ENERGY ALLOCATIONS TO XENOBIOTIC TRANSPORT AND BIOTRANSFORMATION REACTIONS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) DURING ENERGY INTAKE RESTRICTION

by

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ABSTRACT

Energy restrictions can down-regulate cellular defence mechanisms, or if maintained, result in energy trade-offs with other processes. Trout were fed full-rations (1.17% body-weight/day), half-rations (0.59% body-weight/day), or fasted for 9 weeks followed by re-feeding full-rations. Body-weight, liver somatic index, and P-glycoprotein (P-gp), Ethoxyresorufin-*O*-deethylase (EROD), and Glutathione-*S*-transferase (GST) activities were monitored to investigate their maintenance under limited resources. P-gp and EROD activities were maintained in ration-restricted (P-gp: 119±29 pg/min/mg cells, EROD: 0.58±0.17 nmol/min/mg protein) and fasted fish (P-gp: 120±14 pg/min/mg cells, EROD: 0.47±0.14 nmol/min/mg protein). GST activity was significantly attenuated (34%) within 6 weeks of fasting, but recoverable to baseline values after re-feeding. Significant reductions in condition indices of calorie-restricted (BW: 16%; LSI: 33%) and fasted trout (BW: 38%; LSI: 44%) suggest mobilization of body stores to support these systems. Condition indices and defence activities also varied over time, suggesting that water temperature change (range: 13-17°C) may modulate these parameters.

Keywords: diet; energy; fasting; P-glycoprotein; Ethoxyresorufin-O-deethylase; Glutathione-S-transferase;

DEDICATION

To my parents Connie and Mike; my siblings Chris, Pat, and Kelly; and Chris Thachuk; for their strength, support, and enthusiasm throughout my academic endeavours.

"Only when the last tree has died and
the last river been poisoned and the last
fish been caught will we realize
we cannot eat money."

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LIST OF ABBREVIATIONS

BW.	Body-weight
EROD	Ethoxyresorufin-*O*-deethylase
GST	Glutathione-*S*-transferase
LSI	Liver somatic index
P-gp	P-glycoprotein
R123	Rhodamine 123

CHAPTER 1: THESIS INTRODUCTION

Background on bioenergetic models

Diet is not only a means of garnering essential carbon skeletons, amino acids and vitamins, but by providing energy-yielding molecules for catabolism, diet also fuels endergonic reactions within organisms (Bureau et al. 2002). These endergonic reactions are components of larger physiological processes (e.g. digestion, osmoregulation, locomotion, growth and development, reproduction) necessary for life (De Silva and Anderson 1995). How energy provided from diet is allocated to various processes in an organism is a daunting but crucial question applicable to all aspects of biology, one that remains relatively unanswered by current research. Though physiological processes such as reproduction, excretion, respiration etc. are shared by all animals, each organism will have unique and specific costs associated with these processes. Considering fish as a model system, intake energy from the diet can be lost as faecal waste, as well as absorbed and allocated to costs of urinary and branchial waste excretion, and heat loss associated with food processing (Caddy and Sharp 1986; Cho 1992; De Silva and Anderson 1995; Jobling 1994). The energy available from the diet after these processes is the net intake energy. A proportion of this net intake energy is partitioned to maintenance metabolism for

regulation of basic functions and food processing; any remaining dietary energy can be recovered and allocated to processes of growth and development, voluntary activity and reproduction (Caddy and Sharp 1986; Cho 1992; De Silva and Anderson 1995; Jobling 1994). Factors that can affect energy costs associated with processes of ingestion, metabolism, and waste excretion include diet quantity and composition, fish size, as well as abiotic conditions of temperature, photoperiod, and the degree of oxygen saturation of water (Jobling 1994). To better understand energy partitioning in an organism and how it may change under variable environmental parameters, we need to begin quantifying the costs of these processes; to this end, *in vivo* and *in vitro* experimentation and bioenergetic modeling can be useful.

Periods of starvation and fasting are natural and can be common in heterotrophic organisms; to withstand these situations, a number of animals decrease voluntary activities and metabolic requirements, increase the catabolism of body reserves, or both (McCue 2007). Throughout their lifetime, teleost fish species can naturally experience periods of fasting and severely restricted energy intake during which body energy reserves are mobilized to meet a portion of energetic costs (Andersson et al. 1985; Boujard et al. 2000; Peragon et al. 1999; Ruane et al. 2002; Salem et al. 2007). How energy partitioning in fish is affected when gross intake energy is reduced by change in diet quality or quantity is unknown, and is an area of active research pertinent to aquaculture as well as ecological and toxicological study (Azevedo et al. 1998; Cho 1990, Furne et al. 2008; Lanno et al. 1989).

The applicability and ecological relevance of a bioenergetics approach seems most apparent in its recent implementation into stressor-response models for terrestrial and aquatic organisms living in present-day polluted and constantly changing environments. Beyers et al. (1999) have applied this type of model in largemouth bass (*Micropterus salmoides*) as a means of organizing cumulative and relative impacts of natural (e.g. fasting) and anthropogenic (e.g. chemical) stressors on the energy balance in fish, and the consequent effects on biologically important endpoints (e.g. growth, reproduction). Recent work in daphnia (*Daphnia magna*) and lizards (*Sceloporus occidalis*) further support the concept of complex energy trade-offs within organisms exposed to environmental change by demonstrating that exposure to chemical stressors elicited increased energy consumption as well as reductions in voluntary activity, available lipid, protein and carbohydrate reserves, and consequent delays/arrest of reproductive functions (De Coen and Janssen 2003; DuRant et al. 2007). Considering these studies, fasting periods in heterotrophic organisms (e.g. teleost fish) may divert energy resources from storage reserves and other physiological processes which could have ramifications on the organism and possibly the population (De Coen and Janssen 2003; DuRant et al 2007). Natural situations of fasting or limited intake energy may also compromise the ability of an organism to compensate for unaccounted for additional energy costs in a variable environment (e.g. chemical stress, infection) which must be overcome for survival.

Contamination in aquatic environments

Fish species are ecologically (e.g. keystone species) and economically (e.g. aquaculture/fishing industries) relevant. In present-day aquatic environments, fish can be acutely or chronically exposed to natural toxins (e.g. algae and shellfish toxins) as well as anthropogenic contaminants through respiratory, dermal and dietary pathways (Bard 2000; Smital et al. 2004). Much of this environmental degradation is linked to contaminant release from anthropogenic sources into water systems by runoff, effluent, atmospheric deposition, and groundwater pathways (Erickson et al. 2008). Pertinent aquatic pollutants can include release of metal ions from mining operations (e.g. copper), pesticide runoff from agricultural uses (e.g. synthetic pyrethroids), and halogenated aromatic hydrogen exposure in industrialized harbors (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (Coats 2008; Luoma et al. 2008; Tillitt et al. 2008). New and emerging contaminants present in very low concentrations in wastewater effluents and landfill leachate include pharmaceuticals and personal care products (e.g. synthetic musk fragrances, xeno-estrogens) (Daughton and Ternes 1999; Smital et al. 2004). The level of exposure to a toxicant, the ability of the toxicant to affect change in cells, and the energy available to fish at the time of these exposures will contribute to the way in which organisms respond to environmental changes and the extent to which they can use available energy to mount a cellular defence against chemical insult.

Defence systems in organisms

Herbivorous organisms can protect themselves from ingested allelochemical toxins produced by plants to avoid predation, using cellular defence/detoxification mechanisms with associated energetic costs, and thus requiring a change in the energy partitioning to other processes (e.g. growth) in the organism (Cresswell et al. 1992). Heterotrophic organisms have also evolved defence and detoxification mechanisms to protect themselves from chemical attacks by microbes, plants, or animals, which may result from competition for space and limited resources in an ecosystem (Bard 2000; Smital et al. 2004). Such defence and detoxification mechanisms are common to terrestrial and aquatic organisms (e.g. bivalves, fish, rodents, humans) and can be conceptualized into consecutive lines of cellular defence (Bard 2000; Oberdorster et al. 1998).

Recently Sakardi et al. (2006) have added to the idea of consecutive mechanisms of cellular defence, and proposed a concept of “chemo-immunity”, likening the response of cellular defence mechanisms to the innate and adaptive responses of the classical immune system.

The innate response in the classical immune system consists of an immediate non-specific response to an acute threat, whereas the adaptive response tends to occur over multiple exposures and involves a modulation of the defence system that recognizes specific foreign molecules and mounts an amplified response (Sakardi et al. 2006). Challenging fish with chemical exposure has elicited similar innate responses, where a wide range of

compounds from different contaminant classes, and varying structures, can be detoxified or bio-transformed by a general cell defence mechanism. Exposure to xenobiotics can also elicit adaptive changes in cellular defence mechanisms where specific isozymes involved in defence can be up-regulated by the presence of certain contaminants (Bard 2000; Matteson et al. 2001; Perez-Lopez et al. 2000; Stegeman et al.1992).

Each of these cell defence mechanisms has a specific related energetic cost, such that the presence of chemical stressors represents an energy cost, which may necessitate energy re-allocation and amplified expenditure. In scenarios where energy supply is already restricted, it is postulated that resources may be allocated to detoxification and defence pathways most pertinent to survival. This energetic prioritization is based on the idea that reductions in other fitness parameters are recoverable, whereas the effectiveness of successive barrier systems and metabolic processing of exogenous toxins or endogenous by-products have immediate implications on survival (Blom et al. 2000; Kennedy et al. 2004; Morrow et al. 2004).

Barrier functions (Phase 3/ P-glycoprotein)

Although all organs contain cellular defence mechanisms, the liver plays a key role in first pass metabolism and biotransformation of xenobiotics, and hepatocytes are excellent models for illustrating the successive defence systems protecting cells. Together the activities of P-gp and MRP transporter families are

components of a “Phase III” barrier-like defence system capable of modulating the toxic effects of compounds by manipulating their disposition and bioavailability in organisms (Bard 2000; Zaja et al. 2007).

The P-glycoprotein (P-gp) of hepatocytes represents a front-line cellular defence mechanism. As a 170 kDa ATP-dependant efflux pump embedded in the cell membrane, it comprises a barrier function and is aptly named for its membrane ‘permeability altering properties’ for a vast range of planar, moderately hydrophobic, compounds (Bard 2000; Epel 1998). This pump is a member of the ATP-binding cassette (ABC) super-family of proteins present ubiquitously in organisms ranging from bacteria to humans (Epel 1998; Jones and George 2004). A major role of this membrane bound pump is likely preventing the accumulation of natural toxins encountered in the diet or environment, by facilitating the removal of parent compound and metabolites into bile and urine (Bard 2000; Epel 1998; Jones and George 2004; Loo and Clarke 2005). To this end, P-gp-like proteins in terrestrial and aquatic organisms are localized in high concentrations in organs with secretory, excretory, and barrier functions. In teleost fish, isoforms of P-gp are expressed in epithelial, kidney, gill, intestine, pancreatic, and liver cells (Bard 2000; Shuilleabhain et al. 2005). One isoform of P-gp, fpgp B, embedded in the plasma membrane of fish hepatocytes is capable of effluxing unchanged and metabolized xenobiotics into bile, and possibly mediating endogenous lipid transport (Bard 2000; Loo and Clarke 2005; Sturm et al. 2001a; Sturm et al. 2001). A second P-gp isoform, fpgp A, known as

“sister of P-gp” is likely involved in bile salt efflux and similarly localized adjacent to bile caniculi (Bard 2000; Sturm et al. 2001).

