THE EFFECTS OF DIET COMPOSITION AND RATION ON BIOTRANSFORMATION ENZYMES AND STRESS PARAMETERS IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

by

Michelle Dayna Morrow
B.Sc. (Hons.), University of Western Ontario, 2000

RESEARCH PROJECT SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENVIRONMENTAL TOXICOLOGY

In the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY

December 2003

APPROVAL

Name: Michelle Dayna Morrow

Degree: Master of Environmental Toxicology

Title of Project:

The effects of diet composition and ration on biotransformation enzymes and stress parameters in rainbow trout, *Oncorhynchus mykiss*.

Examining Committee:

Chair: Dr. A. Harestad

Dr. C. Kennedy, Associate Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. T. Williams, Professor
Department of Biological Sciences, S.F.U.

Dr. M. Moore, Associate Professor
Department of Biological Sciences, S.F.U.
Public Examiner

5 December 2003
Date Approved

PARTIAL COPYRIGHT LICENCE

Title of Thesis/Project/Extended Essay

The effects of diet composition and ration on biotransformation enzymes and stress parameters in rainbow trout, *Oncorhynchus mykiss*.

Author: _____
(signature)

Michelle Dayna Morrow
(name)

Dec 11, 2003
(date)

ABSTRACT

Nutritional quality and quantity play significant roles in the amount of energy an animal assimilates and in the distribution of that energy. Furthermore, energy is allocated in a hierarchical fashion beginning with basal metabolism and then successively to other physiological processes depending on other energetic requirements of the animal. To date, little information exists on the effects of diet and ration on the stress response and detoxification system in fish. Therefore, the purpose of this study was to measure the effects of dietary composition and ration on the biotransformation and stress response systems in juvenile rainbow trout.

Juvenile rainbow trout (*Oncorhynchus mykiss*) were fed one of three isoenergetic diets varying in protein (35 to 55%) and lipid content (8 to 18%), at full satiation or partial ration for 6 weeks in order to investigate the effects of diet on baseline stress parameters and biotransformation enzyme activity. Growth was highest in fish fed low protein, high lipid diets at both ration levels and was highest overall in fish fed to satiation. Stress indicators, including plasma lactate, glucose and cortisol concentrations were not significantly affected by dietary treatment or ration. Basal biotransformation enzyme activity, including glutathione S-transferase (GST) and ethoxyresorufin *O*-deethylase (EROD) activity, were also unaffected by dietary treatment. Fish exposed to the biotransformation enzyme inducer ß-napthoflavone did not exhibit an alteration in stress indicators or GST activity; however, EROD activity was increased (87 to 210-fold) in fish receiving all diets and rations demonstrating the importance of this system even under limited energy intake.

The results of the present study indicate that, unlike mammals, fish may be more recalcitrant to different levels of ingestion (above maintenance ration) of isoenergetic diets varying in protein and lipid concentration with respect to stress responses and the

maintenance of basal titres of biotransformation enzymes and their induction. Although no differences in detoxification enzyme activity or stress indicators were found between fish fed the different dietary treatments in this study, possibly because dietary treatments did not vary enough from each other, other studies have reported differences when having used diet compositions and rations that varied more drastically. These findings signifying the importance of appreciating the nutritional history of experimental fish prior to testing especially if the intention is to use them as bioindicators within the natural setting.

DEDICATION

For my parents, Colleen and Roland, for their endless emotional, spiritual and financial support in all my academic and personal endeavours.

ACKNOWLEDGEMENTS

Several people have provided me with much support and encouragement throughout my graduate degree. However, I must express my greatest gratitude and thanks to my senior supervisor, Dr. Kennedy. His guidance, motivation and confidence in my abilities as a graduate student were invaluable in the completion of my graduate degree.

I must also express my thanks to Dr. Williams for his valuable comments and suggestions during the writing process, which helped to greatly improve the quality of my final product.

The formulation and preparation of the three custom diets used in this study was made possible the help of Dr. Higgs, at the Department of Fisheries and Oceans. Additionally, Mahmoud Rowsandeli provided me with a great deal of technical assistance during the diet preparation and analysis processes and was a vast resource of nutritional information.

There are great number of other people were essential in providing me with support and assistance during my time at Simon Fraser. It is my friends and colleagues that have made my time here most enjoyable and memorable. I must thank Danielle, Deb, Harp, Helena, Hugh, Jim, Jonathan, Kat, Tara and Wade for their friendship and encouragement.

I must finally express my gratitude to the many other people outside of the Simon Fraser community who were a constant source of encouragement over the past three years and helped made this degree achievable, including my parents (Colleen and Roland), my siblings (Nicole and Chris) and several other friends. I must also express a special thanks to Mark who came into my life during the most stressful part of this degree and provided much needed support and understanding.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ß-napthoflavone	ß-NF
Condition Factor	CF
Ethoxyresorufin O-deethylase	EROD
Glutathione S-transferase	GST
Hepatosomatic Index	HSI

Introduction

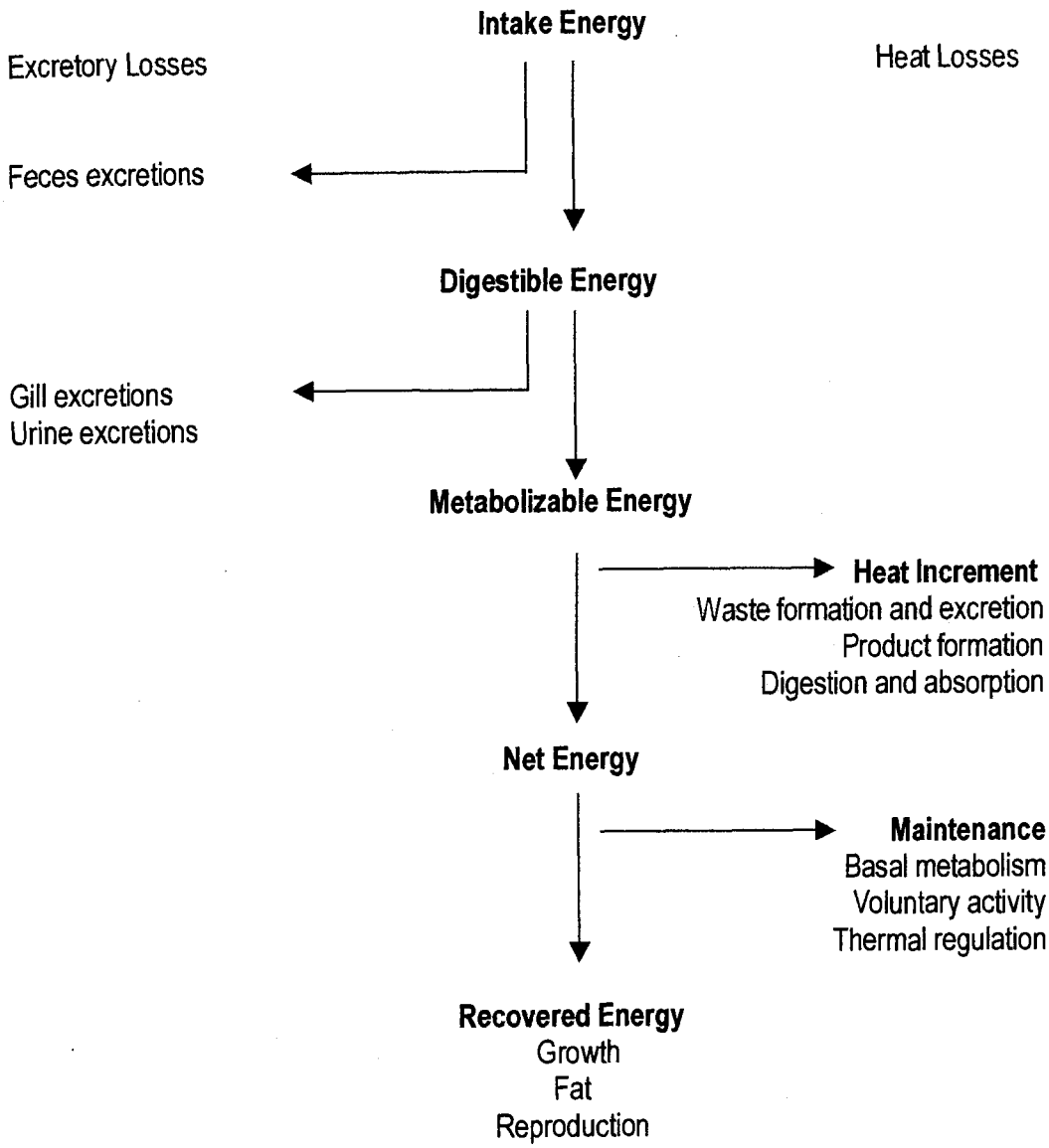
Energy

All animals obtain energy and the nutrients necessary for survival from the food they ingest. Thus, an appropriate composition of dietary constituents including energy, protein, lipid, carbohydrate, vitamins and minerals, is necessary to maximize animal health, growth and reproduction while minimizing energy losses through excretions. In particular, the quality and quantity of a diet can influence the metabolic partitioning of dietary components between anabolism for storage and catabolism for fuels.

Energy is not a nutrient, rather, it is released from the diet through the metabolic oxidation of dietary carbohydrates, proteins and lipids. In fish, the majority of this energy is derived from proteins and lipids, as carbohydrates make a comparatively small contribution due to the limited ability of fish to digest them (NRC, 1993). Food contains a measurable amount of energy based on the proportion of these energy-yielding compounds. The gross energy of a diet may be calculated from the mean heat of combustion values for protein, lipid, and carbohydrate being 23.6, 39.5, and 17.2 kJ/g, respectively (Bureau et al., 2002). However, the calculated gross energy contained in a diet is not equal to that amount used by the body for two reasons; first, not all gross energy may be bioavailable to the animal, and second, there are many points between intake and energy recovery where energy is lost.

An organism's allotment and loss of energy is termed the energy budget and may be schematically represented in an energy flow diagram (Figure 1). As stated previously, not all of the energy ingested as food is available for use by an organism and consequently, any food that cannot be digested will be lost directly in the feces (Bureau et al., 2002). The amount of energy lost at this point may vary based on feeding rate and the nature of dietary ingredients. For example, because fish have a limited

Figure 1. Schematic representation of dietary energy allocation (modified from NRC, 1993).


Intake Energy
Excretory Losses
Heat Losses
Feces excretions
Digestible Energy
Gill excretions
Urine excretions
Metabolizable Energy
Heat Increment
Waste formation and excretion
Product formation
Digestion and absorption
Net Energy
Maintenance
Basal metabolism
Voluntary activity
Thermal regulation
Recovered Energy
Growth
Fat
Reproduction

ability to break down carbohydrates, increasing the carbohydrate content of a diet will decrease that proportion of energy retained from the diet resulting in an increase of energy lost in the feces (Cho and Kaushik, 1990). The energy that is bioavailable to the animal is designated digestible energy.

The digestion of proteins, lipids and carbohydrates, respectively, produce amino acids, fatty acids and glucose, all of which are absorbed and used as metabolic fuels (Halver and Hardy, 2002). During their digestion, all three components produce carbon dioxide and water as waste byproducts that are easily managed by the body. In fish, protein digestion additionally produces ammonia and urea in comparatively small amounts, as waste byproducts that are excreted via the kidney and gills. These excretions are regarded as losses of digestible energy because both ammonia and urea contain nitrogen, which if it had been assimilated, otherwise could have been incorporated into amino acids (Cho and Kaushik, 1990).

Energy remaining after all excretory losses is termed metabolizable energy, and is essentially the physiologically utilizable fuel available from the diet (Steffens, 1989). A large proportion of metabolizable energy is lost as heat from the catabolism of energy sources used to do physiological work. Energy losses occur during two key events: feeding and maintenance (NRC, 1993). During feeding, heat loss occurs due to digestion and absorption processes, the transformation of substrates and their retention in tissues as well as during the formation of metabolic wastes. Collectively, this heat loss is known as the `heat increment of feeding'. Maintenance energy, however, is that energy required to maintain the body functions immediately essential to life *while fasting* and includes basal (or standard) metabolic rate, active metabolism including voluntary and resting activity, and thermal regulation in some species. Basal metabolic rate takes top priority, as this is the energy required to maintain the minimum rate of metabolic activity necessary to sustain structure and function of tissues through activities such as

respiration, ion and metabolite transport, body constituent turnover, and circulation, while at complete rest and not digesting any food (Cho and Kaushik, 1990). Although some level detoxification enzyme activity may be maintained during basal metabolism, the amount of oxygen consumption contributed to this process is uncertain. Fish and other poikilotherms require considerably less fasting maintenance energy (40 kJ/kg $BW^{0.824}$ day^{-1} for rainbow trout at 15^0C) than homeotherms, which require approximately 300 kJ/kg $BW^{0.75}$ day^{-1} (Bureau et al., 2002). The lower energy needs of fish result from the lack of need to expend energy on regulating body temperature, their ability to maintain neutral buoyancy thus lack of need to oppose gravity, and their mode of waste nitrogen excretion (i.e. ammoniotelism). Any energy remaining after maintenance is subsequently allocated to growth and reproduction.

Dietary Composition

A diet consists of five main components: proteins, lipids, carbohydrates, minerals and vitamins. Proteins are valuable dietary constituents, which require great consideration due to their uses as energy sources and investment resources. After ingestion, proteins are broken down by digestive enzymes into 20 individual amino acids that are subsequently absorbed into the gastrointestinal tract. Amino acids are used in the synthesis and repair of tissues and are important in enzyme production (Wilson, 2002). Of these 20 amino acids, 10 are termed 'essential' amino acids, meaning they cannot be synthesized by fish and must be provided through the diet. However, a diet must be balanced with respect to all 20 amino acids in order to prevent the loss of the 10 non-essential amino acids by their conversion to the 10 essential ones. This may result in a diminished number of amino acids available for growth and energy uses, even if the overall protein content of the diet is satisfactory (Steffens, 1989). Furthermore, an inadequate supply of protein results in the catabolism of less vital tissues to maintain the

function of more crucial tissues, resulting in reduced growth rate and even weight loss (Wilson, 2002). Finally, a diet must contain proteins that are highly digestible in order minimize nitrogen excretion.

Since dietary protein is an important component for growth and is a valuable energy source, the digestible protein:digestible energy ratio (DP:DE) greatly influences the partitioning of protein between its energetic and investment uses (Cho, 1992). For example, if DP:DE is lowered, growth rates will decrease as the majority of the protein will be used to fulfil energy requirements and only the small remaining proportion will be available for growth. However if the ratio is too high, fish may not consume enough food to provide adequate protein for normal growth because fish eat to satisfy their energetic requirements and not to meet their growth needs, and therefore, will cease feeding when their energetic needs have been met (Rienitz et al., 1978; Lee et al., 2002). Since protein is the most costly part of diet, it is ideal for fish producers to maximum growth while minimizing protein use as an energy source.

The DP:DE ratio may be optimised by providing energy in the form of other energy-yielding nutrients. Lipids are most commonly used as the alternative energy source due to the poor digestibility of carbohydrates (Bureau et al, 2002). Optimization of the DP:DE ratio is accomplished by comparing growth values of fish fed diets varying in either protein *or* lipid content (Lee et al., 2002). By increasing the dietary level of non-protein energy-yielding sources (e.g. lipids), energetic needs may be fulfilled by these alternate sources thereby freeing protein for growth (Cho and Kaushik, 1990; Engin and Carter, 2001). This phenomenon has been termed protein sparing and has been demonstrated in many fish species (Reintz et al., 1978; Cho and Kaushik, 1990; Cho, 1992; Lee et al., 2002; Yang et al., 2003). Although, variations in optimal DP:DE ratios have been found in several studies (NRC, 1993), they are suggested to be due to

differences in species and strain and fish age (Einen and Roem, 1997), as well as rearing condition differences (Catacutan and Coloso, 1995).

Lipids are utilized as a major energy source in fish as they contain almost twice as much energy per unit weight as proteins (Hilton and Slinger, 1981). Lipids are also important in the formation of cellular structure, preservation of biomembrane integrity, and are involved in maintaining optimal health, growth and reproduction (Hilton and Slinger, 1981; NRC, 1981). However, an excess of lipid in the diet may result in high levels of carcass fat especially in the visceral cavity. Lipids provide fish with two fatty acids, specifically linoleic and linolenic acid, that must be supplied in the diet as fish cannot synthesize them (Kanazawa, 1985). Thus, consideration must not only be given to the quantity of lipid in the diet but also to the types of lipid provided to ensure that essential fatty acid requirements are met.

The ability to use dietary carbohydrates varies among fish species. Although no true carbohydrate requirement has been demonstrated in fish, their absence in diets increases the catabolism of other compounds, such as protein and lipid, for energy and other biological components usually derived from carbohydrates (Bureau et al., 2002). Furthermore, carbohydrates are often incorporated into diets as a binder for dietary ingredients in pelleted and extruded feeds (NRC, 1993). In general, formulated salmonid diets usually contain less than 15% carbohydrate due to the limited capacity of the fish to break them down.

