PROTEOMIC ANALYSIS OF THE EFFECTS OF DIET IN ZEBRAFISH LIVER

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David R. Jury
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PROTEOMIC ANALYSIS OF THE EFFECTS OF DIET IN ZEBRAFISH LIVER

David R. Jury

Thesis

Approved: ___________________________ Accepted: ___________________________

Advisor
Dr. Richard L. Londraville

Dean of the College
Dr. Ronald Levant

Committee Member
Dr. Brian P. Bagatto

Dean of the Graduate School
Dr. George R. Newkome

Committee Member
Dr. Qin Liu

Date

Interim Department Chair
Dr. Richard L. Londraville
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CHAPTER I
INTRODUCTION

Calorie availability pivotally affects metabolic rate, reproductive fitness, growth, and survival in fish (Tocher, 2003). Calories in the form of fatty acids are the most significant source of ATP for many species of fish. Accordingly, fish manipulate storage and mobilization of fatty acids as part of their natural history resulting in a variety of outcomes. Striped bass increase intracellular lipid droplets 13-fold in red muscle during cold acclimation (Egginton and Sidell, 1989) and salmon increase serum cholesterol without eating during spawning migrations (Farrell and Munt, 1983). The expression of fat metabolism genes is regulated by free long chain fatty acids and their metabolic by-products, however the definitive mechanism by which they do so remains elusive (Duplus et al., 2000). Characterizing the molecular signaling behind these changes in fat metabolism has traditionally been approached by looking at candidate proteins, (e.g. fatty acid binding proteins (FABPs) (Londraville and Sidell, 1995)) organelle function (peroxisomes (Crockett and Sidell, 1993)), and enzymatic indicators of fatty acid flux (Sidell et al., 1995). However, these are specific indicators, not an integrated global mechanism. One family of transcription factors, the peroxisome proliferated-activated receptors (PPARs), has the potential to attach a mechanism to the metabolic changes detailed above.
Acting as lipid sensors, PPARs are a nuclear hormone receptor superfamily of transcriptional regulators that are responsive to polyunsaturated fatty acids (PUFAs) (Issemann and Green, 1990), and their metabolic derivatives that act to orchestrate metabolism (Evans et al., 2004). Various PPAR isoforms have been linked to regulation of lipid metabolism from their associated pathologies in mammals (Bilsen et al., 2002). PPAR\(\alpha\) is a critical mediator in the rodent liver, responding to perturbation of lipid metabolism but also directly and indirectly directing genes involved in hepatocyte growth (Corton et al., 2000). PPAR's from fish were first identified in Atlantic salmon (Ruyter et al., 1997) and subsequently cloned (Andersen et al., 2000). PPAR's have also been identified in zebrafish (Escriva et al., 1997) with several homologous mammalian isoforms (PPAR\(\alpha\), PPAR\(\beta\), and PPAR\(\gamma\)) being conclusively identified by immunohistochemistry (Ibabe et al., 2002). I hypothesized that high-calorie diets in zebrafish will up-regulate activity of PPARs. I tested this hypothesis using proteomics to identify which proteins change with diet. I did not find an up-regulation of proteins under the control of PPARs but I did identify several proteins associated with stress.

Studying the effects of a single protein in isolation is limited in explanatory power because of the ways proteins interact in vivo. Proteomics quantitatively estimates alterations in protein expression within a tissue at a moment in time (Abbott, 1999). Its' power is that it focuses on the part of the genome that is expressed, (including the unexpected), thus more effectively estimating differences in expression among different groups or experimental
Proteomics’ utility is in sifting through thousands of proteins to find likely targets for further study. My approach was a hypothesis-generating step, to find these targets that could then be rigorously characterized with immunoblotting, nucleic acid hybridization, cloning, and sequence-functional expression in future studies.

**Lipid Metabolism**

Lipid homeostasis is the equilibrium among dietary influx and *de novo* biosynthesis, remodeling, storage, mobilization, and net metabolism (Tocher, 2003). Each process has individual regulators on a cellular, tissue, and organ level as well as overall regulation of all the processes for the entire organism. Individual regulation can be performed by saturated and unsaturated long-chain fatty acids turning on genes such as liver fatty acid binding protein (FABP) and carnitine acyl transferase (*Duplus et al.*, 2000). Overall regulation is accomplished in mammals by polyunsaturated fatty acids (PUFAs) that articulate gene transcription in the liver through direct and indirect mechanisms that affect membrane composition, eicosanoid production, oxidant stress, nuclear receptor activation and modification of transcription factors (Jump and Clarke, 1999). PUFAs bind and activate the PPARs transcriptional regulators (*Dreyer et al.*, 1992; *Zhang et al.*, 1992; *Lee et al.*, 1995; *Schoonjans et al.*, 1995). PPARs were originally discovered to be responsive to hypolipidemic drugs in rat liver (*Isseman and Green*, 1990). Among the genes regulated by PPARs, as demonstrated in mammals, are acyl-CoA oxidase, bifunctional enzyme, thiolase, and long-chain
fatty acid acetyl-CoA synthetase (Dreyer et al., 1992; Zhang et al., 1992; Lee et al., 1995; Schoonjans et al., 1995), fatty acid transport protein, fatty acid translocase, liver cytosolic fatty acid-binding protein, and lipoprotein lipase (Motojima et al., 1998).

Lipids are generally classed as either free fatty acids, polar phospholipids, which are critical to cell membrane function, or neutral triacylglycerols, (three fatty acids esterified to a glycerol) which are the storage form used by most vertebrates (Zakim and Boyer, 2003). Lipids, particularly triacylglycerol and its component fatty acids, are the primary source of metabolic energy for growth, reproduction, and swimming in fish. Fish lipid catabolism, absorption and mechanical transport are similar to mammalian physiology (Tocher, 2003). As in mammals, fish store lipids as triglycerides which can be transported systemically via albumin and chylomicra (Sheridan, 1994).

