYP1A1) activity was evaluated as 7-ethoxyresorufin-*O*-deethylas (EROD) activity in the s9 liver fraction; obtained by homogenizing liver in HEPES (4-2-hydroxyethyl-piperazine-1-ethanesulfonic acid) grinding buffer (50 mg of tissue in 250 ul of buffer 0.1 M pH = 7.8) and centrifuged at 10,000 g for 20 min at 4° C. The supernatant was collected in 1.5 mL micro-centrifuge tubes and stored at -80° C until further analysis.

HEPES grinding buffer was prepared by dissolving 5.592 g potassium chloride and 2.603 g HEPES in 500 mL of Mili-Q water and adjusted to pH 7.5, then stored at 4°C. HEPES buffer was prepared by dissolving 13.015 g Hepes in 500 mL of Mili-Q water and adjusted to pH 7.8, then stored at 4° C. 7ER/HEPES buffer was prepared by dissolving 0.022 mg of 7-ethoxyresorufin (7ER) in 1 mL dimethyl sulfoxide (DMSO). This solution was then measured in a cuvette at 461 nm and diluted with DMSO until the absorbance read between 1.60 and 1.70 absorption units. To create 7ER/HEPES buffer, 550 µL 7ER was mixed with 4550 µL HEPES buffer for each plate being run. This was created in a 15 mL falcon tube and foiled to prevent degradation by light.

Resorufin standards were prepared by first creating a super stock solution (5.0 mg resorufin in 1 mL DMSO). A working solution was created by diluting 1 mL of super stock in 9 mL of DMSO. Both super and working stock were foiled and stored at -20° C. To create the standard curve 10 µL of working solution was diluted in 4990 µL of HEPES grinding buffer and aliquoted into five 1.5 mL micro-tubes creating a standard curve of 0.0 to 4.0 µg/mL resorufin.

Fifty µl homogenate/standard (in triplicate) was transferred to a nonbinding black polystyrene 96-well plate (Costar, distributed by Fisher scientific, Ottawa, ON, Canada), 50 µl of 7-ethoxyresorufin in HEPES buffer (0.0024 mg/ml) was added. The plate was incubated in the dark at room temperature for 10 minutes. The reaction was started by adding 10 µl of NADPH and the reaction was followed using a 530- and 590-nm excitation and emission filter, in a Synergy HT Multidetection Microplate Reader (Bio-Tek instruments, Winooski, VT, USA) once per minute for 15 minutes at 25° C. Final EROD activity was expressed as pmol/min/mg of protein, following the protocol used by Orrego *et al.,* (2008).

4.13 Enzyme linked immunosorbent assay (ELISA) for fish vitellogenin

Plasma VTG concentrations were obtained using an enzyme-linked immunosorbent assay (ELISA) (Orrego et al.,, 2006). Half area, high binding, polystyrene 96-well microplates (Costar distributed by Fisher scientific, Ottawa, ON, Canada) were incubated with a vitellogenin standard curve (1-1000 ng) (Biosense laboratories, Bergen, Norway) and trout plasma samples. The plasma samples were diluted to a protein concentration of 2.00 µg/mL in coating buffer (1.59 g sodium carbonate and 2.93 g sodium bicarbonate dissolved in 1 L Mili-Q water and adjusted to a pH of 9.3).

A vitellogenin standard curve was prepared by dissolving 12 µg of VTG in 1000 µL PBS. It was then diluted in PBS to create a curve from 25-1000 ng/mL. 50 µL of sample/standard in triplicate was added to the 96 well microplate, 50 µL of coating buffer was then added to each well. The plate was covered and incubated for 24 hours at 4° C. After the incubation period the plate was washed

three times with 0.05 % TWEEN-20 in phosphate buffered saline (PBS: 1.15 g of Na_2HPO_4, 0.2 g KH_2PO_4, 8.0 g NaCl, 0.2 g KCl dissolved in 1 L Mili-Q water and adjusted to pH 7.3) (TPBS: 100 mL PBS with 500 µL Tween-20 and 900 mL Mili-Q water). During the third wash the plate was allowed to sit for 5 minutes with the TPBS before shaking it out.

The plates were then blocked with 100 µL bovine serum albumin (BSA 2% in 1x PBS) and incubated for 1 hour at room temperature. After incubation the plates were washed as above. Plates were then coated with anti-Sea Bream Vitellogenin polyclonal antibody (Biosense Laboratories). This primary antibody was used at a concentration of 1/1000 in 1 % BSA in PBS. This was added at a volume of 50 µL per well and the plate was covered and incubated for 24 hours at 4° C.

After the incubation the plates were washed as above. Following this 50 µL of goat anti-rabbit immunoglobulin G peroxidise conjugated as a secondary antibody at a concentration of 1/5000 in 1 % BSA in PBS was added to each well. The plate was then covered and incubated for 1 hour at room temperature. Following incubation the plate was washed as above.

Finally 50 µL of developing solution was added to each well consisting of 0.1 M Citric acid, 0.2 M phosphate disodic, 0.012% H_2O_2 and 0.04% O-Phenylenediamine. The reaction was allowed to develop for 30 minutes at 37°C and then was stopped by then adding 50 µL of 1.8 M H_2SO_4 to each well. Finally the plates were measured at 490 nm in a Synergy HT Multidetection Microplate

Reader (Bio-Tek Instruments Inc.). The VTG concentration was then determined from the standard calibration curve.

4.14 Data analysis

Prior to the experimental setup a power analysis was performed in order to determine sample size required for statistical significance. With 11 treatments, 5 sampling times, a sample variation of 15% and a type 1 error (alpha – α) of 0.05 it was determined that a minimum of 27 fish per treatment would be required for 80% power. For 90% power 33 fish would be required. Consequently a sample size of 30 fish was chosen.

Data was assessed for normality using a Shapiro-Wilk W test and for homogeneity using a Brown & Forsythe's test. For the ISP it was first determined if there are any statistical differences between the control CO and the two hormone controls T and E2. Since no statistical differences are detected between controls only the data corresponding to the sampling time corn oil injected control fish was used for comparison of respective treatments. Differences between lab treatments and the control were assessed using a two way analysis of variance (ANOVA, $p \leq 0.05$) confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

For DHAA, some treatments needed to be normalized. The HSI and LDH data was logged, the EROD data was normalized by a cube root was, the SDH by a ln transformation, and the CS by a square root. It should also be noted that for plasma protein the data showed two separations/groupings in terms of

normality with samples with less than 50 mg/ml protein following one normal pattern, and anything > 50 mg/ml following a different pattern. As a result of the low variance in data it was decided that the data was robust enough to conduct a parametric ANOVA. Once normality and homogeneity was established data was analysed using either a one way (comparing just treatments) and two way (treatments and days) analysis of variance (ANOVA, $p \leq 0.05$), confirmed by Tukey Post – hoc Test, $p \leq 0.05$. If there was no difference between the TZ and day 7 CO samples the TZ samples were discarded.

All statistical analyses were performed using STATISTICA 9.0 software. All graphs were created using SigmaPlot 11.0 graphing software.

5.0 RESULTS DHAA

5.1 Mortality

There were 3 mortalities that occurred during the 28 day exposure, and all occurred on or shortly after the second injection. Two fish from 5ppm DHAA/BS (one from replicate a, one from replicate b) and one fish from the 5ppm DHAA/ E2 treatment. All three were thought to have been a result of improper injection, as they died after or shortly after receiving their second injection. As a result of this, the day 14 5ppm DHAA/BS treatment had4 replicates, and the day 14 5ppm DHAA/E2 treatment had 5 replicates.

5.2 Water parameters

The water temperature during the 28 day exposure was 12.98 +/- 1.06 º C. The acclimation tank water temperature in the aquatic toxicology lab is was ~12 +/- 0.4º C. When weekly temperature is examined it is clear that water temperature increased during the 28 day exposure: 11.80 +/- 0.47 º C week 1, 12.60 +/- 0.67 º C week 2, 14.27 +/- 0.16 º C week 3 and 13.27 +/- 0.76 º C week 4. Despite this small increase of a few degrees in water temperature, the temperatures all stayed within the optimal range of rainbow trout of 10 - 15 º C. Water pH stayed relatively constant at 7.11 +/- 0.37 which falls within the ideal pH range for rainbow trout (7.0 - 7.5).

5.3 Growth (wet weight)

As the trout were individually labelled, it was possible to follow their changes in wet weight throughout this experiment. There were significant differences in wet weight found between days (i.e. day 7 fish grew but not as much as day 14 fish, while the day 28 fish were the largest) (Fig. 6). However, there were no differences in wet weight of fish between the different treatments and the corn oil control, when compared to their specific days ($p > 0.05$). Both E2 and BS had very similar growth patterns to the corn oil control. However, most of the DHAA treatments and the mixed DHAA treatments (for both E2 and BS) showed a slight non-significant reduction in wet weight compared to the corn oil controls on day 7. This effect was not as pronounced on days 14 or 28.

5.4 Physiological indices:

5.4.1 GSI

There were no changes in gonadosomatic index (GSI) observed across all treatments or times ($p > 0.05$, Appendix 1). There was large variation seen across all treatments.

5.4.2 K

There were no changes in condition factor compared to controls (K) across all treatments or times ($p > 0.05$, Appendix 2). Condition factors remained close to 1 which indicated that the fish were in good health.

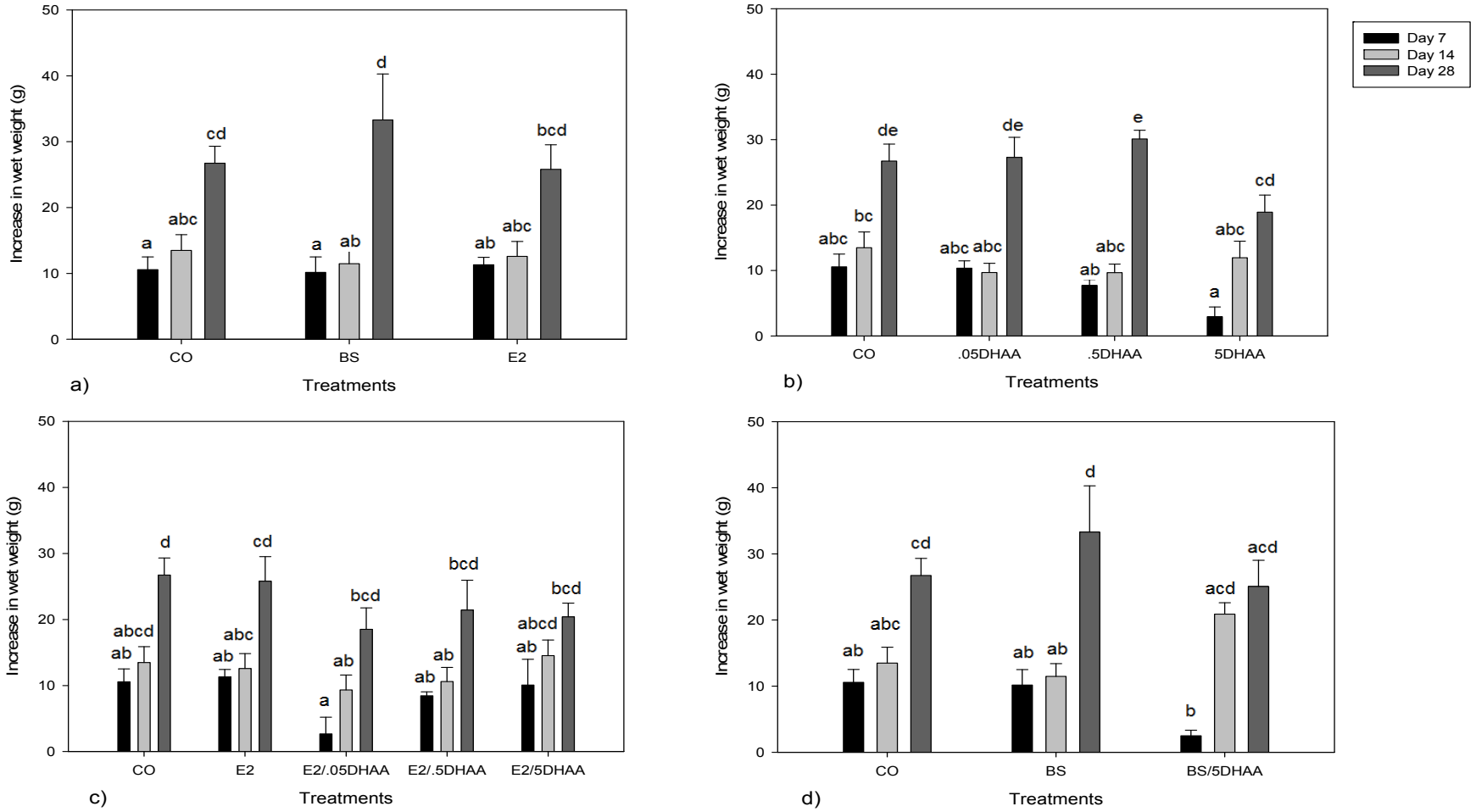


Figure 6: Increase in wet weight (g) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and day, following i.p. injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

5.4.3 HSI

The data was first examined as mean treatments (where the days were pooled) to see if there were any general effects (Fig. 7). There was a significant increase from the control observed in the E2 treatments ($p \leq 0.05$). This effect appears to have been reduced in the mixed treatments when compared to the control (E2/0.05ppm DHAA and E2/5ppm DHAA, $p > 0.05$). While slightly reduced, the E2/0.5ppm DHAA treatment was still significantly higher than the control ($p \leq 0.05$). None of the other treatments showed any statistical difference from the control ($p > 0.05$).

Subsequently, the data were split into groups and analysed by two- way factorial ANOVA, comparing both treatment and time (Fig. 8 a-d). As exposure time increased there was an increase in HSI of E2 treated fish compared to the corn oil control ($p \leq 0.05$). While E2 treatment alone increased the HSI, this effect was lessened by mixing E2 and DHAA, with all three concentrations not being significantly different from corn oil control (Fig. 8c). Neither BS nor 5ppm DHAA/BS had an effect on HSI (Fig. 8d).

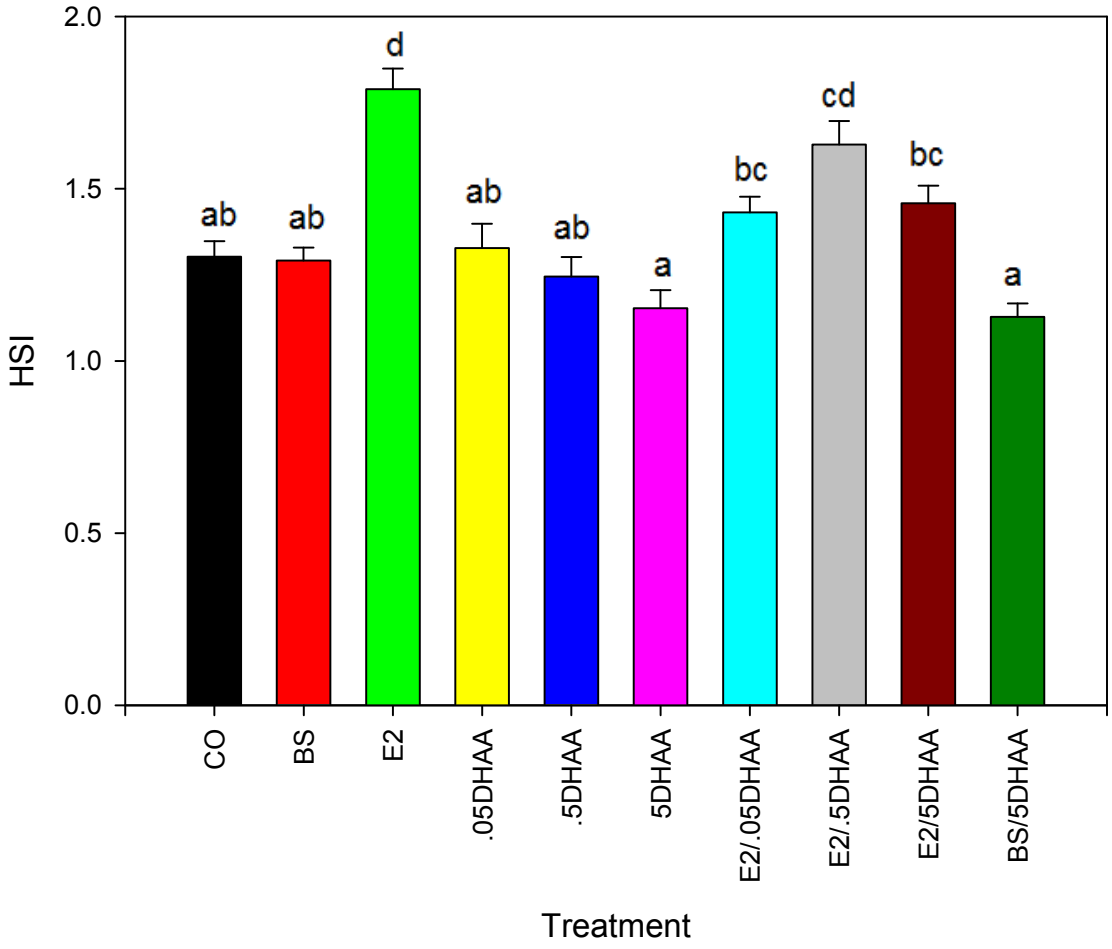


Figure 7: Hepatosomatic index (HSI) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment, following i.p. injection to various treatments. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed log(HSI).