Although dietary vitamins and minerals are not used as energy sources, they are required for the normal functioning of animals. Minerals are inorganic elements, including calcium, magnesium, sodium, potassium, iron, zinc, copper and selenium, which fish may absorb from both their diet and the external environment (Lovell, 1998). Minerals are used in formation of skeletal structure, electron transfer, regulation of acid-base equilibrium, and osmoregulation. They are also important components of

hormones and enzymes (Lall, 2002). Vitamins are organic compounds, distinct from amino acids, lipids and carbohydrates, which are required in trace amounts from exogenous sources for normal growth, reproduction and health (Lovell, 1998). Vitamins may have coenzyme functions or functions independent of enzymes, such as the requirement of folic acid for normal blood cell formation (Halver, 2002). In mammals, vitamin deficiency leads to characteristic diseases, but in fish, deficiency diseases are less specifically defined (NRC, 1993). A variety of detrimental symptoms may be experienced by fish, dependent on which vitamins are in deficiency, and include reduced growth, hemorrhaging, tissue lesions, atrophy, coordination and equilibrium difficulties, and ocular damage, among other symptoms (Halver, 2002).

In wild populations of fish, changes in dietary composition occur over time as fish grow, develop superior locomotory and sensory skills for prey capture, and as prey availability changes (Keast, 1978; Wootton, 1998). These dietary changes may occur gradually where the replacement of prey items occurs in an overlapping fashion, or abruptly when the shift in prey items is rapid (Keast, 1985). In addition to ontogenic changes in diet composition, diel and seasonal differences in diets also occur (Wootton, 1998). For example, the pumpkinseed sunfish (*Lepomis gibbosus*) feeds on amphipods and chironomid pupae in the early morning, bivalves, chironomid larvae, isopods and zygopteran nymphs in the late morning, and chironomid larvae and bivalves in the afternoon (Keast and Welsh, 1968). These differences reflect changes in activity and subsequent vulnerability of the prey over the course of the day. Seasonally, changes in diet composition occur due to differences in habitat available for foraging, life history patterns of the prey, and feeding activities of the fish themselves (Wootton, 1998). For instance, fish are found to make feeding decisions based on prey abundance and size, therefore the growth of a major prey item such as an insect nymph has the ability to alter the diet composition of a fish (Keast, 1978). Different prey items have different

nutritional value and are variably digestible, consequently the consumption of various prey items may have effects on dietary energy conversion (Higgs et al., 1995).

Although variations in dietary quality have a significant role in fish health and growth, diet quantity must also be considered. Since energy is additionally limited by the quantity of food consumed, a reduction in feed intake will necessitate the allocation of proportionally more dietary energy to metabolism and result in less remaining for growth and reproduction (Brett and Groves, 1979). Several factors may contribute to variations in quantity of food consumption throughout different stages in the life of a fish. In temperate regions, many fish species experience periods of extremely reduced feeding, and even starvation, during the cold months of the winter (Andersson et al., 1985a; Beauchamp, 1990). Spawning migration physiology also signals many fish species to cease feeding (Wootton, 1998). Additionally, external circumstances such as diminished prey availability, prey vulnerability, and increases in predation competition may reduce the ability of fish to maximize energy intake (Beauchamp, 1990). All of these factors could lead to consumption of suboptimal prey and reduced energy conversion (Higgs et al., 1995). During these periods of reduced feeding, fish must rely on their internal stores of energy to survive and subsequently may experience reduced growth rates (Sumpter et al., 1991).

Variations in dietary content and ration (i.e. quality and quantity) play considerable roles in the fitness and health of fish. Dietary nutrients and energy are important considerations when performing nutrition studies as these two factors can greatly influence the assimilation and partitioning of energy in the body. Energy attained from a diet is budgeted and stored in a hierarchical manner. Within this hierarchy, energy is allocated first to basal metabolism and then to successive physiological processes based on the organism's maintenance requirements (Brett and Groves, 1979). Moreover, the principle of allocation states that if an organism can only attain a limited

amount of materials and energy for which two processes are competing, then an increase in allocation to one process must result in a decreased allocation to the other. Since coping with changing environmental conditions continually requires additional energy expenditures, which can result in energetic tradeoffs, it is of great interest to determine where various biological systems lie within the energy hierarchy.

In nature, fish are exposed to a myriad of physical, biological and chemical conditions, which, individually or together, can impose considerable challenges to physiological systems that strive to maintain homeostasis. Anthropogenic activities have further complicated these challenges, especially through the introduction of synthetic chemicals into the environment. Fortunately, organisms possess mechanisms by which they may cope with rapidly changing environmental conditions. The systems that aid in enhancing the potential for optimum performance and survival under varying conditions, are the stress response system, the xenobiotic biotransformation system, and the immune system. From an energetic point of view, the stress response mobilizes stored energy, biotransformation uses energy to convert harmful chemicals into less toxic metabolites, and the immune response requires energy to identify and destroy foreign micro-organisms, bacteria, viruses and other antigens. Being the sole energy source in animals, nutrition is intimately tied to all of these responses as well as all other biological functions.

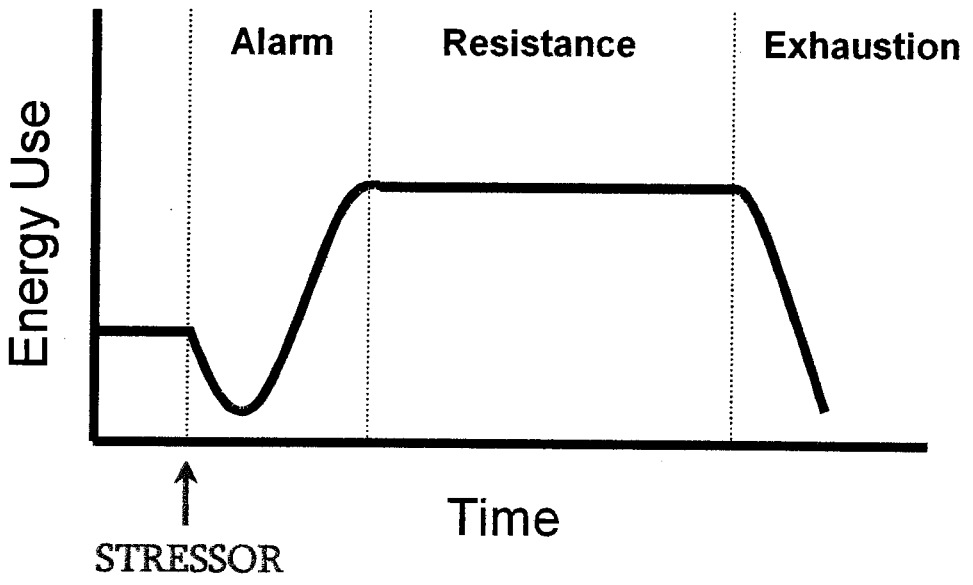
Stress Response

Over the course of an organism's life, the ability to store and release energy will play an integral role in its ability to deal with stressors. Stress is defined as a diversion of metabolic energy away from an animal's normal level of homeostasis (Barton and Schreck, 1987). It may be brought on by a wide variety of intrinsic or extrinsic stressors, which are not typically managed within the normal operating framework of the animal.

These conditions initiate an integrated stress response: a set of compensating and adaptive responses that readjust metabolic processes in order to cope with the effects of a stressor and return the animal to some level of homeostasis (Bonga, 1997). Compensating for the effects of a stressor results in a reallocation of metabolic energy away from investment activities and toward activities that work to restore homeostasis. In fact, the restoration of homeostasis in a stressed individual is associated with an increase in metabolic rate over the non-stressed condition (Beyers et al., 1999).

The physiology of energy reallocation in an animal under stress follows a well-defined pattern consisting of three sequential stages that cumulatively make up the general adaptation syndrome (GAS) of stress (Seyle, 1956): alarm, resistance and exhaustion (Figure 2). Under ordinary conditions, the energy required by an organism to sustain everyday homeostasis is maintained at a relatively steady state, however, once a stressor is introduced, the animal enters a period of alarm. Alarm, which may last from a few seconds to several minutes, occurs when the animal initially perceives the stressor and experiences a state of physiological shock as homeostasis is disrupted. Following this state of shock, the animal's physiological systems are adjusted to compensate for the stressor and to return the body to a state of homeostasis. The next stage of the GAS, resistance, is characterized by the restoration of homeostasis. Resistance is often associated with an increase in metabolic rate, as compared to the pre-stress rate due to the added energetic costs of compensating for the stressor in addition to the energy required to maintain homeostasis. If the organism is unable to adapt to the physiological demands imposed by the stressor, the animal will enter the exhaustion phase of the GAS. During this phase, physiological compensation is no longer possible and ultimately results in the mortality of the animal (Seyle, 1956). Thus, stress only becomes detrimental if the demands of the stressor are too severe or too long lasting and is

Figure 2. Conceptual representation of the three stages of the general adaptation syndrome, alarm, resistance and exhaustion, and theoretical energy use during each stage in order to maintain homeostasis in response to a stressor (modified from Beyers et al., 1999).


Alarm
Resistance
Exhaustion
Energy Use
Time
STRESSOR

otherwise beneficial to the survival of the organism as it works to maintain a level of homeostasis.

The process of homeostatic compensation just described is achieved through a series of events taking place at various levels of biological organization throughout the stress response. These events fall into three phases: primary, secondary and tertiary responses (Pickering, 1981). The primary stress response consists of the activation of the neuroendocrine systems. When a teleost fish perceives a stressor, the brain triggers the activation of two pathways, the hypothalamic-sympathetic-chromaffin cell axis and the hypothalamic-pituitary-interrenal cell (HPI) axis. The activation of these two pathways results in the release of catecholamines and cortisol into the blood and initiates the secondary stress response (Bonga, 1997).

In the hypothalamic-sympathetic-chromaffin cell axis, hypothalamic stimulation of sympathetic nerves results in the release of three catecholamines (dopamine, epinephrine and norepinephrine) from their storage sites in chromaffin cells of the head kidney into the blood stream (Randall and Perry, 1992). Catecholamines rapidly, within a matter of seconds to minutes, affect circulation, respiration, and energy accessibility. Specifically, catecholamines increase energy availability by escalating the rate of gluconeogenesis and glycogenolysis in liver cells, thereby enhancing blood glucose levels (Bonga, 1997). Glycogenolysis, the more prominent of the two processes, is up-regulated as a result of catecholamine binding to ß-adrenoreceptors on the surface of liver cells. This triggers the stimulation of glycogen phosphorylase, which ultimately mediates the conversion of glycogen into glucose (Wright et al., 1989). Gluconeogenesis is considered to play a relatively lesser role in glucose availability, especially while glycogen stores are still able to maintain an elevate level blood glucose during stress (Janssens and Waterman, 1988). In addition to increasing energy availability, oxygen uptake and its rate of delivery are also amplified by catecholamine

binding to ß-adrenoreceptors at various sites (Randall and Perry, 1992). Catecholamines significantly increase surface area available for gas exchange in the gill vasculature through the vasodilation of gill lamellae, as well as increasing the permeability of gill epithelium to oxygen. However, increased gill permeability also indirectly allows uncontrolled ion exchange and, as a result, osmoregulation of the fish is perturbed during this period (Isaia et al., 1978). Further, catecholamines enhance oxygen supply to the tissues by increasing both oxygen delivery rate and the blood's oxygen carrying capacity (Hazon and Balmont, 1998; Soldatav, 1996). The rate of oxygen delivery to tissues is enhanced through catecholamine-mediated increases in heart rate and blood pressure and decreases in peripheral resistance (Mazeaud and Mazeaud, 1981). Oxygen carrying capacity is enhanced through ß-adrenergic activation of the red blood cell Na^+/H^+ antiporter, which leads to an increase in intracellular pH of the erythrocytes and subsequently the affinity and capacity for oxygen binding of hemoglobin. Moreover, catecholamines stimulate the splenic release of red blood cells into circulation (Randall and Perry, 1992). The catecholamine response is generated quickly and is relatively short lived due to the depletion of their stores. However, when catecholamine activity begins to decline, the HPI axis response takes over (Bonga, 1997).

Activation of the HPI axis commences with the release of corticotrophin-releasing hormone (CRH) from the hypothalamus in the brain (Mommsen et al., 1999). CRH subsequently stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into circulation. Upon reaching the head kidney, ACTH initiates the production and release of cortisol from the interrenal cells that lie in close proximity to the chromaffin cells (Mommsen et al., 1999). Cortisol is released into the blood stream and is thought to enter into cells by passive diffusion due to its lipophilic nature or by a low-affinity carrier protein located in the cellular membrane (Porthé-Nibelle and

Lahlou, 1981). Once cortisol has entered a cell, it binds with a glucocorticoid receptor within the cytosol. Acting as a transcription factor, this hormone-receptor complex binds to specific DNA sequences and increases gene expression. The overall effects include an increase in glucose levels, and subsequently its availability, from the bloodstream and restoration of the hydromineral imbalance caused by catecholamines (Bamberger et al., 1996). In order to increase circulating glucose levels, cortisol is postulated to increase the levels and activities of several glycogenolysis enzymes in hepatocytes (Mommsen et al., 1999). Additionally, cortisol may increase gluconeogenic activity in the liver and promote the release of gluconeogenic precursors from protein and lipid stores within the body (Fujiwara et al., 1996). Furthermore, cortisol stimulates gill Na^+/K^+ - ATPase activities and chloride cell proliferation, which in stressed saltwater fish pumps ions out and in stressed freshwater fish brings ions in, thereby restoring the disrupted hydromineral balance caused by catecholamines (Avella et al., 1991).

In summary, catecholamine and cortisol secretion initiate the secondary stress responses, which include an increased cardiac output, an increased respiration rate, a mobilization of energy stores and, in fish, a short-term disruption, and subsequent restoration, of the hydromineral balance. Together, these actions increase the availability and delivery of oxygen and glucose to the physiological systems working to maintain homeostasis.

Finally, tertiary stress responses are manifested at the level of the whole animal. These effects include decreased growth rate, reduced organ condition, decreased immune response, and decreased fitness (Bonga, 1997). Each of the tertiary responses results from increased energy utilization for maintenance and the subsequent reduction in energy allocated to these investment processes.

The fundamental rationale of the stress response is that metabolic energy is directed away from investment activities and toward activities that restore homeostasis.

Consequently, stress in fish has been shown to have measurable costs including decreased growth rates, reduced reproduction capacities, and influences on various other growth indices, all of which demonstrate a reduction in energy availability for investment activities (Goede and Barton, 1990). Due to the increased energy needs to maintain homeostasis, stress may not only decrease growth rates, it can also induce cellular atrophy of various tissues in order to release energy (Pickering and Pottinger, 1989). For example, fish stressed during sexual maturation have been found to have a diminished reproductive capacity, as demonstrated through reduced levels of androgens, testosterone and 11-ketotestosterone (Pickering et al., 1987). Finally, stress has been shown be associated with reduced feed consumption rates in various species, thereby further decreasing energy supplies (Pickering et al., 1982; Pickering and Stewart, 1984).

Several studies focussing on diet restriction and stress in fish have been published, however, variable effects of starvation in teleosts on the most commonly cited indicators of stress, namely plasma cortisol and glucose concentrations, have been reported (Barton et al., 1988; Farbridge and Leatherland, 1992; Vijayan and Moon, 1992; Blom et al., 2000). Basal plasma cortisol levels in food deprived teleosts have been found to increase to levels approximately 2000 % higher than those found in fed fish after 21 days (Blom et al., 2000), decrease to 20 % of the level found in fed fish after 20 days (Barton et al., 1988) and have levels comparable to fed fish following six weeks (Farbridge and Leatherland, 1992) and 4 weeks (Vijayan and Moon, 1992) of food deprivation, thereby suggesting a complicated relationship between food deprivation and its perception as a stressor. However, in all of these studies, both the starved and fed fish were able to mount cortisol stress responses of similar magnitude thereby demonstrating that the cortisol response was not inhibited by food deprivation.

Studies investigating the relationship between starvation and basal plasma glucose in salmonids have also provided varying results. Vijayan and Moon (1992) reported no difference in resting glucose levels after 30 days of food deprivation. However, Barton et al. (1988) and Farbridge and Leatherland (1992) observed significantly depressed glucose levels (84 % and 62 % of control) after 20 days and 6 weeks of starvation, respectively. The effects of food deprivation on glucose levels in response to acute stress have also provided varying results, with starved fish attaining levels similar to fed fish (Vijayan and Moon, 1992) or significantly lower than starved fish (Barton et al., 1988; Farbridge and Leatherland, 1992). Several factors may be responsible for these differences in cortisol and glucose responses such as differences in species, age, season, water temperature, duration of fast and prior rearing condition of the fish.

The effects of dietary quality on stress in fish are much less reported. Barton et al. (1988) investigated the effects of varying lipid concentrations (7, 13 and 19%) in the diet while maintaining 45% protein on the stress response in Chinook salmon. Their results showed no difference in basal glucose or cortisol levels after 21 weeks on each of the diets. Furthermore, the response of cortisol to an acute stressor, was not affected by the amount of lipid in the diet, however, increases in plasma glucose were significantly higher in the fish fed a high lipid diet than in the fish fed a low lipid diet between 3 and 24 hours post-stress. This lead the authors to suggest that feeding regimes may play a role in hyperglycemic stress responses and prior nutrition should be considered when analyzing results of stress experiments.