Fatty acids and amino acids signal the release of bile, which acts as an amphipathic surfactant in partnership with phospholipids and monoglycerides to emulsify fats in preparation for digestion by forming micelles, as in mammals (Zakim and Boyer, 2003). Micelles diffuse through the intestinal mucosa in the area of greatest lypolytic activity in the proximal small intestine and pyloric caeca if existent in the species by passive diffusion (Kapoor et al., 1975). In the endoplasmic reticulum of intestinal cells, the free fatty acids are reesterified with the glycerols, acyl glycerols, and lysosphosholipids to reintegrate these components back into triacylglycerols and phosphoglycerides (Sargent et al., 1989). Wax esters or sterols may be formed but this happens to a much lesser
degree than conversion to the other forms of lipid (Bauermeister and Sargent, 1979). Once this repackaging has occurred lipids are transported from the intestine in the form of lipoproteins. As in mammals the majority of these intestinal lipoproteins are carried through the lymphatic system with some being carried through a portal system directly to the liver (Sheridan et al., 1985). These lipoproteins have a range in size, structure, carrying capacity, and lipid affinities for the different classes (Sheridan, 1988; Babin and Vernier, 1989). The carrier protein for the fish is a plasma protein with a similar size, solubility, and electrophoretic mobility as serum albumin (Desmet, 1978). One lipoprotein unique to fish is vitellogenin, which has an even higher density than high-density lipoprotein (HDL) and is found in oviparous females and specimens injected with estrogen. Vitellogenin is synthesized in the liver and is transported to the ovaries (Wallace, 1985).

Free HDLs are taken up by the liver where the intracellular transport of component fatty acids and other hydrophobic ligands is facilitated by highly conserved cytoplasmic proteins known as fatty acid binding proteins (FABPs). There are various tissue-specific isoforms of FABP found notably in liver, adipose, brain and heart (Zakim and Boyer, 2003). Zebrafish liver FABP was similar to the FABPs that were found in the liver of catfish and chicken (Denovan-Wright et al., 2000).

In the liver fatty acids are reformed into triacylglycerols. Triacylglycerol synthesis is iterative esterification of fatty acids to glycerol-3-phosphate (G3P) via glycerophosphate acyltransferase followed by diacylglycerol acyltransferase in
mammals (Zakim and Boyer, 2003). G3P acyltransferase as well as the recovery of labeled intermediates were found in rainbow trout liver suggesting that fish possess a similar mechanism (Holub et al., 1975). Catabolically, triacylglycerol lipases have been demonstrated to be activated by bile salts in both marine and fresh water fish (Olsen and Ringo, 1997). Within the liver β-adrenergic and hormonal stimulation from epinephrine, norepinephrine, glucagon, cortisol, adrenocorticotropic hormone (ACTH), growth hormone, somatostatin, thyroxin, and prolactin, provoke triacylglycerol recruitment through hormone sensitive lipase, whereas insulin has the reverse effect (Sheridan, 1994).

Fatty acids from triglycerides are broken down by acetyl units in mitochondria and peroxisomes by a process known as β-oxidation. Medium and short chain FA can immediately enter the mitochondria but long chain FA bound to carnitine are coported by a carnitine acyl transferase in exchange for free carnitine (Zakim and Boyer, 2003). In Atlantic salmon, β-oxidation via peroxisomes was highest in liver, per gram of wet tissue (Froyland et al., 2000) and may account for up to 30% of liver β-oxidation activity in some species of Antarctic fish (Crockett and Sidell, 1993).

In fish 16:0 (16 carbons and 0 double bonds) and 18:0 are the dominant species of saturated FAs as they are the primary constituents of cell membranes in fish and other animals. But species of lengths of 12-24 can be observed (Tocher, 2003). The production of unsaturated fats 16:1 (n-7) (7 carbon atoms from the last double bond to the terminal methyl group) from 16:0 and 18:1 (n-9) from 18:0 takes place in fish, as all animals, in the endoplasmic reticulum in an
aerobic process with other oxidizing agents in various tissues (Zakim and Boyer, 2003). A second desaturation of the 18-carbon fatty acids is impossible in fish, as in all vertebrates and therefore 18:2 (n-6) and 18:3 (n-3) are essential fatty acids that must be present in the diet. However further elongation and desaturation of these essential fatty acids to 20:4 (n-6), 20:5 (n-3), and 22:6 (n-3) is possible and the ability to do so is inversely proportional to their ability to acquire them from their diets (Tocher, 2003). Interestingly most animals have desaturase enzyme activity at the 5th to 6th bond (as measured from the carboxyl group carbon) or at the 6th to 7th bond to accomplish their lipid rearranging. A zebrafish desaturase enzyme has been cloned that demonstrates activity for both, with twice the 6th to 7th activity than 5th to 6th activity (Hastings et al., 2001).

Phosphoglycerides are an important dietary lipid in fish as an essential element in biological cell membranes. Typically phosphoglycerides and fatty acid concentrations are more consistent then that of triacylglycerols (Tocher, 2003). Phospholipids anabolic processes are not well characterized in fish (Greene and Selivonchick, 1987) but the existing evidence would suggest that the pathways are similar to the better-understood mammalian model (Sargent et al., 1989).

Lipogenesis is the de novo formation of endogenous lipid from carbohydrate or protein sources. When circulating levels of glucose are high, insulin from the pancreas stimulates the liver to absorb glucose. Within the liver glucose can either be converted and stored as glycogen or oxidized to acyl-CoA. However, this is not reversible. In a high-calorie diet, a carbohydrate deficiency is created because there is no metabolically significant pathway for the conversion
of fat to carbohydrate. This can cause the liver to swell with fat, which physically removes storage capacity of glycogen and lead to ketosis (Zakim and Boyer, 2003). Generally, ketones are not metabolically important to teleost fish (Henderson and Sargent, 1985).