A point that is both interesting and pertinent to aquatic toxicity is the responsive nature of P-gp expression under environmental change (Bard 2000; Smital et al. 2004). Laboratory and field studies in aquatic organisms, including fish, observed enhanced efflux activities and P-gp expression in response to stressors such as increased contaminant exposure, reactive oxygen species, and water temperature (Bard 2000). This modulated response suggests that cellular defence mechanisms giving rise to/capable of producing “chemo-immunity” are highly regulated and adapt to provide a specific and appropriate level of defence activity (Chin et al. 1990; Hong et al. 2006; Sakardi et al. 2006).

Other members of the ABC protein super family involved in cellular defence include multidrug resistance proteins (MRPs) of which at least two isoforms are involved in the efflux of organic anions, neutral compounds, and glutathione, glucuronate, and sulfate conjugated compounds into blood and bile (Borst et al. 2000; Saurborn et al. 2004; Zaja et al. 2007). These transporters have been identified in bivalves, nematodes, cartilaginous and teleost fish (Borst et al. 2000; Saurborn et al. 2004; Zaja et al. 2007). In a sense, MRPs are a 3rd line of defence providing an excretion route for conjugated xenobiotics, which due to their hydrophilic nature cannot cross the cell membrane (Borst et al. 2000). MRPs were not investigated in the present work due to time constraints in the sampling period and the fact that methods for investigating MRP protein activity in fish are in the primary stages of development.

Biotransformation reactions (Phase I/CYP450 1A)

Hydrophobic xenobiotics that enter cells via passive diffusion can be modified to render them increasingly water-soluble, which facilitates their excretion from the body. The cytochrome P450 mono-oxygenase system is already known for its role as a “Phase 1” biotransformation mechanism, but its ability to transform and detoxify hydrophobic xenobiotics that have entered the cell also makes it representative of a secondary line of cellular defence (Bard 2000; Guengerich 1993; Van der Oost 2003). This system of proteins bound in the membrane of the endoplasmic reticulum, works in concert with NADPH-cytochrome P450 reductase and cytochrome b5 to catalyze oxidative reactions (hydroxylation, epoxidation, and dealkylation) involving the incorporation of an oxygen in exogenous as well as endogenous substrates (Goksoyr and Husoy 1998; Stegeman and Hahn 1994; Van der Oost 2003).

CYP 450 enzymes are ubiquitous to all biological kingdoms, including prokaryotes (Guengerich 1993). The superfamily of cytochrome P450 monoxygenase enzymes may have evolved as a means of synthesizing/metabolizing endogenous steroids; but it likely diverged in function to accommodate environmental exposure to pyrogenic, biogenic, and diagenic compounds, as well as enabling the synthesis/metabolism of toxic compounds in prey and predators, respectively (Goksoyr and Husoy 1998; Stegeman 1993). Members of the CYP1A subfamily (isoforms of CYP 1A1 and 1A2) are of great importance in xenobiotic biotransformation, being known to biotransform environmental contaminants (e.g. polycyclic aromatic hydrocarbons (PAHs),

polyhalogenated aromatic hydrocarbons (PHAHs)), and have been identified in over 30 species of fish (Goksoyr and Husoy 1998; Van Veld and Nacci 2008; Whyte 2000). The rainbow trout (*Onchorhynchus mykiss*) isoform of CYP 1A1 (previously known as LM4b and P450E), is often referred to as CYP1A due to its homology to sections of both mammalian CYP1A1 and 1A2, and the difficulty in distinguishing its activity and function from the trout 1A3 isoform (Lester et al. 1993; Goksoyr and Husoy 1998; Stegeman 1993; Whyte 2000). In keeping with their function, trout CYP1A proteins are expressed in many organs (e.g. heart, gills, kidney, brain), but they exist in greatest concentration in the liver (Campbell and Devlin 1996; Lester et al. 1993). Determination of CYP1A enzyme activity can be done in a number of ways, including correlating the formation rate of a specific substrate metabolite with CYP 450 protein content (Stegeman and Hahn 1994). In this regard, microsomal *O*-dealkylation of the substrate 7-ethoxyresorufin by the CYP1A isoform in an ethoxyresorufin-*O*-deethylation assay (EROD) is often used as a proximate measure of CYP1A catalytic activity (Burke and Mayer 1974; Stegeman and Hahn 1994).

Immuno-histochemistry and activity assays have shown that expression of CYP1A in organs can be induced by a number of contaminants (e.g. PAHs and PHAHs) in a concentration dependant manner; and is illustrative of an adaptive response to environmental change by a key Phase I biotransformation enzyme (Goksoyr and Husoy 1998; Sakardi et al. 2006; Whyte 2000). This amplification can be mediated by the aryl hydrocarbon receptor (AhR) pathway and has been linked to alterations in cellular homeostasis and initiation of carcinogenesis in

organisms (Whyte 2000). Further evidence that this enzyme may be tightly regulated and responsive to cellular change comes from evidence that EROD activities can be modulated by temperature, exposure to natural and synthetic estrogens, or high doses of PAHs and PHAHs (Campbell and Devlin 1996; Goksoyr and Husoy 1998; Safe and Wormke 2003; Whyte 2000).

Conjugation mechanisms (Phase 2/Glutathione-S-Transferase)

Acetylation of amino groups, amino acid conjugation to carboxylic acids, glucuronic acid transfer to aglycones, sulfate transfer to amino or carboxyl groups, and conjugation of a peptide to electrophilic N, S or C atoms are all reactions that can further enhance the hydrophilicity and excretion of xenobiotic and endogenous metabolites in the cell (Daughton et al. 1999; Kennedy 1995; Schlenk et al. 2008). For their role in these reactions, glutathione-S-transferase (GST) enzymes are representative of a “Phase II” conjugating system, and components of a third line of consecutive cellular defence. GST enzymes have been identified ubiquitously in organisms from prokaryotes to mammals and are associated with a number of functions, the most important being detoxification of reactive metabolites into eventual mercapturic acid derivatives for excretion (Schlenk et al. 2008). Other functions of GST enzyme isoforms include carrying out biosynthesis of prostaglandins, leukotrienes, and steroids, reversibly binding and controlling transport of lipophilic compounds and endogenous products in cells, conjugating glutathione to ROS-damaged cell components, and covalently

binding to electrophiles (e.g. epoxides) to prevent adduct formation (George 1994; Kennedy 1995; Nimmo 1987; Schlenk et al. 2008; Stegeman et al 1992).

Soluble, cytosolic GST enzymes have been sub-divided into homodimers or heterodimers belonging to 7 different classes (alpha (α), mu (μ), omega (ω), pi (π), theta (θ), sigma (σ), and zeta (ζ)), of which α, μ, π, and θ isoforms have been identified in fish species (Schlenk et al. 2008). GST enzymes tend to be present in organs exposed to the environment; π (dominant isoform) and α enzymes have been identified in rainbow trout; and GST enzyme activity, measured as a rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation, has been noted in the spleen, intestine, pyloric caeca, kidney, liver, and gills (Leaver and George 1998; Nimmo 1987; Schlenk et al. 2008). These isoform classes correlate with conjugation activities towards electrophilic compounds (e.g. benzo[a]pyrene epoxide) and lipid peroxidation products, highlighting their roles in cellular defence against oxidative stress (Schlenk et al. 2008).

Similar to P-glycoprotein and the many isoforms of CYP450 enzymes, GST enzymes exhibit a broad substrate specificity, as well as many isozymes to handle specialized functions; these features are characteristic of the ‘wide-range recognition’ of an innate defence system (Sakardi et al. 2006). Further in keeping with the concept of chemo-immunity in cellular defence mechanisms, GST activity and expression in fish appears to be highly regulated, and ‘adaptive’ to change in cellular conditions (Schlenk et al. 2008). GST activity can be induced by exposure to some contaminants (e.g. PAHs and PHAHs) through activation of the aryl hydrocarbon receptor gene battery (Van der Oost et al. 2003). Activities

of fish GST isoforms can be further modulated by the presence of bioactivated CYP 450 metabolites, contaminants that generate reactive oxygen species (e.g. heavy metals, pesticides) and damaged cellular components from oxidative stress (Morales et al. 2004; Schlenk et al. 2008; Van der Oost et al. 2003). Reactive oxygen species (ROS) and the redox status of cells may influence GST activities and those of antioxidant defences systems of the cell (e.g. glutathione peroxidase and reductase) *via* their sharing of the common substrate reduced glutathione, dependence on a constant source of reduced NADPH, and the likely presence of antioxidant response elements in promoter regions of piscine GST genes (George 1994; Gurderley et al. 2003; Morales et al. 2004; Schlenk et al. 2008; Van der Oost 2003).

Objectives of study

Literature suggests that the activities of representative cellular defence systems (P-gp, CYP1A, and GST) in teleost fish are regulated and responsive to changes in the external and internal environment. Prior research in contaminant exposed insects (*Spodoptera eridania*; *Daphnia magna*), lizards (*S. occidalis*), and largemouth bass (*M. salmoides*) suggest that cellular defence and detoxification mechanisms have associated energy costs, and that environmental stressors requiring change in activity of these mechanisms to maintain homeostasis will require energy trade-offs between processes which may affect partitioning to biologically important endpoints (Beyers et al. 1999; Cresswell et al. 1992; De Coen and Janssen 2003; DuRant et al. 2007). Today's aquatic

systems realistically represent situations of concurrent diminishing energy intake and chemical stressors; as natural periods of fasting through winter months further limit the available energy of teleost fish they likely modulate energy trade-offs between body stores and other physiological processes in contaminated environments. Severe energy budgeting that results in down-regulated activities of cell defence and detoxification proteins may exacerbate effects of exogenous or endogenous compounds normally metabolized through these pathways. Alternatively, energy limiting conditions may elicit trade-offs with other fitness parameters (e.g. growth) to maximize the efficiency of metabolic processes and maintain defence and detoxification mechanisms for immediate survival. To begin addressing the interactions between bioenergetics and cellular defence, the current study aimed to investigate if limiting intake energy through diet could change the activities of three key defence and detoxification proteins representative of consecutive lines of cellular defence using rainbow trout hepatocytes as a model system. To accomplish this, juvenile rainbow trout were fed full or half-rations of a commercial diet, or fasted, as whole body condition indices of body-weight and liver somatic index, and P-gp, EROD, and GST activities were monitored over a nine-week period. A further goal of this investigation was to determine if observed alterations in condition indices or cell defence activities were recoverable to baseline levels after re-feeding on full rations.

CHAPTER 2: INTRODUCTION

Chapters 2 to 5 are from the manuscript “Energy allocations to xenobiotic transport and biotransformation reactions in rainbow trout (*Oncorhynchus mykiss*) during energy intake restriction.” accepted to Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology.