The initiation of the stress response causes the release of energy stored within the body. Since diet is the sole source of energy in animals it is logical that nutritional state and the integrity of the stress response may be linked. However, few studies have investigated the relationships between nutrition and stress in fish, and unfortunately, these experiments have provided extremely varied results. Although differences may be

due to species, strain, age, experimental conditions and prior life experiences, more research in this area is necessary to fully understand any relationships that may occur between nutritional state and the stress response in fish.

Biotransformation System

Wild organisms are constantly exposed to xenobiotics, including chemicals, of anthropogenic and natural origin. The chemical properties of these compounds often allow them to be readily absorbed but not as easily removed, thus resulting in their accumulation within the body. Fortunately, animals have developed processes to transform these compounds into increasingly water-soluble forms thereby facilitating their excretion from the body by renal or biliary routes (Sipes and Gandolfi, 1986). The majority of biotransformation occurs in the liver, although biotransformation capacity also exists in the brain, lungs, skin, kidney and intestine as well as numerous other tissues (Sipes and Gandolfi, 1986; Zakrzewski, 1997). While the majority of biotransformation reactions result in increasing the water-solubility of a compound along with its subsequent excretion, some of these reactions increase the toxicity of a chemical by a process termed bioactivation (Sipes and Gandolfi, 1986).

Two distinct modes of biotransformation work to detoxify chemicals in animals: namely Phase I and Phase II reactions. Phase I biotransformation of xenobiotics renders hydrophobic chemicals more water soluble. This is achieved by the addition or unmasking of a functional group (e.g. -OH, -NH_2, -SH or –COOH) via hydrolytic, oxidative or reductive reactions (Sipes and Gandolfi, 1986). These reactions are usually carried out by the cytochrome P450 enzyme system, a large family of structurally and functionally related hemoproteins. The broad substrate range of these enzymes includes a variety of endogenous (e.g. fatty acids and steroids) and exogenous (e.g. chemicals) substrates. Therefore, one P450 isoenzyme may accept several different

substrates and conversely, one substrate may be transformed by more than one isoenzyme (Nerbert, 1979).

Cytochrome P450 consists of a coupled enzyme system made up of cytochrome P450 (a heme-containing enzyme) and NADPH-cytochrome P450 reductase, both of which are located within endoplasmic reticulum (Sipes and Gandolfi, 1986). The biotransformation of compounds by cytochrome P450 requires both molecular oxygen and two electrons, derived from energy coupling systems driven by digested food, in order for the reaction to go to completion (Gokøsyr and Förlin, 1992). Through an oxidation reaction, cytochrome P450 binds both the substrate and molecular oxygen, while NADPH donates the necessary electrons through a coupled electron transport system of reductases. The result of these reactions is insertion of one atom of oxygen into the substrate and reduction of the second oxygen atom to form water. This incorporation of oxygen increases the hydrophilicity of the molecule and exposes functional groups (-OH) for Phase II reactions (Sipes and Gandolfi, 1986).

Phase II reactions involve the conjugation of an endogenous moiety to a functional group on the xenobiotic. Most commonly the functional group has been added or exposed during Phase I metabolism (Sipes and Gandolfi, 1986). Since these Phase II biotransformations are biosynthetic, they require energy to drive the reaction. Conjugation with endogenous molecules further increases the water solubility of the compound. Additionally, this promotes the metabolite's movement across cellular membranes including transport out of the cells in which they were produced and transport across hepatic, renal and intestinal membranes for excretion. Therefore, linking xenobiotics with endogenous moieties allows otherwise harmful compounds to utilize ATP-dependent transport systems designed for endogenous molecules. Phase II reactions are diverse and include glucuronidations, sulfations, methylations, acetylations, amino acid conjugations and glutathione conjugations.

The majority of teleost research concerning diet quantity and detoxification ability has focused on the relationship with starvation. The effects of depriving teleosts of food for periods between 15 days and 12 weeks on their biotransformation enzyme abilities have produced varying results (Buhler and Rasmusson, 1968; Andersson et al., 1985a; Viganò et al., 1993; Wall and Crivello, 1999; Blom et al., 2000). After two weeks of starvation, juvenile rainbow trout biotransformation enzymes including, hepatic 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione *S*-transferase (GST) and benzo(a)pyrene hydroxylase (AHH) activities were maintained while UDP glucuronyltransferase (UDP-GT), and glutathione reductase (GR) activities were significantly decreased and cytochrome P450 activity was increased when compared to fish fed *ad libitum* (Viganò et al., 1993). In other studies with rainbow trout (Huuskonen et al., 1995) and winter flounder (*Pleuronectus americanus*) (Wall and Crivello, 1999), EROD activity was significantly reduced after 2 weeks of starvation. The effects of longer-term starvation are also inconsistent. Buhler and Rasmusson (1968) reported no change in hepatic cytochrome P450 activity of rainbow trout after 8 weeks of starvation, while Blom et al. (2000) reported significantly lower EROD, GST and GR activities after just 7 weeks of starvation. These findings have lead researchers to suggest that some forms of biotransformation enzymes may be decreased by starvation while other, enzymes may maintain, or even increase activity levels (Andersson et al., 1985a; Blom et al., 2000). Moreover, Andersson et al. (1985a) reported that while activity levels of 7-ethoxycoumarin-*O*-deethylase (ECOD), EROD, UDP-GT and AHH were unaffected by 6 weeks of starvation, their activity was significantly decreased after 12 weeks. This reduction in activity by week 12 may illustrate a manner of reducing metabolic costs during starvation. However, more research is needed in both of these areas to fully understand the relationship between food restriction and detoxification enzyme induction and reductions.

Although several mammalian studies have investigated the effects of dietary quantity and detoxification abilities (Campbell and Hayes, 1974; Sachan, 1975; Shavila et al., 1994), information on the effects of dietary composition on fish biotransformation enzymes is extremely limited. A small number of experiments using fish have compared the effects of commercial diets on biotransformation enzymes but these have not systematically altered dietary composition. Nevertheless, consumption by fish of these different commercial diets, which varied in all aspects of proximate composition, resulted in the reduction and induction of assorted biotransformation enzymes depending on which diet was received (Viganò et al., 1993). Although differences in enzyme activity were found between diets in this study, it is impossible to discern the precise of the changes as diets were not modified one step at a time.

In more regimented dietary studies, the effects of lipid type and concentration have been investigated. Increasing the lipid concentration (0.4, 7, 14, 21%) of diets fed to juvenile red drum (*Sciaenops ocellatus*) resulted in a significant (3-fold) increase in hepatic EROD activity between the diets with the lowest and highest lipid content (Craig et al., 1999). Moreover, varying the nature of the lipid, polyunsaturated v. saturated and type of fatty acid, has been shown to variably increase basal EROD and GST activities in the channel catfish (*Ictalurus punctatus*) (Ankley et al., 1989). Therefore, dietary content can affect detoxification enzyme activity in fish, although the extent of the relationship remains unclear.

Objectives

The purpose of this investigation was to determine the effects of diet treatment on basal levels of stress parameters and biotransformation enzyme activities in the rainbow trout, *Oncorhynchus mykiss*. To accomplish this, groups of juvenile rainbow trout were fed isoenergetic diets varying in lipid and protein content, at two different ration levels.

The specific research questions were: 1) Can dietary ration and/or quality affect basal stress parameter levels or act as stressors in rainbow trout? 2) Does dietary quality and/or quantity affect basal biotransformation enzyme activity? 3) If biotransformation enzyme activity is affected, is there a threshold protein or energy value below which detoxification is not sustained or is there a graded reduction in detoxification activity? 4) Is the induction of detoxification systems maintained under limited dietary conditions and composition?

Materials and Methods

Diets

Three isoenergetic diets varying in protein and lipid content, but with constant digestible carbohydrate content (13%), were formulated and prepared at the West Vancouver Laboratory of the Department of Fisheries and Oceans. The diets were formulated so that diet 1 would contain high protein and low lipid i.e. 55% and 8% respectively, diet 2 would contain intermediate protein and lipid levels i.e. 45% and 14% respectively and diet 3 would contain a low amount of protein and high lipid i.e. 35% and 20% respectively. Diet ingredients and composition are shown in Table 1. Tables 2 and 3 provide the compositions of the vitamin and mineral supplements, respectively.

All dry ingredients were obtained from ICN Biomedicals Inc. (Coast Mesa, CA) and were added one at a time, after which the entire mixture was blended with either all (diet 1) or a portion of the required anchovy oil (diets 2 and 3; all diets adjusted to 8% lipid before pelleting), for 15 minutes in a Hobart Commercial Mixer (Hobart Manufacturing Company, Troy, OH). Subsequently, each diet was steam pelleted using a Californica model CL type 2 laboratory pellet mill (San Francisco, CA), that was equipped with a 2.38 mm die. The pellets were dried in a custom-made vertical pellet cooler, immediately after formation, for 5-10 minutes. After drying, anchovy oil was applied to the surface of the diet 2 and 3 pellets and then stored overnight to allow complete lipid absorption. The finished diets were stored in an air tight containers at 4^0 C until use. All diets were later analyzed for percent moisture, ash, lipid, protein and energy content.

Table 1. Ingredient composition of the experimental diets (HP:LL – High protein/Low lipid; IP:IL – Intermediate protein/Intermediate lipid; LP:HL – Low protein/High lipid). All diets were formulated to contain equivalent digestible energy content (approx. 17 MJ/kg) and carbohydrate content (13.0%), but varying proportions of protein and lipid.

Ingredient	Diet (g/kg air dry basis)		
	1 (HP:LL)	2 (IP:IL)	3 (LP:HL)
Austral fish meal	587.6	485.9	381.9
Blood flour (spray-dried)	51.2	42.3	33.3
Squid meal	51.1	42.3	33.3
Krill hydrolysate	21.8	18.0	14.2
Wheat gluten meal	50.7	41.9	32.9
Pregelatinized wheat starch	80.1	81.2	82.3
Raw wheat strarch	88.4	90.2	91.9
Vitamin supplement	18.9	19.2	19.3
Mineral supplement	18.9	19.2	19.3
Anchovy oil (stabilized)	2.8	18.3	29.3
Soybean lecithin	9.4	9.5	9.6
Choline chloride (60%)	4.7	4.8	4.8
Vitamin C, monophosphate (35%)	2.7	2.7	2.8
Permapell (lignin sulphonate binder)	9.4	9.5	9.6
DL-methionine	2.1	1.8	1.4
α - cellulose	-	59.9	121.3
Anchovy paste (spray)	0	5.7	12.7

Table 2. Vitamin composition of the experimental diets.

Vitamin	g/kg diet
D-calcium pantothenate	0.1684
Pyridoxine HCl	0.0493
Riboflavin	0.0678
Folic acid	0.0188
Thiamine mononitrate	0.056
Biotin	0.075
Vitamin B_{12}	0.090
Vitamin K (as MSBC)	0.0546
Vitamin E	0.600
Vitamin D_3	0.0048
Vitamin A	0.010
Inositol	0.400
Niacin	0.3046
BHT	0.022
Whetstar 3 (raw wheat starch)	18.0787

Table 3. Mineral composition of the experimental diets (HP:LL – High protein/Low lipid; IP:IL – Intermediate protein/Intermediate lipid; LP:HL – Low protein/High lipid).

Mineral	Diet (mg/kg dry weight basis)		
	1 (HP:LL)	2 (IP:IL)	3 (LP:HL)
$MnSO_4 \cdot H_2O$	231.0	231.0	231.0
$ZnSO_4 \cdot 7H_2O$	240.0	315.0	391.0
$CoCl_2 \cdot 6H_2O$	12.1	12.1	12.1
$CuSO_4 \cdot 5H_2O$	19.6	19.6	19.6
$FeSO_4 \cdot 7H_20$	249.0	249.0	249.0
KIO_3	8.4	8.4	8.4
KI	6.5	6.5	6.5
NaF	11.0	11.0	11.0
Na_2SeO_3	0.44	0.44	0.44
$MgSO_4 \cdot 7H_2O$	4053.0	4053.0	4053.0
K_2SO_4	-	1458.0	3116.0
K_2CO_3	-	1156.0	2471.0
α - cellulose	5169.0	12481.0	9432.0

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) from the same cohort were obtained from Sun Valley Trout Farms in Mission, British Columbia. At the start of the experimental period, the fish weighed 48.70 ± 10.21 grams and had fork lengths of 130.73 ± 10.21 mm (mean ± SD). Twenty-seven fish were arbitrarily assigned into each of twelve 160L fibreglass tanks. The tanks were supplied with fresh, flowing, dechlorinated municipal water at seasonal temperatures (14–15 ^{0}C), pH 6.4 and hardness of 5.2 to 6.0 mg/L $CaCO_3$. The fish were housed in a 12 hour light:dark cycle. All fish were fed *ad libum* daily with 1.5 mm EWOS Canada Trout feed (Surrey, BC) prior to the start of the experiment. All excess pellets were removed from the tank by suction after each feeding to acclimate the fish to this aspect of the subsequent experimental procedures.

Feeding

Each of the three diets were fed at two ration levels: satiation (Ration 1) and half satiation (Ration 2). Thus, six dietary treatments were used during the experiment: diet 1 to satiation, diet 2 to satiation, diet 3 to satiation, diet 1 at half-satiation, diet 2 at half-satiation, diet 3 at half-satiation. These six treatments were subsequently randomly assigned, in duplicate, to the twelve tanks of fish.

To establish the half ration feeding rate, the full ration fish were fed by hand slowly until they no longer consumed the pellets as they sunk, fed off the bottom or regurgitated ingested pellets. The pellets remaining in the bottom of the tank were then suctioned out and counted in order to determine the weight of the wasted pellets. This value was then multiplied by the mean air-weight of the pellets and subtracted from the total weight of pellets used at each feeding occasion to determine daily weight of ingested feed per fish.

This information was then used to determine the amount of feed (in grams) to be fed to the half ration fish.

During the course of the experiment, the fish were kept on a rigorous feeding regime that consisted of being fed twice daily during the weekdays and once each day of the weekend. Feeding took place beginning at 09:00 and at 15:00 during the week and mid-day (beginning at 11:00) on the weekends.

To better estimate the final feeding rations, the average amount of food eaten per fish on a daily basis as % body mass was calculated at the end of the experiment. To calculate this, the standard growth rate (SGR), a measure of growth as a percent body mass gained per day, was first calculated. The SGR value was then used to estimate of the weight of fish on each day of the experiment. To determine the amount of food eaten per day as percent body weight, the average amount of food eaten/day/fish (g) was divided by the estimated weight of individual fish for each day of the experiment. This was then averaged over the 42 days to estimate the average amount of feed eaten per day as percent body weight.

Tissue and Blood Sampling

To establish the effects of the dietary treatments on various growth parameters, basal blood and stress parameters and enzyme activity, six fish were taken from each tank on days 0, 21 and 42 of the experiment. This was carried out by corralling the fish in one end of the tank and arbitrarily selecting individuals for analysis of the each of the endpoints. Fish were quickly netted out of the tank and immediately euthanized in 0.5 g/l MS-222 (3-aminobenzoic acid ethyl ester methane sulphonate; Argent Chemical Laboratory's, Redmond, WA) buffered with 1.0 g/l sodium bicarbonate (BDH Inc., Toronto) followed by a sharp blow to the head. All sampling was completed before the fish were fed in the morning.

Fork length and total wet weight of each fish was measured immediately after they were euthanized. These values were then used to determine growth and to calculate the condition factor (K = weight/(length)^3)) of each fish. Whole livers were quickly removed, blotted dry and weighed, from which the hepatosomatic indices (HSI) for each of the fish were calculated (HSI = (liver weight/body weight) x 100). Livers were kept on ice for preparation of hepatic tissue for enzyme analyses.

Whole blood samples were collected from the caudal vasculature of each fish immediately after euthanization. Blood was collected in 1.1 mm heparinized capillary tubes from each fish by severing the caudal peduncle. Samples were kept on ice until being spun at 13,460 *x g* in an IEC Micro-MB Centrifuge (International Equipment Company, Needham Heights, MA) for 3 - 5 min. Percent white blood cells (leucocrit) and red blood cells (hematocrit) were determined manually using digital calipers and a micro-hematocrit capillary tube reader, respectively. The tubes were then cut so that only the segment of the tubes containing plasma were sealed and stored. The plasma was stored at –86°C until further analysis.