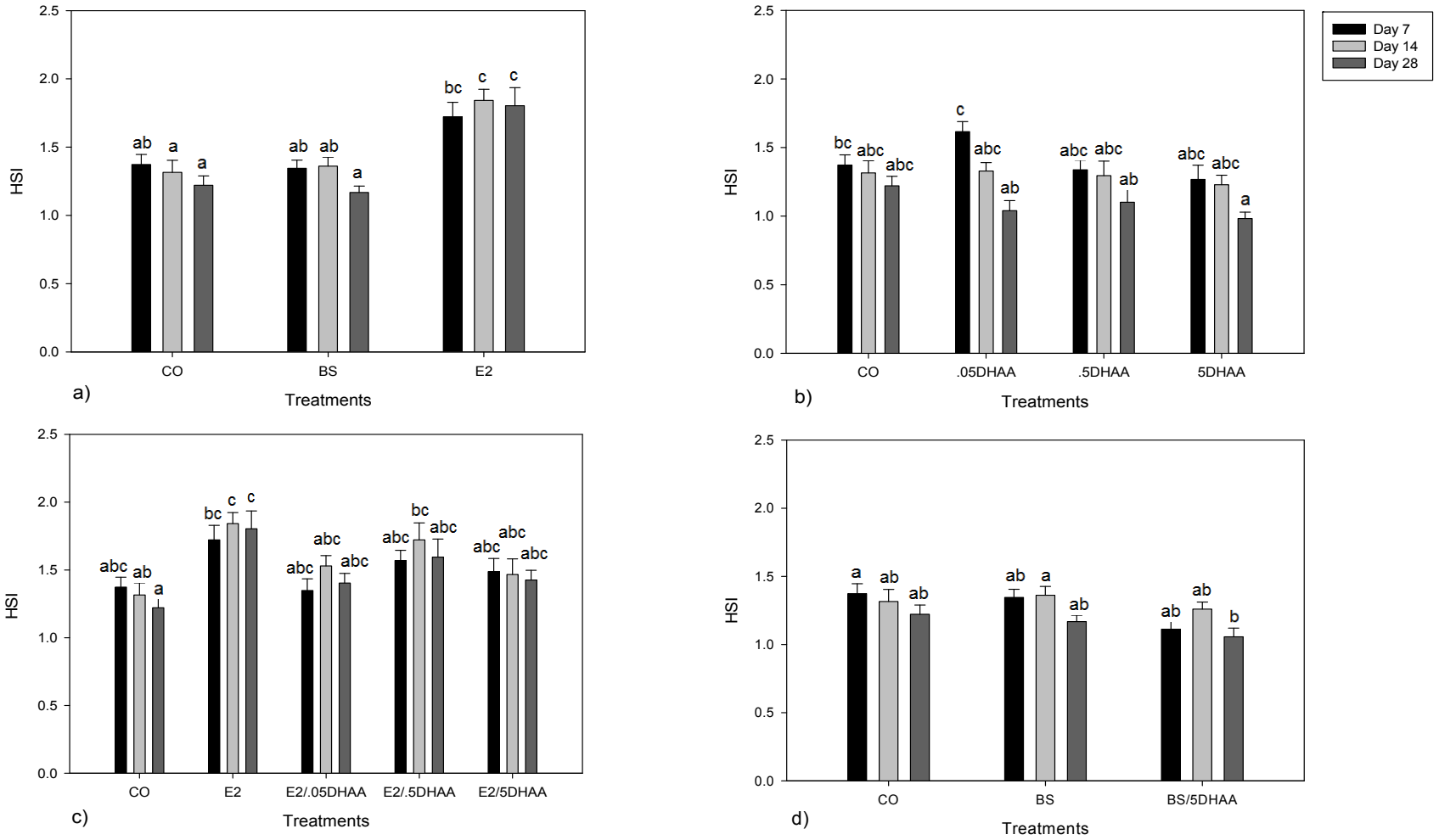


Figure 8: HSI of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days, following i.p. injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed using log (HSI).

5.5 Liver metabolic enzymes

Liver metabolic enzyme activities (LDH and CS) were expressed as fold of control. Raw data are found in Appendix 3 and 4 for CS and LDH, respectively there was a small increase in control LDH and CS activity as time increased. The variability was too high between days for both LDH and CS to find any significant differences between treatments when days were pooled (Fig. 9).

However, when comparing treatments and days, some effects were observed. When examined by days, CS had lower variability than LDH. The DHAA treatments had significantly higher CS activity (3.17, 3.86 and 3.39 for 0.05, 0.5 and 5ppm DHAA respectively) on day 7 relative to controls (Fig. 10, $p \leq 0.05$). However, CS activity was lower than controls on day 14 and 28. LDH activity was more variable, but increased activity (>1 fold) was observed on days 7 and 14 for 0.05, 0.5 and 5ppm DHAA, followed by a decrease in activity on day 28 (0.48, 0.53 and 0.31 fold 0.05, 0.5 and 5ppm DHAA, respectively).

E2 generally did not affect either LDH or CS activity, and was not significantly different from the corn oil controls (p. >0.05, Fig. 11). However, when combined with E2, all mixed DHAA treatments had significantly increased CS activity on day 7 (4.90, 4.85, and 4.63 fold, 0.05 DHAA/E2, 0.5 DHAA/E2 and 5 DHAA/E2, respectively).

The mixed treatments also showed a slight reduction in CS activity on days 14 and 28. The mixed treatments also had increased LDH activity (> 2 fold control) on day 14. While BS had no significant variation in CS activity, the mixed BS/DHAA treatment showed an increase in activity on day 7 (Fig. 12, 4.18

fold control). Interestingly, like the E2 mixed treatments, the DHAA/BS treatment also had increased LDH activity on day 14 ($p \leq 0.05$).

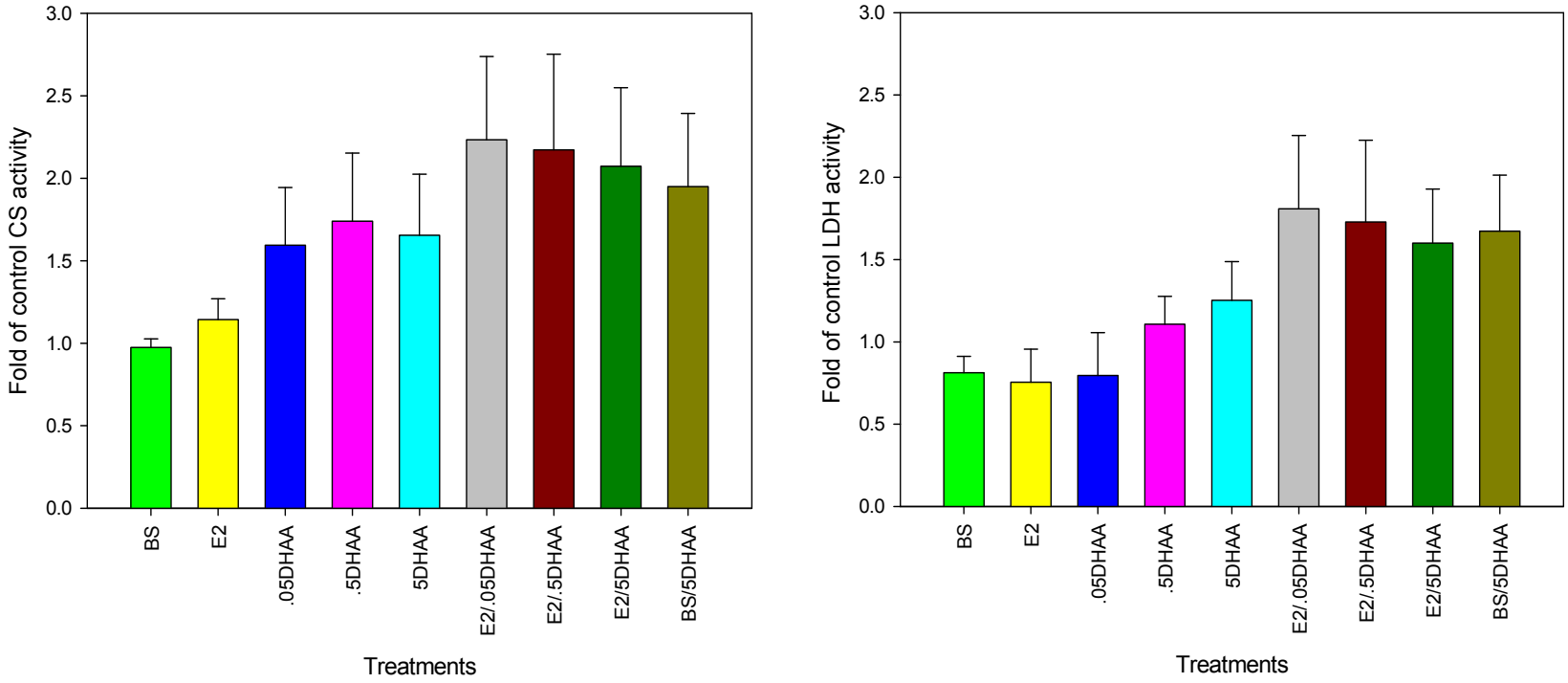


Figure 9: A comparison of liver LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment, following i.p. injection to various treatments; expressed as folds of control. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). No significant differences were observed across treatments (ANOVA, p >0.05). *Normalized data was analyzed using log (LDH) and sqrt (CS)

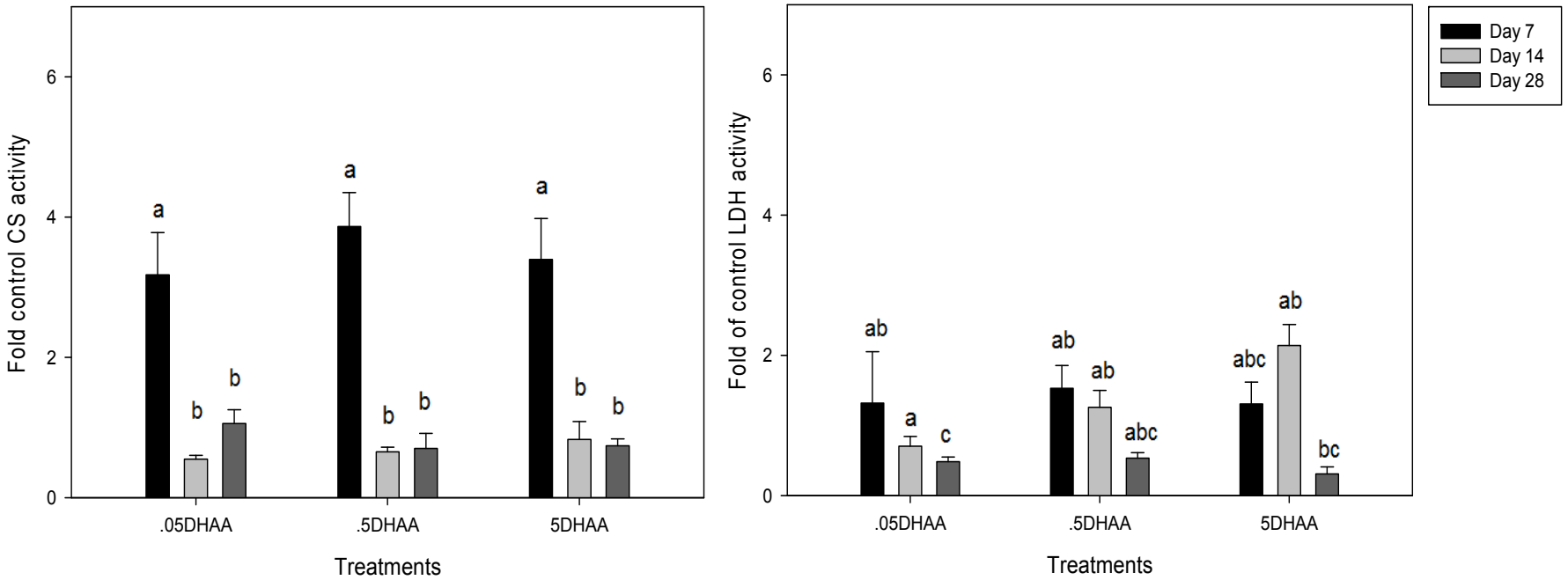


Figure 10: Hormonal effects on LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed using log (LDH) and sqrt (CS).

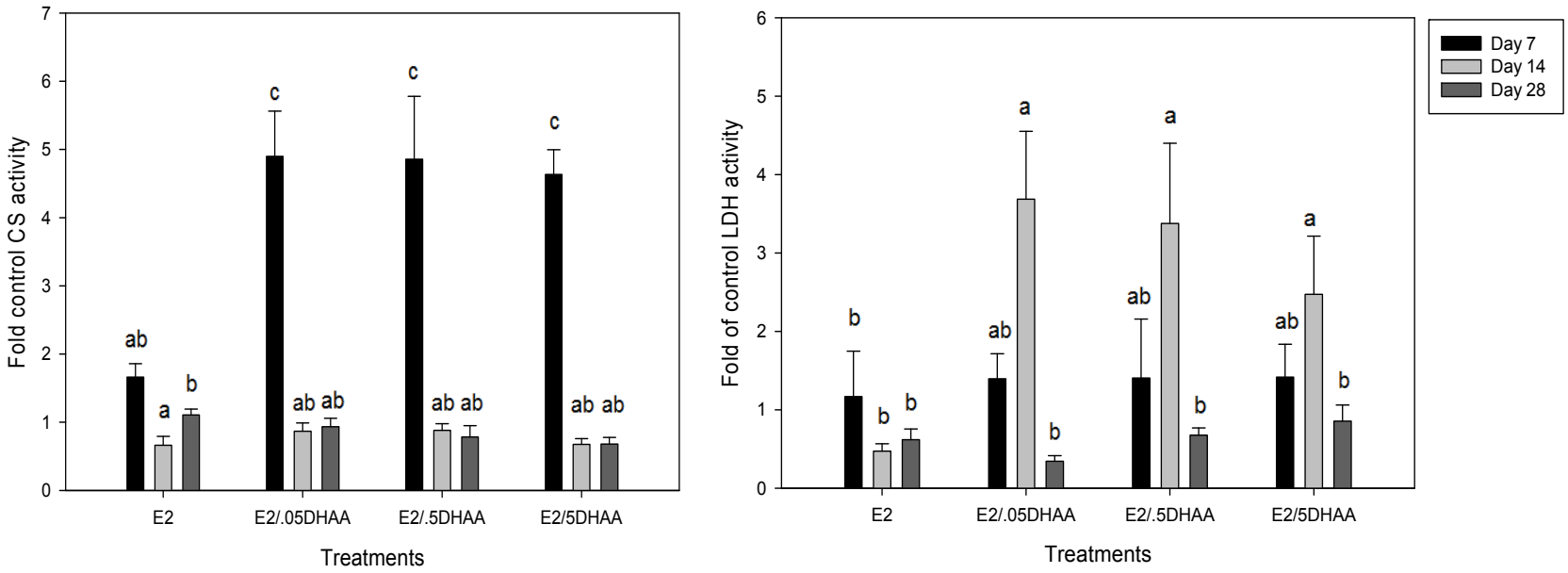


Figure 11: Effect of E2 and E2/DHAA treatments on liver LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed using log (LDH) and sqrt (CS).