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Teleost fish are often exposed to natural conditions of fluctuating energy availability and intake. The question of how resources are allocated to different endergonic pathways under dynamic internal and external environmental conditions in organisms is one of the main goals of bioenergetics (Bureau et al. 2002). Energy partition models in rainbow trout (*O. mykiss*) suggest that at 15°C, approximately 69% of energy taken in is available for allocation to body maintenance, activity, growth and development, or reproductive investments (Caddy and Sharp 1986; Cho 1992). The patterns of allocation of this assimilated energy changes with a number of factors including life stage. For example, juvenile fish have high metabolic costs due to rapid growth and development, whereas mature fish have high costs associated with migration and spawning, competition, and gonadal development (Vijayan et al. 1993; Thorpe 1994; Bains and Kennedy 2005). Portioning within organisms may also be affected by factors which alter the availability of assimilated energy. Many teleost species annually undergo fasting and starvation periods during over-wintering, sexual maturation, and migration during which endogenous stores and body tissues are catabolized to meet energy demands (Andersson et al. 1985; Peragon et al. 1999; Boujard et al. 2000; Ruane et al. 2002; Salem et al. 2007). In addition to natural periods of limited energy, exposure to chemical stressors can increase energy demand causing decreases of available energy for other purposes resulting in, for example, reductions in voluntary activity, mobilization of available energy reserves, and delayed reproductive activities (De Coen and Janssen 2003; DuRant et al. 2007; McCue 2007). Such trade-offs may impinge on the success

of the organism in foraging, migration and escape behaviors, and the population in terms of reproductive capacity (De Coen and Janssen 2003; DuRant et al. 2007).

Contaminated aquatic habitats can harbor natural toxins as well as anthropogenic stressors which may degrade the quantity and quality of food they contain. Information regarding the effects of decreased energy intake or poor nutrition on the ability of fish to defend themselves against xenobiotic exposure is limited. Teleosts exhibit both innate and inducible defence systems that can be conceptualized into consecutive lines of cellular defence (Bard 2000), each with specific related energy costs which may be affected by degraded habitats.

One of the primary lines of cellular defence is the 170 kDa ATP-dependant efflux pump P-glycoprotein (P-gp/ABCB1), a member of the ABC protein super-family capable of transporting a wide range of planar, moderately hydrophobic xenobiotics (Bard 2000). P-gp, in conjunction with other transporter families, has been considered a “Phase III” defence system capable of modulating the toxic effects of compounds by manipulating their bioavailability and disposition (Bard 2000; Zaja et al. 2007). Biotransformation includes oxidation, reduction, and hydrolysis reactions that typically result in more water-soluble metabolites to facilitate their excretion; the role of the cytochrome P450 mono-oxygenase system in these reactions identifies it as a secondary line of cellular defense and a “Phase I” biotransformation mechanism (Bard 2000; Gibson and Skett 2001; Van der Oost et al. 2003). Members of the CYP1A protein subfamily are integral in metabolizing environmental contaminants and are highly expressed in various

organs of fish (Lester et al. 1993; Stegeman 1993). Excretion of xenobiotics and Phase I metabolites can be further enhanced through conjugation to sugars, peptides, methyl, acetyl and sulfate groups (Kennedy 1995). Given their role in these reactions, cytosolic glutathione-S-transferase (GST) enzymes are an important component of a third line of cellular defence, the “Phase II” conjugating system. Not only do these enzymes assist in detoxification by transforming reactive metabolites into eventual mercapturic acids, they can also increase the bioavailability of lipophilic contaminants to Phase I biotransformation enzymes, and assist in the recycling of cell components damaged by oxidative stress (Nimmo 1987; Stegeman et al. 1992; Kennedy 1995; Leaver and George 1998).

When intake energy is restricted, it is likely that resources are preferentially allocated to key xenobiotic defence pathways given that reductions in parameters such as growth and reproduction are recoverable, whereas the effectiveness of barrier systems and biotransformation processes have immediate implications for survival (Blom et al. 2000; Kennedy et al. 2004; Morrow et al. 2004).

The effects of food scarcity requiring energy re-allocations on the activities of these three phases of cellular defence are, however, unknown. If energy allocations to defence systems are reduced, then the susceptibility to exogenous and endogenous compounds may be exacerbated. If defence activities are maintained, they may result in energy trade-offs with other physiological systems. The present study investigated effects of restricted energy intake on whole body condition indices (BW and LSI) and the activities of three key proteins

representing consecutive lines of cellular xenobiotic defence. Whole body indices, and P-gp, EROD, GST activities were measured in juvenile rainbow trout fed full-rations, half-rations, or fasted. Fish were also subjected to a re-feeding period on full-rations to determine how quickly the activities of altered parameters were recoverable when energy was again made available. For clarity, the terms fasting and starving (both of which occur in the time-frame of the experiment), are used synonymously within the text (Farbridge and Leatherland 1992).

CHAPTER 3: MATERIALS AND METHODS

Chapters 2 to 5 are from the manuscript “Energy allocations to xenobiotic transport and biotransformation reactions in rainbow trout (*Oncorhynchus mykiss*) during energy intake restriction.” accepted to Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology.

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Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*, n=120) of the same strain and cohort, initially weighing 150-300 g were obtained from Miracle Springs Trout Farm (Mission, BC). Fish (n=20) were weighed and randomly assigned to each of six 500 L fiberglass flow-through tanks supplied with aerated, carbon-filtered, de-chlorinated water in a randomized block design of three treatment groups, in duplicate. A simulated 12 hour light:dark photoperiod and water quality parameters of pH 6.7, water hardness 6.3 mg/L $CaCO_3$, and oxygen saturation >90% were maintained throughout the experiment. Water temperature was recorded weekly over the experimental period. Fish were allowed to acclimate to tank and water conditions for four weeks prior to treatment initiation, and during this period fish were hand fed a commercial pellet diet *ad libitum*.

Diet

Through the acclimation and experimental periods, fish were fed a commercial diet of EWOS Pacific 2.0 mm pellet feed (Surrey, BC) containing 39.8% digestible protein (DP), 16.6% digestible lipid (DL), and approximately 17.2 MJ digestible energy (DE)/kg. This feed was chosen because it closely resembles the proposed protein/lipid feed composition for optimum energy utilization in rainbow trout at 15°C (36% DP, 16% DL, and a minimum energy density of 15 MJ DE/kg as reported by Cho (1992)). This pellet size was chosen to exclude feed containing pigments which can potentially affect enzyme activities. Feed was stored in airtight containers at -20°C, and in darkness until use.

Feeding

Fish were hand-fed as described in Morrow et al. (2004). Briefly, fish were exposed to a consistent twice-daily feeding regime (0900 h and 1700 h) of full-rations, half-rations, or food-deprivation diet. Slow hand feeding was halted when satiation endpoints were noted; regurgitation of ingested pellets, cessation in consumption of sinking pellets, and feed left untouched on the tank floor for at least 30 sec. Diets were initially calculated from the average body-weights of fish following the acclimation period; tanks receiving full-rations were fed at a rate of 1.17% BW/day (minimum daily digestible energy requirement), and used as a control for the experimental period (Cho 1992). Accordingly, duplicate tanks receiving half-rations were fed 0.59% BW/day diet based on the average body weight of the control group. The remaining two tanks were fasted over the initial 9-week sampling period. Every two weeks, a sub-sample of fish (n=4) were weighed to re-calculate and adjust feed rations accordingly.

Sampling and calculation of LSI

Sampling occurred at 0, 3, 6, and 9 weeks from the experimental start date, after which all tanks were fed full-feed rations for one week; fish were sampled again at week 12. At each time point, two fish from each treatment tank were euthanized in 0.5 g/L buffered tricaine methanesulfonate (MS 222) (Argent Chemical Laboratories, Redmond, WA). Body-weight, liver-weight and sex were

recorded and used to determine change in body mass and liver somatic index. LSI was calculated by dividing liver-weight by body-weight, and multiplying the subsequent ratio by 100. Livers were immediately used for EROD and GST activity assays. Two additional fish were removed from each tank, anesthetized with 0.3 g/L buffered MS 222, weighed, and livers excised for hepatocyte isolation and assessment of P-glycoprotein activity.

Microsomal and cytosolic preparation

Excised livers were rinsed in an ice-cold 0.2 M HEPES buffered 5 mM EDTA, 0.15 M KCl solution, pH 7.4. Cytosolic and microsomal fractions were prepared as in Morrow et al. (2004). All homogenization and microsomal preparation steps were carried out on ice. Livers were homogenized (0.25 g liver/mL buffer) with a glass-teflon homogenizer (7 strokes/2500 rpm); homogenate was centrifuged twice at 10, 000 x *g* for 20 min. at 4°C. After removal of the floating lipid layer, the post-mitochondrial supernatant was pipetted off and re-centrifuged at 100, 000 x *g* for 65 min at 4°C. The resulting cytosolic supernatant was decanted and stored at -80 °C until analyzed for GST activity. The microsomal pellet was rinsed with homogenization buffer before re-suspension in 1 mL aliquots of a 1.0 mM EDTA, 50 mM Tris-HCl, 1.0 mM dithiothreitol, 20% glycerol buffer solution, pH 7.4, and stored at -80 °C until analyzed for EROD activity.

EROD activity and microsomal protein content

EROD activity and microsomal protein content were determined simultaneously using a fluorometric microplate procedure adapted from Kennedy et al. (1994). Microsomal samples, reagent blanks, and standards were run in duplicate in 96-well plates. BSA [Sigma] (2.0 mg/mL) and resorufin (16.75 µM) in methanol were diluted to achieve final protein (0-150 µg/mL) and resorufin (0-0.5 µM) concentrations in standard wells. Sample wells contained 150 µL of NaH_2PO_4 buffer (50 mM, pH 8.0) and 10 µL microsomal suspension. Ethoxyresorufin (871 µM) was diluted 13-fold in NaH_2PO_4 buffer before addition to all wells for a final well concentration of 10 µM. Plates were incubated for 10 min at 21 °C prior to initiation of the reaction by addition of 25 µL of 13.4 mM NADPH [Sigma] in NaH_2PO_4 buffer to all wells. Reactions were stopped after 6 min with 100 µL of fluorescamine in acetonitrile (300 µg/mL). Well plates were incubated for a further 15 min at 21° C before measurement of resorufin fluorescence (ex. λ=530 nm, em. λ=590 nm), and measurement of fluorescamine-protein fluorescence (ex. λ=400 nm, em. λ=460 nm) using a Carey Eclipse fluorescence spectrophotometer (Varian, Inc., Mississauga, ON).

GST activity and cytosolic protein content

GST activity and protein content of cytosolic samples were determined using spectrophotometric microplate assays described in Sharma et al. (1997). To determine GST activity, 180 µL of a reaction mixture consisting of 16 mL of 0.1 M KH_2PO_4, 1 mM EDTA buffer (pH=6.5), 1 mL of 20 mM glutathione, and 1

mL of 20 mM CDNB in ethanol was added to triplicate blank wells containing 20 µL of a 0.1 M KH_2PO_4, 1 mM EDTA buffer, and triplicate sample wells containing 20 µL diluted cytosolic supernatant in 96-well plates. The change in absorbance within the wells was measured at 1 min intervals for 5 min at λ=340 nm using a microplate scanning spectrophotometer (BioTek Powerwave 340, BioTek Instruments Ltd., [Winooski, VT]). Protein content of the cytosolic supernatent was determined using diluted BioRad protein dye reagent (BioRad, [Hercules, CA]). 200 µL of diluted dye reagent was added to 20 µL of sample, blank or BSA standards and run in triplicate in 96-well plates. Plates were incubated at 21 °C for 5 min before reading on a microplate scanning spectrophotometer at λ= 600 nm.