In the second part of the experiment, the effects of diet and ration on the activities of biotransformation enzymes after induction was examined. The remaining animals were given an intraperitoneal injection of 50 mg/kg ß-napthoflavone (ß-NF; Sigma Chemical Co., St. Louis, MO), a potent inducer of some biotransformation enzymes, dissolved in peanut oil and injected (0.2 mL/100 g) on day 49. Peanut oil has previously been shown to have no effects on activities of the biotransformation enzymes examined (Zhang et al., 1990) and was not found to affect any parameters in vehicle control trials. Maximal EROD induction has been reported to occur 3 days following injection at this dose (Andersson, et. al., 1985b), therefore injected fish were sampled on day 52 to determine if enzyme induction occurred in fish and to determine if ß-NF was perceived as a stressor. The day 42 values of the various parameters were used as the controls for this

experiment. Sampling procedures for this experiment were identical to those for the first experiment.

Feed Proximate Analysis

Moisture

The moisture content of each of the diets was determined through removal of water and other volatiles by means of heating the samples under partial vacuum. Approximately 2 g of ground feed was placed into a previously labelled and weighed crucible, and an exact weight of the food was determined. The crucibles were placed into an Isotemp Oven (Fischer-Scientific, Pittsburgh, PA) at 100^{0}C where they were left undisturbed for 16 hours. The crucibles then were removed, placed into a desiccator and reweighed when cool. The percentage moisture was calculated by subtracting lost weight.

Ash and Organic Matter

Oxidization of all the organic matter in the samples by incineration allowed for determination of ash (or mineral content) in the feed samples. The cooled samples from the moisture analysis were placed into a muffle furnace (Isotemp Muffle Furnace, Fischer Scientific, Pittsburgh, PA) at 600^{0}C for 2 hours. The crucibles were then placed into a dessicator, cooled, reweighed and percent ash was determined based on lost weight. The percent organic matter was calculated by subtracting percent ash from 100 %.

Lipid

Crude lipid content was analyzed according to the methods described by Bligh and Dyer (1959). In this regard, 2 g of feed was combined with 10 mL of chloroform (>99.8%; Anachemia, Richmond, BC), 20 mL of methanol (>99.8%; Anachemia, Richmond, BC) and 8 mL of distilled H_2O to create a homogenous mixture, which

extracts the lipids from the samples. The samples were homogenized using a Sorvall Omni-mixer (Ivan Sorvall Inc., Norwalk, CT) for approximately 120 seconds followed by the addition of another 10 mL of chloroform in order to separate the phases. The samples were then blended for 30 seconds and after the addition of 8 mL of distilled water, were blended for another 30 seconds. The mixtures were decanted into a vacuumed Buchner funnel lined with distilled water dampened filter paper (Whatman No.1) and was kept under vacuum until the filter paper dried. The filtrate was transferred into a 50 ml graduated cylinder, covered with foil and allowed to settle into a biphasic solution for 1 hour. The volume of the lower, chloroform layer was recorded while the upper, methanol layer, was suctioned off. Five milliliters of the chloroform layer was pipetted into a preweighed foil dish and weighed. The dish was then heated in a fume hood to facilitate the evaporation of the chloroform, leaving only a thin lipid layer in the dish. The dish was transferred to a drying oven (Isotemp Oven, Fischer Scientific, Pittsburgh, PA) set to 100^0C for one hour to remove any residual chloroform. The dish was then reweighed and the weight loss was used to determine the amount of lipid in the original feed sample.

Protein

Protein content was assessed according to the Kjeldahl method (Bradstreet, 1965), which determines protein based on the total nitrogen content of the sample. Approximately 0.2 g of feed was transferred onto 6 cm^2 pieces of nitrogen-free weigh paper, folded and placed into labelled digesting tubes. Six boiling stones (Anachemia Science, Richmond, BC), which prevent rapid bubbling, 1 crushed Kjeltab (Pro-pac Tablets CT-37; 3.5 g K_2SO_4, 0.4 g $COSO_4$) (Alfie Packers Inc., Omaha, NE), to act as a reaction catalyst and to provide indicator colouration, and 10 mL of concentrated sulphuric acid (Anachemia Science, Richmond, BC) for feed digestion were added to each tube. Blanks were prepared by placing an empty, folded sheet of weigh paper into

the tubes. Five mL of hydrogen peroxide (Anachemia Science, Richmond, BC) was added to each tube, 1 mL at a time and shaking between, to aid in the organic matter digestion and to minimize foaming. The tubes were placed into a preheated (410 to 430^{0}C) BD-20 Block Digestor (Technicon Industrial Systems, Tarrytown, NY) until the samples were digested (i.e. the solution had turned black), approximately 35 minutes. After the tubes were removed and cooled, 40 mL of distilled water was added, the solutions were mixed by vortexing and cooled again. This step was repeated with the addition of 30 mL of distilled water. All the tubes were then filled with distilled water to the 75 mL mark and mixed thoroughly by inversion. Samples were read spectrophotometrically at 660 nm using a Technicon 2 AutoAnalyzer (Technicon Industrial Systems, Tarrytown, NY). A nitrogen standard curve was prepared by adding ammonium sulfate [$(NH_4)_2SO_4$] at various concentrations to tubes prepared without feed sample.

Energy

Feed energy (cal/g) was determined by bomb calorimetry using an IKA Calorimeter System C5003 (IKA Werke Inc, Wilmington, NC). The integrity of the bomb was tested using an IKA Werke Standard tablet (benzoic acid, palleted; IKA Werke Inc, Wilmington, NC) before readings of feed were taken. To determine the energy of the feed samples, approximately 0.5 g of sample was ground to a fine powder and weighed into the bomb crucibles. A cotton twist (batch 050; IKA Werke Inc Wilmington, NC) was used as a wick to ignite the samples. The samples were then placed into the calorimeter system and burned. Energy was electronically calculated by the Calorimeter System.

Assays

All assays were completed once and then results were verified by randomly selecting a subset of samples and repeating the assay.

Microsomal Preparations

Liver microsomes were prepared according to the method of Kennedy (1994). Liver tissue was minced and homogenized as 25 g tissue/ml in ice cold 0.2M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) (Merck KgaA, Darmstadt, Germany) buffer: 0.15M KCl (BDH Inc, Toronto), 5mM ethylenediamine-tetraacetic acid (EDTA; Fischer Scientific, Nepean, ON); pH 7.4). The homogenate was centrifuged in precooled test tubes for 20 min at 10,000 *x g* in a Model L7 Beckman ultracentrifuge (Beckman Instruments Inc., Mississauga, ON) at 4^0C. After discarding the lipid layer, the supernatant was decanted into clean, cooled test tubes and recentrifuged for 65 min at 100,000 *x g* at 4 ^{0}C. Once again, the lipid layer was discarded and the supernatant transferred into ice cooled plastic test tubes and capped. The microsomal pellet was resuspended in 1.5 mL resuspension buffer (0.15 M KCl, 100mM Tris-HCl (BDH Inc., Toronto, ON); pH 7.4, 20% glycerol (BDH Inc., Toronto, ON) v/v) in a cooled 1.5 mL Eppendorf ultracentrifuge tube. Both the supernatant and microsomal fractions were stored at -86^0C until further analysis.

Protein

Microsomal and cytosolic protein content of each liver was determined by the methods of Bradford (1976). This assay is based on the absorbance maximum of the acidic solution (Coomassie Brilliant Blue G-250) shifting to 595 nm as binding to protein occurs. The differential change in absorbance is related to the amount of protein contained in the sample. A standard curve was prepared with standards made using

bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in a range of concentrations from 50 – 200 μg/mL. Cytosolic and microsomal samples were diluted 1:100 in dH_20 prior to the assay. A microplate assay was carried out in which 200 μL of Bio-rad protein dye solution (Bio-rad, Hercules, CA) and 10 μL of standard/sample were pipetted into the wells of a Falcon 96 well polyvinyl chloride microplate and mixed. The plate was incubated at room temperature for 5 min and read on a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 595 nm. Absorbance values obtained from the spectrophotometer were used to determine protein concentrations based on the values obtained for the standard curve. Blanks contained distilled water in place of liver fraction samples.

Glutathione S-transferase (GST)

Glutathione-S-transferase (GST) (a Phase II enzyme) activity in the liver cytosolic fraction was determined by the spectrophotometric methods described in Habig et al. (1974). GST is a ubiquitous enzyme that protects cells from toxicants by conjugating them to glutathione, thereby neutralizing electrophilic sites and rendering the molecule more water-soluble (Sipes and Gandolfi, 1986). This assay utilizes an increase in absorbance at 340 nm which accompanies the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione. The activity of GST is directly proportional to the rate of absorbance increase. The reaction mixture consisted of 1.95 mL of 50 mM HEPES, pH 7.5, 40 μL of 50 mM CDNB (Sigma Chemical Co., St. Louis, MO) in ethanol and 25 μL of cytosolic supernatant. A baseline reading of the mixture was taken over 1 minute at 340 nm on a Beckman DU 650 Spectrophotometer (Beckman Instruments Inc., Mississauga, ON). The reaction was started by the addition of 25 μL of 50 mM reduced glutathione (GSH; Sigma Chemical Co., St. Louis, MO) in

HEPES buffer. The change in absorbance was followed for 1 min at 340 nm and compared to readings for controls, which were completed without supernatant.

Ethoxyresorufin O-deethylase (EROD)

The activity of ethoxyresorufin O-deethylase (EROD), a Phase I enzyme, was determined as per the methods of Burke and Mayer (1974). This method takes advantage of the intense fluorescence of resorufin, which results from the O-deethylation of 7-ethoxyresorufin, thus directly measuring enzyme activity. Fluorescence was determined using a Perkin Elmer fluorescence spectrophotometer (LS 50; Perkin Elmer Inc., Wellesley, MA) linked to an IBM 50 Z computer. Samples were prepared by adding 50 µl of microsomal suspension to 1100µl of 0.1 M HEPES buffer solution pH 7.8, 10 µl of 2.57M $MgSO_4$ (BDH Inc., Toronto, ON), 50 µl of 40 mg/ml BSA (Sigma Chemical Co, St. Louis, MO) and 30 µl of 0.5mM NADPH (Sigma Chemical Co., St. Louis, MO). The solutions were incubated for 5 minutes at 25^0C followed by the addition of 10 µl of 0.19mM 7-ethoxyresorufin (Sigma Chemical Co., St. Louis, MO) in dimethyl sulfoxide (DMSO; Fischer Scientific, Nepean, ON). The tubes were allowed to incubate for exactly 2 minutes at which time the reaction was stopped by the addition of 2.5 mL of methanol. Controls were prepared in a similar fashion to the samples, however the reactions were prevented by the addition of methanol prior to the 7-ethoxyresorufin. Both samples and controls were centrifuged at 25,000 *x g* in an IEC Centra MP4R (IEC, Needham Heights, MA) centrifuge in order to remove any proteins precipitated by the methanol. The supernatants were transferred into quartz cuvettes and fluorescence was determined at an excitation fluorescence of 530 nm and an emission fluorescence of 585 nm. A standard curve was prepared by following the same set of methods, however, the addition of 10 µl of resorufin (Sigma Chemical Co., St. Louis, MO) standards (in DMSO)

between the concentrations of 0.001 and 0.5 μg/L replaced 7-ethyoxyresorufin in reaction mixtures which had been eradicated by the prior addition of 2.5 mL of methanol.

Blood Assays

Similar to the enzyme assays, all blood assays were completed once and then repeated using a selected subsample and in order to confirm the results.

Glucose

Blood plasma glucose was determined using a Sigma Diagnostics Infinity Glucose Reagent Kit (Sigma Chemical Co., St. Louis, MO). The test is based on the catalyzed formation of NADH from glucose in the plasma sample, where the amount of NADH is directly proportional to the concentration of glucose in the plasma sample and may be spectrophotometrically measured. Plasma samples were diluted 1:100 by the provided kit reagent in a 96-well Falcon microplate and allowed to incubate for 3 min at 37^0C. The sample absorbances were read at 340 nm on a Spectra Max 340 microplate reader. Control wells were prepared by replacing plasma with double distilled water (ddH_2O). A standard curve was prepared using Sigma Diagnostics Glucose/Urea Nitrogen Combined Standards (100, 300 and 800 mg/dL; Sigma Chemical Co, St. Louis, MO) and blanks were prepared using ddH_2O.

Lactate

Blood plasma lactate was determined using Sigma Diagnostics kits (Sigma Chemical Co., St. Louis, MO). Plasma lactate concentration is based on the degree of the dye colour change due to the peroxidation of the enzymatically produced, lactate decomposition product, hydrogen peroxide. The increase in absorbance produced by this change is directly proportional to the amount of lactate in the sample. Lactate

reagent solutions and standards (20, 80 and 120 mg/dL) were prepared from the kit provided materials. Plasma was diluted 1:100 with the reagent solution in a 96-well Falcon microplate. The plate was incubated at room temperature for 5 to 10 min. The absorbance of the solution in the wells was read at 540 in a Spectra Max 340 microplate reader. Control wells were prepared using ddH_2O instead of plasma.

Osmolality

Plasma osmolality is an expression of the total concentration of solutes in a sample, including sodium, potassium, chloride, bicarbonate and glucose. The method used to assess osmolality in this study utilized a thermocouple hydrometer to measure the dew point of the sample, thereby determining the concentration of solutes in the sample. Osmolality was measured in duplicate by pipetting 10 μL of sample onto solute-free paper and was analyzed using a Wescor Vapour Pressure Osmometer, Model 5500 (Wescor, Logan, UT). The osmometer was calibrated using Wescor 290 and 1000 mOsm standards (Logan, UT).

Cortisol

Plasma cortisol was determined using an enzyme-linked immunosorbent assay kit from Neogen Cortisol ELISA kit (Product # 402710; Neogen Corporation, Lansing, MI). Cortisol analysis is based on the competition of the cortisol in the plasma sample and with an enzyme conjugate for binding sites on the ELISA plate. The colour change in this assay is due to the reagent binding to the enzyme conjugate thus, the greater the cortisol concentration in the samples, the lower the absorbance, because of the lower amount of enzyme conjugate bound. Plasma was diluted 1:80 with prepared extraction buffer. The sample/standard (50 μl) was added to each well of the provided microplate after which 50 μl of diluted enzyme conjugate was added. The plate was mixed and allowed to incubate at room temperature for one hour after which time the contents of

the wells were discarded and the plate was washed three times with diluted wash buffer. The enzyme substrate (150 μl), 3,3',5,5' tetramethylbenzidine (TMB) plus hydrogen peroxide was added to each of the wells containing standards or samples. Control wells were prepared by the addition of substrate into wells, which contained neither standard nor sample. The plate was incubated at room temperature for 30 min. The absorbance of each well was read spectrophotometrically by a Spectra Max 340 microplate reader at 650 nm. A standard curve was prepared by diluting the cortisol standard provided in the kit.

Statistical Analysis

The data from the duplicate tanks for each dietary treatment was pooled for every parameter evaluated. The effects of the dietary treatments on each of the parameters tested during the experiment were assessed by multiple comparisons using a split plot in time analysis (Schwarz, 2000). This test allowed for the simultaneous 3-way ANOVA comparisons of treatments within a single sampling time period and also for comparisons between treatments across sampling periods. A Bonferroni correction was applied to each comparison in order to adjust the alpha level to account for the number of comparisons being completed and in doing so, lowers the chance of Type 1 error.

The analysis was completed using SAS 8.0 (SAS Institute Inc., Cary, NC). Values in each case are presented as means ± S.E. Statistical significance was established at $P < 0.05$.

RESULTS

Proximate Analysis

Results of the proximate analysis for the three diets are given in Table 4. The determined protein and lipid contents of the test diets on a dry weight basis were: diet 1 (56 % and 8 %), diet 2 (45 % and 14 %) and diet 3 (36 % and 18 %), which fell within 1 % of their intended values. The measured gross energy content of diets 1, 2 and 3 on a dry weight basis were 18.27, 19.56 and 20.73 MJ/kg, respectively, and were not expected to significantly affect the energy partitioning in the fish. The ash and organic matter contents of the diets varied within only 4 % of each other and are not considered to differ significantly.

Feeding Rates and Morphometric Parameters

Due to a fallen outflow standpipe in one of the diet 2, ration 2 replicate tanks on day 41 all fish in this tank were lost.

Fish fed at full ration consumed approximately 3.1 % body weight (bw)/day while the half ration fish consumed 1.2 % bw/day of each diet. Although the half ration fish consumed slightly less than one half of the satiated fish, this is not considered to be detrimental to the experiment design as the purpose was to limited energy intake, which was accomplished.

Morphometric parameters for the fish given each of the six dietary regimes over the 6 weeks are provided in Table 5. Fish in all treatment groups gained a significant amount of weight over the course of the experiment. Weight increases over initial values were significantly higher in fish fed full rations (197 to 224 %) compared to those fed restricted rations (99 to 142 %) for each of the three diets by day 42. In addition, fish fed diet 3 gained more weight than fish fed either diet 1 or 2 by day 42 regardless of ration.

Hepatosomatic index values increased 37 to 61 % between days 0 and 21 in all groups. By day 42, HSI (hepatosomatic index) values had reduced to intermediate levels in some treatment groups, however they remained 16 to 38 % higher than day 0 values. All fish attained an increase in condition factor by day 42 regardless of dietary regime (10 to 20 %). This increase was significant in all groups except those fed diet 2, ration 2.