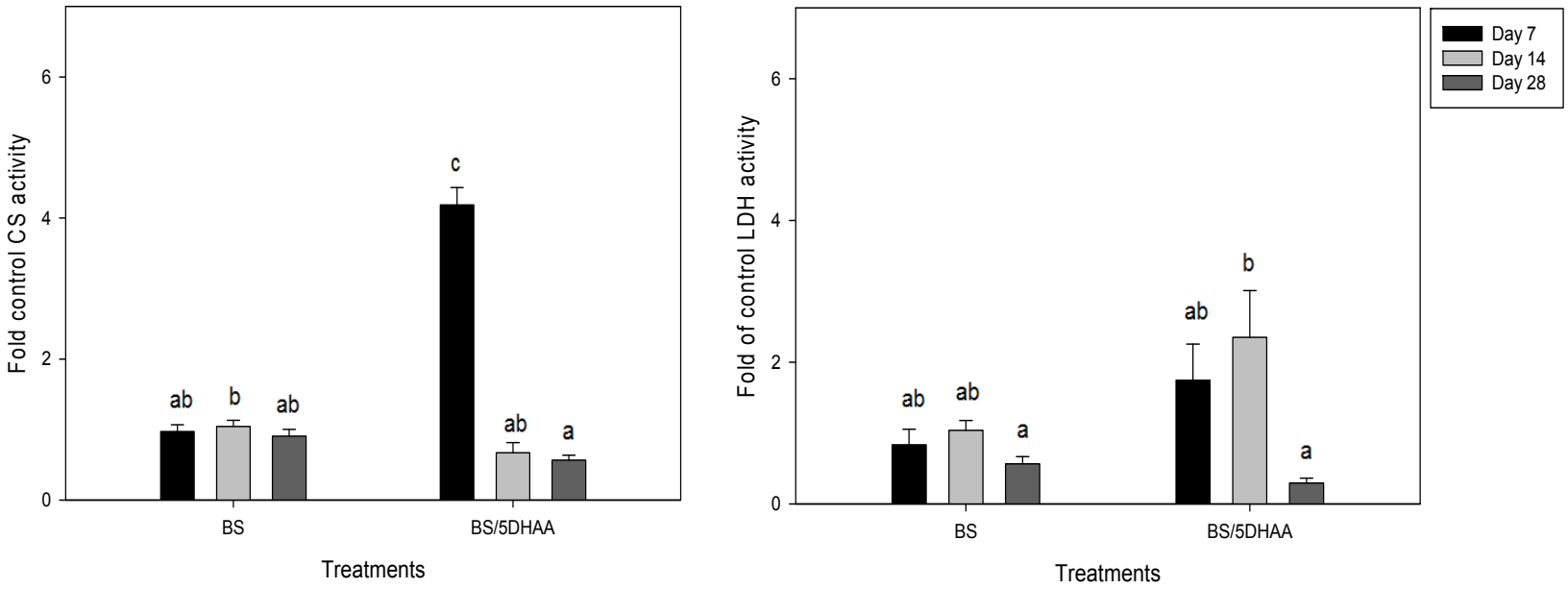


Figure 12: Effect of BS and BS/DHAA treatments on LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed using log (LDH) and sqrt (CS).

5.6 EROD

EROD variability was very high for all treatments (pooled days, Fig. 13) and there were no significant differences between treatments ($p > 0.05$). When EROD was compared over treatments and days, a clear pattern emerged. EROD activity increased across all treatments as the exposure increased (Fig. 14). Generally, activity was at its lowest on day 7, and then increased steadily on days 14 and 28. This effect was also observed in the controls and data were expressed as fold of controls (Fig. 15).

All EROD activity increased, in the 5ppm DHAA treatment compared to the control ($p \leq 0.05$). This effect of DHAA on EROD activity was also observed in all the E2 mixed treatments, as well as the BS/DHAA mixed treatments. Overall, DHAA exposure greatly increased EROD activity on day 7, followed by a moderate increase on day 14 and a slight reduction in EROD on day 28. When present alone, there was a concentration-dependant increase in EROD activity with DHAA exposure on day 7 of 1.85, 3.79 and 4.95 fold of control for 0.05, 0.5 and 5ppm DHAA, respectively. This effect was not observed in the DHAA/E2 treatments, which on day 7 all had higher EROD activities of 3.47, 4.30 and 3.16 fold for E2/0.05, E2/0.5 and E2/5ppm DHAA, respectively. Neither E2 nor BS increased EROD activity relative to controls ($p > 0.05$).

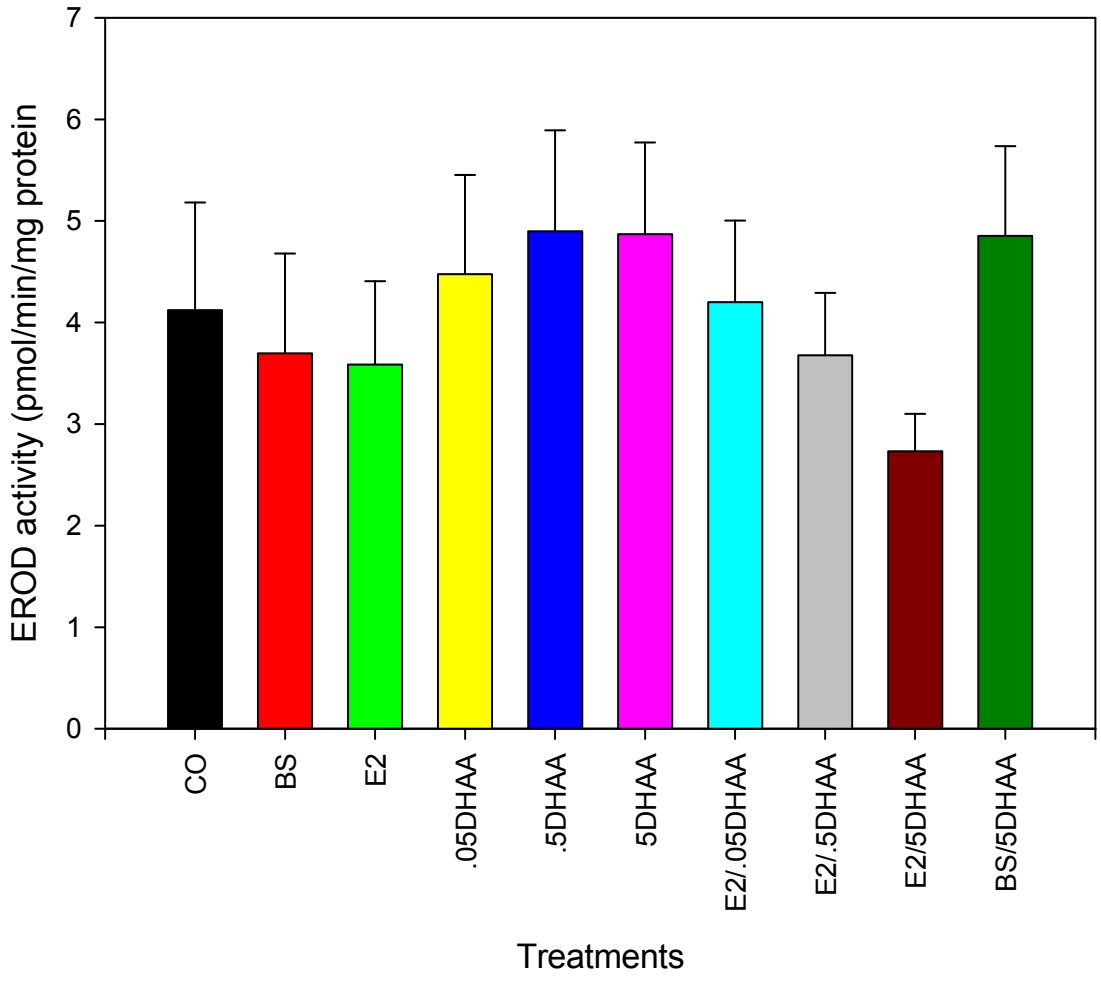


Figure 13: Ethoxyresorufin-O-deethylase (EROD) activity (pmol/min/mg protein) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). No significant differences between treatments were observed (ANOVA, $p > 0.05$).*Normalized data was analyzed using (EROD)^(1/3).

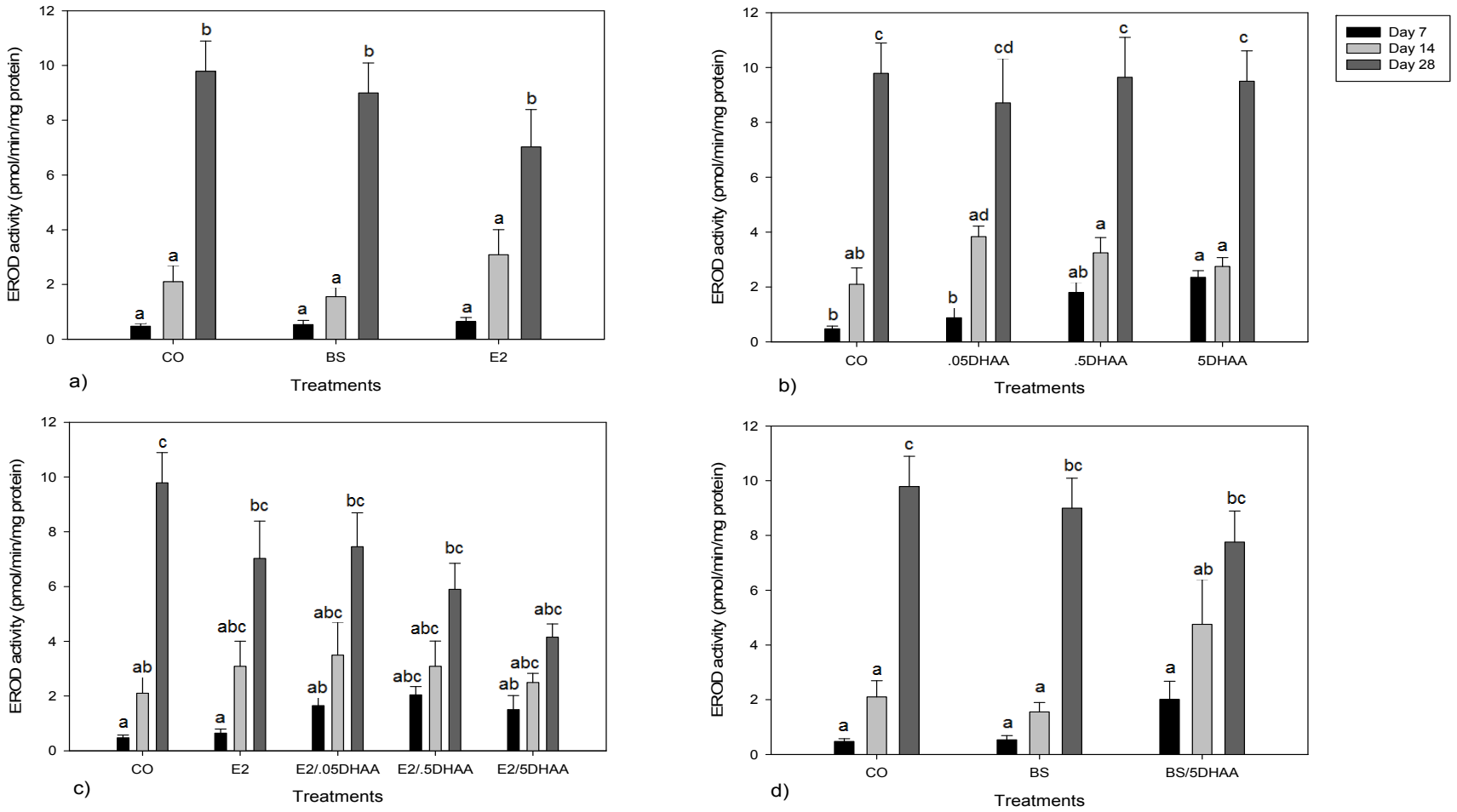


Figure 14: EROD activity (pmol/min/mg protein) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Normalized data was analyzed using (EROD)^(1/3).

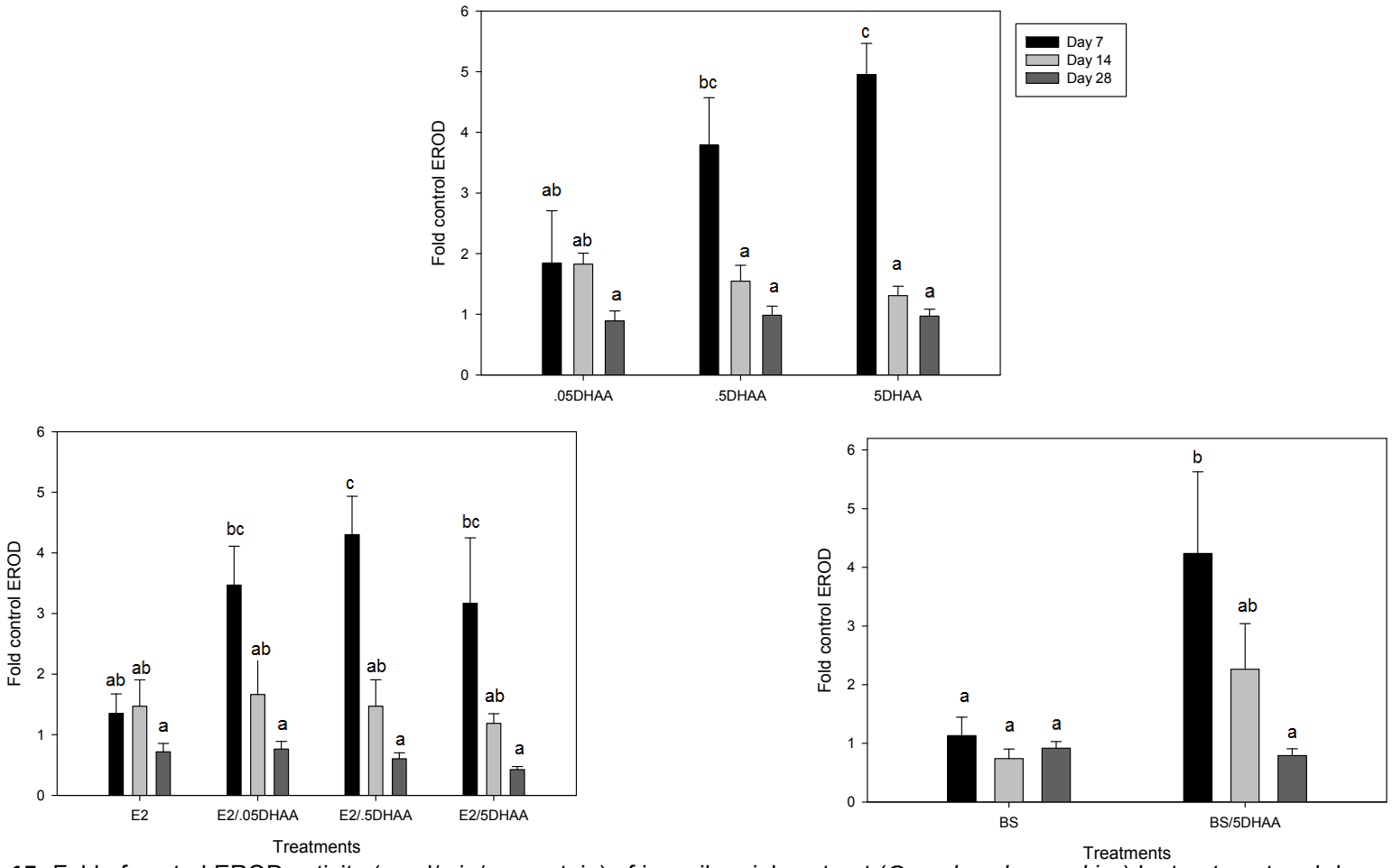


Figure 15: Fold of control EROD activity (pmol/min/mg protein) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). On each graph, bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

5.7 Plasma protein

E2 exposure significantly increased the concentration of plasma protein (Fig. 16 $p \leq 0.05$). DHAA did not affect plasma protein. When combined with E2, there was an inverse relationship between DHAA concentration and the effect of E2, with both 0.05 and 0.5 ppm DHAA/E2 plasma protein concentrations being significantly higher than then controls, while 5ppm DHAA/E2 plasma protein concentration was not significantly different.