Isolation and primary culture of hepatocytes

Hepatocytes were isolated as in Moon et al. (1985). Livers were perfused with well-oxygenated, ice-cold HSSA solution (Hank's balanced salts solution [Ca^{2+}, Mg^{2+} free] [Sigma, St. Louis, MO]) which contained 10 mM HEPES, 0.81 mM $MgSO_4$, 4.167 mM $NaHCO_3$, pH=7.5) at a flow rate of 2.0 mL/min/g liver. Perfusion was continued with HSSC solution (0.6 g/L Type IV collagenase [Sigma] in HSSA), after which the liver was excised and minced in HSSA. This suspension was gently passed through two screens of nylon mesh (253 µm and 73 µm) and separated by low speed centrifugation (37 x *g* for 4 min at 4°C) using a Beckman Coulter CS-15R centrifuge. Hepatocytes were re-suspended and washed twice in ice-cold HSSB solution (0.11 g/L $CaCl_2$, 2% fatty acid-free bovine serum albumin [Sigma] in HSSA) by low speed centrifugation. Final

hepatocyte suspensions were standardized to 12.5 mg wet weight cells/mL as per Siebert (1985), and Bains and Kennedy (2005) and acclimated for 1 h at ambient tank temperatures prior to Rhodamine 123 (R123) accumulation assay. Cell viability was assessed by trypan blue exclusion and the culture discarded if viability≤70%.

R123 accumulation assay

The functional activity of P-gp in hepatocytes was measured by examining the accumulation of the model P-gp substrate R123 (Bains and Kennedy 2005; Sturm et al. 2001). Briefly, 5 µL of R123 in DMSO was added to duplicate culture vials (25 mg wet weight cells/vial, final concentration 5 µM R123), and gently vortexed. Cells were incubated on an orbital shaker for up to 120 min at ambient tank temperatures. At 20 min intervals, duplicate culture vials were removed, and cell suspensions were centrifuged (37 x *g* for 4 min at 4°C). Cell pellets were washed twice in HSSB solution before being stored at -20°C. Accumulated R123 was extracted from thawed cell pellets into two volumes of 2.0 mL *n*-butanol (Bains and Kennedy 2005). Triplicate 250 µL aliquots of extracts for each time point were analyzed for fluorescence (ex. λ=517 nm, em. λ=532 nm) in 96-well plates using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Mississauga, ON).

Statistics

To determine P-gp functional activity, initial accumulation rates were calculated from the first 40 min. of R123 accumulation in primary cell cultures, and reported as pg R123/min/mg wet weight cells, where higher rates of accumulation reflect lower efflux activity (Bains and Kennedy 2005). EROD activity was determined measuring resorufin production with time and values were standardized to microsomal protein content and expressed as (nmol/min/mg microsomal protein). Specific GST activities of samples were expressed as (μmol/min/mg cytosolic protein). Results are expressed as mean ± S.E.M. The assumptions of equal variances and normal distributions in residuals were tested and data was log transformed as necessary. Data was analyzed in a two-factor (treatment x time) analysis of variance that compared mean values between treatment groups, and within groups over time. Fish did not exhibit signs of sexual maturation until the week 12 sampling interval; when gender was included as a variable in the statistical model; neither EROD nor P-gp efflux activity was affected. Differences between sampling days were investigated and accounted for in the statistical model. When significant differences ($p<0.05$) in ANOVAs were noted, Tukey's HSD multiple comparison test ($\alpha=0.05$) was performed. All data was analyzed using JMP Version 7.0.2 software and graphs were produced using Graphpad Prism Version 4.03 software.

CHAPTER 4: RESULTS

Chapters 2 to 5 are from the manuscript “Energy allocations to xenobiotic transport and biotransformation reactions in rainbow trout (*Oncorhynchus mykiss*) during energy intake restriction.” accepted to Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology.

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Temperature

Ambient water temperature fluctuated (range=13 to 17 °C; mean=14±0.49 °C) over the experimental period. Water temperature peaked at the week 6 sampling interval (17 °C) after which it declined to the end of the week 12 sampling interval (Fig. 1).

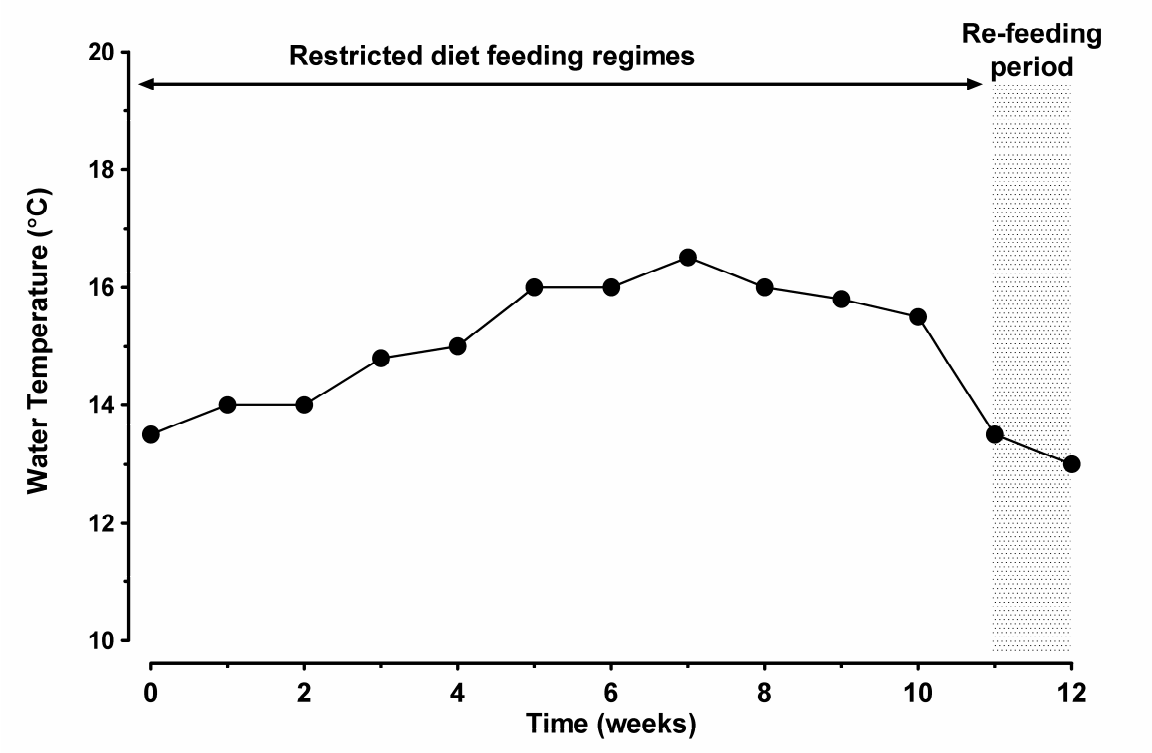

Figure 1 Water temperatures over the 12 week sampling period (July-November 2007).

Body-weight

At the start of the experimental period fish mean BW was 518±23 g, variation in BW was likely due to social hierarchies established within tanks during acclimation. Mean body-weights of trout fed full rations varied significantly over time ($p < 0.001$, overall 258% weight gain) (Fig. 2).Trout receiving full

rations exhibited the greatest overall rate of mass increase, which was significant at week 9 through 12. Restricting food also had a significant effect ($p<0.001$) on mean body-weight (Fig. 2). Mean body-weight of fish receiving half-rations did not increase significantly over time until weeks 9-12, however at week 12 mean body-weight of the half ration group was still significantly lower (30.0%) compared to the full ration group. Fasted fish exhibited a 29.7% loss in body mass from week 0 to week 9, but the difference over time was not statistically significant. Fasted fish exhibited significantly reduced body-weights compared to the full-ration (40% decrease) and half-ration (38% decrease) treatment groups beginning at weeks 3 and 6, respectively. Finally, one week of re-feeding fish in both the half-ration and fasted treatment groups did not result in significant increases in mean body-weight in these groups by sampling at week 12.

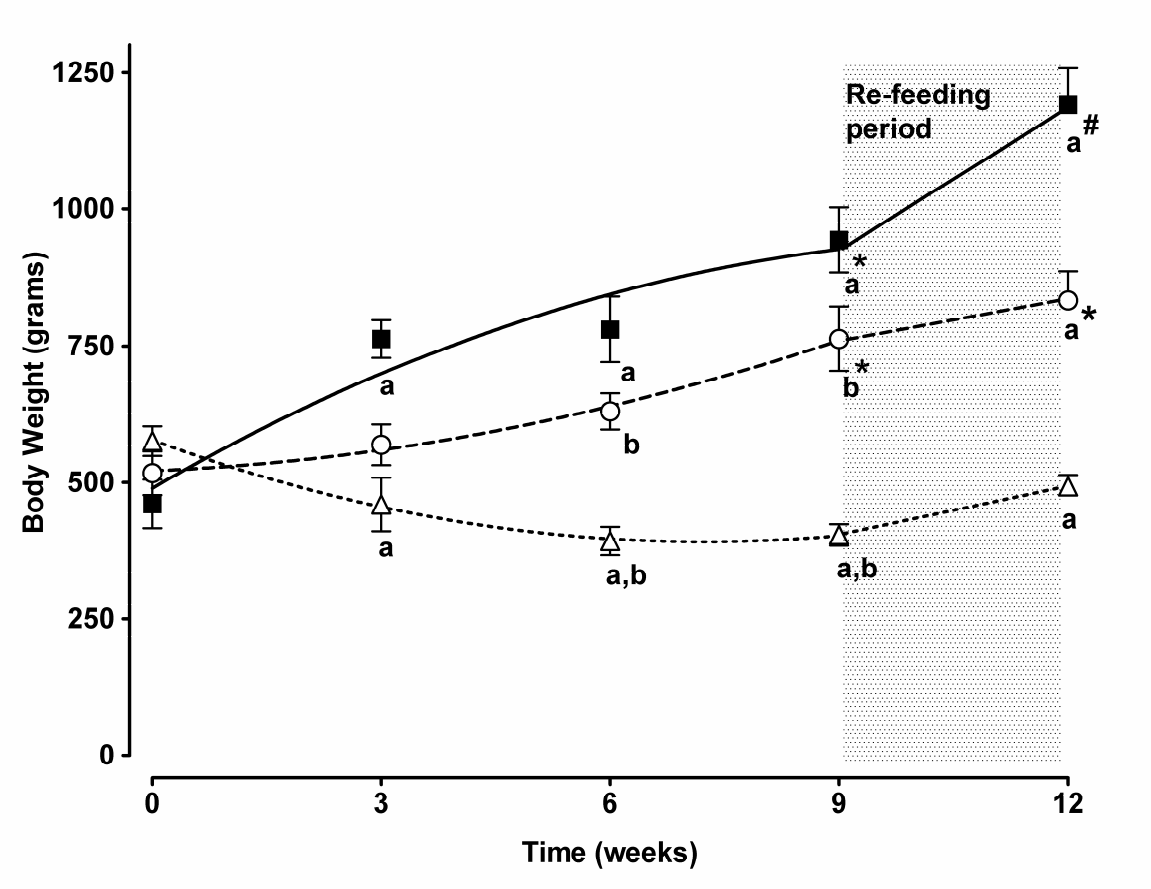

Figure 2 Change in body weight of rainbow trout exposed to three diet regimes [(■) full-rations, y = 489.5 + 80.40 (x) + (-3.541) x^2, r^2= 0.5271; (○) half-rations, y = 521.1 + 5.715 (x) + (2.320) x^2, r^2= 0.3587; (Δ) fasted, y = 577.3 - 51.24 (x) + (3.545) x^2, r^2 = 0.3824] for nine weeks followed by re-feeding of all treatment groups on full-rations. Data points with different symbols denote significant differences between time points within a treatment group ($p<0.05$). Data points with the same letters denote significant differences between treatment groups ($p<0.05$).