Basal blood parameters

The values for blood hematocrit, leucocrit and plasma osmolality are shown in Figure 3. Hematocrit levels were generally unaffected over the course of the experiment with the exception of a decrease in hematocrit between days 0 and 21 in fish fed diet 3 to satiation and diet 1 on the restricted basis. Leucocrit levels were variable throughout the experiment, however, very few significant differences were observed between treatments. Fish fed diet 3 to satiation and restriction rations of diet 1 encountered a significant increase in leucocrit between days 0 and 21, while on day 42, a significant difference in leucocrit levels was observed between diet 3 at the two ration levels. Blood osmolality was stable over the 42 days as no differences in levels were observed with respect to diet, ration or time.

Table 4. Proximate composition of the experimental diets.

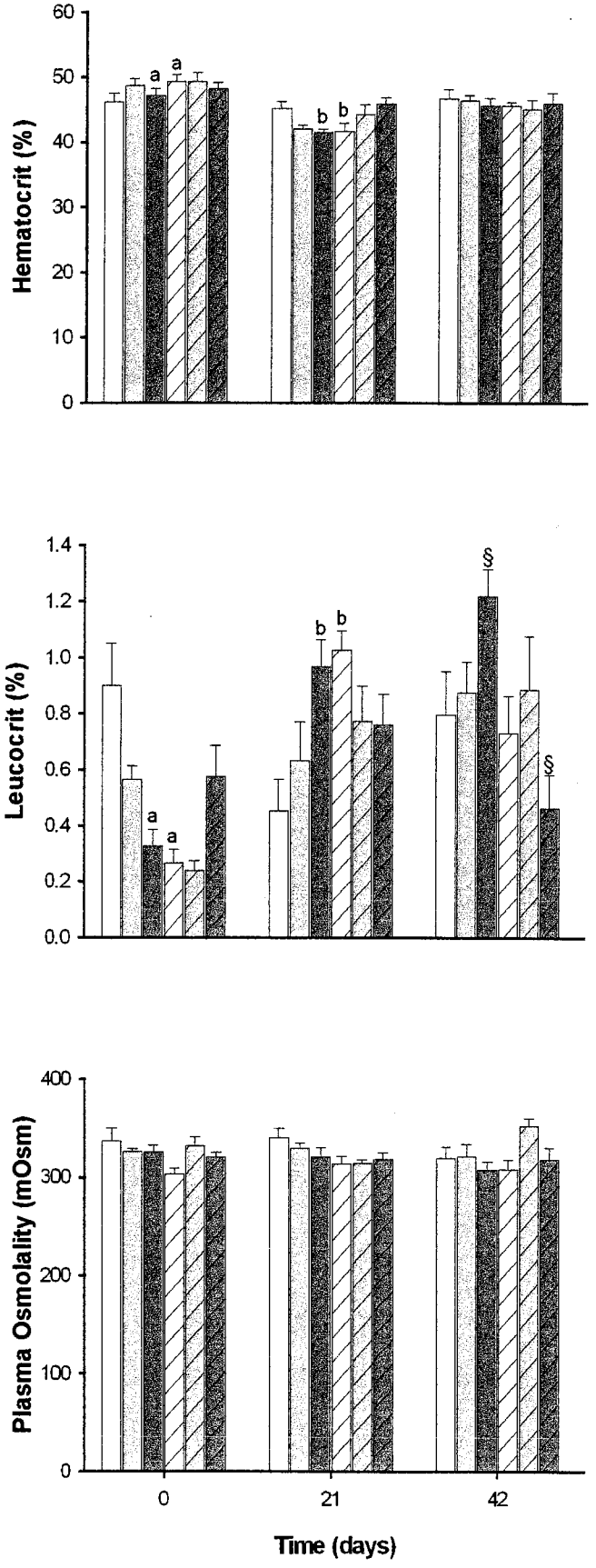
Diet	% Dry Matter	% Ash[1]	% Organic Matter[1]	% Lipid[1]	% Protein[1]	Energy[2]
1	88.97	12.30	87.70	8.12	55.54	18.27
2	91.45	10.17	89.83	13.71	44.68	19.56
3	91.23	8.38	91.62	18.35	36.01	20.73

[1] Expressed as g kg^{-1} of dietary dry matter.
[2] Expressed as MJ kg^{-1} of dietary dry matter.

Table 5. Morphometric parameters of rainbow trout fed the various dietary regimes over 42 days. Values represent pooled data means ± S.E. from duplicate tanks. Different letters represent statistical differences in means within a dietary regime over time. Similar symbols indicate statistical differences in ration means of a diet at a single time point. All statistical significances tested at $P < 0.05$. $n = 12$ in all groups except Diet 2, Ration 2 on day 42 where $n = 6$.

		Dietary Regime					
	Day	Diet 1, Ration 1	Diet 2, Ration 1	Diet 3, Ration 1	Diet 1, Ration 2	Diet 2, Ration 2	Diet 3, Ration 2
Body Weight (g)	0	42.20 ± 1.35^{a}	45.55 ± 2.74^{a}	47.91 ± 1.55^{a}	42.78 ± 1.96^{a}	50.02 ± 1.87^{a}	46.19 ± 2.06^{a}
	21	79.10 ± 2.40^{b}	86.03 ± 6.12^{b*}	90.09 ± 5.17^{b§}	65.89 ± 1.85^{a}	68.99 ± 3.25^{a*}	64.69 ± 2.28^{a§}
	42	132.95 ± 8.34$^{c¥}$	135.15 ± 9.29^{c*}	155.28 ± 10.32^{c§}	99.81 ± 2.32$^{b¥}$	99.77 ± 4.77^{b*}	111.80 ± 5.88^{b§}
HSI (%)	0	1.14 ± 0.08^{a}	1.26 ± 0.03^{a}	1.18 ± 0.03^{a}	1.09 ± 0.04^{a}	1.06 ± 0.05^{a}	1.08 ± 0.05^{a}
	21	1.84 ± 0.07$^{b¥}$	1.73 ± 0.09^{b}	1.84 ± 0.06^{b}	1.52 ± 0.03$^{b¥}$	1.44 ± 0.05^{b}	1.70 ± 0.07^{b}
	42	1.56 ± 0.06^{b}	1.46 ± 0.04	1.57 ± 0.08^{b}	1.32 ± 0.06	1.39 ± 0.06	1.49 ± 0.05^{b}
Condition Factor	0	1.26 ± 0.017^{a}	1.19 ± 0.022^{a}	1.29 ± 0.022^{a}	1.19 ± 0.021^{a}	1.24 ± 0.026	1.21 ± 0.030^{a}
	21	1.36 ± 0.023	1.43 ± 0.022^{b*}	1.45 ± 0.054^{b}	1.35 ± 0.036^{b}	1.28 ± 0.022*	1.27 ± 0.026^{a}
	42	1.40 ± 0.022^{b}	1.43 ± 0.021^{b}	1.53 ± 0.030^{b}	1.38 ± 0.021^{b}	1.37 ± 0.022	1.41 ± 0.023^{b}

Figure 3. Blood parameters (hematocrit, leucocrit and osmolality) of rainbow trout fed the various dietary regimes on days 0, 21 and 42. Values represent pooled data means ± S.E. from duplicate tanks. (□) Diet 1, Ration 1; (▨) Diet 2, Ration 1; (▩) Diet 3, Ration 1; (▨) Diet 1, Ration 2; (▨) Diet 2, Ration 2; (▩) Diet 3, Ration 2. Different letters represent statistical differences in means within a dietary regime over time. Similar symbols indicate statistical differences in ration means of a diet at a single time point. All statistical significances tested at $P < 0.05$. $n = 10$ to 12 with the exception the following groups in which $n = 6$: Diet 2, Ration 2 on day 42 (all parameters) and Diet 1, Ration 1 on day 0, Diet 2, Ration 1 on day 21 (osmolality only).


Hematocrit (%)
60
50
40
30
20
10
0
a
a
b
b
Leucocrit (%)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
§
§
Plasma Osmolality (mOsm)
400
300
200
100
0
0
21
42
Time (days)

Basal blood plasma stress parameters

The concentrations of plasma lactate, glucose and cortisol in fish given each of the diet treatments are shown in Figure 4. Plasma cortisol concentrations generally increased with time in all groups however, these increases where not found to be significant. No differences in cortisol levels were observed with respect to diet or ration. Plasma glucose values were unaffected by dietary treatments over the experiment. However, a low glucose concentration in the fish fed diet 1 on the restricted basis on day 0 attained concentrations comparable to the other treatment groups by day 21 and 42 and consequently resulted in differences over time within this group. Lactate concentrations did not vary significantly with diet, ration or time over the 42 day experiment.

Basal biotransformation parameters

Liver cytosolic and microsomal protein concentrations are shown in Figure 5. Cytosolic protein concentrations were not significantly affected by treatment over the experiment. Similarly, there was no significant effect of diet, ration or time on microsomal protein concentrations. Liver biotransformation enzyme activities are shown in Figure 6. GST activities were unaffected by diet, ration or time during the experiment. Liver EROD activity was also unaffected by diet, ration or time.

Figure 4. Blood stress parameters (cortisol, glucose and lactate) of rainbow trout fed the various dietary regimes on days 0, 21 and 42. Values represent pooled data means ± S.E. from duplicate tanks. (□) Diet 1, Ration 1; (▫) Diet 2, Ration 1; (▩) Diet 3, Ration 1; (▨) Diet 1, Ration 2; (▨) Diet 2, Ration 2; (▩) Diet 3, Ration 2. Different letters represent statistical differences in means within a dietary regime over time. All statistical significances tested at $P < 0.05$. $n = 10$ to 12 with the exception the following groups in which $n = 6$: Diet 1, Ration 1 on day 0, Diet 2, Ration 1 on day 21 and Diet 2, Ration 2 on day 42.

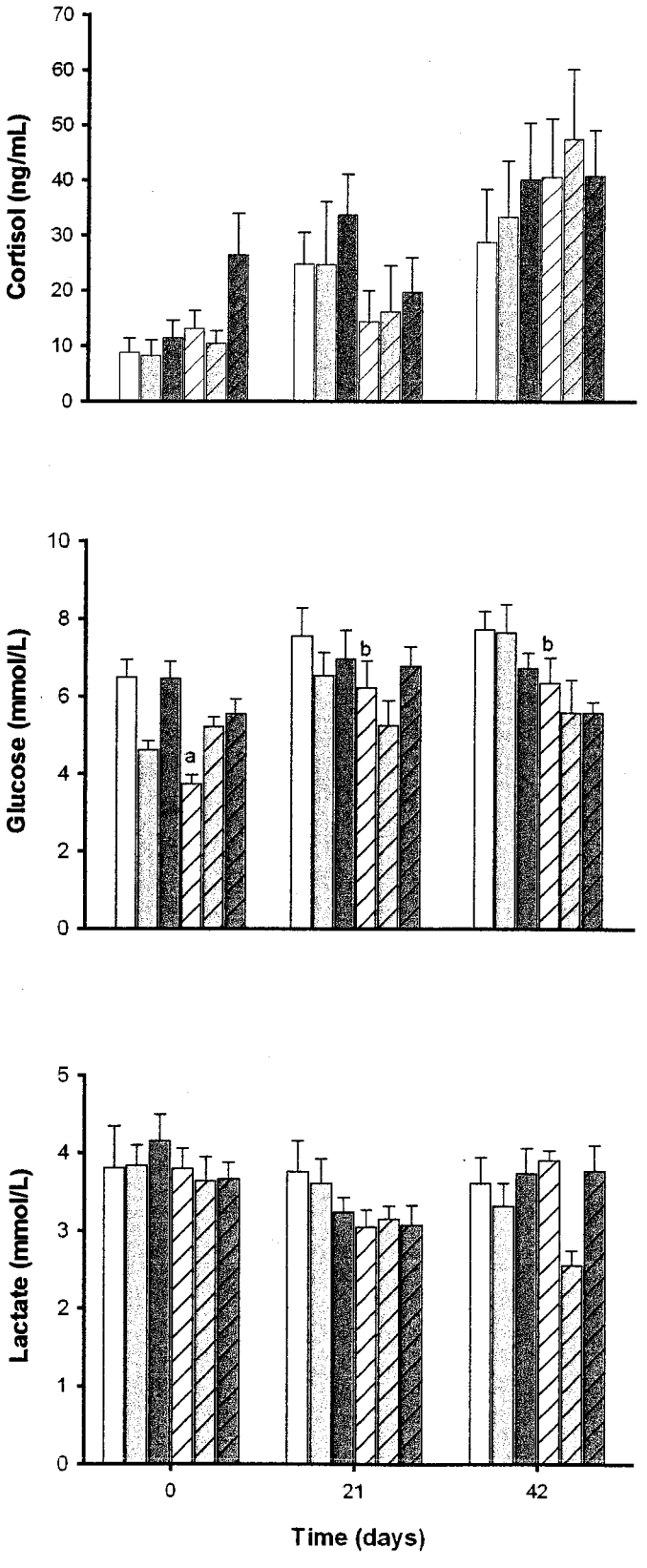

Cortisol (ng/mL)
70
60
50
40
30
20
10
0
Glucose (mmol/L)
10
8
6
4
2
0
a
b
b
Lactate (mmol/L)
5
4
3
2
1
0
0
21
42
Time (days)

Figure 5. Liver cytsosolic and microsomal protein levels of rainbow trout fed the various dietary regimes on days 0, 21 and 42. Values represent pooled data means ± S.E. from duplicate tanks. (□) Diet 1, Ration 1; (▨) Diet 2, Ration 1; (■) Diet 3, Ration 1; (◰) Diet 1, Ration 2; (◰) Diet 2, Ration 2; (◪) Diet 3, Ration 2. All statistical significances tested at $P < 0.05$. $n = 12$ with the exception of Diet 2, Ration 2 on day 42 where $n = 6$.