DHAA, DHAA/BS and BS did not significantly affect plasma protein concentration (Fig. 17b and d). However, E2 exposure significantly increased plasma protein on days 14 and 28 ($p \leq 0.05$, Fig. 17a), with the highest concentration observed on day 28 (81.2 +/- 4.88 mg/mL). Protein concentration was similarly increased on days 14 and 28 for the mixed E2/DHAA treatments (Fig. 17c). These were only significant from their respective controls on day 28 for all mixed treatments and on day 14 for E2/0.5ppm DHAA ($p \leq 0.05$, Fig. 17c).

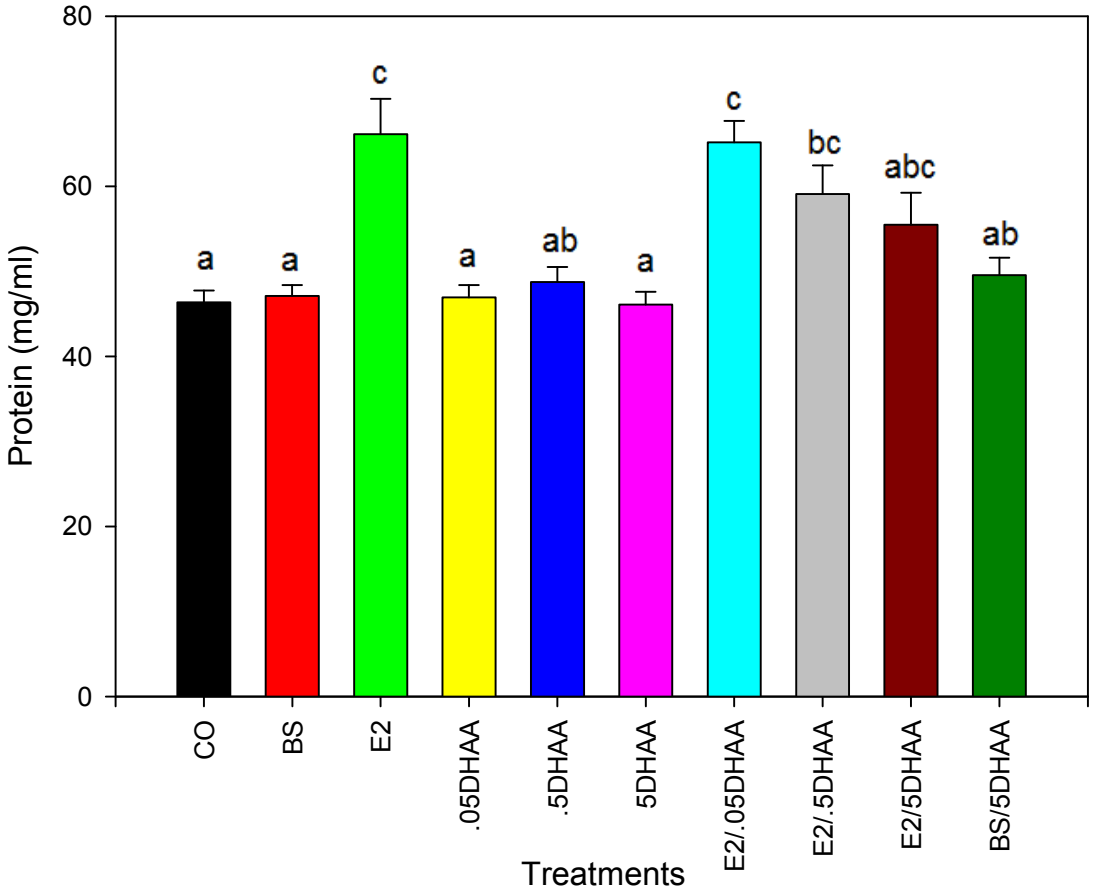


Figure 16: Plasma protein concentration (mg/mL) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post–hoc Test, $p \leq 0.05$.

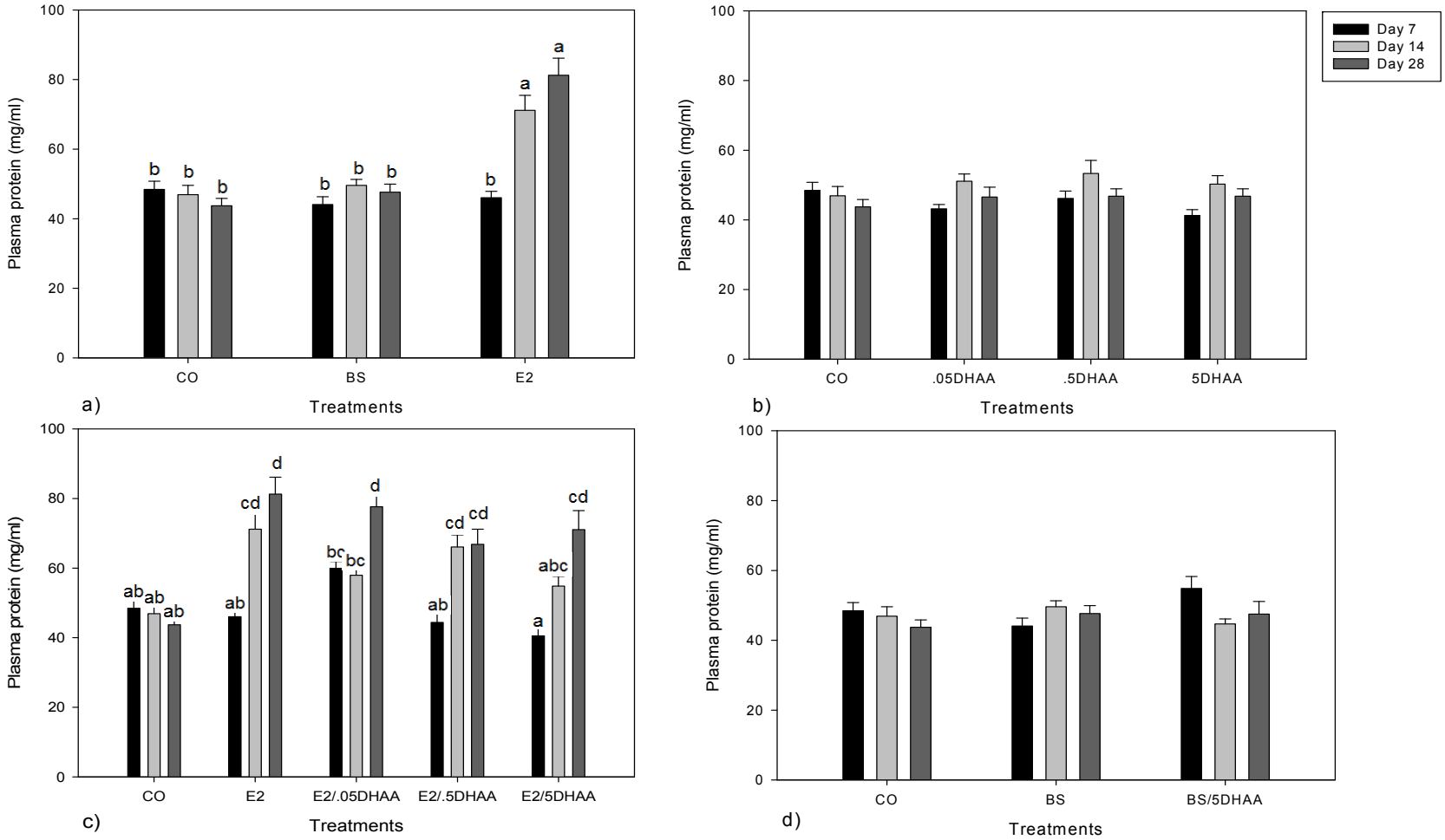


Figure 17: Plasma protein concentration (mg/mL) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

5.8 VTG

E2, BS, 0.5ppm and 5ppm DHAA exposure all significantly increased the concentration of trout plasma VTG (expressed in mg/mL) compared to the controls (Fig. 18 $p \leq 0.05$). Interestingly, BS showed a small induction of VTG while E2 had a larger effect.

VTG in the corn oil controls did not vary much with days, with an average of 15.9, 15.2 and 25.3 mg/mL plasma VTG on days 7, 14 and 28, respectively (Fig. 19a). E2 caused the highest induction of VTG, achieving its highest levels on day 14 before dropping slightly on day 28 (26.7, 38.6 and 31.9 mg/mL for days 7, 14 and 28, respectively). BS induced VTG production when compared to the corn oil control with an average concentration of 19.3, 28.6 and 32.3 mg/mL, respectively. Although the response was slower, BS exposure caused the same increase in plasma VTG as 17β-estradiol by day 28. Both BS and E2 significantly induced plasma VTG production compared to the corn oil controls on day 14 ($p \leq 0.05$).

Only the 0.5ppm DHAA treatment significantly induced plasma VTG compared to the corn oil control ($p \leq 0.05$, Fig. 19b). However, there was a pattern of increased plasma VTG for all DHAA treatments on day 7 (27.2, 29.7 and 26.5 mg/mL, 0.05, 0.5 and 5ppm respectively), with no significant differences on day 28.

The E2/0.05ppm DHAA mixed treatment VTG was not significantly different from the corn oil control VTG levels on any day ($p > 0.05$, Fig. 19c). However, the E2/0.5ppm DHAA mixed treatment VTG was significantly different

than the corn oil control VTG on all three days ($p \leq 0.05$, Fig. 17b). The highest mixed concentration of E2/DHAA VTG was significantly higher than the corn oil control VTG levels on day 7 and 14 ($p \leq 0.05$) but not on day 28 ($p > 0.05$). Both the E2/0.5 and the E2/5ppm DHAA mixed treatments had VTG induction on day 7 followed by a reduction in VTG concentration on day 14. The 5ppm DHAA mixed treatment slightly reduced plasma VTG on day 28, while the 0.5ppm DHAA mixed treatment increased VTG.

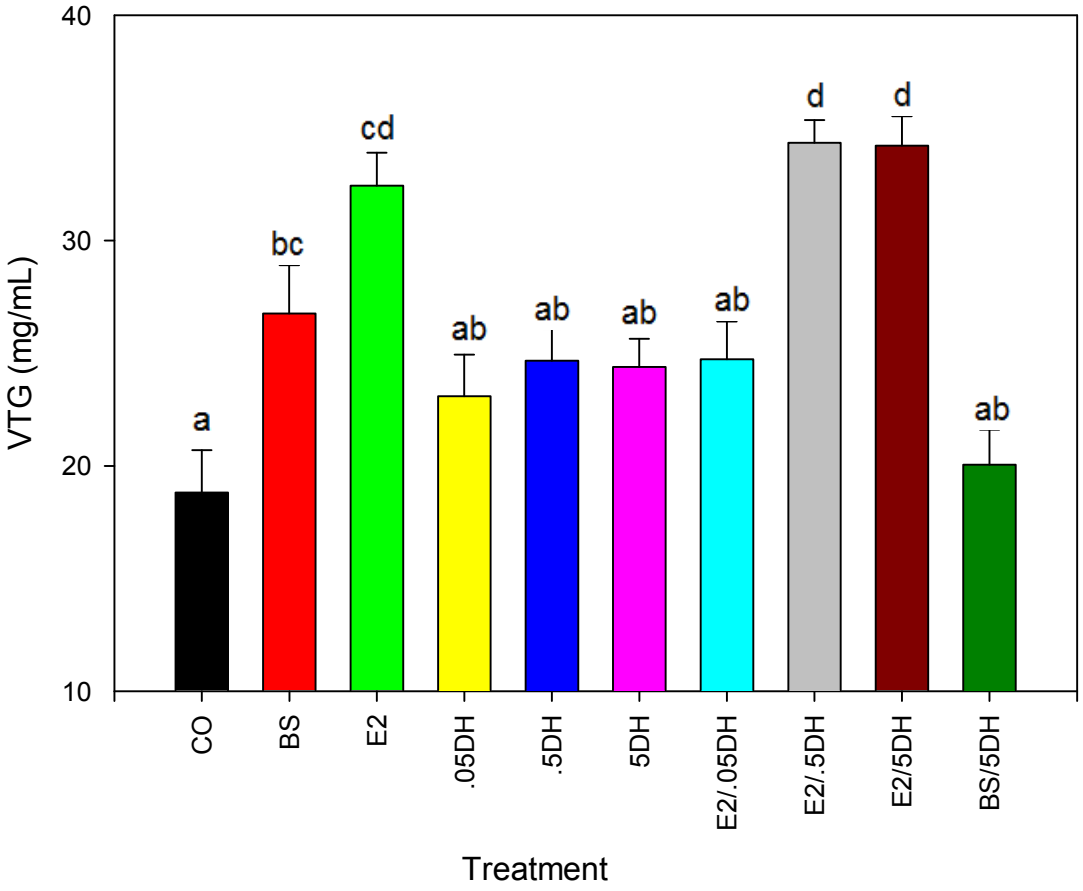


Figure 18: Vitellogenin (VTG) plasma concentration (mg/mL) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