Protein metabolism in hepatocytes deaminates and oxidizes amino acids to acetyl-CoA (Zakim and Boyer, 2003). From the available pool of acetyl-CoA, cytosolic fatty acid synthetase (FAS) assembles fatty acids. FAS is the most important pathway in lipogenesis and has been described in fish, producing in particular, palmitic acid and stearic acid (Sargent et al., 1989). Other parts of this complex pathway such as the generation of NADPH are accomplished in fish most significantly by G6P dehydrogenase, and to a lesser degree malate dehydrogenase and various enzymes of the pentose pathway shunt (Dias et al., 1999 as cited in Tocher, 2003). These pathways are likely more pronounced in fresh water species than in marine due to fact that lipid in the diet is far less for the freshwater species (Tocher, 2003).

Liver: Function and Form

Liver is the primary organ of metabolism and largest gland in vertebrates (reviewed in Zakim and Boyer, 2003). Hepatocytes of liver tissue are rich in organelles, particularly peroxisomes for the metabolism of lipids. Hepatocytes are also extremely active in synthesis of plasma proteins, secretory and membrane bound proteins, the production of bile, endogenous and exogenous substance metabolism, and production of ascorbic acid (vitamin C). Liver is comprised of
several cell types, which include the hepatocyte, sinusoidal endothelial cells, Kupffer cells and Stellate or Ito cells. The three peripheral types of cells populate the lumen border of the hepatic sinusoids. Sinusoidal epithelial (SE) cells lack basement membranes providing fenestrations or pores for the passage of macromolecules between the space of Disse and the sinusoids. SE cells take part in the metabolism of cholesterol, lipoproteins and vitamin A, and produce prostaglandins. Kupffer cells are macrophages that occupy but do not completely block the fenestrations of the SEs and absorb damaged or unnecessary molecules. Stellate cells also known as perisinusoidal cells occupy space between the hepatocytes within the space of Disse just deep to the sinusoidal lumen and store fat and vitamin A. Also populating the sinusoids are lymphoid cells that provide the liver and ultimately the entire animal with an early defense against gut-derived and circulating antigens (Zakim and Boyer, 2003).

Liver hepatocyte function is intrinsically tied to its anatomical arrangement. The liver is organized into lobules that are hexagonically packed with the hepatic acini, the functional units of the liver. Within each acinus the labyrinth structure of sinusoids allows blood from branches of the portal vein to flow past hepatic cells to a central vein of each lobule. The high permeability of the sinusoids from the large gaps of the endothelial lining coupled with a single layer of hepatocytes on each sinusoid maximizes perfusion area (Zakim and Boyer, 2003).

Zebrafish hepatocyte plasmalemmal folds opposite to the endothelial lining of the sinusoids form a relatively small, as compared to other vertebrates,
perisinusoidal space of Disse. There are desmosomal connections between the hepatocytes and the mesothelial cells of the adjacent pancreas. Zebrafish hepatocytes lack basement membranes and instead fine filaments accumulate parallel to the intracellular and extracellular canaliculi. Starved zebrafish undergo hepatic atrophy as would be expected with the consumption of glycogen stores (Weis, 1972).

**Zebrafish**

Zebrafish (*Danio rerio*) are a tropical Cypriniform of the family Cyprinidae (Hamilton, 1822 as cited in Laale, 1977). Taxonomical characters of *D. rerio* include pharyngeal teeth in three rows, a terminal and superior mouth, an absent or incomplete lateral line, and a lack of preorbital process or frontal spines (Laale, 1977). Zebrafish are native to the Ganges and Brahmaputra rivers and their tributaries throughout Burma, Thailand, Indochina India, Northern Pakistan, Nepal, and Bhutan in Southeast Asia (Axelrod and Schultz, 1955). Zebrafish are omnivorous and thrive on diets of commercial food, plankton, *Paramecium*, white worms, mosquito larvae, tubifex, brine shrimp, daphnia or *Drosophila* (Laale, 1977).

Reproduction is energetically demanding for fish. The lipid content of striped bass eggs, to be used as an energy source for the developing embryo, is in excess of 55% of total egg weight (Eldridge *et al.*, 1983). The freshwater burbot (*Lota lota*, L) prepares for reproduction by storing large amounts of lipid in its liver (Karhapää, 1978 as cited in Mustonen *et al*. 2002), which are mobilized
prior to spawning for use in the gonads (Muss, 1968 as cited in Mustonen et al. 2002). In both cases lipid metabolism and net requirements are important. Zebrafish are an oviparous species with spawning occurring typically after lengths of 24.9 mm are reached in the female population and 23.1 mm in the male population, approximately 75 days after culture at 25.5 ± .2 ºC (Eaton and Farley, 1974a). Per spawning, typically between 150-400 but as many as 1800 eggs are laid 5 to 45 days from the last oviposition (Hisaoka and Firlit, 1962). Frequency of spawning and the number of eggs per pairing increases with the age of the fish if left continuously with a mate. Egg development is triggered by contact with the male and thus eggs cannot be taken from the female until male contact has occurred (Eaton and Farley, 1974b). The period of development from fertilization to hatching is 96 hours at 26°C (Hamilton, 1822 as cited in Laale, 1977).

Zebrafish have been used extensively in research due to ease of procurement and care and their ability to be kept together in large numbers incurring low maintenance costs. Under suitable conditions zebrafish will provide large numbers of emersible, non-adherent eggs that develop outside the mother's body allowing convenient accessibility for treatment or attention. The eggs are transparent allowing real time observation of development of internal organs (Briggs, 2002). Zebrafish are used in the study of development (Ingham, 1997), gene expression, toxicology, nervous tissue function, aging (Gerhard, 2003), blood and circulation (Berman et al., 2003; Lawson and Weinstein, 2002), behavior (Guo, 2004), hearing (Whitfield, 2002), cancer (Stern and Zon, 2003),
circadian rhythms (Pando and Sassone-Corsi, 2002), and small molecule studies (MacRae and Peterson, 2003). To my knowledge no group has investigated zebrafish liver using proteomics.