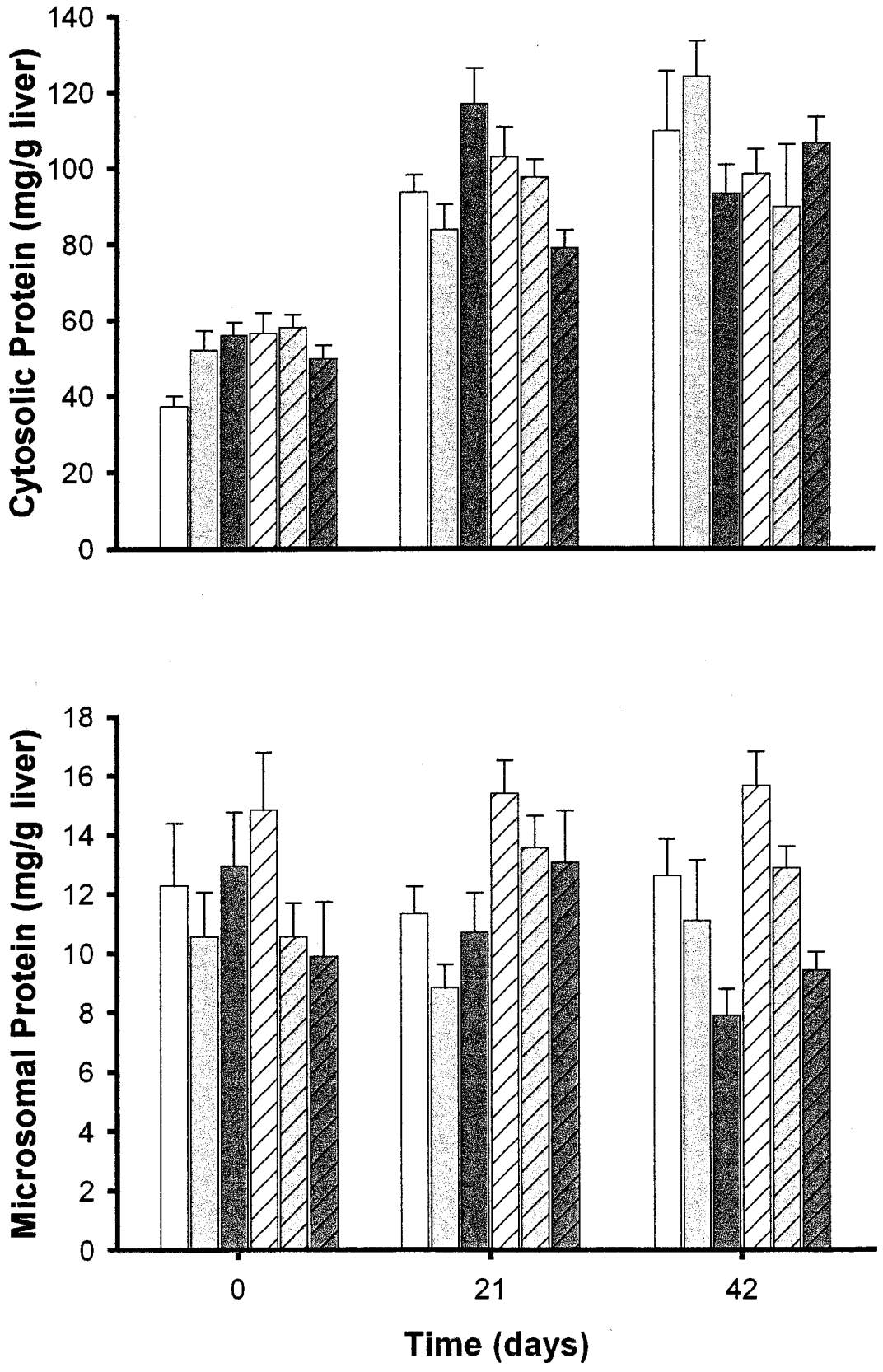
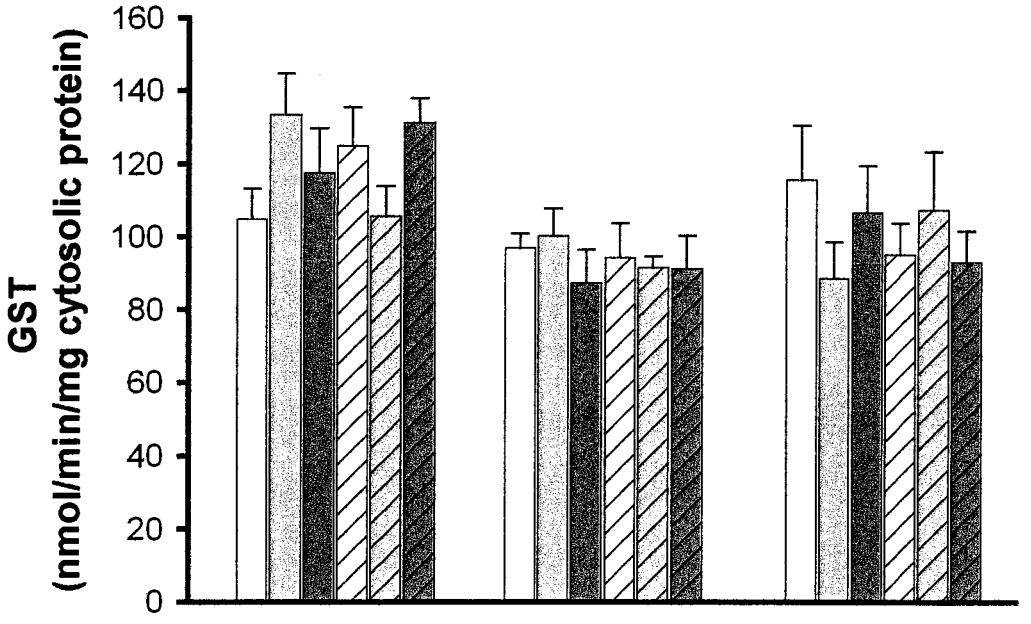
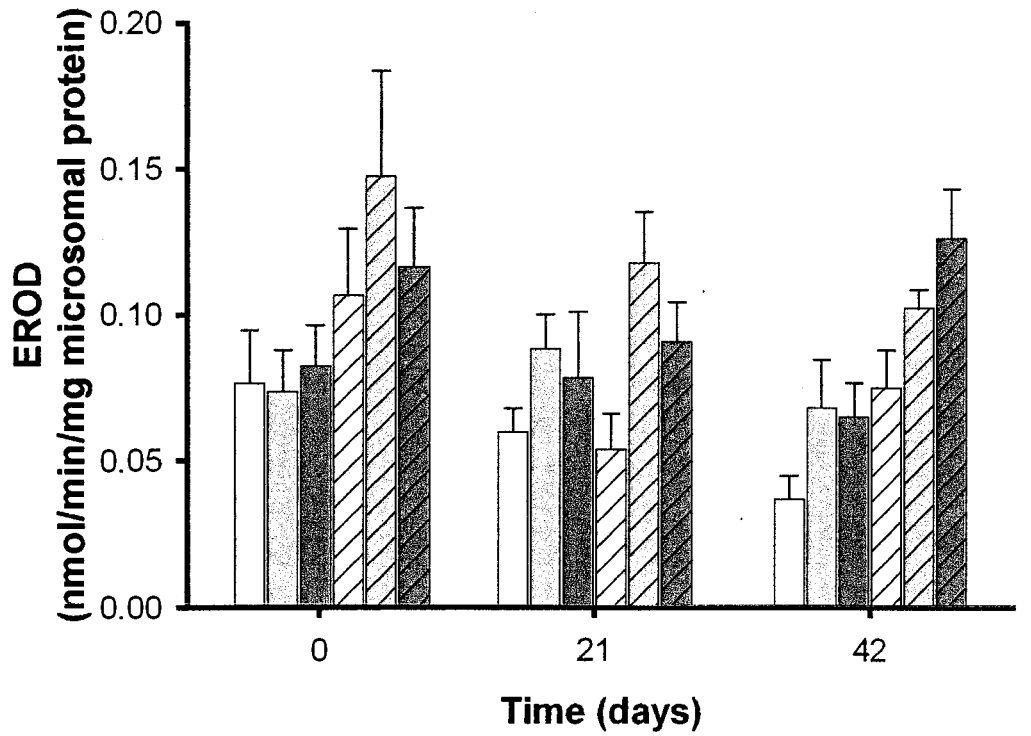

Cytosolic Protein (mg/g liver)
140
120
100
80
60
40
20
0
Microsomal Protein (mg/g liver)
18
16
14
12
10
8
6
4
2
0
0
21
42
Time (days)

Figure 6. Liver biotransformation enzyme (GST and EROD) activities of rainbow trout fed the various dietary regimes on days 0, 21 and 42. Values represent pooled data means ± S.E. from duplicate tanks. (□) Diet 1, Ration 1; (□) Diet 2, Ration 1; (■) Diet 3, Ration 1; (▨) Diet 1, Ration 2; (▨) Diet 2, Ration 2; (▨) Diet 3, Ration 2. All statistical significances tested at $P < 0.05$. n = 10 to 12 with the exception of Diet 2, Ration 2 on day 42 where n = 6.


GST
(nmol/min/mg cytosolic protein)
160
140
120
100
80
60
40
20
0


EROD
(nmol/min/mg microsomal protein)
0.20
0.15
0.10
0.05
0.00
0
21
42
Time (days)

ß-NF effects on blood parameters and enzyme activities

The effects of ß-napthoflavone (ß-NF) on blood parameters are shown in Table 6. Hematocrit values were significantly depressed following ß-NF injection in fish fed diets 1 and 2 at restricted ration. No other effects of ß-NF were identified with respect to hematocrit. Leucocrit values demonstrated a decreasing trend in response to ß-NF treatment, however, levels were only significantly depressed in fish fed diet 1 and 3 at satiation. ß-NF treatment had no effect on plasma osmolality. Table 7 provides the results of ß-NF treatment on the measured blood stress parameters. ß-NF injection had no significant effect on plasma lactate, glucose or cortisol. The concentrations and activities of hepatic cytosolic, microsomal protein, and biotransformation enzymes following ß-NF injection are summarized in Table 8. ß-NF did not affect cytosolic or microsomal protein. GST activity remained unchanged following ß-NF injection, however, EROD activity was significantly elevated (22 to 88-fold) in all dietary treatment groups.

Table 6. Blood parameters of rainbow trout fed the different dietary regimes before and 3 days following an intraperitoneal injection of ß-NF. Values represent means ± S.E. from duplicate tanks. Different letters represent statistical differences in means within a dietary regime before and after injection. All statistical significances tested at $P < 0.05$. n = 11 to 12 in all groups except Diet 2, Ration 2 where n = 6.

	Blood Parameters					
	Control			ß-NF Injected		
Dietary Regime	Hematocrit (%)	Leucocrit (%)	Osmolality (mOsm)	Hematocrit (%)	Leucocrit (%)	Osmolality (mOsm)
Diet 1, Ration 1	46.67 ± 1.37	0.795 ± 0.155[a]	319.8 ± 11.3	45.08 ± 1.25	0.371 ± 0.076[b]	330.1 ± 11.3
Diet 2, Ration 1	46.33 ± 0.81	0.875 ± 0.109	321.3 ± 12.9	46.25 ± 1.82	0.613 ± 0.109	341.8 ± 9.3
Diet 3, Ration 1	45.60 ± 1.17	1.218 ± 0.096[a]	307.5 ± 8.5	42.67 ± 1.63	0.728 ± 0.100[b]	324.4 ± 5.0
Diet 1, Ration 2	45.60 ± 0.48[a]	0.731 ± 0.131	308.5 ± 9.2	41.33 ± 0.74[b]	0.423 ± 0.084	321.8 ± 8.1
Diet 2, Ration 2	45.00 ± 1.46[a]	0.887 ± 0.189	352.8 ± 7.7	38.00 ± 0.97[b]	0.568 ± 0.074	328.5 ± 23.8
Diet 3, Ration 2	45.90 ± 1.62	0.463 ± 0.119	318.3 ± 12.0	43.33 ± 1.63	0.331 ± 0.055	298.8 ± 13.2

Table 7. Stress parameters of rainbow trout fed the different dietary regimes before and 3 days following an intraperitoneal injection of ß-NF. Values represent means ± S.E. from duplicate tanks. All statistical significances tested at $P < 0.05$. n = 11 to 12 in all groups except Diet 2, Ration 2 where n = 6.

	Blood Stress Parameters					
	Control			ß-NF Injected		
Dietary Regime	Cortisol (ng/mL)	Glucose (mmol/L)	Lactate (mmol/L)	Cortisol (ng/mL)	Glucose (mmol/L)	Lactate (mmol/L)
Diet 1, Ration 1	27.78 ± 9.67	7.72 ± 0.48	3.61 ± 0.33	40.79 ± 13.32	5.11 ± 0.43	3.78 ± 0.28
Diet 2, Ration 1	33.40 ± 10.17	7.63 ± 0.74	3.32 ± 0.29	47.82 ± 11.18	4.77 ± 0.29	3.73 ± 0.39
Diet 3, Ration 1	40.16 ± 12.73	6.71 ± 0.40	3.73 ± 0.32	54.62 ± 12.04	5.58 ± 0.77	3.99 ± 0.40
Diet 1, Ration 2	40.56 ± 10.59	6.34 ± 0.64	3.90 ± 0.11	46.64 ± 13.85	4.72 ± 0.26	2.87 ± 0.25
Diet 2, Ration 2	47.47 ± 12.79	5.58 ± 0.84	2.56 ± 0.19	45.91 ± 18.92	3.86 ± 1.22	3.68 ± 0.45
Diet 3, Ration 2	40.78 ± 8.28	5.58 ± 0.28	3.77 ± 0.33	44.06 ± 9.71	5.70 ± 0.56	3.30 ± 0.30

Table 8. Liver biotransformation parameters of rainbow trout fed the different dietary regimes before and 3 days following an intraperitoneal injection of ß-NF. Values represent means ± S.E. from duplicate tanks. Different letters represent statistical differences in means within a dietary regime over time. All statistical significances tested at $P < 0.05$. $n = 11$ to 12 in all groups except Diet 2, Ration 2 where $n = 6$.

	Biotransformation Parameters				
	Dietary Regime	Cytosolic Protein[1]	Microsomal Protein[1]	GST[2]	EROD[3]
Control	Diet 1, Ration 1	109.71 ± 10.93	12.63 ± 0.88	115.40 ± 11.45	0.04 ± 0.01[a]
	Diet 2, Ration 1	124.00 ± 7.38	11.11 ± 1.50	88.51 ± 7.04	0.07 ± 0.02[a]
	Diet 3, Ration 1	93.27 ± 5.69	7.90 ± 0.63	107.68 ± 9.32	0.07 ± 0.01[a]
	Diet 1, Ration 2	98.38 ± 4.71	15.66 ± 0.87	94.94 ± 6.15	0.08 ± 0.01[a]
	Diet 2, Ration 2	89.72 ± 11.04	12.89 ± 0.90	107.12 ± 11.16	0.12 ± 0.02[a]
	Diet 3, Ration 2	106.45 ± 5.43	9.43 ± 0.60	92.80 ± 6.38	0.13 ± 0.02[a]
ß-NF Injected	Diet 1, Ration 1	112.08 ± 6.23	9.68 ± 0.58	62.09 ± 2.08	3.52 ± 0.37[b]
	Diet 2, Ration 1	128.08 ± 4.45	10.09 ± 0.69	65.17 ± 3.16	3.47 ± 0.49[b]
	Diet 3, Ration 1	140.17 ± 8.13	8.83 ± 0.49	74.87 ± 4.42	2.67 ± 0.40[b]
	Diet 1, Ration 2	108.45 ± 3.40	11.53 ± 0.88	75.05 ± 4.50	3.83 ± 0.58[b]
	Diet 2, Ration 2	51.40 ± 4.78	8.30 ± 1.34	60.08 ± 2.96	2.82 ± 0.39[b]
	Diet 3, Ration 2	64.69 ± 6.65	10.21 ± 0.75	67.59 ± 2.36	2.87 ± 0.44[b]

[1] mg/g liver
[2] nmol/min/mg cytosolic protein
[3] nmol/min/mg microsomal protein

Discussion

Dietary composition and ration play fundamental roles in energy intake and its allocation to various physiological systems, including the hypothalamic-pituitary-interrenal (HPI) axis and xenobiotic biotransformation system. Both of the systems are essential to chemical defense wherein the biotransformation system detoxifies and excretes xenobiotics from the body and the HPI axis increases the energy available to complete these processes. This study reports the concentrations of baseline stress parameters and detoxification enzyme activities, and their response to ß-napthoflavone treatment, in fish fed isoenergetic diets varying considerably in protein and lipid content, while maintaining a constant carbohydrate content, at full or half ration.

Dietary ration and composition were found to have significant effects on all of the growth indices examined. Feeding ration significantly affected the growth of fish, as fish fed to satiation attained significantly more mass by day 42 than those fed restricted rations. This finding is not surprising as greater feeding rations increased the amount of energy supplied to the energy budget and enhanced the proportion of energy allocated to growth. Although fish fed the restricted ration gained less weight that the fish fed to satiation, they still received energy in excess of the amount required for physiological maintenance based on the significant increases in weight over initial values in all groups. Dietary composition also seemed to have some influence on growth. For instance, fish fed diet 3 (low protein:high lipid) to satiation and on the restricted basis were heavier, although not statistically, than those fed the other diets at similar rations. This finding suggests that the dietary protein needs of the rainbow trout in this study were met by a diet that contained 36% crude protein and about 20.7 MJ of gross energy/kg with most of the non-protein energy originating from lipid. Hence, diets of higher protein content with equivalent energy content were unnecessary to enhance the growth of trout in this study.

Previous studies have reported that fish growth is enhanced with increases in dietary protein concentration, but the results are dependent upon the fish species and its life history stage and various other factors (Chan et al., 2002; Lee et al., 2002), thus it is necessary to consider these modifying factors when designing a custom diet for a specific group of fish.

Dietary proteins are known to provide fish with high levels of energy and carbon, which may be used for both metabolism and growth (Kim et al., 1991). However, by increasing the lipid content of a diet, protein sparing may occur and thus lipid is used for energy allowing for a more efficient use of protein in growth processes (Lee et al., 2002). Previous studies have found that the most efficient combination for protein sparing in rainbow trout were diets containing 35 to 36% protein and 15 to 20% lipid (Watanabe et al., 1979; Cho and Kaushik, 1990). Although the lipid content of 18% in diet 3 may have allowed this phenomenon to occur, a factorial design to test the effect of the three protein (35, 45 and 55%) and lipid (8, 13 and 18%) contents of these diets on fish growth would be required to draw any definitive conclusions.

During periods of high energy intake, both the liver and muscle may accumulate stored energy which may be measured as an increase in mass. Condition factor ($K = \text{weight}/(\text{length})^3$) reflects the nutritional state of an individual as varying energy intake affects an individual's tissue growth. Therefore, the weight of individual fish of similar length may vary based on nutritional status (Busacker et al., 1990). Additionally, during periods of high energy intake, fish store energy in the liver in the form of glycogen, therefore, liver weight correlated to body weight may also be used as an indication of the nutritional state of the animals. Other researchers have used hepatosomatic values and condition factors as additional indices of growth and nutritional status (Busacker et al., 1990). In the present study, both of these indices showed a significant increase between day 0 and 42 in fish of all dietary regimes. This demonstrates that all the diets

provided energy beyond the needs of physiological metabolism, thus allowing for energy storage.

Analysis of hematological parameters are useful in the detection of metabolic disturbances, nutritional disturbances and disease processes (Spannhof et al., 1979; Sandnes et al., 1988); however, research directly relating hematocrit, leucocrit and plasma osmolality to specific dietary change is scarce. In this study, dietary treatments were not found to affect hematological parameters. Hematocrit values were well within the normal ranges reported for rainbow trout (24 to 48%) (Miller et al., 1983) and it may be concluded that diet, ration and time had no effects on hematocrit. Leucocrit values appeared to be moderately variable throughout the entire study however, neither diet content nor ration had effects on leucocrit values. Similarly, plasma osmolality was unaffected by dietary treatments as levels remained constant throughout the duration of the experiment.