Liver somatic index

Liver somatic index (LSI) varied in fish with time, in all treatment groups. Mean LSI was not significantly different between treatment groups at week 0; but limiting feed began significantly ($p<0.0001$) reducing mean LSI values in fasted and half-ration groups from fish receiving full rations by week 3 (Fig. 3). In fish

fed full-rations, mean LSI values exhibited a decreasing trend (26% from week 0) up to week 6, after which values increased 32% between sampling at weeks 9 and 12, and to a final value of 5.5% over baseline values at week 0 (Fig. 3). Fish receiving half-rations also exhibited a trend of reduced LSI values (31%) up to week 6 of the experiment. In fasted fish, this trend was exaggerated and LSI values were significantly diminished (44%) between weeks 3-9. After the one week re-feeding period, mean LSI was not significantly different between treatment groups, with the greatest increase in LSI noted after re-feeding in the fasted group (98% increase over mean week 9 values), and the least increase noted after re-feeding in the half-rations group (5% increase over mean week 9 values).

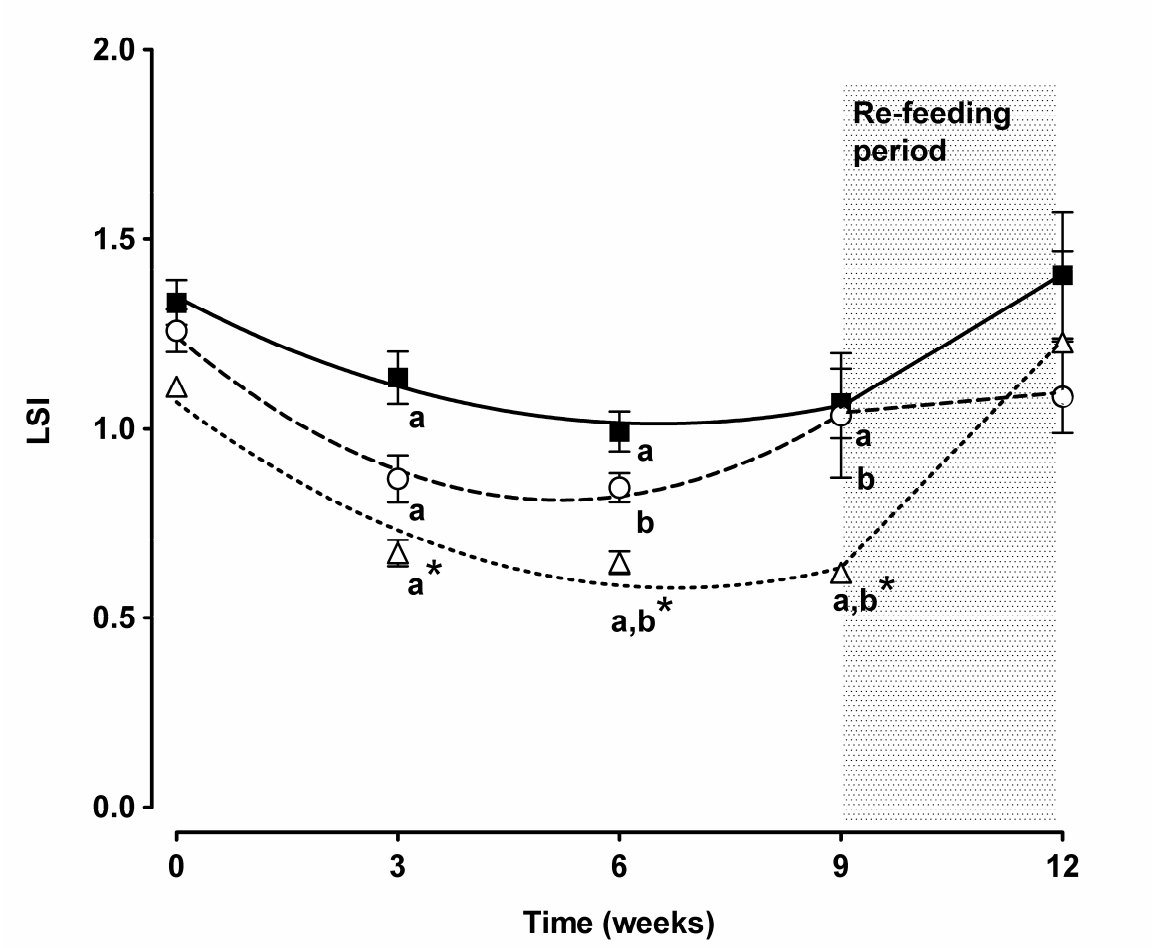

Figure 3 Liver somatic index of rainbow trout exposed to three diet regimes [(■) full-rations, y = 1.348 + (-0.1023)x + (0.007835) x^2, r^2= 0.3106; (○) half rations, y = 1.244+(-0.1658)x + (0.01590) x^2, r^2= 0.2481; (Δ) fasted, y = 1.070+ (-0.1451) x + (0.01072) x^2, r^2= 0.8275] for nine weeks followed by re-feeding of all treatment groups on full-rations. Data points with asterisks (*) denote significant differences between time points within a treatment group ($p<0.05$). Data points with the same letters denote significant differences between treatment groups ($p<0.05$).

Determination of P-gp activity

The mean viability of isolated hepatocytes was always >84%. Rates of R123 accumulation in hepatocytes in all groups varied with time. Hepatocytes from fish fed full-rations exhibited a significant trend of diminishing R123

accumulation rates (increased R123 efflux and P-gp activity) which was reduced by 71% at week 9, compared to baseline time 0 values (195±22 pg R123/min/mg). By week 12, R123 accumulation rate in this group increased 146% over mean activity at week 9, and was not significantly different from baseline activity at week 0. R123 accumulation rates in hepatocytes from fish receiving half-rations were also significantly attenuated (68% decrease compared to baseline activity at week 0) by week 9. Fasted fish exhibited a trend of diminishing R123 accumulation rates between weeks 3 to 9, but these activities were not significantly reduced compared to baseline activity at week 0. In both half-ration and fasted groups, R123 accumulation rates increased (37% and 16%, respectively) between weeks 9 to 12 and were comparable to basal activity levels. Limiting energy intake through feed-restriction and fasting did not significantly ($p=0.8127$) affect mean R123 accumulation rates (119 ± 29 and 120 ±14 pg/min/mg cells, respectively) compared to rates in fish fed full-rations (115 ± 25 pg/min/mg cells) (Fig. 4). However, fish receiving half-rations and fasted fish exhibited a non-significant trend of increased R123 accumulation rates that was most pronounced at week 9, suggesting a potential for decreased R123 efflux capacity under severe feed restrictions.

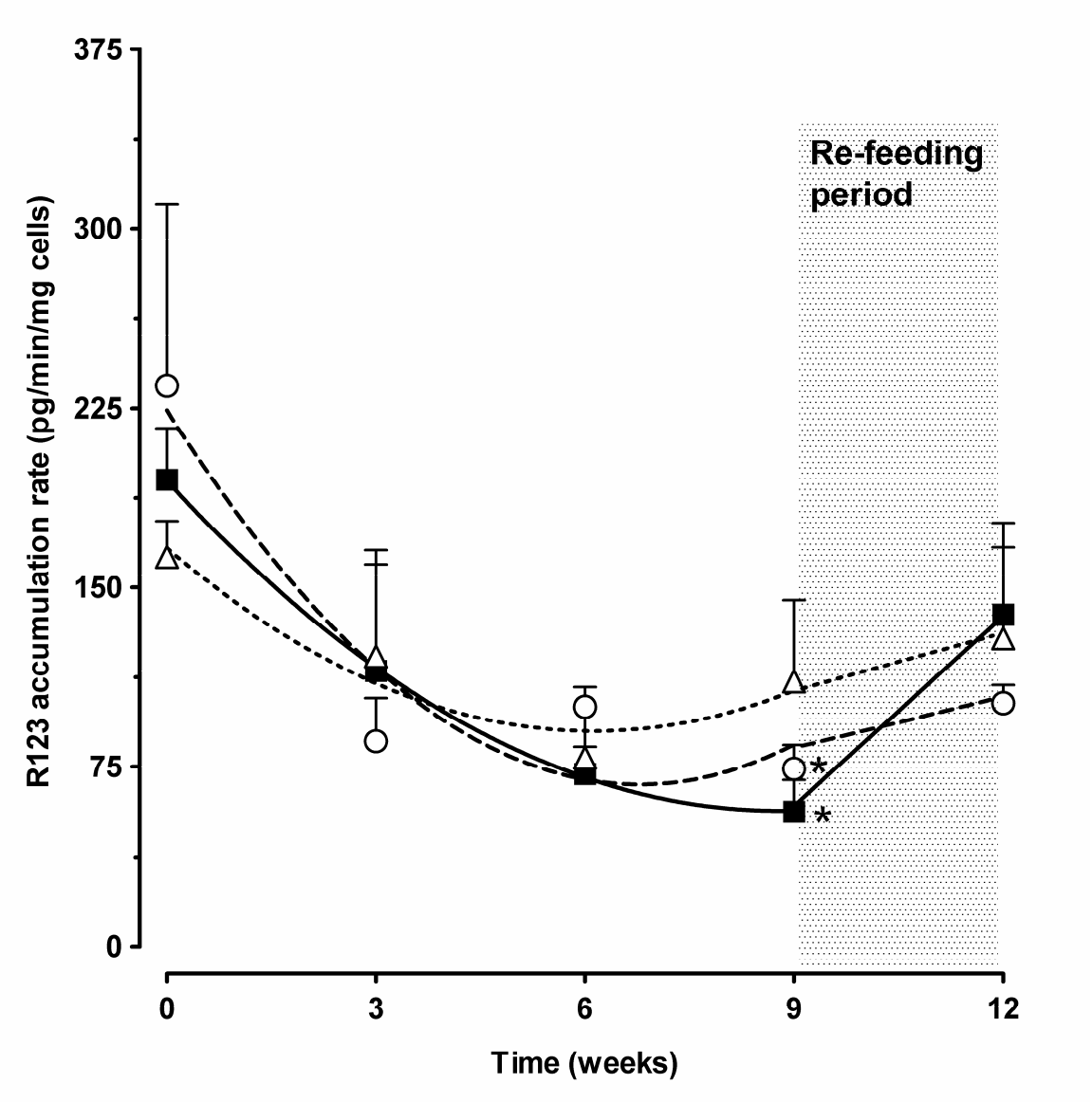

Figure 4 Accumulation rate of R123 in hepatocytes of trout exposed to three diet regimes [(■) full rations, y = 194.6 + (-31.38) x + (1.785) x^2, r^2= 0.5366; (○) half rations, 224.2 + (-46.17) x + (3.403) x^2, r^2= 0.4129; (Δ) fasted, 166.5 (-25.00) x + (2.043) x^2, r^2= 0.2470] for nine weeks followed by re-feeding of all treatment groups on full-rations. Data points with asterisks (*) denote significant differences between time points within a treatment group ($p<0.05$).