Proteomics

The fundamental experimental procedures of proteomics encompass first separating all proteins with two-dimensional electrophoresis gels followed by analysis of particular bands on those gels. Two-dimensional electrophoresis involves the separating of protein, first by charge and then by mass. Protein is first extracted from the tissue then the protein pellet is resuspended in a solution that denatures the protein while maintaining charge and facilitates absorption into an isoelectric focusing strip. This focusing strip contains an immobilized pH gradient, and when placed in an electric field, the proteins will separate and migrate based on their net charge until they reach the pH where net charge is zero (isoelectric point). Isoelectric focusing (IEF) can resolve proteins with high fidelity, (e.g. extraction of total protein from the same type of tissue gives consistently repeatable results, (Oguri et al., 2002). Proteins that have been separated with IEF can then be separated in the second dimension based on their molecular weights. The IEF strip is placed on a polyacrylamide gel with protein molecular weight standards. When put in an electric field the proteins will migrate from the IEF strip into a traditional SDS-PAGE gel and then separate according to their mass. This sequential resolution of proteins by charge and mass yields a ‘map’ of proteins spots or bands that can achieve separation of as
many as 1000 proteins simultaneously. After the two dimensional map is made
the next step is to identify individual proteins with mass spectrometry (Davidsson
et al., 2001).

Proteins are digested with a protease that generates component
peptides. The mass of each of these peptides is measured by mass
spectrometry, which measures the mass to charge ratio of gaseous ions. This
results in a mass spectrum (collection of masses of each peptide from the
digested protein), which is unique for each protein. Proteins can be identified by
their mass spectra by comparison to all proteins sequenced in a database (these
are ‘virtually’ digested and their mass spectra compared to that of the sample).
Proteins can be identified also by their component peptide amino acid sequences
via comparison to known sequence in a database (e.g. Genbank). Peptides are
ionized and fragmented with a shear gas, generating shorter peptides that differ
from one another by single amino acids. Measuring the precise mass of each of
these sub-peptides allows one to deduce amino acid sequence (i.e. the longest
peptide is 57 Da larger than the next largest fragmented peptide, and 57 Da is
the exact molecular weight of glycine, therefore glycine is the residue at that
position.) Using this approach the whole peptide is conceptually reconstructed
and the amino acid sequence is determined (Kinter and Sherman, 2000). Novel
proteins (unknown to databases) can be characterized using their peptide
sequence data to design cloning experiments with the reverse transcriptase
polymerase chain reaction.
Since its advent in 1975, proteomics has provided a tool for the purification and discrete separation of a heterogeneous mixture of proteins. Three primary caveats that hindered proteomic utility and deterred research were (1) a lack of genomic database information, (2) low throughput of mass spectrometry and (3) the difficulties in reproducing consistent gels.

Recent advances in molecular biology such as PCR (Taylor and Robinson, 1998), X-ray crystallography, DNA sequencing, transgenic animals (Galas and McCormack, 2003), gene microarrays, (Troyanskaya, 2005) and computer application power (Altman et al., 2002) have made an explosion in the knowledge base of genomics. Several genomes have been completely sequenced including human, (Collins et al., 2003) mouse, (Mouse Genome Sequencing Consortium, 2002) Drosophila, (Adams et al., 2000) and Fugu (Aparicio et al., 2002). The zebrafish genome is over half complete at 857,102,509 of 1,563,966,025 nucleotides as of 6/19/05 (Sanger Institute) and is predicted to be finished in 2006. As this data accumulates central storehouses of genes and gene fragments called expressed sequence tags (ESTs) have greater and greater power to correlate and identify new sequences. Thus peptide sequences identified through mass spectrometry can be quickly, and easily identified.

Mass spectrometry in recent years been heralded as an unrivaled tool to structurally characterize proteins with high sensitivity. Whether to investigate specific protein complexes or pursue more general profiling as a response to a treatment is an objective choice (Chalmers and Gaskell, 2000). If profiling a
system is the goal then the need for multiple analyses and importance of high-throughput becomes paramount. The recent improvements of the MALDI-TOF or matrix-assisted laser desorption ionization time-of-flight have hastened the speed of the old-style quadrupole filter by an order of magnitude (Griffin et al., 2001) and sensitivity by two orders of magnitude (Chalmers and Gaskell, 2000).

Producing repeatable gels is difficult even under ideal circumstances. Two things have been developed that minimize this problem. The first is the development and perfection of stabilized pH gradients utilized in the first step of isoelectrofocusing. Prior to 1982 the pH gradient was formed with carrier ampholytes that did not reliably position themselves in a consistent manner due to cathodic drift (Bjellqvist et al., 1982). Proteins would migrate along the gradient to their isoelectric point but from established gradient to another there was a high degree of variability (Görg et al., 1985). With the advent of commercially available stabilized gradients this problem was circumvented. The other problem was the subjective analysis of data collection from gels. Visual inspection can identify candidate bands that may exhibit a change between groups but being highly subjective was not reproducible from observer to observer or even necessarily by the same individual day to day. Several proteomic software packages exist that allow an unbiased algorithmic identification of bands from one gel to the next within a group. This allows the information about band size and density to be objectively gathered for analysis even from gels that are not perfectly or totally resolved.
CHAPTER II

MATERIALS AND METHODS

Animals

Wild-type, age-matched adult *Danio rerio* were purchased from Scientific Hatcheries Inc. (Huntington Beach, CA). A total of 104 Zebrafish were kept separated by gender and treatment (4 groups) in 10-gallon tanks with filtered, dechlorinated tap water at a constant 28.5°C. (26 fish per tank). Water quality was monitored for ammonia levels and temperature daily. Diets consisted of Scientific Hatcheries 3-pigment mash fed 3x/day (high-calorie) or 1x/week (low-calorie) at ~1% of body weight per feeding event via automatic feeders for 5 weeks. In the high-calorie treatment, this left a minimal excess of food, ensuring fish ate to satiety (excess food and feces removed daily). After completion of the diet treatment animals were euthanized by overdose of clove oil.