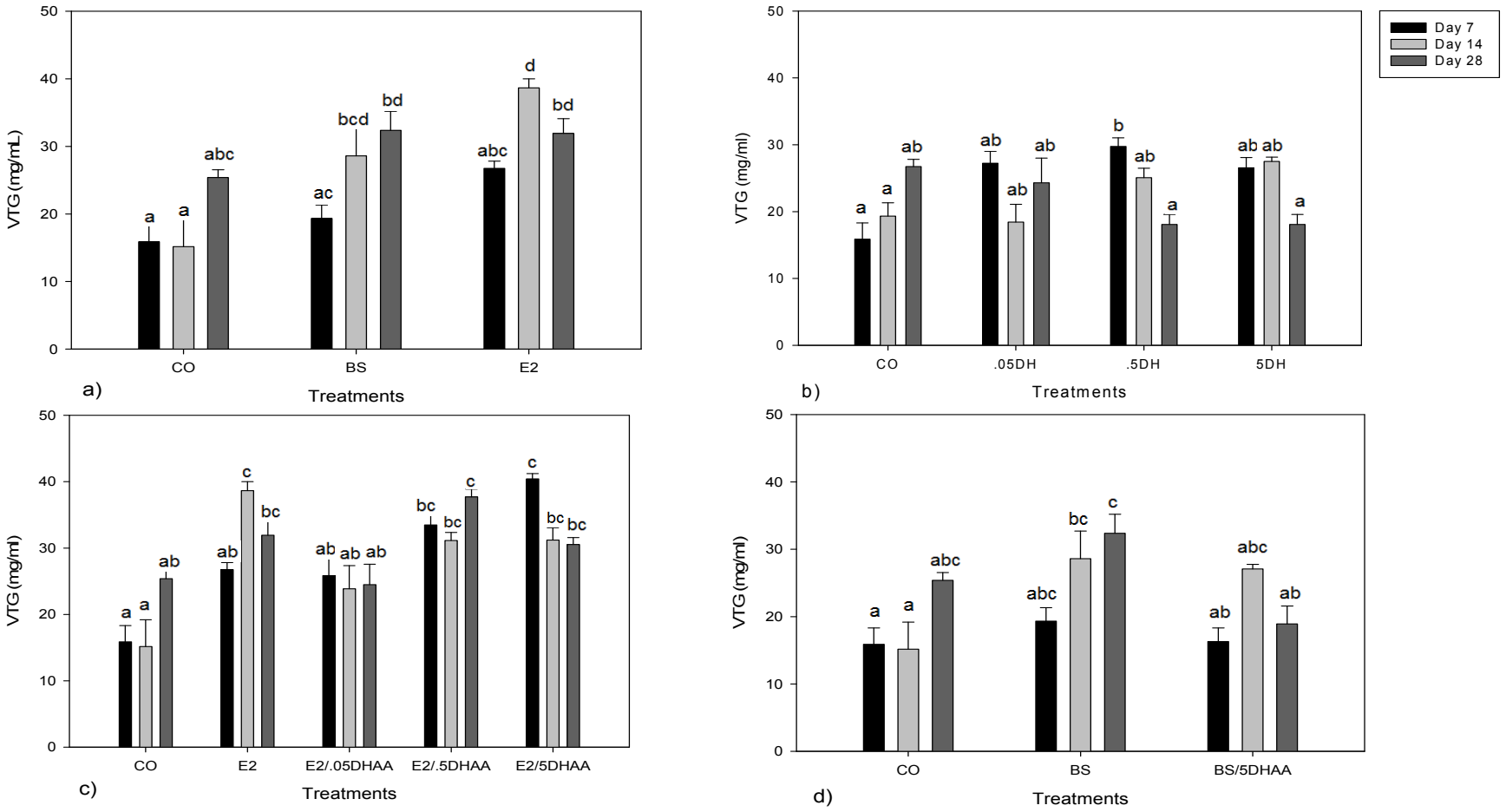


Figure 19: VTG plasma concentration (mg/mL) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$.

However, within their respective treatments (Fig. 20), it is clear that the 5ppm DHAA mixed solution caused the largest reduction in plasma VTG (a reduction of 9.2 mg/mL on day 14 and 9.9 mg/mL or ~25% on day 28) compared to day 7. The 0.5 ppm DHAA mixed treatment caused a small reduction in VTG on day 14 (2.3 mg/mL), but then increased VTG by 4.2 mg/mL on day 28 compared to their respective day 7 values. The 0.05 ppm DHAA mixed treatment had the smallest effect, reducing plasma VTG level by 1.9 mg/mL on day 14 and 1.3 mg/mL on day 28 compared to their day 7 values.

Finally the DHAA/ BS mixed treatment did not cause any significant change in VTG relative to the corn oil control ($p > 0.05$, Fig. 19d). Compared to the BS treatment, the mixed 5ppm DHAA/BS treatment reduced the amount of plasma VTG on all three days, with the most significant reduction being on day 28.

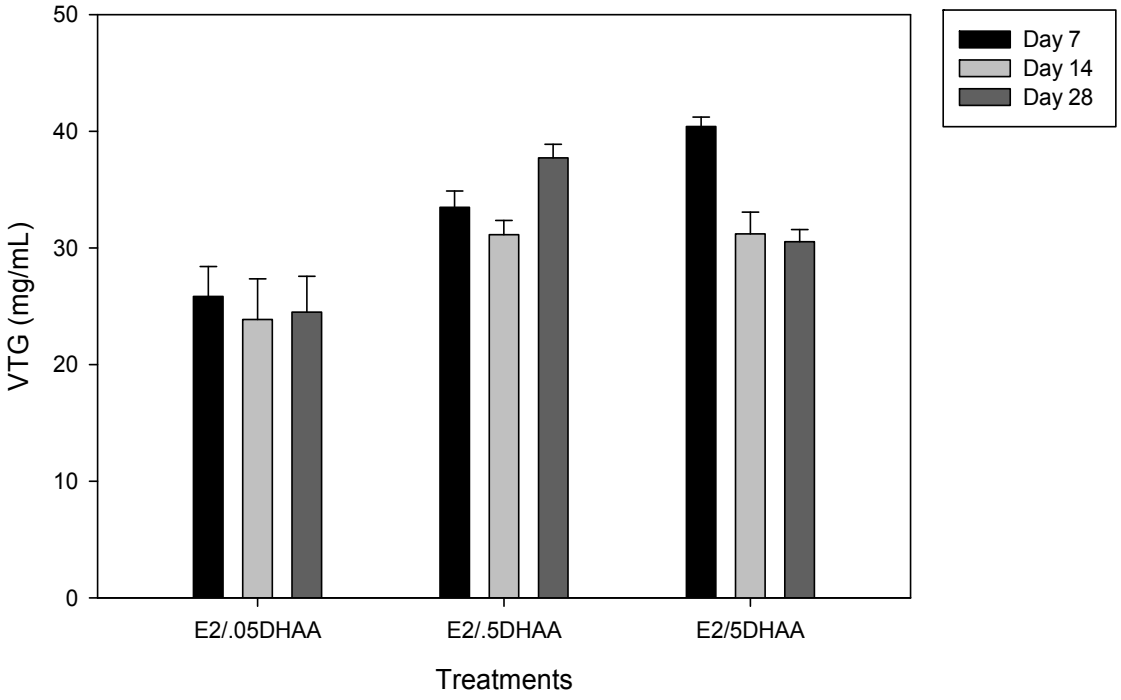


Figure 20: Effect of combining DHAA with E2 on plasma VTG concentration (mg/mL) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE).

5.9 SDH

E2 exposure significantly induced SDH activity ($p \leq 0.05$, Fig. 21). There were no effects of any other treatments on SDH activity relative to the corn oil controls, possibly due to the large variation in SDH activity.

Corn oil controls had the lowest experimental fish SDH activity of 23.6, 7.4 and 20.8 mU on days 7, 14, and 28, respectively (Fig. 22a). However, half (3) of the control fish were below the detection limit for SDH on days 7 and 14. All fish had measurable plasma SDH on day 28. E2 treated fish had the highest SDH activities of 123.5, 109.8 and 123.5 on days 7, 14, and 28, respectively, significantly higher than the respective corn oil controls ($p \leq 0.05$, Fig. 22a). BS exposed fish had increased SDH activities on day 7 and day 14 ($p \leq 0.05$) and then returned to control values on day 28 (Fig. 22a). Exposure to BS increased SDH only on day 14 relative to the respective CO controls ($p \leq 0.05$, Fig. 22a).

There were no effects of DHAA treatments on SDH relative to controls on any sampling day ($p > 0.05$, Fig. 22b). The 0.05 ppm DHAA/E2 and 0.5 ppm DHAA/E2 mixed treatments had significantly increased SDH relative to controls ($p \leq 0.05$, Fig. 22c). Mixed treatment SDH increases in SDH on day 7 were followed by a reduction in SDH activity for all mixed DHAA treatments on day 14 with only 0.05 ppm DHAA/E2 mixed treatment SDH activity significantly higher than the day 14 control ($p \leq 0.05$). Both 0.5 and 5ppm DHAA/E2 treatments were significantly reduced when compared to the day 14 E2 treatment ($p \leq 0.05$), but not different from the controls ($p > 0.05$). On day 28 the 0.5 ppm DHAA/E2

treatment had no effect on SDH activity relative to the corn oil controls and the E2 treatment ($p > 0.05$). However, both the 0.05 and 5 ppm DHAA/E2 treatment SDH levels were significantly reduced on day 28 compared to the E2 treatment SDH activity ($p \leq 0.05$), but they were not different from the controls ($p > 0.05$).

DHAA/BS treatment effects on SDH activity followed a similar pattern to BS, but the SDH activity was slightly lower on all days (Fig. 22d). The DHAA/BS treatment SDH activity was not significantly different from the controls on any days ($p > 0.05$).

A multimetric cluster analysis was conducted in order to determine how the different biomarkers related to each other (Fig. 23). It was found that GSI and K related closely, and that they formed a separate branch with the other physiological parameter HSI. A second branch was formed with all the biomarkers found in the plasma including SDH, plasma protein and VTG. Finally, the liver metabolic enzymes formed a third branch.

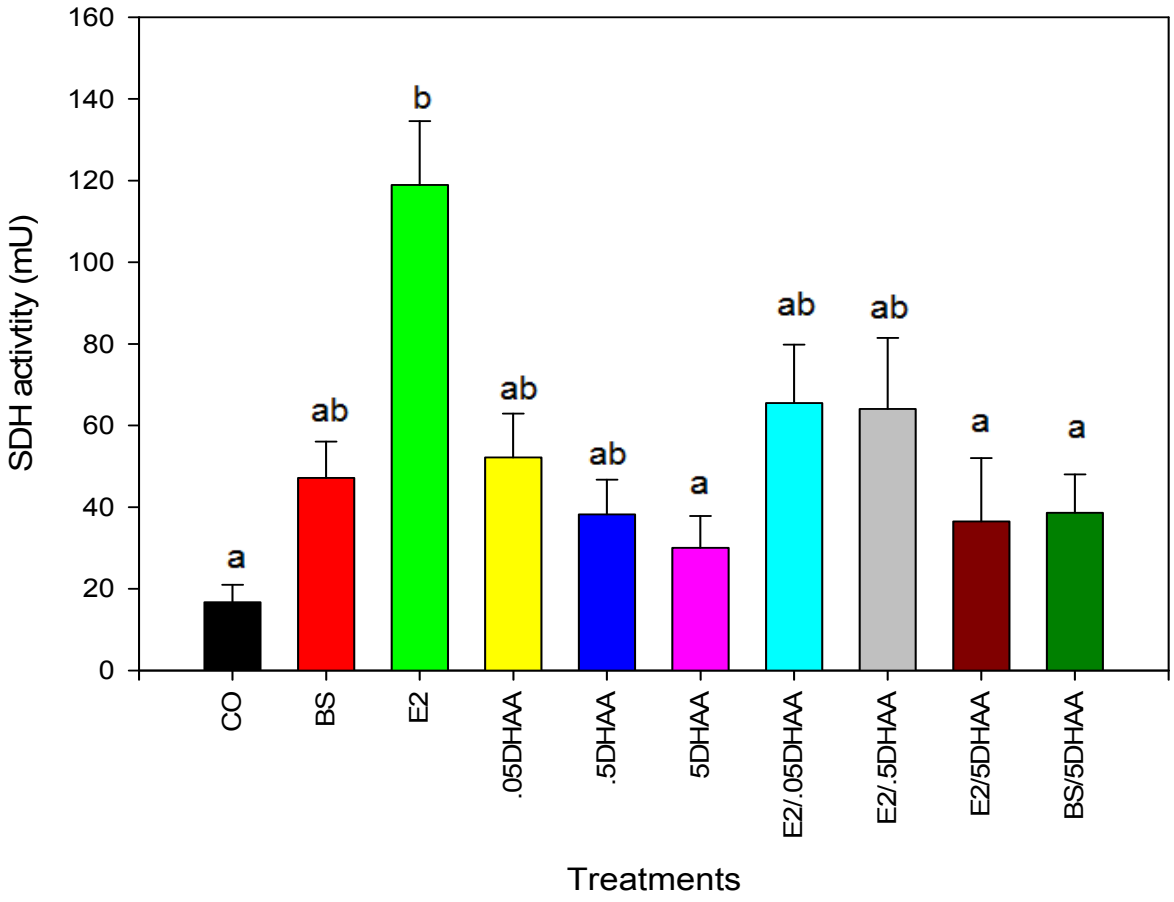


Figure 21: Sorbitol plasma dehydrogenase (SDH) activity (mU) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment. Eighteen trout were sacrificed per treatment; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Note normalized data was analyzed using ln (SDH).

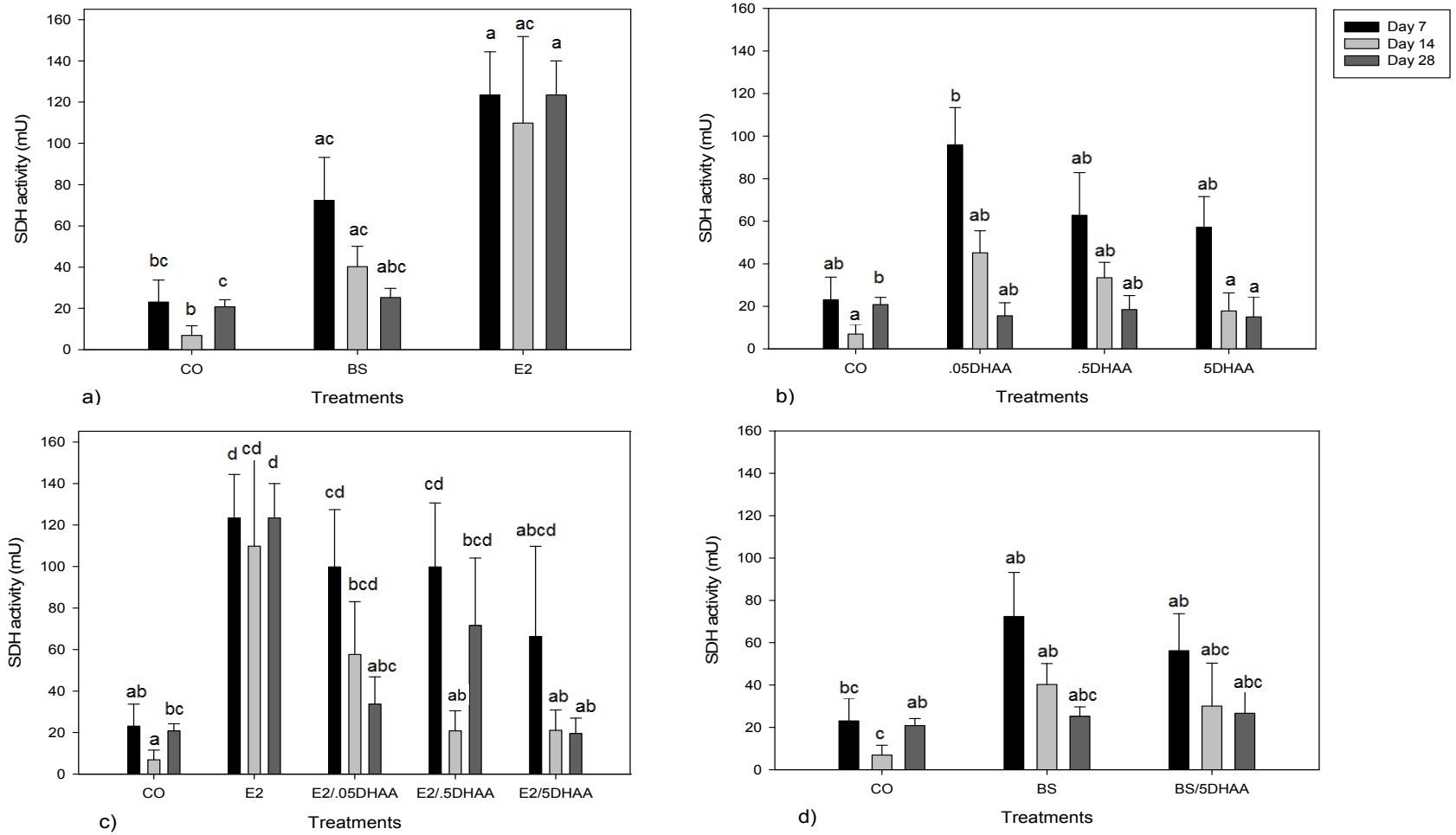


Figure 22: Plasma SDH activity (mU) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE). Bars with common letters are not significantly different ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test, $p \leq 0.05$. *Note normalized data was analyzed using ln (SDH).