Determination of specific CYP1A (EROD) activity

Fish receiving full-rations exhibited a trend of increased EROD activity (526% over a baseline value of 0.10 ± 0.012 nmol/min/mg at week 0) from week 0 to 6, followed by a decline (48% decrease from mean week 6 activity) to basal levels by the end of the experiment (Fig. 5). Fish from the fasted and ration-restricted treatment groups exhibited even greater increases in EROD activity over baseline activities between weeks 0 to 6 (708% and 610%, respectively), a trend that was significant in the fasted treatment group. This decreased by week 12 to rates not significantly different from those seen at week 0. Limiting feed ration did not significantly affect ($p=0.0933$) mean EROD activity of fish, however it did elicit a slight trend of elevated overall EROD activity in calorie-restricted and fasted fish (73% and 38% increase, respectively) compared to fish receiving full-rations.

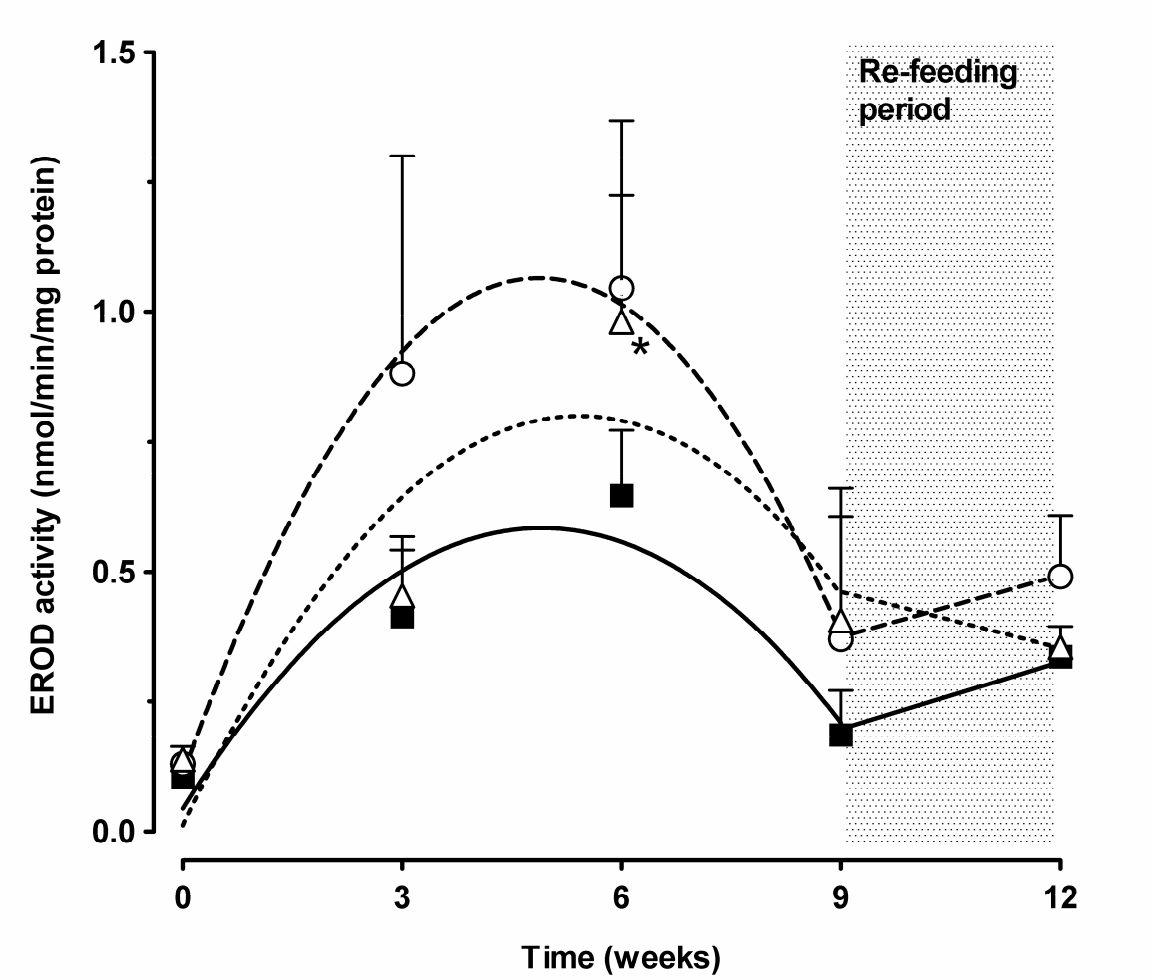

Figure 5 EROD activity of rainbow trout exposed to three diet regimes [(■) full-rations, non-linear regression y = 0.04397+ (0.2201)x + (-0.02242) x^2, r^2= 0.4408; (○) half-rations, non-linear regression y = 0.1082+ (0.3929)x + (-0.04032) x^2, r^2= 0.2996; (Δ) fasted, non-linear regression y = 0.01200+ (0.2910)x + (-0.02685) x^2, r^2= 0.3135] for nine weeks followed by re-feeding of all treatment groups on full-rations. Data points with asterisks (*) denote significant differences from initial EROD activity within a treatment group (p<0.05).

Determination of specific GST activity

Hepatic GST activities of fish fed full-rations were significantly modulated over time (p≤0.0001), with mean GST activity peaking at week 3 (35% increase over a baseline activity of 3.5 ± 0.14 µmol/min/mg) before declining to week 9,

after which mean activity increased 8.2% back to basal levels by week 12. Fish receiving half-rations and fasted fish exhibited peaks in mean GST activity at week 3, though 5% and 31% less than fish fed control rations, respectively. Following week 3, the half-ration and fasted groups also exhibited reductions in mean GST activity to week 9. Restricting feed ration significantly ($p=0.0092$) attenuated the overall mean GST activity of fasted fish (2.4 ± 0.31 μmol/min/mg) from that of fish offered full-rations (3.3 ± 0.40 μmol/min/mg) and half-rations (3.3 ± 0.41 μmol/min/mg) (Fig. 6). By week 6, mean GST activity in fasted fish was reduced 34% from activities of fish fed full-rations and 28% from activities of those receiving half-rations. Following one week of re-feeding mean GST activities increased 69% from week 9 activity in the half-ration group, and 14% from week 9 activity in the starved group to be not significantly different from basal activities at week 0. Fish began exhibiting signs of sexual maturation at week 12. Accordingly, gender was included as a component of the statistical model at this sampling interval. GST activities in females at week 12 were significantly different from males (50% decrease compared to males).

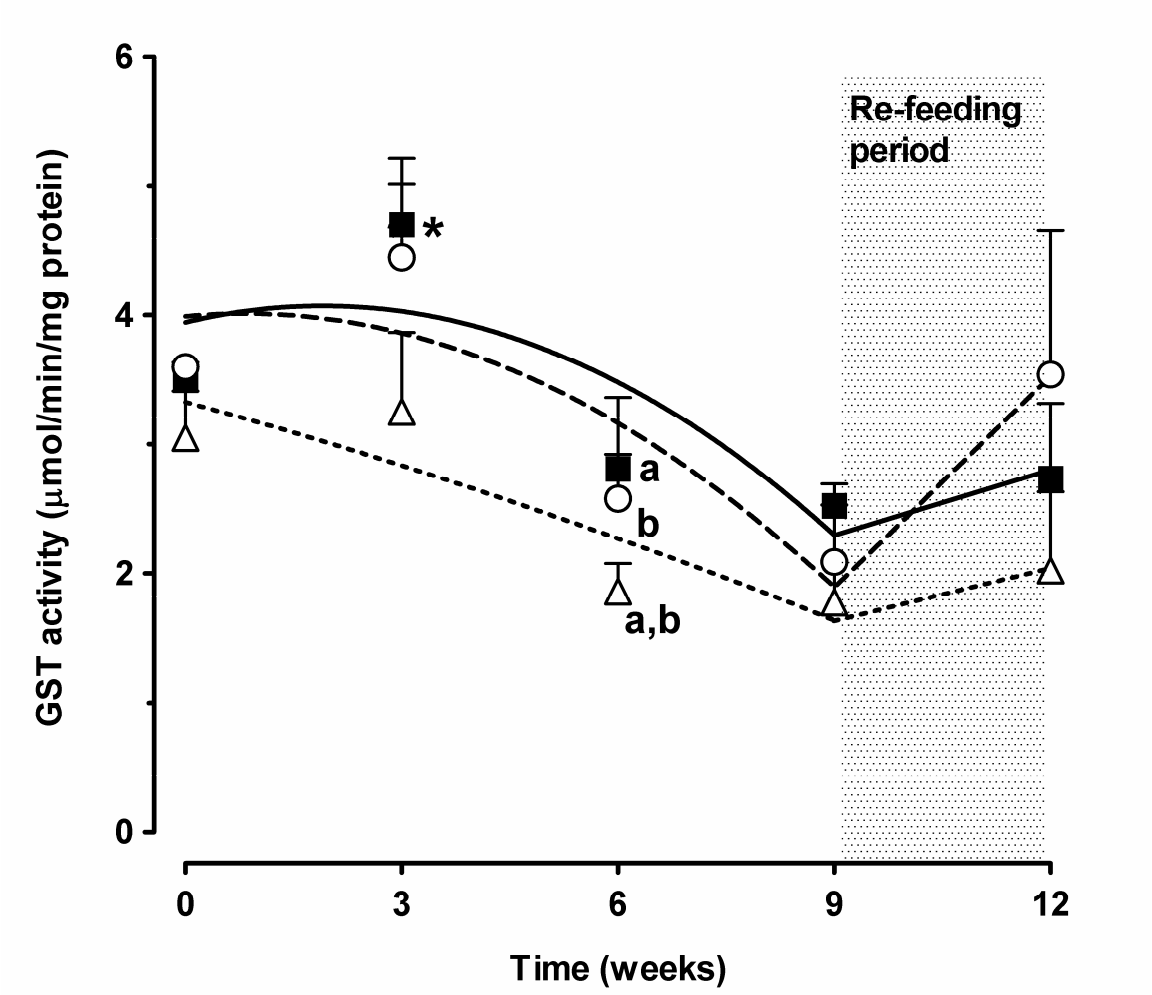

Figure 6 GST activity of rainbow trout exposed to three diet regimes [(■) full-rations, non-linear regression y = 3.944+ (0.1340)x + (-0.03516) x^2, r^2=0.3792; (○) half-rations, non-linear regression y = 3.990+ (0.05248)x + (-0.03168) x^2, r^2 = 0.4738; or (Δ) fasted, non-linear regression y = 3.326+ (-0.1511)x + (-0.004065) x^2, r^2 = 0.3695] over nine weeks followed by re-feeding of all treatment groups on full-rations. Data points with asterisks (*) denote significant differences between time points within a treatment group ($p<0.05$). Data points with the same letters denote significant differences between treatment groups ($p<0.05$).

CHAPTER 5: DISCUSSION

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It is currently unknown if energy is preferentially allocated to cellular defence mechanisms under limited energy intake over other physiological systems or processes. In order to determine this, the effects of restricted feed ration on the activities of key cellular defence proteins involved in rainbow trout hepatocytes were examined. Whole body indices (BW and LSI) were monitored in concert with activity assays to investigate how body energy stores provide energy under dietary restriction that may be used towards maintaining defence activities.