Although the ß-NF injection significantly decreased hematocrit levels in two dietary treatment groups and leucocrit values in another two groups, generally, the lack of significant changes following ß-NF treatment suggests that there was no appreciable effect of ß-NF on these parameters. Plasma osmolality was unaffected by the ß-NF treatment. Overall, blood chemistry was unaffected by dietary treatment during both the basal and induction parts of the experiment, demonstrating that blood chemistry is resilient to nutritional variation within the confines of these diets.

The stress response provides a mechanism by which body energy stores may be accessed and used to aid the animal in coping with stressors. In this study, glucose and lactate were unaffected by dietary treatment or ration over the 42 days. However, cortisol levels demonstrated an increasing trend over the sampling period despite diet or ration, although this trend was not found to be significant. Ration did not have an effect on fish stress parameter responses in this study since the levels in fish fed full and

restricted rations of similar diets were comparable. In previous studies, long periods of starvation, up to 6 weeks, were not shown to affect cortisol levels in juvenile salmonids (Farbridge and Leatherland, 1992; Vijayan and Moon, 1992). Similarly, glucose levels, although not affected after 30 days of starvation (Vijayan and Moon, 1992), were significantly reduced after 6 weeks of starvation (Farbridge and Leatherland, 1992). Long term food restriction in rats has been shown to elevate basal levels of ACTH and corticosterone while the levels of circulating glucose remain below those in fed rats (Wan et al., 2003). These findings suggest an increased utilization of glucose during the period of food restriction. However, during the current study, food was not appreciably restricted as demonstrated by the weight gain experienced by fish in all groups.

Different dietary nutrient compositions are generally not perceived as a stressor since basal corticosterone and glucose levels are shown to be unaffected in most studies. Barton et al. (1988) found that a dietary lipid content of 7, 13 or 19% had no influence on glucose or cortisol levels in juvenile Chinook salmon after 21 weeks on the diets. Several mammalian studies have also focused on the effects of high fat diets. Basal corticosterone levels were shown not to be affected in rats fed high fat diets (59% of calories) as compared to high starch diets (70% of calories) by Pascoe et al. (1991), while Tannenbaum et al. (1997) showed that rats fed diets high in fat (20%) had significantly elevated corticosterone levels as compared to rats fed control diets (5% fat). Resting glucose levels similarly unaffected by high dietary fat intake in several studies (40% of calories, Hulsmann, 1978; 70% of calories, Pascoe et al., 1991; 20% of diet, Tannenbaum et al., 1997). In the present study, dietary composition did not influence cortisol or glucose levels over the 42 days and consequently, the diets were not perceived as stressors to the animals.

The administration of ß-napthoflavone to induce biotransformation enzyme activity did not caase changes in plasma stress parameters. Routine handling procedures, such as

those necessary to inject the fish with ß-NF, are known to induce stress within one hour post-handling and the levels of indicators typically decrease to pre-stress levels after 48 hours (Barton et al., 1988; Ackerman et al., 2000). Since sampling was completed 72 hours following ß-NF injection, it is possible that cortisol, glucose and lactate concentrations may have returned to control levels by sampling time, if elevation due to handling had occurred. Alternatively, Wilson et al. (1998) reported that 3 days following ß-NF injection, cortisol concentrations in rainbow trout were similar to controls, and in addition they demonstrated that interrenal cell sensitivity to adrenocorticotrophic hormone, the primary stimulator of cortisol release, was abolished by an effect of ß-NF on either ACTH receptor dynamics and/or steroidogenic pathways. This finding was further by the observation that significantly lower cortisol levels were achieved following an acute handling stress 3 days following the ß-NF injection. In our experiments, it is unclear as to whether a stress response was initiated in reaction to the handling procedures and levels of glucose and cortisol returned to control levels by sampling time or whether the stress response was inhibited by ß-NF. It would be interesting to further investigate this issue by repeating the experiment and include a 3-minute handling stress and subsequent time course study of the stress parameters.

Although the costs of stress may be estimated through growth rates, organismal parameters (i.e. hepatosomatic indices) and biochemical measurements, these elements may have the potential to misrepresent stress costs, for instance, some indices used to assess the costs of chemical stressors may be complicated by toxic effects. One example of this is the use of the hepatosomatic index, where the value may increase if liver weight increases due to hyperplasia (increase in cell number) or hypertrophy (increase in cell size) as an adaptive response to the capacity of the liver to detoxify chemicals (Heath, 1995). Additionally, cortisol secretion in fish is known to be controlled by negative feedback mechanisms at the level of the hypothalamus and pituitary,

thereby modulating ACTH release. This may be the case in chronically stressed fish which demonstrate a decreased sensitivity to ACTH and consequently lower cortisol levels in response to an acute stressor as compared to previously unstressed fish (Vijayan and Leatherland, 1990). Thus, measuring routine metabolism through oxygen consumption rates may provide a more integrative assessment of metabolic costs.

The mixed-function oxidase enzymes play various roles in animals, from the detoxification of exogenous compounds to the breakdown of endogenous molecules. This suggests that the maintenance of detoxification enzyme activity in the energy budget at the expense of other, less critical systems, during periods of decreased energy intake might be expected. In the present study, the activities of the CYP 1A1-mediated reaction, EROD, and the conjugating enzyme, GST, were maintained at constant levels all ration levels suggesting the importance of these systems under the various dietary treatments. This is further supported by the maintenance of these, and other biotransformation enzymes, including cytochrome P450, benzo(a)pyrene hydroxylase (AAH), UDP-glucuronosyl transferase (UDP-GT), during periods of teleost starvation (Buhler and Rasmusson, 1968; Andersson et al., 1985a; Viganò et al., 1993). The maintenance of biotransformation enzyme activities under these conditions is expected to have adaptive significance for fish, which often undergo recurring periods of starvation, while mammals challenged with starvation demonstrate extremely variable liver cytochrome P450 dependent activities (Campbell and Hayes, 1974). Furthermore, reductions in detoxification enzymes in mammals have been observed during starvation periods as short as 36 hours.

Diet composition, similar to ration, was not found to affect the activities of the biotransformation enzymes examined in this study. Very little information is available on the effects of dietary composition on fish detoxification abilities, however studies on the red drum, *Sciaenops ocellatus*, have demonstrated that by increasing dietary lipid

content by 21%, hepatic EROD activity increased three-fold, suggesting that the high dietary lipid content augmented the synthesis of the enzyme (Craig et al., 1999). More studies have been completed with mammals demonstrating that the amount of protein (Marshall and McLean, 1969; Wood and Woodcock, 1970; Paine and McLean, 1973; Sachan, 1975) and the type and concentration of lipid (Ammouche et al., 1993, Shavila et al., 1994) in a diet can differentially affect basal mixed-function oxidase systems.

Reports on the effects of dietary protein have been met with variable results. Paine and McLean (1973) found significantly lower hepatic AHH activity in rats fed no or low protein diets as compared to rats fed stock diets. Similar results were found with respect to cytochrome P450 activities (Marshall and McLean, 1969). Wood and Woodcock (1970) found that removing protein from the diet significantly increased the activity of UDP-GT in rats. In the parsnip worm, *Depressaria pastinacella*, a 75% decrease in dietary protein, produced a 60% decrease in their growth while the level of xanthotoxin detoxification was maintained. Furthermore, detoxification only became affected when protein was completely absent from the diet (Berenbaum and Zangerl, 1994), suggesting the importance of the detoxification system within the energy hierarchy.

Dietary lipid content and type have been found to differentially affect biotransformation enzymes. Increasing the dietary lipid content of a ferret, *Mustella putorius furo*, by adding 20% beef suet, increased EROD activity by 90% while no other enzymes, including total P450, were affected (Shavila et al., 1994). Ammouche et al. (1993) reported differential increases and reductions in cytochrome P450 concentration with respect to type of dietary lipid (fat free, peanut-rapeseed, sunflower and fish oil). The differences in enzyme activities with respect to type and quantity of dietary lipid may be attributable to the role of lipids in biomembrane phospholipids and their capacity to alter membrane formation and fluidity, and thus the associated activities of membrane-bound detoxification enzyme content, their binding abilities and subsequent

biotransformation activities (Ammouche et al., 1993). Since no modifications to enzyme activity were detected with altered dietary composition in our study, it may be possible that the differences in dietary protein and lipid content (protein: 35 to 55%; lipid: 8 to 18%) were not extreme enough to cause such changes. Future research may further limit composition, and ration, to determine if nutritional threshold values exists with respect to detoxification enzyme activities.

The induction of xenobiotic metabolizing systems is often considered as evidence that maintaining high cytochrome P450 titers is metabolically costly (Brattsten et al., 1977). Therefore, the induction process is believed to have evolved through selective pressures to reduce the metabolic costs of detoxification. The results of the ß-napthoflavone treatment in this study show that GST activity was unaffected, while the activities of EROD were significantly increased across dietary groups. Although GST can be induced by ß-NF to approximately 100 to 200% over controls, maximal activity is usually achieved one to three weeks following induction (Andersson et al., 1985b; Celander et al., 1993; Noble et al., 1996; Noble et al., 1998). The magnitude of EROD induction (approx. 21 to 87-fold) over control levels observed here is similar to increases seen in other studies with fed rainbow trout where the highest activity levels were achieved 3 days following treatment (Andersson et al., 1985b; Zhang et al., 1990; Pretti et al., 2001). Moreover, although the basal activity of several enzyme activities including EROD, UDP-GT, ECOD, AHH and P450, are depressed during long periods of starvation, induction to levels comparable to those achieved by fed fish is possible (Andersson et al., 1985a), thereby, demonstrating the importance of this system even under extremely limited energy intake.

Reports regarding the effect of dietary composition on detoxification enzyme induction are limited. In channel catfish, *Ictalurus punctatus*, fed two commercial diets and one non-commercial diet, differences in the induction of EROD, ECOD and GST by

Aroclor 1254, a PCB congener, were demonstrated (Ankley and Blazer, 1988). This lead the researchers to suggest that dietary composition may significantly affect detoxification activity and induction differentially in response to type of inducer used. In mammals, similar results have been reported. In rats, intraperitoneal administration of phenobarbital induced cytochrome P450 activity in rats fed low (3%), medium (23%) and high (38%) protein by 91, 68 and 41%, respectively (Sachan, 1975), although no explanation for these differences was offered. Since no differences in β-NF-induced EROD activity were observed between the three diets in the present study, it can be concluded that dietary compositions may not have varied significantly enough to produce a differential induction pattern.

Detoxification enzyme activity were maintained over the range of dietary composition and ration used in this study. However, this study did not fully challenge the energy budget because energy was still available for growth under all dietary treatment groups. The approach taken in this study, that is examining resting levels of detoxification enzymes in relation to varying energy inputs based only on feeding rate and dietary protein/lipid content, is an improvement over studies completed in the past, which have used exposure to xenobiotics to test the biotransformation energetics thereby possibly confounding results with toxicological impacts. However, recent work in a variety of species, including birds (Guglielmo et al., 1996), marsupials (Dash, 1988; Foley, 1992), and mammals (Lindroth and Batzli, 1984), has successfully attempted to estimate detoxification costs by directly measuring the energy content of excreted metabolites and thereby estimating the associated energy losses. For example, Ruffed grouse (*Bonasa umbellus*) fed diets high in coniferyl benzoate (CB), a natural ester found in some of their food, resulted in an additional cost of 10 to 14% to their metabolizable energy source over diets low in CB, as determined by measuring the energy of individual conjugation metabolites recovered from excreta (Guglielmo et al., 1996). Specifically,

the energetic costs of detoxification are due to energy uses for: 1) energy use for enzymatic conversions of the parent and Phase I metabolites, 2) nutrient drains experienced from using endogenous molecules as conjugation substrates e.g. glucuronic acid used in the formation of glucuronides are carboxylic acid derivatives of glucose, 3) maintenance of the acid-base balance which is altered by the production of acidic organic metabolites, and 4) transportation of metabolites across biomembranes to facilitate excretion, e.g., active metabolism in urinary excretion (Sipes and Gandolfi, 1986; Foley, 1992).

Although differences in dietary quality have been shown to increase energetic losses through excreta and reduced growth, this is often due to reduced conversion efficiency/digestibility of the diets with increased toxic content. For example, although overall metabolizable energy was similar between ringtail possums, (*Pseudocheirus peregrinus*), fed diets high and low in tannic acid, possums fed the high concentration diets consumed a greater amount of food due to a reduced conversion efficiency associated with the high tannic acid content (Foley, 1992). Thus, to make truly insightful conclusions of the relationship between nutrition and detoxification, it is necessary to analytically determine the energy assimilated from the diet for physiological work.

Summary and Conclusion

The ability of an animal to compensate for changing environmental conditions relies heavily upon life history and energy reserves, thus nutrition plays a critical role in fitness. Animals attain energy from the food they ingest, however, nutrient composition and quantity directly affect the amount of energy obtained from a given diet. Energy is limited and must be shared amongst all physiological processes within the body. Thus the order of allocation and factors determining how much energy is allocated to certain systems is of great interest, especially to systems enabling the organism to deal with extrinsic challenges.

This thesis demonstrated the effects of varying dietary nutrient content and ration on basal levels of two physiologically important systems for dealing with environmental challenges, the stress response and the detoxification system. Basal levels of stress parameters were unaffected during this study and further, the stress response was not activated signifying that neither diet content nor ration was perceived as a stressor by the fish. Detoxification activity was maintained at similar rates regardless of dietary treatment while fish growth was reduced thus, suggesting that detoxification has an important position within the energy budget of the rainbow trout. Additionally, the fish maintained the ability to induce biotransformation enzymes under these conditions, further indicating the significance of this system even under limited energy intake.

Although no effects of dietary nutrient content or ration size were observed in this study, other studies have demonstrated that both dietary nutrient content and dietary quantity may have significant effects on an animal's stress response (Barton et al., 1988; Farbridge and Leatherland, 1992) and on detoxification ability (Ankley et al., 1989, Viganò et al., 1993; Blom et al., 2000). However, results of these studies have provided equivocal results to the degree of the effects and demonstrated that more research is

needed in this area. It is probable that no effects of dietary treatment were observed in this study because dietary treatments did not differ drastically enough from each other.

Since the biotransformation enzymes and stress parameters are considered to be useful in the field of environmental monitoring it is imperative to be familiar with the extent of the effects modifying factors, including age, species, sex and nutrition, may have on animals. Nutrition and feeding regimes are important factors to bear in mind in any study which plans to investigate detoxification abilities or stress responses because, as demonstrated in the literature, dietary composition and quantity may affect both stress response and detoxification abilities. Without taking nutritional history into consideration during these types of studies, the possibility of reporting confounded results exists. Additionally, the aquaculture industry may also use nutritional information to provide farmed fish with diets that will not hinder their ability to deal with the stressors and toxicants they are exposed in open water pens.

REFERENCES

Ackerman, P.A., Forsyth, R.B., Mazur, C.F., Iwama, G.K., 2000. Stress hormones and the cellular stress response in salmonids. Fish Physiol. Biochem. 23, 327-336.

Ammouche, A., Dinh, L., Youyou, A., Clement, M., Bourre, J.M., 1993. Rate of alteration of hepatic mixed-function oxidase system in rats fed different dietary fats. Biochem. Cell. Biol. 71, 530-537.

Andersson, T., Koivusaari, U., Förlin, L., 1985a. Xenobiotic biotransformation in the rainbow trout liver and kidney during starvation. Comp. Biochem. Physiol. 82C, 221-225.

Andersson, T., Pesonen, M., Johansson, C., 1985b. Differential induction of cytochrome P-450-dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyl transferase activities in the liver of the rainbow trout by ß-napthoflavone or clophen A50. Biochem. Pharm. 34, 3309-3314.

Ankley, G.T., Blazer, V.S., 1988. Effects of diet on PCB-induced changes in xenobiotic metabolism in the liver of the channel catfish (*Ictalurus punctatus*). Can. J. Fish. Aquat. Sci. 45, 132-137.

Ankley, G.T., Blazer, V.S., Plakas, S.M., Reinert, R.E., 1989. Dietary lipid as a factor modulating xenobiotic metabolism in channel catfish (*Ictalurus punctatus*). Can. J. Fish. Aquat. Sci. 46, 1141-1146.

Avella, M., Schreck, C.B., Prunet, P., 1991. Plasma prolactin and cortisol concentrations of stressed coho salmon, *Oncorhynchus kisutch*, in freshwater or saltwater. Gen. Comp. Endocrinol. 81, 21-27.

Bamberger, C.M., Schulte, H.M., Chrousos, G.P., 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr. Rev. 17, 245-261.

Barton, B.A., Schreck, C.B., 1987. Metabolic cost of acute physical stress in juvenile steelhead. Trans. Am. Fish. Soc. 116, 257-263.

Barton, B.A., Schreck, C.B., Fowler, L.G., 1988. Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile Chinook salmon. Prog. Fish-Cult. 50, 16-22.

Beauchamp, D.A., 1990. Seasonal and diel food habits of rainbow trout stocked as juveniles in Lake Washington. Trans. Am. Fish. Soc. 119, 475-482.