From all four groups, length and weight Size Indexing Variables (SIVs) were recorded from individuals allowing calculation and comparison of condition factor between treatments. From a subset of each population, whole-body total fat mass was determined via petroleum ether extraction (Folch, *et al.*, 1957) and total protein by Bradford assay (Bradford, 1976). From the remaining individuals, liver, heart and brain were harvested and snap frozen in liquid nitrogen immediately.
2-D electrophoresis

A 10% (w/v) homogenate of each zebrafish liver sample in 50 mM Tris (pH 7.8) was made and followed by sonication (5 sec at 5 W) to disrupt the cells within the sample and extract protein. Preliminary data indicated that individual liver homogenates were insufficient to achieve optimal protein loading hence I utilized from each treatment a series of discrete samples each composed of 2 to 3 randomly pooled liver homogenates. Relative protein concentration was determined with a protein assay (Bradford, 1976). I extracted the protein for the first dimension following the manufacturer's protocol for the Perfect-FOCUS™ kit (GenoTech, St. Louis, MO).

Extracted protein pellets were resuspended in a 2D suspension buffer consisting of 7 M Urea, 2 M Thiourea, 1% (v/v) t-octylphenoxypolyethoxyethanol (Triton X-100), 100 mM DTT, 1% (v/v) Pharmalyte, 1% (w/v) CHAPS, 1% (v/v) of Sigma Protease inhibitor cocktail, and a trace of Bromophenol blue. I actively rehydrated the resuspended protein into isoelectric strips at 50V overnight (BioRad Protein IEF Cell) to maximize absorbance into the IEF strip. I then focused the proteins to their isoelectric point for a total of 10000 Vhrs (gradient of 0-1000V for approximately 2.5 hours). Following focusing I equilibrated the strips in 50 mM Tris, 6 M urea, 30% (v/v) glycerol, and 1% (w/v) SDS, and 3.3 mM DTT for 10 minutes. A second equilibration was done in 50 mM Tris, 6 M urea, 35% v/v glycerol, and 1% (w/v) SDS, and 12 mM iodoacetamide for 10 minutes. The entire strip was placed on a 15% (BioRad Criterion) polyacrylamide gel for the second dimension next to a lane of protein molecular weight standards. The strip
was sealed with molten agarose and electrophoresed at 200V until the indicator dye ran off the gel. Gels were then fixed 50% ethanol, 10% acetic acid (v/v) overnight, followed by 3 X 10 min in distilled water wash, and stained with a colloidal Coomassie blue stain for approximately 72 hours with gentle agitation (Pierce GelCode Blue, Rockford, IL).

Stained gels were scanned with a transmittance scanner (Epson 2450, 3.3 Dynamic range) and analyzed with Melanie software (version 4.0, Genebio, Geneva, Switzerland). This software can quantify band volume (area*density) and normalize values relative to total staining intensity, correcting for gel-to-gel variation in total protein loaded. Band selecting algorithms matched proteins among replicate gels from established landmarks, accounting for local distortions in electrophoretic migration and pairing them to a master gel. Individual band volume data was pooled and manually selected to maximize sample size ($n \geq 2$) and minimize variation (standard deviation/average < .6) within a treatment (i.e. the same bands detected on multiple gels with minimal variation within a treatment). From this subset, proteins with greater than a two-fold treatment change factor were identified and submitted for identification using mass spectrometry.

**Mass spectrometry**

Each band to be identified was first digested by cutting it from the gel in a manner to minimize excess polyacrylamide; afterwards it was washed and destained in two aliquots of 50% ethanol/5% acetic acid for 1 hour each. The
bands were dehydrated in acetonitrile, washed in 0.1 M ammonium bicarbonate, and again dehydrated in acetonitrile before drying in a Speed-vac. Trypsin permeated the bands by rehydration in 5 µL of 20 ng/µL trypsin in 50 mM ammonium bicarbonate at room temperature for 15 min, followed by another 10 µL of 50 mM ammonium bicarbonate. Digestion was run to completion 5 hours to overnight at room temperature. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30µL 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to approximately 15µL for LC-MS analysis.

The tandem mass spectrometry LC-MS system is a Finnigan LTQ linear ion trap mass spectrometer (Finnigan LCQdeca ion trap, San Jose, CA) utilizing an operator packed 8 cm x 75 um id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. The individual extracts were injected and the peptides eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of about 1 µL/min. The microelectrospray ion source was operated at 4.0 kV. Digests were analyzed using the data dependent multitask capability of the instrument, acquiring full scan mass spectra to determine peptide molecular weights and collisionally induced dissociation (CID) product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 2000 CID spectra of ions ranging in abundance over several orders of magnitude, not all of which are derived from peptides.

The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program.
Mascot. All matching spectra were verified by manual interpretation. If the
search program was unable to identify the protein based on these spectra, then
the CID spectra were interpreted manually by a published strategy (Kinter and
Sherman, 2000) to determine the amino acid sequence.
CHAPTER III

RESULTS

Evaluating expression differences

Relative volume (pixel area * pixel density) of each band identified with Melanie was exported to Excel and sorted to eliminate bands that had less than a 200% difference, were not found in at least two gels from each treatment, and had more than 60% within treatment-variability (as quantified by standard deviation percent of mean :SDPM). A total of 28 bands met these criteria. For these 28 bands the average SDPM was 37.2% within the high-calorie group and 36.2% within the low-calorie group (e.g. on average, pixel volume for a given band within the high-calorie treatment varies 37.2% among gels). Thus to estimate a treatment effect, the difference between treatments must be larger than the highest SPDM within a treatment. I set this difference between treatments conservatively at 200%. In the above case, 37% of that difference is experimental or interindividual variation, and ~160% is due to the treatment. Due to time constraints, 20 proteins with the greatest fold change of the 28 were selected for mass spectrometry analysis. (Figure 1)
Comparison of high-calorie diet vs. low-calorie diet

The high-calorie diet treatment was compared to the low-calorie diet treatment in females to determine changes in protein. Bands focused in a minimum of two gels per treatment, and that had a 2.0 fold or greater change in expression were considered to be of interest. A total of 28 proteins met these criteria of which 20 were submitted. (Table 1, Figure 2). Previously identified zebrafish proteins (Londraville, pers. comm.), are included for reference.