Change in Whole Body Indices (Body Weight/LSI)

In fully-fed fish, BW did not increase significantly until weeks 9 to 12, whereas mean LSI exhibited a trend of decreasing 25% between weeks 0 and 6, before increasing 32% between weeks 9 to 12. These differences over time were not caused by dietary treatment, but are likely responses to seasonal changes in water temperature that occurred over the experimental period. The observed changes are similar to results in other species that show temperature-responsive growth rates which tend to increase with rising spring temperatures before becoming inversely proportional to summer temperatures, and peaking again in fall as water temperatures drop (Swift 1961; Adams and McLean 1985). Furthermore, observed changes in LSI followed a similar pattern over time, which may be attributed to mobilizing liver resources to deal with increased metabolic demands and maintaining homeostatic mechanisms at warmer temperatures (Adams and McLean 1985). Fish in the half-ration treatment group did not exhibit

significant increases in BW until weeks 9 to 12, and exhibited a trend of diminished mean LSI in weeks 3 to 6. As well, fasted fish showed no significant change in body-weight over time, but a significantly diminished mean LSI until re-feeding. Responses to seasonal temperature changes in these groups may be minimized as endogenous energy stores are increasingly mobilized under dietary restriction.

By week 6, mean BW of fasted fish was significantly reduced from control and ration-restricted trout. Dietary restriction had an even greater influence on mean LSI, as both ration-restricted and fasted groups exhibited a significantly reduced mean LSI from the control group as early as week 3. Whereas growth accounts for the cumulative effects of all factors acting on an organism, liver somatic index has been considered a 'faster, more sensitive response' to alterations in the environment (Adams and McLean 1985). It is not surprising that in the present study LSI responded more rapidly than BW to changing dietary conditions. These results are in agreement with other literature on feed-deprived rainbow trout, in which BW decreased within 4 to 6 weeks, and reductions in LSI became significant within much shorter time frames (2 days to 3 weeks of dietary restriction) (Farbridge and Leatherland 1992; Vigano et al. 1993; Blom et al. 2001; Mattson et al. 2001). The rapid change in LSI in early fasting is likely due to a cortisol-induced mobilization of liver glycogen and lipid stores; as trout fasted for 3 to 4 weeks exhibit enhanced gene expression of key enzymes in gluconeogenesis, and a down-regulation of glycolysis and the pentose phosphate pathways (Blom et al. 2000; Simpkins and Hubert 2003; Johansen

and Overturf 2006; Salem et al. 2007). As the body adjusts to long-term fasting, visceral fat and muscle lipid stores begin to be catabolized for maintenance energy in preference to exhausting hepatic lipid stores; this can be exhibited as a resistance to further reductions in LSI that have been noted in past studies, and in the present study appears as little reduction in mean LSI of fasted fish after week 3 (Adams and McLean 1985; Farbridge and Leatherland 1992; Simpkins and Hubert 2003; Salem et al. 2007). Coordinately, protein accretion is attenuated and catabolism of skeletal muscles and gut tissue is induced in an effort to preserve hepatic protein content for as long as possible (Peragon et al. 1999; Simpkins and Hubert 2003; Azevedo et al. 2004; Salem et al. 2007).

Ration-restricted and fasted trout did not display a significant rapid gain in body-weight after one week of re-feeding. This may be due to the fact that fish were re-fed a consistent ration, whereas studies illustrating compensatory growth re-fed fish *ad libitum*, allowing hyper-phagia to occur (Boujard et al. 2000). Furthermore, significant compensatory growth in rainbow trout, has been observed after 4 weeks of re-feeding, such that one week may be too short for this effect to be notable (Weatherly and Gill 1981). However fasted trout did display a compensatory gain in liver weight as evidenced by a 98% increase in LSI after re-feeding; this result is in agreement with previous literature, and may occur as hypertrophy of cells via up-regulated fatty acid synthesis and protein accretion in the liver (Weatherly and Gill 1981; Farbridge and Leatherland 1992; Peragon et al. 1999; Boujard et al. 2000; Johansen and Overturf 2006).

R123 accumulation in cells as a measure of P-gp activity

In trout fed full-rations, R123 accumulation rates (P-gp activity) varied inversely to changes in temperature over the experimental period, suggesting that increased P-gp efflux activity may be correlated with increasing temperatures; the literature is equivocal in this regard (Lin et al. 2000; Parasrampuria et al. 2001). In oyster gills, it has been suggested that transcriptional activation plays a role in enhancing P-gp expression with increasing water temperature (Bard 2000). Further work is needed to determine if greater efflux capacities at high temperatures may be due to increased catalytic efficiencies, or indirect up regulation of P-gp expression through cellular stress responses involving heat shock proteins or oxidative stress signalling cascades (Chin et al. 1990; Minier et al. 2000; Hong et al. 2006). Trout in half-ration and fasted treatment groups also exhibited diminished R123 accumulation rates up to week 9 although to a lesser extent than in the control group, suggesting that the P-gp responses to seasonal temperature changes may be modulated in fish under dietary restriction.

Limited information exists regarding P-gp activity and dietary energy intake. Increased P-gp expression has been observed in killifish hepatocytes cultured for 9 hours under nutrient deficient conditions (Albertus and Laine 2001). However, the present study illustrates that severe and chronic feed restrictions in rainbow trout do not significantly affect P-gp activity in hepatocytes. Maintenance of this efflux pump activity under 9 weeks of starvation suggests that P-gp activity remains a prioritized system. Recent work by Gatlik-Landwojtowicz et al. (2004)

supports this idea; cells in carbon-starved cultures maintained P-gp activity at the cost of a greater proportion of cellular metabolic reserves. Nonetheless, it is of interest that ration-restricted and deprived groups in the current study exhibited a slight trend of decreasing P-gp activity in cells under increasingly severe diet restrictions, suggesting that under these conditions efflux pump activity may not be maintained indefinitely.

EROD activity

Trout in the full-feed treatment group exhibited increases in EROD activity that peaked at week 6 and then returned to baseline levels, changes which correlate with changes in ambient water temperature. The effects of temperature on biotransformation enzyme activities, including cytochrome P450-mediated (e.g. EROD) reactions, are well-documented (Kennedy et al. 1991; Andersson and Forlin 1992). Acute temperature increase has been shown to result in an increase in benzo[a]pyrene hydroxylase activity and other P450-mediated transformations, however, given a sufficient acclimation period, activities return to baseline levels through compensatory mechanisms (Andersson and Koivusaari 1986; Gill and Walsh 1990; Andersson and Forlin 1992, Kennedy and Walsh 1994; Lange et al. 1998). Fish in the fasted and half-ration treatment groups exhibited peak EROD activities at week 6 which were greater than those noted in fish fed full-rations, before returning to basal levels by week 12. These results suggest that variations in EROD activity due to seasonal temperature changes are modulated in fish also subject to dietary restriction.

Reported basal EROD activities in rainbow trout range widely (Gregus et al. 1983; Weber et al. 2002); mean basal activity in the current study (0.34 ± 0.095 nmol/min/mg protein) is within this range using similar methods (Paige and Davies 2002). Fasted trout and those fed half-rations maintained EROD activities that were not significantly different from fish receiving full-rations despite severe feed restrictions for at least 9 weeks implying that CYP1A activity is also a prioritized system. This work supports a theory of recalcitrance to change suggested in prior studies by sustained EROD activities of up to 3 to 7 weeks, depending on the severity of feed deprivation (Andersson et al. 1985; Vigano et al. 1993; Blom et al. 2000; Morrow et al. 2004).

Of extreme interest is the strong trend ($p=0.093$) of up-regulated EROD activity in ration-restricted trout compared to controls, suggesting that CYP1A activity is an increasingly prioritized biotransformation mechanism under conditions of diminishing energy reserves. Similar results have been reported in the literature; where enhanced EROD activity was seen in rainbow trout following 2 to 7 weeks of caloric restriction (Vigano et al. 1993; Blom et al. 2000; Mattsson et al. 2001; Kennedy et al. 2004; Morrow et al. 2004). The mechanism of induced EROD activity may be attributed to nutritional stress enhancing glucocorticoid biosynthesis and consequently potentiating induced CYP1A gene expression (Devaux et al 1992; Blom et al. 2000; Ferraro et al. 2003). In rats, the presence of glucocorticoid response elements in CYP1A genes, highlights the potential for cross-talk between these two pathways (Celander et al. 1989; Stegman 1993; Longo et al. 2000). In support of this, rainbow trout CYP 1A1 and 3A4 gene

expression have been shown to be up-regulated following 3 weeks of fasting (Salem et al. 2007).

GST activity

Fish receiving full-rations exhibited mean GST activities that peaked at week 3 before declining to week 9, after which mean activity increased 8.2% to reach baseline levels by week 12. These results suggest GST activity is relatively insensitive to transient temperature changes possibly due to 'positive thermal modulation' whereby Km increases with increasing temperatures (Kennedy et al 1991; Kennedy and Walsh 1994). However, long-term variation in GST activity over seasons has been noted in other aquatic organisms and may be linked to increased metabolic rates at high temperatures (Wilhelm Filho et al. 2001; Kaur et al. 2005). Furthermore, published work in eelpout and juvenile rainbow trout has shown seasonal reductions in GST activity during the fall-winter seasonal transition (Ronisz et al. 1999). The transient increases in GST activity observed in the current study at week 3 may be attributed to increased production of reactive oxygen species at higher temperatures (increasing metabolic rate) eliciting antioxidant responses that may interfere with concurrent thermo-modulatory adaptations (Kaur et al. 2005). Ration-restricted and fasted fish also exhibit peak GST activities at week 3, but to a lesser extent than seen in control fish. In addition, these treatment groups exhibited exaggerated reductions in GST activity up to week 9, as energy intake remained reduced. These results suggest

that changes in GST activity in response to seasonal temperature changes, and possibly oxidative stress, are modulated in fish under diet constraint.

The mean basal GST activity in this study (3.3 ± 0.40 μmol/min/mg) is well within the range of literature values (Gregus et al. 1983; Petrivalsky et al. 1997). Feed deprivation significantly attenuated the GST activity of fasted fish compared to those fed full and half-rations within 6 weeks of treatment, suggesting that maintenance of this phase II enzyme activity may not be supportable when intake energy is severely limited for this length of time. In fasted dentex (*Dentex dentex*), increasingly mobilized liver and adipose stores and the down-regulation of NADPH and fatty acid biosynthesis pathways diminishes NADPH stores, indirectly limiting the activity of antioxidant and detoxification enzymes dependant on reduced glutathione (Morales et al. 2004). In rainbow trout, significant reductions in GST activity and gene expression have been seen within 3 to 6 weeks of fasting (Andersson et al. 1985; Blom et al. 2000; Kennedy et al. 2004; Morrow et al. 2004; Salem et al. 2007). Prior to reductions in GST activity, pathways mobilizing body energy stores that assist NADPH regeneration are likely sustaining GSSG-GSH recycling, and consequently GST activity during acute starvation (Andersson 1986; Vigano et al. 1993; Kennedy et al. 2004). Re-feeding of feed-restricted and fasted trout initiated a 69% and 14% increase in GST activity, returning it to baseline levels, a trend also noted in dentex (*Dentex dentex*) and gilthead seabream (*Sparus aurata*) (Pascual et al. 2003; Morales et al. 2004). These results suggest that renewed energy intake provides the energy necessary to restore support mechanisms for this Phase II defence activity

(Morales et al. 2004). At the week 12 interval, trout began exhibiting signs of sexual maturation, and GST activities in females were significantly reduced from males. This observation is in keeping with sex effects noted in past mammalian research, and warrants further research in fish species (Stegeman et al. 1992, McFarland et al. 1999, Schlenk et al. 2008).