Berenbaum, M.R., Zangerl, A.R., 1994. Costs of inducible defense: protein limitation, growth, and detoxification in parsnip webworms. Ecology 75, 2311-2317.

Beyers, D.W., Rice, J.A., Clements, W.H., Henry, C.J., 1999. Estimating physiological cost of chemical exposure: integrating energetics and stress to quantify toxic effects in fish. Can. J. Fish. Aquat. Sci. 56, 814-822.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Blom, S., Andersson, T.B., Förlin, L., 2000. Effects of food deprivation and handling stress on head kidney 17α-hydroxyprogesterone 21-hydroxylase activity, plasma cortisol and the activities of liver detoxification enzymes in rainbow trout. Aquat. Toxicol. 48, 265-274.

Bonga, S.E.W., 1997. The stress response in fish. Physiol. Rev. 77, 591-625.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bradstreet, R.B., 1965. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York, NY.

Brattsten, L.B., Wilkinson, C.F., Eisner, T., 1977. Herbivore-plant interactions: mixed-function oxidases and secondary plant substances. Science 196, 1349-1352.

Brett, J.R., Groves, T.D.D., 1979. Physiological Energetics. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish physiology, vol. 8. Academic Press, New York, pp. 279-352.

Buhler, D.R., Rasmusson, M.E., 1968. The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25, 223-239.

Bureau, D.P., Kaushik, S.J., Cho, C.Y., 2002. Bioenergetics. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, New York, pp. 1-59.

Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorometric assay of microsomal O-dealkylation which is preferentially induced by 3-methylcolanthrene. Drug Metab. Disp. 2, 239-256.

Busacker, G.P., Adelman, I.R., Goolish, E.M., 1990. Growth. In: Schreck, C.B., Moyle, P.B. (Eds.), Methods for Fish Biology. American Fisheries Society, Bethesda, Maryland, pp. 363-387.

Campbell, T.C., Hayes, J.R., 1974. Role of nutrition in the drug-metabolizing enzyme system. Pharmac. Rev. 25, 171-197.

Catacutan, M.R., Coloso, T.M.T., 1995. Effect of dietary protein to energy ratios on growth, survival, and body composition of juvenile Asian seabass, *Lates calcarifer*. Aquaculture 131, 125-133.

Celander, M., Leaver, M.J., George, S.G., Förlin, L., 1993. Induction of cytochrome P450 1A1 and conjugating enzymes in rainbow trout (*Oncorhynchus mykiss*) liver: A time course study. Comp. Biochem. Physiol. 106C, 343-349.

Chan, J.C.K., Mann, J., Skura, B.J., Rowshandeli, M., Rowshandeli, N., Higgs, D.A., 2002. Effects of feeding diets containing various dietary protein and lipid ratios on the growth performance and pigmentation of post-juvenile coho salmon *Oncorhynchus kisutch* reared in sea water. Aquaculture Res. 33, 1137-1156.

Cho, C.Y., 1992. Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. Aquaculture 100, 107-123.

Cho, C.Y., Kaushik, S.J., 1990. Nutritional energetics in fish: Energy and protein utilization in rainbow trout (*Salmo gairdneri*). In: Bourne, G.H. (Ed.), Aspects of Food Production, Consumption and Energy Values. Vol. 61. World Rev. Nutr. Diet. Basel, Karger. pp, 132-171.

Craig, S.R., Washburn, B.S., Gatlin III, D.M., 1999. Effects of dietary lipids on body composition and liver function in juvenile red drum, *Sciaenops ocellatus*. Fish Physiol. Biochem. 21, 249-255.

Dash, J.A., 1988. Effect of dietary terpenes on glucuronic acid excretion and ascorbic acid turnover in the brushtail possum (*Trichosurus vulpecula*). Comp. Biochem. Physiol. 89B, 221-226.

Einen, O., Roem, A.J., 1997. Dietary protein/energy ratios for Atlantic salmon in relation to fish size:growth, feed utilization and slaughter quality. Aquaculture Nutr. 3, 115-126.

Engin, K., Carter, C.G., 2001. Ammonia and urea excretion rates of juvenile Australian short-finned eel (*Anguilla australis australis*) as influenced by dietary protein level. Aquaculture. 194, 123-126.

Farbridge, K.J., Leatherland, J.F., 1992. Plasma growth hormone levels in fed and fasted rainbow trout (*Oncorhynchus mykiss*) are decreased following handling stress. Fish Physiol. Biochem. 10, 67-73.

Foley, W.J., 1992. Nitrogen and energy retention and acid-base status in the common ringtail possum (*Pseudocheirus peregrinus*): Evidence of the effects of absorbed allelochemicals. Physiol. Zool. 65, 403-421.

Fujiwara, T., Cherrington, A.D., Neal. D.H., McGuinness, O.P., 1996. Role of cortisol in the metabolic response to stress hormone infusion in the conscious dog. Metabolism. 45, 571-578.

Goede, R.W., Barton, B.A., 1990. Organismic indices and autopsy-based assessment as indicators of health and condition of fish. American Fisheries Society Symposium. 8, 93-108.

Gokøsyr, A., Förlin, L., 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol. 22, 287-312.

Guglielmo, G.C., Karasov, W.H., Jakubas, W.J., 1996. Nutritional costs of plant secondary metabolite explain selective foraging by ruffed grouse. Ecology 77, 1103-1115.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Halver, J.E., 2002. The Vitamins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, New York. pp. 1-59.

Halver, J.E., Hardy, R.W., 2002. Fish Nutrition. Academic Press, New York.

Hazon, N., Balment, R.J., 1998. Endocrinology. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press, Boca Raton. pp. 441-464.

Heath, A.G., 1995. Water pollution and fish physiology. CRC Press, Boca Raton, Florida.

Higgs, D.A., MacDonald, J.S., Levings, C.D., Dosanjh, B.S., 1995. Nutrition and feeding habits in relation to life history stage. In: Groot, C., Margolis, L., Clarke, W.C. (Eds.), Physiological ecology of pacific salmon. UBC Press, Vancouver. pp. 159-316.

Hilton, J.W., Slinger, S.J., 1981. Nutrition and feeding of rainbow trout. Can. Spec. Publ. Aquat. Sci. 55, 15 p.

Hulsmann, W.C., 1978. Abnormal stress reactions after feeding diets rich in (very) long chain fatty acids: high levels of corticosterone and testosterone. Mol. Cell. Endocrinol. 12, 1-8.

Huuskonen, S., Rasanen, T., Koponen, K., Lindstrom-Seppa, P.L., 1995. Time-course studies of the biotransformation enzymes in control rainbow trout when adjusting to new habitats. Mar. Environ. Res. 39, 79-83.

Isaia, J., Maetz, J., Haywood, G.P., 1978. Effects of epinephrine on branchial nonelectrolyte permeability in rainbow trout. J. Exp. Biol. 74, 227-238.

Janssens, P.A., Waterman, J., 1988. Hormonal regulation of gluconeogenesis and glycogenolysis in carp (*Cyprinus carpio*) liver pieces cultured *in vitro*. Comp. Biochem. Physiol. 91A, 451-455.

Kanazawa, A., 1985. Essential fatty acid and lipid requirement of fish. In: Cowey, C.B., Mackie, A.M., Bell., J.G. (Eds.), Nutrition and Feeding in Fish. Academic Press, London. pp. 281-298.

Keast, A., 1978. Trophic and spatial interrelationships in the fish species of an Ontario temperate lake. Env. Biol. Fish. 3, 7-31.

Keast, A., 1985. Development of dietary specializations in a summer community of juvenile fishes. Env. Biol. Fishes. 13, 211-224.

Keast, A., Welsh, L., 1968. Daily feeding periodicities, food uptake rates and dietary changes with hour of day in some lake fishes. J. Fish. Res. Bd. Can. 25, 1133-1144.

Kennedy, C.J., 1994. Xenobiotics: designing an *in vitro* system to study enzymes and metabolism. In: Hochachka, P.W. and Mommsen, T.P. (Eds.), Biochemistry and molecular biology of fishes, Vol 3, Elsevier Science, New York. pp. 417-430

Kim, K., Kayes, T.B., Amundson, C.H., 1991. Purified diet development and re-evaluation of the dietary protein requirement of fingerling rainbow trout (*Oncorhynchus mykiss*). Aquaculture 96, 57-67.

Lall, S.P., 2002. The Minerals. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, New York. pp. 259-308.

Lee, S.M., Jeon, I.G., Lee, J.Y., 2002. Effects of digestible protein and lipid levels in practical diets on growth, protein utilization and body composition of juvenile rockfish (*Sevastes schlegeli*). Aquaculture 211, 227-239.

Lindroth, R.L., Batzli, G.O., 1984. Plant phenolics as chemical defenses effects of natural phenolics on survival and growth of prairie voles, *Microtus ochrogaster*. J. Chem. Ecol. 10, 229-244.

Lovell, T., 1998. Nutrition and Feeding of Fish. Kluwer Academic Publishers, Norwell, Massachusetts.

Marshall, W.J., McLean, A.E.M., 1969. The effect of oral phenobarbitone on hepatic microsomal cytochrome P-450 and demethylation activity in rats fed normal and low protein diets. Biochem. Pharmac. 18, 153-157.

Mazeaud, M.M., Mazeaud, F., 1981. Adrenergic responses to stress in fish. In: Pickering, A.D. (Ed.), Stress and Fish. Academic Press, New York. Pp. 49-75.

Miller III, W.R., Hendricks, A.C., Cairns Jr., J., 1983. Normal ranges for diagnostically important hematological and blood chemistry characteristics of rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. 40, 420-425.

Mommsen T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fisher. 9, 211-268.

Nerbert, D.W., 1979. Multiple forms of inducible drug-metabolizing enzymes: A reasonable mechanism by which any organism can cope with adversity. Mol. Cell. Biol. 27, 27-46.

Noble, E., Garin, D., Barré, H., 1996. Effects of hypoproteic soybean diet on energetic parameters and conjugation reactions in carp (*Cyprinus carpio*): Time course response to ß-napthoflavone. Comp. Biochem. Physiol. 113C, 335-343.

Noble, E., Barré, H., Dierickx, P.J., 1998. Effect of diet and ß-napthoflavone on hepatic and renal glutathione S-transferase isoenzymes in carp (*Cyprinus carpio*). Fish Physiol. Biochem. 18, 203-212.

NRC, 1981. Nutrient Requirements of Coldwater Fishes. National Academy Press, Washington, DC.

NRC, 1993. Nutrient Requirements of Fish. National Academy Press, Washington, DC.

Paine, A.J., McLean, A.E.M., 1973. The effect of dietary protein and fat on the activity of aryl hydrocarbon hydroxylase in rat liver, kidney and lung. Biochem. Pharmac. 22, 2875-2880.

Pascoe, W.S., Smythe, G.A., Storlien, L.H., 1991. Enhanced responses to stress induced by fat-feeding in rats: relationship between hypothalamic noradrenaline and blood glucose. Brain Res. 550, 192-196.

Pickering, A.D., 1981. Stress and Fish. Academic Press, London.

Pickering, A.D., Pottinger, T.G., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem. 7, 253-258.

Pickering, A.D., Pottinger, T.G., Carragher, J., Sumpter, J.P., 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 68, 249-259.

Pickering, A.D., Pottinger, T.G., Christie, P.J., 1982. Recovery of the brown trout *Salmo trutta* L. from acute handling stress: A time course study. J. Fish Biol. 20, 229-244.

Pickering, A.D., Stewart, A., 1984. Acclimation of the interrenal tissue of the brown trout, *Salmo trutta* L. to chronic crowding stress. J. Fish Biol. 24, 731-740.

Porthé-Nibelle, J., Lahlou, B., 1981. Mechanisms of glucocorticoid uptake by isolated hepatocytes of the trout. Comp. Biochem. Physiol. 69B, 425-433.

Pretti, C., Salvetti, A., Longo, V., Giorgi, M., Gervasi, P.G., 2001. Effects of ß-napthoflavone on the cytochrome P450 system, and phase II enzymes in the gilthead seabream (*Sparus aurata*). Comp. Biochem. Physiol. 130C, 133-144.

Randall, D.J., Perry, S.F., 1992. Catecholamines. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), Fish Physiology, Vol. XIIB. Academic Press, New York, pp. 255-300.

Reinitz, G.L., Orme, L.E., Lemm, C.A., Hitzel, F.N., 1978. Influence of varying lipid concentrations with two protein concentrations in diets for rainbow trout (*Salmo gairdneri*). Trans. Am. Fish. Soc. 107, 751-754.

Sachan, D.S., 1975. Effects of low and high protein diets on the induction of microsomal drug-metabolizing enzymes in rat liver. J. Nutr. 105, 1631-1639.

Sandnes, K., Lie, Ø., Waagbø, R., 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, *Salmo salar*. J. Fish Biol. 32, 129-136.

Schwarz, C., 2000. Intermediate Sampling and Experimental Design, 01-1 Term. Simon Fraser University.

Selye, H., 1956. The Stress of Life. Mc-Graw-Hill, New York.

Shavila, J., Ioannides, C., King, L.J., Parke, D.V., 1994. Effect of high fat diet on liver microsomal oxygenations in ferret. Xenobiotica 24, 1063-1076.

Sipes, I.G., Gandolfi, A.J., 1986. Biotransformation of Toxicants In: Klassen, C.D., Amdur, M.O., Doull, J. (Eds.), Casarett and Doull's Toxicology, The Basic Science of Poisons. Macmillan Publishing Company, New York. pp. 64-98.

Soldatov, A.A., 1996. The effect of hypoxia on red blood cells of flounder: a morphologic and autoradiographic study. J. Fish Biol. 48, 321-328.

Spannohof, L., Nasev, D., Kreutzmann, H.L., 1979. Early recognition of metabolic disturbances in trout (*Salmo gairdneri* Rich.) stocks. Aquaculture 18, 317-323.

Steffens, W., 1989. Principles of Fish Nutrition. Ellis Horwood Limited, New York.

Sumpter, J.P., Le Bail, P.Y., Pickering, A.D., Pottinger, T.G., Carragher, J.F., 1991. The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout , *Oncorhynchus mykiss*. Gen. Comp. Endocrin. 83, 94-102.

Tannenbaum, B.M., Brindley, D.N., Tannenbaum, G.S., Dallman, M.F., McArthur, M.D., Meaney, M.J., 1997. High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. Endocrinol. Metabol. 273, E1168-E1177.

Viganò, L., Arillo, A., Bagnasco, M., Bennicelli, C., Melodia, F., 1993. Xenobiotic metabolism enzymes in uninduced and induced rainbow trout (*Oncorhynchus mykiss*): effects of diets and food deprivation. Comp. Biochem. Physiol. 104C, 273-277.

Vijayan, M.M., Leatherland, J.F., 1990. High stocking density affects cortisol secretion and tissue distribution in brook charr, *Salvelinus fontinalis*. J. Endocrinol. 124, 311-318.

Vijayan, M.M., Moon, T.W., 1992. Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 49, 2260 - 2266.

Wall, K.L., Crivello, J., 1999. Effects of starvation on liver microsomal P450 activity in juvenile *Pleuronectes americanus.* Comp. Biochem. Physiol. 123C, 273-277.

Wan, R., Camandola, S., Mattson, M.P., 2003. Intermittent food deprivation improves cardiovascular and neuroendocrine response to stress in rats. J. Nutr. 133, 1921-1929.

Watanabe, T., Takeuchi, T., Ogino, C., 1979. Studies on the sparing effect of lipids on dietary protein in rainbow trout (*Salmo gairdneri*). Proc. World Symp. on Finfish Nutrition and Fishfeed Technology, Hamburg, Vol. I. pp 113-125.

Wilson, J.M., Vijayan, M.M., Kennedy, C.J., Iwama, G.K., Moon, T.W., 1998. ß-napthoflavone abolishes interrenal sensitivity to ACTH stimulation in rainbow trout. J. Endocrinol. 157, 63-70.

Wilson, R.P., 2002. Amino Acids and Proteins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, New York. pp. 259-308.

Wood, G.C., Woodcock, B.G., 1970. Effects of dietary protein deficiency on the conjugation of foreign compounds in rat liver. J. Pharm. Pharmac. 22, 60S-63S.

Wootton, R.J., 1998. Ecology of Teleosts Fishes. Kluwer Academic Publishers, Boston, MA.

Wright, P.A., Perry, S.F., Moon, T.W., 1989. Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. J. Exp. Biol. 147, 169-188.

Yang, S-D., Lin, T-S., Liou, C-H., Peng, H-K., 2003. Influence of dietary protein levels on growth performance, carcass composition and liver lipid classes of juvenile *Spinibarbus hollandi* (Oshima). Aquaculture Res. 34, 661-666.

Zakrzewski, S.F., 1997. Principles of Environmental Toxicology. American Chemical Society, Washington, DC.

Zhang, Y.S., Andersson, T., Förlin, L., 1990. Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by ß-napthoflavone. Time course studies. Comp. Biochem. Physiol. 95B, 247-253.