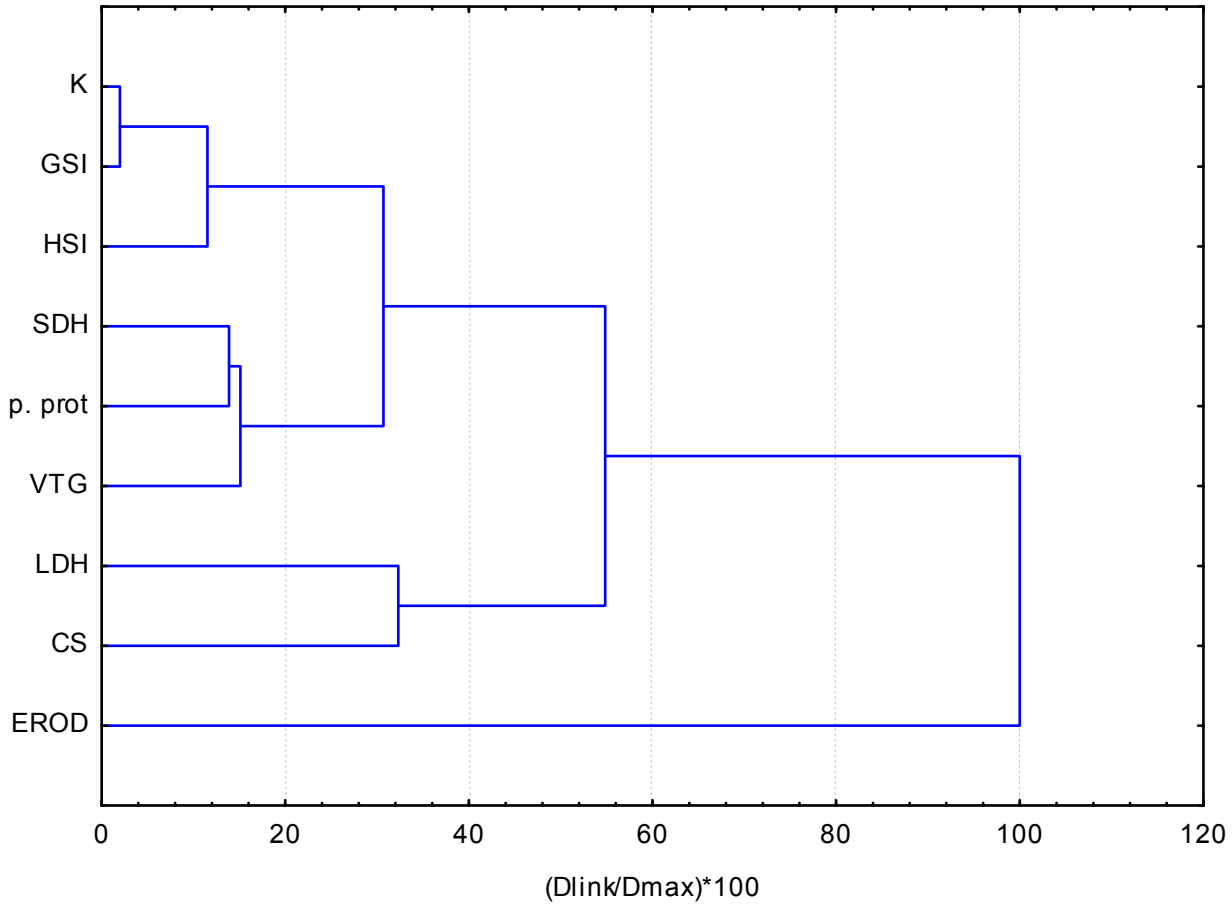


Figure 23: Vertical hierarchical tree plot of multimetric cluster analysis (Euclidean distances) using standardized linkage distance scale (Dlink/Dmax x 100) of all biomarkers analysed.

6.0 INTERNATIONAL COMPARATIVE EXPERIMENT RESULTS

6.1 Liver metabolic effects by country

The effect of i.p. injected pulp mill effluent tended to follow a similar trend for all countries. When the data of the different mills were pooled, it is clear that for CS activity, effluents from both Canada and Brazil had the greatest effects, but these effects were not statistically different from each other (Fig. 24, $p > 0.05$). All three effluents had lower CS activity on day 21 (~0.5 fold control) and higher CS activity on day 28 (3.4, 2.0 and 4.3 for Canada, New Zealand and Brazil respectively). Of all the countries New Zealand seemed to have the lowest effect on CS activity in rainbow trout liver, showing only modest induction on day 28.

The effect of the different mill effluents on LDH had higher variability. LDH activity was higher on most days for all three mills, with day 21 having the greatest increase (3-4 fold control). However, like CS none of these differences were significantly different from the corn oil controls ($p > 0.05$)

6.2 Liver metabolic enzymes by mill and days

Raw liver LDH and CS data are reported in Appendices 5 & 6. However, there were no significant differences in liver LDH and CS activities between the mills on respective sampling days ($p > 0.05$). There was only one significant difference in LDH between any of the mills on any day when compared to the corresponding corn oil (New Zealand B, day 21, $p \leq 0.05$). While the other mills were not found to be significantly different from the controls on this day, all showed increased LDH activity compared to the controls. Interestingly, LDH

activities of fish exposed to effluent from Canadian mills were still induced on day 28, while LDH of those exposed to both Brazilian and New Zealand mills were not.

However, there were many differences in liver CS activities from controls. Generally most mill effluents caused a large increase in CS activity on day 28, while CS activities were reduced on day 21. For the Canadian mills, CA B, CA C and CA D were found to have significantly induced CA activities on day 28 ($p \leq 0.05$, Fig. 25). CS activities for fish exposed to effluent from mill CA A (which typically had the lowest CS activity on most days), was significantly reduced on day 21, relative to controls ($p \leq 0.05$, Fig. 25). Finally fish exposed to CA C had significantly higher CS activity on day 14 ($p \leq 0.05$). Generally, Canadian mill effluents significantly increased liver CS activity on days 4, 14, and 28.

Brazilian mill effluents also followed the same trend as Canadian mills with exposure causing a large increase in trout liver CS activity on day 28 and a slight reduction on day 21. Exposure of fish to effluents from mills BR B, BR C, BR D, and BR E significantly increased liver CS activity ($p \leq 0.05$). Exposure to all mill effluents did not significantly affect liver CS activity on day 21 relative to controls ($p > 0.05$). Similar to fish exposed to Canadian mill effluents, trout exposed to BR D and BR C effluents had increased liver CS activity on days 4 and 14 ($p \leq 0.05$).

The effects of the two New Zealand mill effluents on liver LDH or CS were not as pronounced as for the Canadian or Brazilian mills, and were not significantly different from the corn oil controls on any day ($p > 0.05$). There were no significant differences in liver LDH or CS for fish exposed to the two hormone

treatments and the corn oil control on any day ($p > 0.05$). Mill types were also compared (Soft wood, hard wood and mixed). There were no differences observed in exposed fish liver LDH or CS activities relative to wood type ($p > 0.05$).

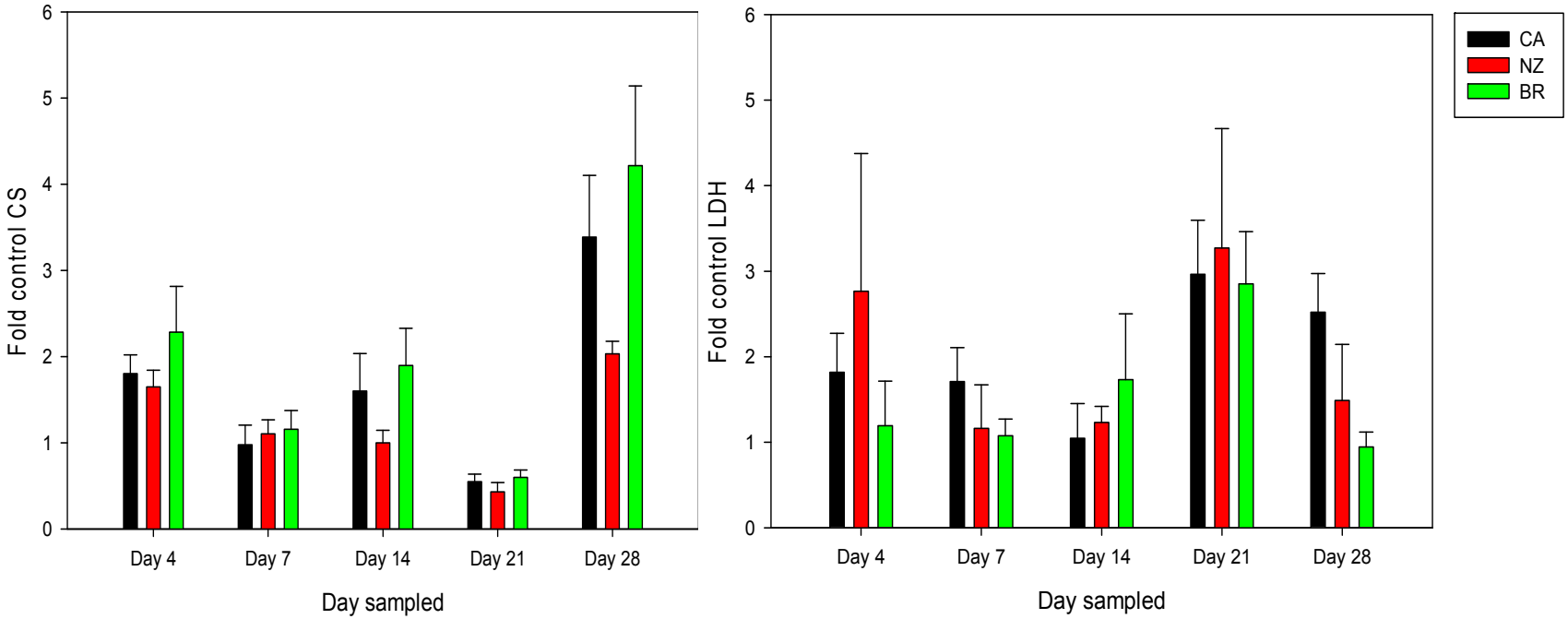


Figure 24: A comparison of liver LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment, following i.p. injection to pulp mill effluents; expressed as folds of control. Each country represents pooled data from three different countries: Brazil (BZ) 5 mills 30 fish/day, New Zealand (NZ) 2 mills 12 fish/day, Canada (CA) 4 mills 24 fish/day; data is displayed as a mean +/- standard error (SE). No significant differences were observed between treatments and their control (ANOVA, p >0.05). *Normalized data was analyzed using log (LDH) and log (CS).

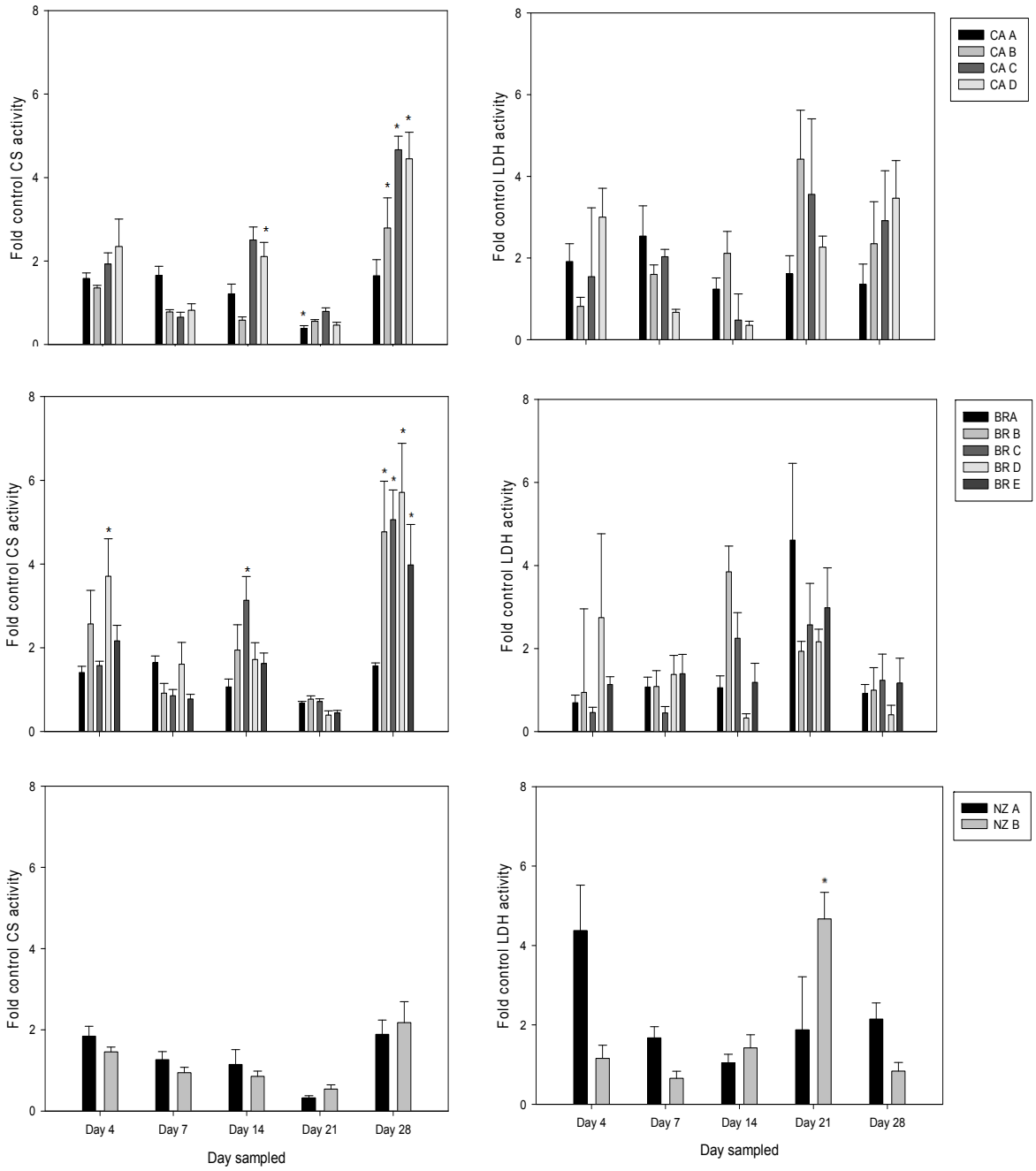


Figure 25: A comparison of liver LDH and CS activity (U) of juvenile rainbow trout (*Oncorhynchus mykiss*) by treatment and days, following i.p. injection to Canadian (CA), Brazilian (BR) and New Zealand (NZ) pulp mill effluents, by day and treatment; expressed as folds of control. Each bar represents the mean of 6 fish sampled per day; data is displayed as a mean +/- standard error (SE). Bars with * are significantly different from their corresponding CO control ANOVA, $p \leq 0.05$ confirmed by Tukey Post – hoc Test *Normalized data was analyzed using log (LDH) and log (CS).