Concluding Remarks

P-gp and EROD activities were maintained in trout fasted for 9 weeks and appear to be prioritized physiological mechanisms even under conditions of long-term fasting. Conversely, GST enzyme activity does not appear to be recalcitrant under fasting conditions, and was significantly attenuated within 6 weeks of treatment. However, down-regulated GST activity was recoverable after one week of re-feeding, demonstrating response plasticity in an organism adapted to tolerate changes in food availability. Calorie-restricted and fasted trout also exhibited dynamic changes in whole body measures over this period suggesting that body stores were catabolized to mobilize energy resources to support these systems, amongst others. New research suggests that scarcity-sensing proteins may regulate these biochemical and physiological changes (Bordone et al. 2005). Signalling molecules of interest include sirtuin proteins, which in starved mammals provide the organism with a greater capacity to metabolize fat and take-up glucose, as well as increase insulin sensitivity, and reduce lipid accumulation in the liver (Bordone et al. 2005; Koo et al. 2006; You et al. 2008). Bile acids have also been suggested as signalling molecules in mammals

capable of modulating transcription of proteins involved in Phase I, II, and III defence systems and metabolic pathways via membrane (e.g. TGR5) and nuclear (e.g. FXR) receptors, both of which have been identified in fish (Lee et al. 2006; Gems 2007; Thomas et al. 2008). Energy trade-offs between breeding cycles/fecundity, and mechanisms promoting body maintenance, longevity, and recalcitrance to environmental stressors have been recognized in a range of organisms (Holliday 2006; Steinberg et al. 2007). Further investigation into signalling molecules as overarching regulators of stress resistance in teleost fish may help explain the capacity of these organisms to survive natural periods of food deprivation and still adapt to today's increasingly variable aquatic environments.

CHAPTER 6: THESIS CONCLUSION AND SUGGESTED FUTURE RESEARCH

Results of the current study suggest that activities of the key defence transporter protein P-gp, and the biotransformation enzyme CYP1A were preserved in fasted trout and appear to be prioritized mechanisms even under conditions of extreme nutrient deficiency. Conversely, the activity of cytosolic GST enzymes does not appear to be recalcitrant to change, as fasted trout exhibited significantly attenuated GST activity. The fact that this down-regulated enzyme activity is recoverable after one week of re-feeding demonstrates a plasticity of response in an organism adapted to weather annual changes in food availability and its natural environment. Energy preserving these activities must come directly or indirectly from endogenous reserves and potential trade-offs with other physiological processes, as suggested by dynamic changes in whole body condition indices (body-weight and liver somatic index) of calorie-restricted and fasted trout.

Applied Aspects of the Study

The applied aspect and eco-relevance of this study is that it highlights potential and realistic risks to teleosts undergoing natural annual cycles of fasting in polluted habitats, or exposed to situations of degraded food quality and

quantity as well as chemical stressors from acute contamination. If dietary energy is severely limited, the current study suggests that GST activity will not be maintained, and will be down regulated in teleost liver.

Although rare, Phase II conjugation reactions catalyzed by GST may bioactivate some environmental contaminants. For example, vicinal halogen alkanes such as 1,2-dibromoethane (a pesticide, and additive to gasoline, dyes and waxes) can be conjugated to glutathione by theta-like GST isoforms to form a reactive, carcinogenic metabolite in teleost fish (ATSDAR [updated 1995]; Schlenk et al. 2008). It is possible that decreasing GST activity in such exposures may limit the formation of this active metabolite and consequently reduce the risk of hepatocarcinoma incidence.

On the other hand, there are many classes of aquatic contaminants that may become more toxic to fish with a decreased ability to utilize this conjugating system.

For example, transition metals present in aquatic systems (e.g. copper, mercury, iron) from mining and other industrial activities can both donate and accept electrons, making them capable of bonding with nucleophilic targets of thiol and amine groups in proteins and nucleic acids in cells, as well as reacting with peroxides to form extremely reactive hydroxyl radicals (Canesi et al. 1999; Schlenk 2001). A reduced GST activity in teleost fish would likely affect the ability of the enzyme to conjugate glutathione to lipid peroxidation products from metal-induced ROS generation. Furthermore, transition metals can form stable complexes with glutathione, preventing their ability to generate ROS and

enhancing their excretion, if the underlying cause of decreased GST activity during fasting is a depletion of reduced glutathione these protective mechanisms may be attenuated in fish (Canesi et al. 1999; Cajaraville et al. 2003).

As well, lipophilic anthropogenic contaminants that can bio-accumulate in cells, and are bio-activated by CYP1A enzyme activity (e.g. benzo[a]pyrene) to form reactive, electrophilic intermediates are of further concern under these conditions. A decrease in GST activity will affect the ability of these enzymes to conjugate glutathione to reactive metabolites for excretion; in addition, GSTs can be covalently bound to electrophiles reducing their ability to react with cell proteins, DNA, and generate ROS (Cajaraville et al. 2003; Schlenk et al 2008). Both triazine herbicides (e.g. atrazine) and organophosphorus pesticides (e.g. Dichlorvos) are prominent aquatic contaminants that can be detoxified in cells by GST-mediated glutathione conjugation (Schlenk et al. 2008). Under conditions of decreased GST activity atrazine may bio-accumulate in fish, enhancing its endocrine disrupting effects, and ability to alter hepatic metabolism at low exposure concentrations (EXTOXNET [updated 1996]; Salaberria et al. 2009). Similarly, if GST activity is directly or indirectly limited (by a depletion of reduced glutathione) during fasting, a greater concentration of cellular Dichlorvos will be available to induce toxic effects in fish by inhibiting acetylcholinesterase, modulating Ca^{2+} in cells, and producing free radicals capable of damaging cell proteins, lipids, and nucleic acids (Pena-Llopis et al. 2003). All of the afore-mentioned contaminants are in some way capable of generating ROS and contributing to oxidative stress in the cell. ROS production that overwhelms

antioxidant and conjugation defences not only affects the redox status of the cell; lipid peroxidation can impair the functioning and permeability of membranes, oxidation of proteins can modulate enzyme activities and structural proteins, and oxidative DNA damage can change gene expression (Cajaraville et al. 2003).

As we become more aware of the pathways and effects of oxidative stress on crucial cell components leading to cell dysfunction (e.g. cancer) and death (e.g. apoptosis, necrosis), peroxisome proliferators (e.g. clofibrate, phthalate esters) and other contaminants which are non-genotoxic, but generate ROS, will become increasingly suspect xenobiotics in aquatic ecosystems (Schlenk 2001; Cajaraville et al. 2003; Schlenk et al. 2008).

It is anticipated that the results of this study and future work in this area will be pertinent to fisheries biologists considering population effects on stocks, risk assessors of contaminated waterways determining chemical sensitivities of teleost fish species during winter months, and government scientists involved in setting regulations and thresholds for industries producing contaminants mediated through these cellular defence pathways.

Contributions to Basic Biology

Fasting-induced changes in the cellular environment can elicit co-ordinate modulation in consecutive lines of cellular defence as well as other physiological processes. P-gp activity and expression in fish has the potential to be modulated by oxidative stress signalling pathways or heat shock proteins considering up-regulated activities and expression in response to ROS exposure

in rat brain endothelial cells, and increases in water temperature in aquatic organisms, respectively (Minier.et al. 2000; Felix and Barrand 2002). Prior work in teleost species and rats has illustrated the possibility that NADPH-dependant CYP1A enzyme activity can be potentiated by circulating stress hormones and the presence of glucocorticoid response elements in the CYP1A gene affecting gene expression (Devaux et al. 1992; Stegeman 1993; Longo et al. 2000; Ferraro et al. 2003; Salem et al. 2007). Furthermore, activities of NADPH-dependant GST enzymes in rainbow trout and other fish species may be responsive to the redox status and reduced glutathione content of the cell, as well as being modulated by antioxidant response elements, or as part of the aryl hydrocarbon receptor gene battery (Morales et al. 2004; Salem et al. 2007;Schlenk et al, 2008). Finally, gene expression changes in fasted rainbow trout over time illustrate concurrent and co-ordinate changes in key metabolic enzyme activities and expression, down-regulating anabolic pathways, and up-regulating catabolism of glycogen, lipid, and protein stores for energy resources (Johansen and Overturf 2006; Salem et al. 2007). The above observations all reiterate the complex regulation and responsiveness of these processes to cellular changes under fasting conditions, and suggest the possibility of “over-arching” signalling molecules initiating co-ordinate and complementary modulations in these systems.

Current research into potential signalling molecules has focused on biochemical and physiological changes in calorie restricted organisms that occur as a “highly regulated process requiring proteins that can sense scarcity and

mount an appropriate physiological response" (Bordone and Guarente 2005). Signalling molecules of interest include NAD^+ dependant sirtuin proteins. These proteins are from a family of evolutionarily conserved genes (with fish expressing orthologous sequences to all seven genes) involved in epigenetic modifications of histone proteins as well as de-acetylation of key transcription factors and proteins of regulatory processes (Bordone and Guarente 2005; Frye 2006). Research in starved mammals indicates that proteins from the SIRT1 gene family may be activated by an increase in NAD^+/NADH ratio in cells and involved in co-ordinating energy provisions by signalling reductions in lipogenesis, increases in circulating stress hormones, mitochondrial biosynthesis, fat and glucose metabolism, as well as increased insulin sensitivity and glucose uptake in muscle and vital organs (Bordone and Guarente. 2005; Koo and Montminy. 2006; You et al. 2008). Circulating bile acids have also been suggested by recent literature as powerful signalling molecules in mammals capable of modulating transcription of Phase 1 and 2 biotransformation enzymes and membrane transporters in the liver, as well as modulating mitochondrial activity, cholesterol, fat and glucose metabolic pathways via membrane G-protein coupled receptors (e.g. TGR5) and nuclear receptors (e.g. FXR), both of which have been identified in fish (Lee et al. 2006; Gems 2007; Thomas et al. 2008). The effect of long term calorie restriction and fasting on circulating bile acid concentrations, and its possible role in modulation of Phase 1, 2, and 3 defence protein activity and expression in teleost fish hepatocytes should be a consideration in future research.

Growth retardation and increased lifespan in ration-restricted guppies, and the diversity of fish species in acidic waters are situations that seem to indicate how a changing environment can elicit alterations in an organism that increase its capacity to survive and resist these fluctuations, likely at the cost of energy partitioning to less crucial processes (e.g. growth, reproduction) (Comfort 1963; Steinberg et al. 2007). A recent article by R. Holliday (2006) supports this idea, drawing attention to organisms that live in environments of variable food supply (e.g. teleost fish, hibernating bears, rodents) and their ability to re-allocate energy from breeding cycles and fecundity, investing it in processes that include body maintenance, stress resistance, and longevity.

Regulated changes in physiological and biochemical processes used by teleost species to survive situations of concurrent chemical stress and energy limitation are of current interest and importance. Identification and investigation into genes and signalling pathways that may be “overarching” regulators of fasting-induced stress resistance in teleost fish should be a priority in future works. Results of this study and future work in the area will assist in deciphering unanswered questions in basic biology: outlining the components of cellular defence systems and their associated energy costs, investigating control of energy partitioning to cellular defence mechanisms among other processes in teleost fish, as well as determining the prioritization of energetic costs under variable environmental parameters (e.g. starvation, chemical stress).

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