CHARACTERIZATION OF VARIATION IN GROWTH PERFORMANCE IN INBRED STRAINS OF ZEBRAFISH (DANIO RERIO)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Although zebrafish have been a widely utilized model organism for several decades, there is little information available on genetic variation underlying physiological variation among the commonly used inbred strains. This study evaluated growth performance in response to fasting in six zebrafish strains [AB, TU, TL, SJA, WIK, and petstore (PET) zebrafish]. Fasting resulted in a decrease in whole blood glucose levels in PET, TL, and TU strains and did not affect glucose levels in AB, SJA, and WIK strains. Similarly, fasting had no effect on myostatin mRNA levels in AB, PET, TU, and WIK strains, but decreased *myostatin-1* and *-2* mRNA levels in SJA zebrafish. Fasting increased *myostatin-2* mRNA levels in TL zebrafish. These data demonstrate that growth performance variation is present between commonly used zebrafish strains and can help future research endeavors by highlighting the attributes of each strain so that the most fitting strain may be utilized.

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1.1. Introduction

Research studies utilizing model organisms are routinely faced with confounding intra-species genetic variation. The genetic lines of many model organisms have been maintained for decades and are subject to inbreeding to maintain a homogenous line for research purposes. Genetic variation that has developed between strains can potentially introduce an inconsistency in data interpretation, as well as results that are not repeatable in other strains. This artifact could be a significant detriment to the greater scientific community if not taken into account.

Zebrafish (*Danio rerio*) have become a popular model organism for developmental, metabolic, and physiological research. Zebrafish genetic stocks are maintained in a similar manner to lab mice (*Mus musculus*) with inbreeding and outbreeding regimes being utilized to maintain certain genetic qualities. Compared to more established model organisms, such as mice, there is less known about the inter-strain variation present in zebrafish strains. With the increase in prevalence of zebrafish research on metabolic and physiologic pathways, there is a need for evaluating potential variation in growth metabolism and physiological status that might be present between commonly used strains of zebrafish. This problem is specifically addressed herein by means of evaluating glucose metabolism and the regulation of a muscle growth regulator, myostatin, in response to caloric restriction.

Metabolic energy production and the regulation of intracellular pathways are essential for the proper maintenance of internal homeostasis. Glucose metabolism plays an important role in metabolic homeostasis and growth physiology, as glucose plays a role in providing energy for metabolic processes and regulates intermediates that take part in physiological pathways.

Glucose is a substrate important in all living organisms and plays vital roles in energy production and cellular respiration, as it contributes to energy production via glycolysis that results in the breakdown of glucose into smaller products and the eventual release of energy in the form of ATP. Pyruvate, the breakdown product from glycolysis contributes to the citric acid cycle that regulates fatty acid synthesis and breakdown, cellular respiration (oxidative phosphorylation), and gluconeogenesis. Glucose, itself, can affect the production of hormones that regulate glucose transport in and out of circulation (insulin and glucagon), which in turn affect several other pathways associated with growth. Given the importance of glucose in energy production, there is a strong case for it being a very important metabolite in growth physiology.

Overall body growth is known to be regulated in a multi-faceted manner by several anabolic (growth hormone, insulin-like growth factors, and testosterone) and catabolic factors (myostatin). Myostatin (mstn) has most commonly been described as a negative muscle growth regulator, but recent evidence has demonstrated that mstn plays many roles in growth and development beyond strictly regulating the cell cycle in muscle tissue (McPherron and Lee, 2002). Myostatin was first described in 1997 and has been observed in many species of animals since, such as mice (McPherron and Lee, 1997), cattle (McPherron et al., 1997), birds (Sato et al., 2006), and fish (Maccatrozzo et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001). The structure of myostatin is highly conserved (McPherron and Lee, 1997) and therefore it is hypothesized that the function is also highly conserved across taxa. A complete genome duplication, termed the fish specific genome duplication, is thought to have resulted in vast teleost diversification and presumptively resulted in paralogous versions of myostatin (Santini et al., 2009). Gene duplications can provide raw material for neofunctionalization, but duplicates can be rapidly lost due to redundancy in function. However, many are retained with new protein

localization or expression properties, as in the ubiquitous and differential expression of mstn in fishes. The multiple myostatin paralogues in fishes could allow for the development of different functions, without losing the original regulatory mechanism. These potential functions may include various energy conservation and physiological adaptations, and increased diversity in mstn function and regulation might be key to phenotypic variation observed within strains of zebrafish.

1.1.1. Overview of glucose

Glucose, the primary simple sugar or monosaccharide found in circulation, serves as the main metabolic energy source for most organisms, from bacteria to humans. In most vertebrates, glucose is the key source of energy providing approximately 3.75 kilocalories of food energy per gram through aerobic respiration. Through glycolysis and the citric acid cycle, glucose is eventually oxidized to form CO_2, water, and energy in the form of ATP. These metabolic pathways are interlinked and provide components for ensuing reactions as is shown in figure 1. The steps of glycolysis ultimately result in the production of pyruvate and ATP among other compounds. Pyruvate is converted to acetyl CoA by means of pyruvate dehydrogenase and is then transported to the mitochondria to become a substrate for the citric acid cycle. The citric acid cycle ultimately produces the reducing agent NADH, among many other components, which are precursors to biological synthesis. The reduction of NADH transports electrons (H^+) across the membrane (electron transport chain) to create an electrochemical proton gradient and chemical energy in the form of ATP. Glucose thus provides metabolic pathways with energy to drive biochemical oxidation/reduction reactions and precursors for synthetic physiological reactions. In addition, glucose serves critical roles in the production of proteins and in lipid metabolism once modified during glycolysis and the citric acid cycle. Many carbon backbones

are produced during glycolysis and cellular respiration that can be used to produce amino acids. The first step of the citric acid cycle produces citrate that can be cleaved with the help of ATP to be returned to acetyl CoA for use in fatty acid synthesis.