7.0 DISCUSSION DHAA

7.1 Physiological indices and growth

One of the main effects of pulp mill effluent seen across Canada is metabolic disruption characterised by an increase in condition factor (K) (Hewitt *el al.,* 2008; Lowell *et al.,* 2005). However, this effect takes years to occur in the field and is not likely to be observed in a chronic 28 day lab exposure. In this experiment there were no significant effects of DHAA or the estrogenic compounds on K. This agrees with other published research using similar compounds and similar conditions which also found no significant change in condition over a 28 day i.p. injection exposure (Orrego *et al.,* 2008; Orrego *et al.,* 2010).

Similarly, there were no significant effects of these chemicals on growth (wet weight) which was also expected. There was a slight non-significant decrease in weight of DHAA injected fish, but this could simply reflect natural variation; although it is also possible that the fish were putting more of their energy towards detoxification rather than growth.

Exposure to DHAA, E2 and BS did not have a significant effect on gonadosomatic index. This is also supported by previous studies which used similar concentrations of DHAA, E2 and BS (Orrego *et al.,* 2010). This is generally expected since juvenile fish were used; it would be unlikely for sexual maturity to occur during the short 28 day experimental exposure.

DHAA did not have any effect on HSI. This agrees with a previous study by Oikari *et al.,* (1982), which showed no significant change in HSI following a 30

day chronic exposure. Exposure to E2 significantly increased the HSI of the juvenile rainbow trout. This effect was not observed in by Orrego *et al.,* (2010), but has been previously reported in wild fish (Lowell *et al.,* 2005). This effect is not unexpected as 17β-estrdadiol and other estrogens are known to be hepatotoxic (Ahmad *et al.,* 2009). Ahmad *et al.,* (2009) found a significant decrease in antioxidant enzymes following i.p. injection of 0.5 and 5 ppm E2 in juvenile sea bass, indicating E2's potential for inducing oxidative stress. E2 can increase the amount of reactive oxygen species (ROS) in the liver which can lead to hepatoxicity (Thilagam *et al.,* 2010).

However, the effect of 17β-Estradiol on HSI is quite variable. In one experiment by Teles *et al.,* (2006) there was a significant increase in HSI following waterborne E2 exposure, but only a non-significant increase in HSI following i.p. injection (E2 concentrations were 0.2 and 2 µg/L in the water and 0.5 and 5 ppm for i.p. injection). In contrast, Mills *et al.,* 2001 reported significant HSI increases in E2 injected fish (2 and 20 ppm i.p. injected) following 4 weeks of exposure. This increase in HSI paralleled high levels of plasma VTG, and was thought to be associated with increased vitellogenesis (Mills *et al.,* 2001).

When 5 ppm E2 was mixed with DHAA, its effect on HSI was reduced in all three concentrations of DHAA (0.05, 0.5 and 5 ppm). While still elevated, the HSI was not significantly different from controls, with the exception of E2/0.5 ppm DHAA on day 14. This demonstrated that DHAA at all three concentrations reduces the effect of E2 on HSI. DHAA has been shown to be an anti-estrogenic compound (Orrego *et al.,* 2010; Teresaki *et al.,* 2009). If the increased HSI

caused by E2 is related to vitellogenesis as Mills *et al.,* (2001) hypothesised, then presumably the reduced HSI observed in the DHAA/E2 mixed treatments is a result of DHAA acting as an anti-estrogen. However, this may not be the case, as Thilagam *et al.,* (2010) found a significant increase in ROS levels in E2 exposed fish. Thus, the increased HSI caused by E2 may also be related to cell damage/oxidative stress.

What is clear is that exposure to E2, BS and DHAA did not affect GSI or K. It was also evident that exposure to E2 significantly increased liver HSI, and this effect was reduced when E2 was mixed with DHAA. Whether this effect is related to endocrine disruption or liver damage will be discussed in greater detail in section 7.3.

7.2 Liver enzymes

The liver is a highly irrigated organ that depends on aerobic metabolism. CS is an important enzyme in aerobic metabolism while LDH is required for anaerobic metabolism, and consequently they tend to be inversely related (Heath, 1988). The Pasteur effect refers to an enhanced rate of glycolysis when oxygen is limiting as a compensation for the decline in ATP generation by oxidative metabolism (Heath, 1988). The rate of ATP generation from anaerobic glycolysis never approaches that of oxidative metabolism (Heath, 1988). Essentially, when an aquatic organism is stressed, a general increase in anaerobic metabolism is expected.

Neither E2 nor BS significantly affected the activity of LDH or CS, which agrees with the previous study by Orrego *et al.,* (2011a) that found no significant difference in either LDH or CS between corn oil injected fish and fish injected with 5 ppm E2 and BS.

There was no early induction of LDH by any of the compounds/mixtures tested. However, this induction of LDH activity tends to occur very quickly, and has been observed after 4 days i.p. injection in rainbow trout but not after 7, 14 or 28 days (Orrego *et al.,* 2011a). Consequently, it is possible that sampling every 7 days is too long to be able to see potential effects on LDH. Exposure to DHAA increased the activity of LDH on day 14. This induction was even more pronounced in the mixed treatments, however, due to the large variability in treatments, only the induction by the 5 ppm DHAA/BS treatments was found to be significant. Thus while the Pasteur effect was not observed in this experiment it is possible that it occurred too early for this design to detect.

A similar study with NaPCP i.p. injected pink snapper (*Pagrus auratus*) also reported increased liver LDH activity after 14 days (Tugiyono and Gagnon, 2002). CS activity significantly decreased on day 14 (compared to day 7), which may have been due to the Pasteur effect. It is possible that due to the multiple injections, there was a development of anaerobic conditions by day 14 at the cellular level in the injected trout.

DHAA significantly increased CS activity on day 7 for all treatments. This increase was not concentration-dependent, as all three concentrations of DHAA (0.05, 0.5 and 5 ppm) increased CS activity by about 4 fold compared to controls.

The 5 ppm DHAA/BS mixture had a similar result. When DHAA was mixed with E2, the induction of CS was slightly higher at ~5 fold of the controls. Both the LDH (day 14) and CS (day 7) results showed a slight additive effect for DHAA/E2 treatments. Despite this large induction of CS activity there was no reduction in LDH activity on this day. In fact LDH activity tended to be slightly induced (~1.2 fold control).

This significant increase in aerobic activity without a decrease in anaerobic activity might reflect an increase in maintenance energy demands and conversion efficiency in this organ (Tugiyono and Gagnon, 2002). Rissanen *et al.* (2003) reported an increase in glycolytic activity and oxygen consumption in rainbow trout hepatocytes exposed to DHAA. As DHAA is also a weak acid, it is likely that it acts as an uncoupler of oxidative phosphorylation, which would increase the respiration rate (Terada, 1990). The increased CS activity observed may reflect this.

After this high induction, CS activity decreased on days 14 and 28. It is possible that the high activity of the CS enzyme on day 7 depleted the available oxygen, forcing the liver to rely more on anaerobic activity by day 14. DHAA is known to deplete ATP, affects oxygen consumption, and causes disturbances in aerobic energy production (Rissanen *et al.,* 2003). By 28 days, despite the multiple injections, both CS and LDH returned to homeostasis and were not significantly different from the corn oil controls. The energetic perturbations early in the experiment (days 7 and 14) followed by a return to baseline by day 28 indicates that the rainbow trout may have adapted to the exposure to DHAA.

In this experiment there was a time-dependent increase in EROD activity. A very similar time-dependent increase in liver EROD activity was also observed in brown trout following an 8 week exposure to pulp and paper mill effluent (Johnsen *et al.,* 1999). It is possible that this effect was seasonally-dependent, as the fish were obtained in the spring and exposed to simulated summer conditions in the lab. Holdway *et al.* (1994) found a significant increase in liver EROD activities in sand flathead (*Platycephalus bassensis*) sampled in September compared to those sampled in March. There are many studies that show a season-dependant effect on EROD both in the field and in the lab (Holdway *et al.,* 1994; Johnsen *et al.,* 1999; Kopecka and Pempkowiak, 2008; Martel *et al.,* 1994). This illustrates the importance of controls, as the data could be expressed as fold of controls to better illustrate the effects of the different treatments.

Neither E2 nor BS had any significant effects on EROD activity. The ability of hormones like E2 to affect EROD activity is somewhat controversial with some studies showing an induction (Orrego *et al.,* 2011b) , others showing no effect (Teles *et al.,* 2006) and further studies showing EROD inhibition (Ahmad *et al.* 2009; Thilagam *et al.,* 2010). The Teles *et al.* (2006) study had multiple exposure routes including both waterborne and i.p. injection and found no change in EROD activity. The Orrego *et al.* studies (2008 and 2011b) demonstrated a weak (<10 fold) induction of EROD following i.p. injection to both BS and E2. It is possible that liver damage was the reason that E2 and BS had no effect on EROD in this experiment. Previous studies have shown that

elevated SDH levels (biomarker of liver damage) are associated with lower liver EROD levels (Holdway *et al.,* 1994; Webb *et al.,* 2007). Since SDH levels were elevated (section 7.3) liver damage may be the reason EROD was not induced. This further agrees with Martel *et al.* (1994) who suggested that high concentrations of hepatotoxic compounds may not cause increased MFO activity.

EROD activity showed a dose-dependant increase with exposure to DHAA on day 7, with 5 ppm DHAA being significantly induced compared to the corn oil controls. EROD activity is commonly induced following exposure to pulp mill effluents (Johnsen *et al.,* 1999; Martel *et al.,* 1994; Orrego *et al.,* 2006; Wartman *et al.,* 2009). However, DHAA's ability to induce EROD varies. Some studies show an induction of EROD activity while others show no effect (Pacheco and Santos, 1999; 1997; Orrego *et al.,* 2011b). While this induction is weak (<10 fold control), it still indicates that DHAA may induce biotransformation and detoxification pathways. However, like CS, this marked increase in EROD was followed by a return to baseline on days 14 and 28, further indicating that the rainbow trout may have adapted to the DHAA exposure.

There was a large variation seen in EROD, LDH and CS activities which made it difficult to show significant differences. This may have been a result of natural variation in fish responses to toxicants. In future studies a larger sample size should be utilized if possible.

7.3 Plasma enzymes and protein

Blood plasma protein can be used as a very crude indicator of effect. Increased plasma levels can indicate that there is a disturbance in homeostasis. Plasma protein was significantly elevated in E2 exposed fish. This effect was lessened by mixing E2 with all DHAA compounds. However, by 28 days, the E2 and all three mixed DHAA/E2 treatments had significantly higher levels than the controls.

The exact proteins that increased in concentration are unknown. However, two of the biomarkers examined in this experiment (SDH and VTG) are both found in the plasma and both were elevated in these fish, so presumably the increase in protein level may be at least partly due to their increased expression. Another possible explanation for increased protein concentrations is a decrease in blood volume (Oikari *et al.,* 1982). A decrease in plasma volume could be related to a change in osmoregulation (Oikari *et al.,* 1982).

It is well established that female hormones such as E2 and BS cause an increase of vitellogenin in trout (Orrego *et al.* 2008, 2011b, Christianson-Heiska and Isomaa 2008). VTG in fish exposed to BS increased as the exposure increased over time, eventually reaching the same level as E2 exposed fish by day 28. VTG in fish exposed to E2 peaked by day 14, and decreased by day 28. This is similar to the E2 effect on VTG observed in rainbow trout by Orrego *et al.* (2011b). This result could likely be explained by the high concentration of E2 injected. Oversaturation of E2 could have eventually led to a slight suppression in vitellogenin production through feedback mechanisms. The control levels of

plasma VTG were comparable to the controls in year-old female Atlantic salmon (King *et al.* 2003).

An unexpected result was that DHAA caused a weak estrogenic effect on day 7 (only significant in one of the concentrations), followed by a return to baseline on days 14 and 28. While Orrego *et al.* (2011b) found no effect of DHAA on VTG, there was a weak increase in VTG concentration. This weak estrogenic effect agrees with a previous *in-vitro* study by Chrisanson-Heiska and Isomaa (2008). However, this same lab found decreased VTG levels in male fish (no change in female fish) (Chrisanson-Heiska *et al.* 2008). The variation in effects of DHAA could be due to different exposure methods employed (i.p. injection, waterborne and cell cultures respectively). However, the effects of DHAA on VTG in this experiment were minor.

When 5 ppm DHAA was combined with BS it caused an anti-estrogenic effect, reducing the level of VTG production to control levels. This agrees with previously published studies that suggest DHAA may act as an anti-estrogen (Chrisanson-Heiska *et al.,* 2008; Orrego et al., 2011b). However, when DHAA was mixed with E2 the results varied. Compared to the corn oil control, VTG in E2/0.05 ppm DHAA exposed fish was not significantly different. However, the higher concentrations of 0.5 and 5ppm DHAA/E2 both showed weak additive effects on VTG compared to the E2 alone on day 7. This is supported by Christianson-Heiska and Isomaa (2008) who demonstrated weak additive effects of wood extractives and E2. However, it conflicts with the previous study by

Orrego *et al.* (2011b) who demonstrated a similar effect on trout VTG relative to the 0.05 ppm/DHAA mixture effects in this experiment.

After induction on day 7, 5 ppm DHAA exposed fish had the largest decrease in VTG production of the 3 mixed concentrations. Thus, the anti-estrogenic effects of DHAA on E2 seem to vary based on concentration and time. The exact mechanism by which DHAA causes its endocrine disrupting effects is unknown. It was recently shown by Teresaki *et al.* (2009) that the estrogenic/anti-estrogenic effect of DHAA is not mediated by the intracellular estrogen receptor. Another explanation could be that DHAA causes its anti-estrogenic effects indirectly through liver damage (Orrego *et al.,* 2011b).

It was mentioned previously that the increased HSI caused by E2 may be related to vitellogenesis, as Mills *et al.* (2001) hypothesized. Some of the results support this, in that E2, 0.5 ppm and 5 ppm DHAA exposed fish all had significantly increased plasma VTG. However, this is not supported by the fact that BS also induced VTG, but had no effect on HSI. Thus, while vitellogenesis may have played a role in the increased HSI, other factors may also have affected HSI.

Another explanation for the elevated HSI is increased liver damage/stress. Dixon *et al.* (1987) demonstrated that liver damage may be marked by both an increase in HSI, and SDH activity. SDH is an indicator of hepatotoxicity on the cellular level (Wiesner *et al.,* 1965). Since E2 had very elevated (6-10 fold control) SDH activity, it is likely that the E2 treated fish were experiencing liver cell damage. This is further supported by the observation that

all the mixed E2/DHAA treatments also had elevated SDH activity on some days. These results further support observations of Dixon *et al.* (1987) that liver damage, as indicated by elevated SDH (day 7), precedes the observation of significant effects in HSI (day 14). This demonstrates that SDH is a very sensitive and useful early biomarker of liver damage. It is possible that both liver damage/stress and vitellogenesis affected the HSI of E2 and DHAA/E2 treated fish, and there may be other factors involved as well.

Most studies have focused on E2 as an endocrine disrupter (Marlatt *et al*., 2006; Orrego *et al.,* 2008). However, other studies using similar concentrations have shown that E2 can also induce toxic effects (Ahmad *et al.,* 2009; Teles *et al.,* 2009; Thilagam *et al.,* 2010). Ahmad *et al.* (2009) demonstrated that a single i.p. injection of E2 of 0.5 and 5 ppm decreased both enzymatic (GPX-glutathione peroxidise, CATcatalase, GR-glutathione reductase and GST-glutathione S-tranferase) and non-enzymatic antioxidants (NP-SH-non protein thiols, GSH-total glutathione). Furthermore, the metabolism of E2 leads to the production of semiquinones and quinones, which then produce free radicals through redox cyclin (Cavalieri *et al.,* 2000). Thus, exposure to E2 may increase oxidative stress in fish by reducing antioxidant defences, while leading to the production of free radicals. This is further supported by Thilagam *et al.* (2010) who found that ROS significantly increased in the liver of E2 exposed fish. They also found that E2 exposure correlated positively with DNA damage, most likely a result of the increased ROS (Thilagam *et al.,* 2010). Cellular ROS accumulation can also

result in tissue damage. Consequently, the cell damage observed in E2 treated trout may have been a result of increased ROS accumulation.