General effects of diet on total protein number

An analysis to evaluate the global difference between treatments was performed. Melanie’s user-operated controls to affect band identification were set at smooth = 4, minimum area = 10, saliency = 150, and minimum % volume at greater than .0035% for all gels. An average of 650±51 (average ± standard error) protein bands were resolved within the low-calorie group and an average of 412±62 protein bands were resolved within the high-calorie group. The standard error as a percent of the mean is 7.9% and 15.1% respectively of total number of bands. A non parametric Wilcoxon Sign ranks test was used to determine the effects of diet on total number of expressed proteins (Figure 3.3). Total number of discrete proteins expressed decreases with high-calorie diet \((P=0.0135)\).
Effects of Diet on Size Indexing Variables and Condition Factor

Diet had an effect on several whole body measurements. Using the Wilcoxon Sign ranks test, high-calorie treatment females total body fat as a percent of dry weight ($P = 0.0275$) and condition factor ($P < 0.0001$) were found to be significantly larger than low-calorie females, but total micrograms protein ($P = 0.3447$) was not. High calorie treatment males were found to have significantly a larger total micrograms of protein ($P = 0.0243$) and condition factor ($P < 0.0001$) than low-calorie males, but a difference in total body fat as a percent of dry weight ($P = 0.2771$) was not detected. (Table 2).
Table 1. Proteins of interest

<table>
<thead>
<tr>
<th>Number</th>
<th>Identity</th>
<th>Accession Number</th>
<th>Fold Change in expression with high-calorie diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>unidentified</td>
<td></td>
<td>- 2.10</td>
</tr>
<tr>
<td>2</td>
<td>unidentified</td>
<td></td>
<td>- 2.14</td>
</tr>
<tr>
<td>3</td>
<td>thioredoxin</td>
<td>27881963</td>
<td>- 2.01</td>
</tr>
<tr>
<td>4</td>
<td>HSC70</td>
<td>1865782</td>
<td>- 2.36</td>
</tr>
<tr>
<td>5</td>
<td>prohibitin</td>
<td>33286931</td>
<td>+ 2.50</td>
</tr>
<tr>
<td>6</td>
<td>unidentified</td>
<td></td>
<td>- 2.75</td>
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<td>7</td>
<td>unknown protein</td>
<td></td>
<td>+ 2.04</td>
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<tr>
<td>8</td>
<td>unidentified</td>
<td></td>
<td>+ 2.50</td>
</tr>
<tr>
<td>9</td>
<td>unknown protein (Flavodoxin 2?)</td>
<td>41350996</td>
<td>+ 2.06</td>
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<td>10</td>
<td>glyoxylase II</td>
<td>37681823</td>
<td>+ 2.06</td>
</tr>
<tr>
<td>11</td>
<td>aldolase B</td>
<td>22671691</td>
<td>+ 3.26</td>
</tr>
</tbody>
</table>

Figure 1. A visual representation of the effect of a high-calorie diet on female zebrafish. The section of gel on the left comes from the high-calorie treatment and the gel section on the right comes from the low-calorie treatment. The numbers represent the proteins from table 1.
<table>
<thead>
<tr>
<th>Number</th>
<th>Identity</th>
<th>Accession Number</th>
<th>Fold Change in expression with high-calorie diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>glutamate oxaloacetate transaminase</td>
<td>29437228</td>
<td>+ 3.55</td>
</tr>
<tr>
<td>13</td>
<td>type I cytokeratin (danio rerio)</td>
<td></td>
<td>- 2.28</td>
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<tr>
<td>14</td>
<td>brain-type fatty acid binding protein</td>
<td>8809798</td>
<td>- 2.36</td>
</tr>
<tr>
<td>15</td>
<td>profilin 2</td>
<td>28278431</td>
<td>- 2.42</td>
</tr>
<tr>
<td>16</td>
<td>unidentified</td>
<td>49900827</td>
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</tr>
<tr>
<td>17</td>
<td>histone</td>
<td>6751</td>
<td>- 4.43</td>
</tr>
<tr>
<td>18</td>
<td>tandem repeat galectin</td>
<td>40288183</td>
<td>+ 2.05</td>
</tr>
<tr>
<td>19</td>
<td>KH domain contain</td>
<td>45709572</td>
<td>+ 2.40</td>
</tr>
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<td>20</td>
<td>NME2 protein</td>
<td>33416409</td>
<td>+ 2.85</td>
</tr>
<tr>
<td>21</td>
<td>transferrin</td>
<td>39645937</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>unidentified</td>
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<td></td>
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<tr>
<td>23</td>
<td>unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>carbonate dehydratase and Triosephosphate isomerase</td>
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<tr>
<td>25</td>
<td>CuZn superoxide dismutase</td>
<td>3021350</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>40S ribosomal protein S12</td>
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<td></td>
</tr>
<tr>
<td>27</td>
<td>HSP 60</td>
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</tr>
<tr>
<td>28</td>
<td>unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>fatty acid binding protein</td>
<td>17530523</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>ribosomal protein L22</td>
<td>15293911</td>
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Table 1. Proteins of interest cont.

<table>
<thead>
<tr>
<th>Number</th>
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<th>Accession Number</th>
<th>Fold Change in expression with high-calorie diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>translationally controlled tumor protein</td>
<td>Q9DGK4</td>
<td></td>
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<tr>
<td>32</td>
<td>eukaryotic translation initiation factor 5a and Ubiquitin</td>
<td>P10159, P5584</td>
<td></td>
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<tr>
<td>33</td>
<td>apolipoprotein</td>
<td>13591600</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>guanidinoacetate n-methyl transferase</td>
<td>33337348</td>
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</tr>
<tr>
<td>35</td>
<td>retinol binding protein</td>
<td>22296469</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>ribosomal protein L22</td>
<td>15293911</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>superoxide dismutase</td>
<td>20139980</td>
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</tr>
<tr>
<td>38</td>
<td>fructose bisphosphate</td>
<td>35902766</td>
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</tr>
<tr>
<td>39</td>
<td>4-hydroxypheny pyruvate dioxygenase</td>
<td>37681833</td>
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<tr>
<td>40</td>
<td>triosephosphate isomerase</td>
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<td></td>
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<tr>
<td>41</td>
<td>unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>myoglobin</td>
<td>127647</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>enolase</td>
<td>11999249</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>not identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>aldolase</td>
<td>226716591</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>cyclophilin?</td>
<td>37590335</td>
<td></td>
</tr>
</tbody>
</table>