SDH is usually negligible in the blood stream (Ozretic and Krajnovic-Ozretic, 1993). The controls still had a measurable level of SDH which was possibly a result of the method of exposure. Biochemical lesions caused by single i.p. injection of toxicants have been shown to release SDH into the serum, preceding histological damage (Dixon *et al.,* 1987). In fact, following a single i.p. injection Dixon *et al.* (1987) found an average activity of 13.0 mU in corn oil injected rainbow trout, which falls within the range reported in this study. This is supported by the observation that all the day 28 fish had a measurable level of SDH, while some of day 7 and 14 fish did not. This indicates that multiple injections cause a small stress on the liver and further illustrates the benefit of using a carrier control, since it allows a baseline to be established and compared to.

DHAA is known to be hepatotoxic to fish at low concentrations (Dixon *et al.,* 1983). As expected, DHAA increased the concentration of SDH in plasma. Conversely to what was expected and despite being many fold above controls, this effect was not statistically significant, likely due to the large variability in SDH. In fact, Dixon *et al.* (1987) suggest a minimum sample size of 10 in order to differentiate SDH activity from controls. Despite being injected every 7 days, the effect of DHAA on SDH was reduced with time. This is very similar to what was found with CS, LDH and EROD, and further indicates that the fish may have adapted during the experiment and been able to mitigate any DHAA effects by 28

days. Future experiments should consider using both plasma SDH activity and histology to provide a clearer explanation.

This result is similar to those reported in a study of the effects of a 30 day exposure of trout to 20 µg/L DHAA which also demonstrated adaptive effects (Oikari *et al.,* 1983). As a result, it is possible that the concentrations of DHAA used in this experiment were close to the minimum effective concentration of this toxicant for i.p. injection.

Contrary to what was hypothesised, mixing DHAA with E2 significantly reduced liver damage. This was most pronounced in the highest concentration of 5 ppm DHAA/E2. It appears that despite being a hepatotoxin, DHAA inhibits the liver toxicity of E2. Consequently, this does not support the hypothesis that DHAA acts as an anti-estrogen indirectly through liver damage. This is corroborated by the fact that at these concentrations, E2 is actually a much more potent hepatotoxin demonstrated by its consistent elevation of SDH activity in exposed fish.

As mentioned above, the metabolism of E2 leads to the production of semiquinones and quinones, which then produce free radicals through redox cycling (Cavalieri *et al.* 2000). If DHAA disrupts the metabolism of E2, it would potentially reduce the production of free radicals which could reduce the amount of cellular damage. Furthermore, it is possible that the early MFO induction and metabolic disruption caused by DHAA reduced the toxicological effects of E2. However, the exact method by which DHAA disrupts the effects of E2 and BS are unknown and warrant further research. The endocrine system is complex.

There are many alternate pathways that warrant future research such as determining if DHAA acts on brain acetylcholinesterase levels, or liver *zona radiata* proteins.

8.0 DISCUSSION INTERNATIONAL COMPARATIVE EXPERIMENT

This experiment was conducted in order to explore the liver metabolic effects of different pulp and paper mills. A previous study examining the liver metabolic effects of Chilean pulp and paper mill effluent found a significant increase in LDH activity on days 4 and 14 of a 28 day exposure (Orrego *et al.,* 2011a). The results of this study support this, as there was a significant increase in LDH activity of fish exposed to the New Zealand mill effluent A on day 14, and there was a general non-significant increase in LDH activity of fish exposed to most mill effluents on day 4.

However, the effect of pulp and paper mill effluent on trout LDH activity was variable with a high error. An induction of LDH activity was also seen in common carp (*Cyprinus carpio*) following waterborne exposure to distillery effluent (Ramakritinan *et al.,* 2005) and in pink snapper (*Pagrus auratus*)injected with sodium pentacholorphenate (Tugiyono and Gagnon, 2002).

It was suggested that the initial weak increase in LDH activity following exposure to mill effluent might be caused by the Pasteur effect (Heath, 1988) as a consequence of stress in the fish and a resultant general increase in anaerobic metabolism. In the Chilean pulp and paper mill study there was a marked increase in CS activity by day 28 (Orrego *et al.,* 2011a). This observed effect

was consistent across many mill effluents in this study, specifically 4/5 Brazilian mills and 3/4 Canadian mills. This increase in CS activity could again be related to metabolic compensation, as the LDH activity appears to have decreased by day 28. The inverse relationship between aerobic and anaerobic metabolism is also apparent on day 21, in which there is a weak induction of LDH and a weak reduction of CS activity. There was some variation between countries, in that New Zealand effluents tended to have the smallest effects on LDH and CS, while Brazilian and Canadian effluents had much more variable effects.

However, what is clear is that there was a large variation in effluent effects between mills and countries. This variation in metabolic effects could contribute to why there is such a variation between mills and countries. This variability has been observed in other biomarkers such as VTG (Kovacs *et al.,* 2005; Orrego *et al.,* 2006; Sepulveda *et al.,* 2004). These differences are likely a result of the large chemical differences between the complex mixtures that make up pulp and paper mill effluents.

Consequently, this study supports the findings by Orrego *et al.* (2011), in that pulp and paper mill effluent affects liver enzyme metabolism. However, the variability in these enzymes for even specific mills was too high for the sample size used in this experiment to prove anything conclusive. Future studies should consider reducing the number of days sampled in order to significantly increase the sample size and thus the statistical power of the experiment to detect such effects.

9.0 CONCLUSIONS

Exposure to 17β-estradiol significantly increased the HSI in female juvenile rainbow trout in this study. It was thought that this increase may have been caused by a combination of increased vitellogenesis marked by increased VTG and liver damage marked by increased SDH. This study also found that an i.p. injection of 5 ppm DHAA will cause metabolic perturbations marked by an increase in CS activity on day 7 and an increase in LDH activity by day 14. Exposure to DHAA was further marked by an elevation in both EROD and SDH activity on day 7.

However, the activity of all these biomarkers was returned to baseline by day 28 which indicates an adaptive response and that this exposure was close to the minimum effective concentration of DHAA through i.p. injection. When mixed with E2, DHAA caused a reduction in the effect of E2 on HSI, liver damage and varied effects on VTG. While this study shows that DHAA does not cause anti-estrogenic effects through liver damage, it does suggest that it may be caused by a disruption in the metabolism of E2 or through early MFO induction.

This study also demonstrated that pulp and paper mill effluent affects liver enzyme metabolism through alterations in the activity of LDH and CS. This activity varied greatly between mills and illustrates the difficulty establishing effects from the complex mixtures that make up pulp and paper mill effluents

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11.0 APPENDICES

Appendix 1

Summary of gonado somatic index (GSI) of juvenile rainbow trout (*Oncorhynchus mykiss*), following intra-peritoneal injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE).

	Day 7	Day 14	Day 28
CO	0.088 (0.012)	0.094 (0.014)	0.080 (0.006)
BS	0.070 (0.012)	0.114 (0.029)	0.074 (0.011)
E2	0.119 (0.026)	0.114 (0.016)	0.078 (0.010)
0.05 ppm DHAA	0.106 (0.016)	0.085 (0.006)	0.097 (0.010)
0.5 ppm DHAA	0.094 (0.011)	0.100 (0.013)	0.083 (0.010)
5 ppm DHAA	0.079 (0.007)	0.071 (0.011)	0.086 (0.010)
0.05 ppm DHAA + E2	0.092 (0.008)	0.096 (0.006)	0.095 (0.018)
0.5 ppm DHAA + E2	0.109 (0.023)	0.080 (0.013)	0.099 (0.008)
5 ppm DHAA + E2	0.083 (0.004)	0.099 (0.014)	0.079 (0.016)
5 DHAA + BS	0.089 (0.019)	0.086 (0.006)	0.088 (0.021)

Appendix 2

Summary of condition factor (K) of juvenile rainbow trout (*Oncorhynchus mykiss*), following intra-peritoneal injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean +/- standard error (SE).

	Day 7	Day 14	Day 28
CO	1.09 (0.03)	1.07 (0.02)	1.04 (0.02)
BS	1.13 (0.04)	1.05 (0.03)	1.05 (0.02)
E2	1.11 (0.05)	1.08 (0.04)	1.08 (0.04)
0.05 ppm DHAA	1.03 (0.04)	1.00 (0.03)	1.00 (0.02)
0.5 ppm DHAA	1.04 (0.04)	1.02 (0.04)	1.07 (0.03)
5 ppm DHAA	1.04 (0.02)	1.05 (0.01)	1.07 (0.03)
0.05 ppm DHAA + E2	1.03 (0.02)	1.02 (0.03)	1.05 (0.01)
0.5 ppm DHAA + E2	1.02 (0.01)	1.02 (0.03)	1.02 (0.03)
5 ppm DHAA + E2	1.10 (0.04)	0.98 (0.03)	1.01 (0.01)
5 DHAA + BS	1.00 (0.04)	1.00 (0.02)	1.06 (0.02)

Appendix 3

Summary of citrate synthase activity (CS) of juvenile rainbow trout (*Oncorhynchus mykiss*) in international units (U), following intra-peritoneal injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean (U) +/- standard error (SE).

	Day 7	Day 14	Day 28
CO	0.21 (0.02)	0.89 (0.08)	1.16 (0.10)
BS	0.21 (0.02)	1.05 (0.11)	1.05 (0.11)
E2	0.35 (0.04)	0.59 (0.12)	1.29 (0.10)
0.05 ppm DHAA	0.67 (0.13)	0.49 (0.05)	1.23 (0.23)
0.5 ppm DHAA	0.82 (0.10)	0.58 (0.06)	0.98 (0.21)
5 ppm DHAA	0.72 (0.12)	0.74 (0.23)	0.86 (0.11)
0.05 ppm DHAA + E2	1.04 (0.14)	0.77 (0.11)	1.08 (0.14)
0.5 ppm DHAA + E2	1.03 (0.20)	0.79 (0.09)	0.91 (0.19)
5 ppm DHAA + E2	0.98 (0.08)	0.60 (0.07)	0.79 (0.12)
5 ppm DHAA + BS	0.89 (0.05)	0.60 (0.13)	0.66 (0.08)

Appendix 4

Summary of lactate dehydrogenase activity (LDH) of juvenile rainbow trout (*Oncorhynchus mykiss*) in international units (U), following intra-peritoneal injection to various treatments. Six trout were sacrificed per treatment per day; data is displayed as a mean (U) +/- standard error (SE).

	Day 7	Day 14	Day 28
CO	0.18 (0.02)	0.22 (0.05)	0.42 (0.12)
BS	0.15 (0.04)	0.23 (0.04)	0.24 (0.04)
E2	0.21 (0.10)	0.10 (0.02)	0.26 (0.06)
0.05 ppm DHAA	0.23 (0.13)	0.13 (0.04)	0.20 (0.03)
0.5 ppm DHAA	0.27 (0.06)	0.28 (0.05)	0.23 (0.03)
5 ppm DHAA	0.23 (0.06)	0.46 (0.07)	0.13 (0.04)
0.05 ppm DHAA + E2	0.25 (0.06)	0.81 (0.19)	0.15 (0.03)
0.5 ppm DHAA + E2	0.25 (0.13)	0.74 (0.22)	0.29 (0.04)
5 ppm DHAA + E2	0.25 (0.07)	0.62 (0.16)	0.31 (0.08)
5 ppm DHAA + BS	0.25 (0.07)	0.76 (0.03)	0.32 (0.20)

Appendix 5

Summary of LDH activity of juvenile rainbow trout (*Oncorhynchus mykiss*) in international units (U), following i.p. injection to various pulp mill effluent extracts. Six trout were sacrificed per treatment per day; data is displayed as a mean (U) +/- standard deviation (SD).

	Day 4	Day 7	Day 14	Day 21	Day 28
CO	0.12 (0.04)	0.26 (0.09)	0.31 (0.08)	0.07 (0.04)	0.13 (0.02)
E2	0.07 (0.01)	0.16 (0.03)	0.19 (0.06)	0.71 (0.20)	0.44 (0.19)
TE	0.04 (0.01)	0.26 (0.07)	0.36 (0.16)	0.31 (0.12)	0.43 (0.18)
CA A	0.23 (0.05)	0.66 (0.17)	0.38 (0.08)	0.11 (0.03)	0.18 (0.06)
CA B	0.10 (0.03)	0.42 (0.06)	0.66 (0.16)	0.31 (0.08)	0.31 (0.13)
CA C	0.19 (0.03)	0.53 (0.05)	0.15 (0.02)	0.25 (0.10)	0.38 (0.16)
CA D	0.36 (0.08)	0.17 (0.02)	0.11 (0.03)	0.16 (0.02)	0.45 (0.12)
BR A	0.08 (0.02)	0.28 (0.06)	0.33 (0.09)	0.32 (0.15)	0.12 (0.03)
BR B	0.11 (0.02)	0.28 (0.10)	1.19 (0.10)	0.14 (0.02)	0.13 (0.07)
BR C	0.06 (0.02)	0.12 (0.04)	0.70 (0.07)	0.18 (0.07)	0.16 (0.08)
BR D	0.33 (0.11)	0.36 (0.12)	0.10 (0.03)	0.15 (0.02)	0.05 (0.03)
BR E	0.14 (0.02)	0.36 (0.12)	0.37 (0.14)	0.21 (0.07)	0.15 (0.08)
NZ A	0.53 (0.12)	0.43 (0.07)	0.32 (0.07)	0.13 (0.05)	0.28 (0.05)
NZ B	0.14 (0.04)	0.17 (0.05)	0.44 (0.10)	0.33 (0.05)	0.11 (0.03)

Appendix 6

Summary of CS activity of juvenile rainbow trout (*Oncorhynchus mykiss*) in international units (U), following i.p. injection to pulp mill effluent extracts Six trout were sacrificed per treatment per day; data is displayed as a mean (U) +/- standard deviation (SD).

	Day 4	Day 7	Day 14	Day 21	Day 28
CO	0.049 (0.007)	0.095 (0.019)	0.093 (0.046)	0.170 (0.034)	0.053 (0.017)
E2	0.040 (0.017)	0.109 (0.032)	0.078 (0.040)	0.164 (0.031)	0.072 (0.001)
TE	0.051 (0.019)	0.195 (0.078)	0.101 (0.065)	0.105 (0.05)	0.073 (0.028)
CA A	0.077 (0.016)	0.157 (0.051)	0.113 (0.054)	0.066 (0.025)	0.087 (0.046)
CA B	0.066 (0.007)	0.074 (0.013)	0.054 (0.018)	0.094 (0.017)	0.391 (0.546)
CA C	0.115 (0.079)	0.078 (0.033)	0.196 (0.070)	0.079 (0.028)	0.236 (0.083)
CA D	0.094 (0.032)	0.062 (0.025)	0.233 (0.072)	0.135 (0.033)	0.247 (0.039)
BR A	0.069 (0.016)	0.156 (0.037)	0.099 (0.044)	0.115 (0.016)	0.083 (0.008)
BR B	0.126 (0.096)	0.087 (0.054)	0.181 (0.137)	0.131 (0.030)	0.253 (0.157)
BR C	0.077 (0.012)	0.081 (0.035)	0.291 (0.129)	0.121 (0.022)	0.268 (0.084)
BR D	0.182 (0.107)	0.153 (0.110)	0.160 (0.091)	0.067 (0.034)	0.303 (0.139)
BR E	0.106 (0.041)	0.074 (0.026)	0.151 (0.057)	0.075 (0.023)	0.211 (0.089)
NZ A	0.090 (0.027)	0.120 (0.046)	0.107 (0.083)	0.055 (0.021)	0.100 (0.046)
NZ B	0.071 (0.013)	0.090 (0.031)	0.079 (0.027)	0.092 (0.044)	0.115 (0.061)