These are from the high-calorie versus low-calorie gels analysis and previously identified proteins.
Figure 2. The 2D-E master gel, a low-calorie diet female zebrafish. Molecular weight standards on the left (kD). Green bands with upward flags indicate proteins that were up-regulated in the high-calorie group. Red bands with downward flags indicate proteins that were down-regulated in the high-calorie group. Purple bands with sideways flags indicate proteins previously identified (Londraville, pers. comm.). Numbers correspond to table 1.
Figure 3 Effects of high-calorie diet on the number of proteins expressed in female zebrafish. For the low-calorie diet n=13 and for the high-calorie diet n=9. Wilcoxon Sign ranks test analysis reveals a high-calorie diet decreases the number of bands ($P=0.0135$).
Table 2. Effects of high-calorie diet on whole body characteristics.

<table>
<thead>
<tr>
<th>Sex</th>
<th>treatment</th>
<th>fat % dry weight</th>
<th>total micrograms protein</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>high-calorie</td>
<td>30.6 ± 2.5 *</td>
<td>15.4 ± 2.0 (14)</td>
<td>1.48 ± 0.05 *</td>
</tr>
<tr>
<td>female</td>
<td>low-calorie</td>
<td>16.0 ± 6.7 (3)</td>
<td>11.8 ± 1.6 (3)</td>
<td>1.11 ± 0.03 (25)</td>
</tr>
<tr>
<td>male</td>
<td>high-calorie</td>
<td>26.7 ± 5.4 (11)</td>
<td>10.7 ± 0.5 * (14)</td>
<td>1.08 ± 0.03 * (26)</td>
</tr>
<tr>
<td>male</td>
<td>low-calorie</td>
<td>16.4 ± 2.7 (7)</td>
<td>8.0 ± 1.2 (8)</td>
<td>0.88 ± 0.02 (26)</td>
</tr>
</tbody>
</table>

The effects of high-calorie diet in zebrafish with respect to fat % dry weight, total protein, and condition factor expressed as mean ± standard error. An “*” indicates a significant difference as detected by the Wilcoxon sign rank test. Sample size for fat % dry weight and total protein and condition factor is given in parenthesis. Condition factor was defined as 100 times the mass divided by the cube of the length.
CHAPTER IV
DISCUSSION

Statistical Challenges

Improvements in proteomic focusing fidelity directly confound the statistical significance of produced data. With every discrete band focused another independent variable is created, building to hundreds of comparisons between treatments. Bonferroni corrections adjust for multiple comparisons, but reduce power, eventually to the point of being impractical. The common statistical assumption that the proteins being compared vary independently of one another is most certainly violated. These problems have been addressed in the literature (Sokal & Rohlf, 1981; Aebersold and Mann, 2003; Nesvizhskii and Aebersold 2004). In our lab we have taken a different approach to the problem by accepting these limitations and instead adopting a different conservative methodology (Edmonds, 2005). I elected to set a difference in band pixel volume (fold-difference) from one treatment to the other, such that it would demonstrate biological variation while accounting for gel-to-gel variation of precision. The gel-to-gel variation was found to be 37.2% within the high-calorie group and 36.2% within the low-calorie group and therefore I set my fold-difference at 200% (a minimum of 162% biological change) as a cutoff to select proteins for further analysis by mass spectrometry. This process generated a finite number of
proteins from which hypotheses can be generated and tested by more precise techniques.

**Stress in Fish**

Stressors, and the genetic potential of individuals to adapt to stressors, act upon every level of organization of life. Stress has a direct impact from the cellular level affecting biochemical reactions (Iwama *et al*., 1997), to individuals affecting behavior (Olla *et al*., 1980 as cited in Iwama *et al*., 1997) to entire populations affecting social hierarchy (Adams, 1990). Stressors such as food availability, water temperature and population density are generally thought to be the primary variables affecting fish growth. Stress in the form of relative health, water quality (e.g. concentration of contaminants), predation, and photoperiod, etc., all influence growth and general metabolism as well. (Weatherley and Gill, 1987).

Stress can be acute, with an instantaneous adaptive response or chronic where the animal is unable to completely acclimate (Wendelaar Bonga, 1997). The immediate acute response is the stimulation of the hypothalamic sympathetic chromaffin axis, which results in secretion of the catecholamines (CAs), epinephrine and norepinephrine from the chromaffin cells. As in mammals, chromaffin cells are modified nervous tissue cells which allow for a response that can be measured in seconds (Mazeaud and Mazeaud, 1981; Hoar *et al*., 1992). However fish lack an adrenal medulla and instead have randomly scattered cells at the head of the kidneys. These CAs act to mobilize fatty acids,
glycogen reserves, and increase oxygen uptake and redirect these resources away from growth and reproduction metabolism to an adaptive stress mechanism (Wendelaar Bonga, 1997). At the cellular level, the acute response to a stressor has been demonstrated to down-regulate expression of numerous individual proteins including proteins associated with protein metabolism, lipid metabolism, mineral metabolism (Ceciliani et al., 2002), growth, carbohydrate metabolism, hormonal regulation, pollutant detoxification, and mitochondrial proteins (Morel and Barouk 1999). Relevant to this study is that many different transcription factors (Morel and Barouk 1999), including PPARs themselves (Beigneux et al., 2000) are down-regulated as well.

The chronic stress response is characterized by in addition to elevated CAs, involvement of the hypothalamic-pituitary-interrenal axis. This axis involves a hormonal cascade of corticotrophic releasing hormone from the hypothalamus stimulating the release of adrenocorticotrophic hormone from the pituitary to stimulate release of cortisol from the interrenal cells that sporadically line the posterior cardinal veins. The response of this system to produce primarily cortisol is measured in minutes but may not achieve a maximal response for up to 4 hours (Hoar et al., 1992).

Cortisol is a consistent product of stress from chronic stressors (Wendelaar Bonga, 1997), and has broad effects on various growth inducing factors including gonadotrophin, estrogen, testosterone, growth hormone, and triiodothyronine (Iwama et al., 1997). Cortisol attenuates levels of gonadotrophin in both the pituitary and plasma (Carragher et al., 1989) and as
well as upon estrogen and testosterone (Foo and Lam, 1993a,b). The affects of stress on growth hormone (GH) are highly variable. Acute stress has been demonstrated to lower GH while chronic stress elevates GH in rainbow trout (Pickering et al., 1991). Where as Cook and Peter (1984) found that acute stress elevates plasma GH in goldfish. Cortisol was found to reduce free plasma triiodothyronine $T_3$ in eel *Anguilla anguilla* (Redding et al., 1986) and salmonids (Redding et al., 1984; Vijayan and Leatherland, 1989; Brown et al., 1991). Cortisol in fish serves as a glucocorticoid activating gluconeogenesis and lipolysis, increasing serum glucose and free fatty acids and as a mineralcorticoid to eliminate Na$^+$ and Cl$^-$ (Wendelaar Bonga, 1997). Treatment with cortisol is consistently associated with growth suppression and hyperglycemia (Butler, 1968; Chan and Woo, 1978; Lidman et al., 1979; Leach and Taylor, 1982; Davis et al., 1985; Barton et al., 1987; Vijayan and Leatherland, 1989; Foo and Lam, 1993a,b).

**Zebrafish on a High-Calorie Diet Expression Pattern Consistent with Stress**

The high-calorie diet treatment in females had significantly less total proteins expressed (see figure 3) as would be predicted from a stress response. Dietary induced obesity in rat has been demonstrated to increase oxidative stress (Beltowski et al., 2000) and low-calorie diets reduce oxidative stress in people with diabetes (Skrha et al., 2005). Low-calorie diets can be protective from other stressors such as irradiation and heat shock in mouse liver (Scrofano et al., 1998). Several specific proteins that fit into the schema of a stress
response were identified from this study (see figure 3.1). Cytokeratin, brain type fatty acid binding protein, profilin 2, and histone are associated with growth and were down regulated as part of the stress response in fish fed the high-calorie diet. Proteins up regulated relating directly to stress were glutamate oxaloacetate transaminase (GOT), glyoxylase II, and prohibitin. Cytokeratin is up-regulated in human neoplasias (Nakanuma et al., 2002) and growth-associated transcription factor binding elements have been identified in the 5’ region of the gene for brain type fatty acid binding protein in zebrafish (Liu et al., 2003). Profilins are involved with growth factor signal transduction (Sohn and Goldschmidt-Clermont 1994), and histone synthesis is necessary for cell replication (Polo and Almouzni, 2005). High levels of glutamate oxaloacetate transaminase has been used as a quantitative measure of stress in rats (Hemalatha et al., 2004). Glyoxylase II is part of a pathway that detoxifies oxidative stress compounds such as glyoxal, which is formed from lipid peroxidation (Thornalley, 1998). Prohibitin is up-regulated in chronically restrained rats (Liu et al., 2004) and quiescent hepatocytes in rats (Tanno et al., 1997).

In this study I hypothesized I would see an upregulation of PPAR regulated proteins. Of the identified proteins the brain-type FABP, that was down regulated in the high-calorie group was the only protein known to be transcriptionally controlled by PPARs. What I saw in the high-calorie diet treatment I interpret as a general stress response as exhibited by a lower total number of discrete expressed proteins. Within the high-calorie diet treatment I also saw specific proteins identified to be up-regulated that would indicate stress,
prohibitin and GOT and a series of proteins that being down-regulated would possibly indicate stress induced retarded growth; cytokeratin, brain type fatty acid binding protein, profilin 2, and histone.

Some Proteins Do Not Fit a General Stress Response

Particular proteins exhibit expression patterns that do not fit into a predictable stress response. Aldolase B, and galectin are positively associated with growth and were up-regulated in individuals fed the high-calorie diet. Aldolase b has been demonstrated to be up-regulated during low cortisol condition in the fish, *Fundulus heteroclitus* (Lawrence *et al.*, 2004) and galectin is up-regulated in human pancreatic tumors (Shen *et al.*, 2004). Thioredoxin and HSC70 were down regulated in fish fed the high-calorie diet. Thioredoxin is an antioxidant indicative of stress in mice (Okuyama *et al.*, 2005) and HSC70 has been measured as an indicator of stress in rats (Cvoro *et al.*, 2004). I would postulate that these proteins do not fit with the very general picture described above because as a broad integrated response not every predictable potential protein would be transcriptionally modulated by the treatment. Other possibilities include potentially new functions for these proteins and/or complicated interactions of pathways simultaneously expressed in the cell (e.g. a general down regulation of growth-associated pathways due to stress, but upregulation of aldolase because of its role in processing an intermediate of a stress pathway). It is almost certain our current understanding of cellular physiology artificially limits the method by which I grouped them, and that as we uncover more of the
pleotropic functions of these proteins (partly through studies like mine), new functions will be assigned to each individual protein.

Further research

This study demonstrated that a high-calorie diet induces a general stress response in female zebrafish. Several avenues of research directly present themselves from these findings. A stress response should elicit an increase in plasma cortisol as well as an increase total gill ventilation and perfusion (Hoar et al., 1992). These whole animal characteristics could be tested to verify what this study found. This study did not find an up-regulation of proteins known to be under the control of PPARs. However this may be in fact a limit of our knowledge in the actual domain of PPAR regulation rather than a lack of a PPAR activity from my treatment. Verifying this could be accomplished by injection of PPAR agonists (e.g. clofibrates) into matched populations of zebrafish and repeating the proteomics experiments. There were also several proteins not identified despite having good CID spectra. These proteins could be isolated by antibody studies and eventually cloned.