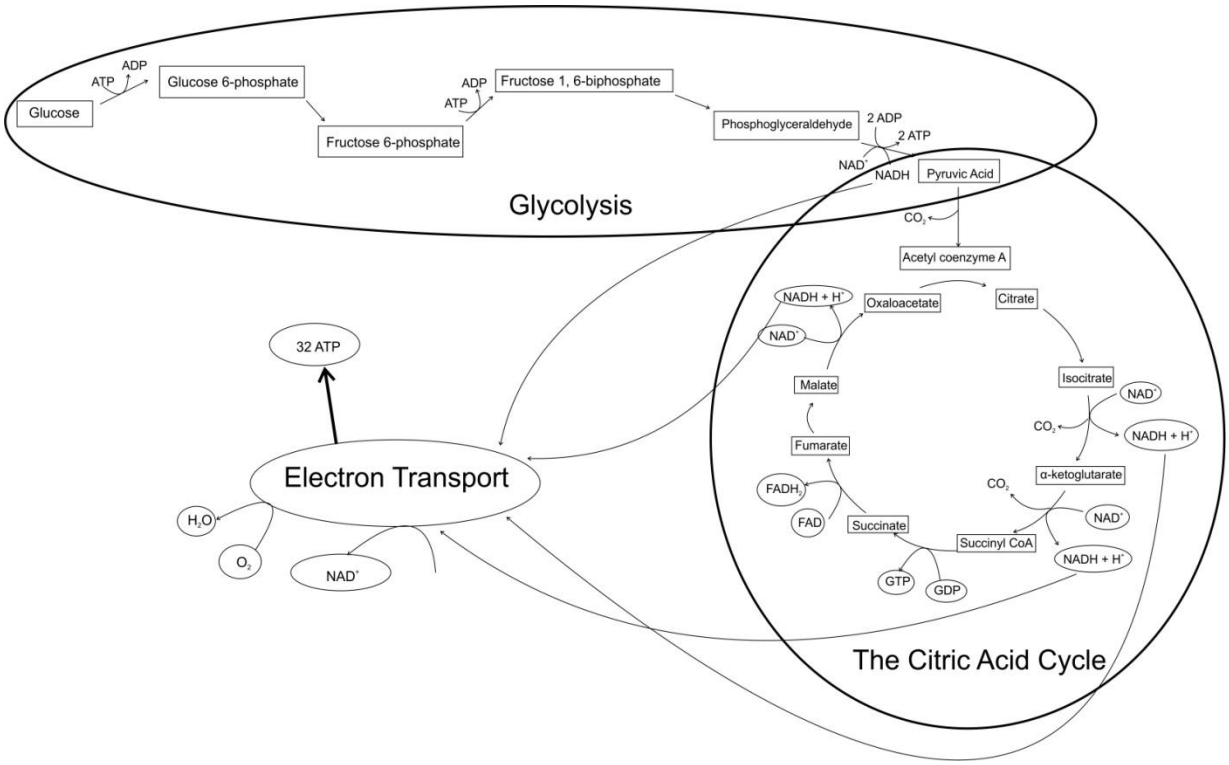

Figure 1: Cellular respiration. This figure shows the general outline of cellular respiration from glycolysis to the citric acid cycle to the electron transport chain ultimately resulting in energy in the form of ATP.

In mammals, changes in glucose concentrations in any small volume of blood depends on the rate at which glucose is introduced into circulating blood, the rate at which it is distributed throughout the blood circulation, and the rate at which it is removed from circulation (Zierler, 1999). Circulating glucose levels are generally tested in an intermediate level between feeding and starvation in what is known as the basal level. The basal level or state is simply a late postprandial period, or early starvation, in which stability in circulating glucose levels are

maintained to support steady-state energy utilization by the body's cells. Changes in steady-state blood glucose levels can then be utilized as a measure of the rate of glycolysis and therefore as a measure of metabolic status. Feeding status directly regulates circulating glucose levels and mechanisms for increasing blood glucose can be activated if too much time passes between feedings.

During periods of fasting, some organisms regulate circulating glucose levels by converting stored glycogen polymers into glucose monomers (glycogenolysis). This process normally takes place in the liver or muscle tissues where glycogen is stored, and it is triggered by epinephrine or glucagon in response to low blood glucose levels. In addition, organisms will regulate blood glucose levels by synthesizing glucose from non-carbohydrate carbon based substrates using a process called gluconeogenesis. This process is ubiquitous in animals and often takes place in the liver during periods of dietary restriction. The role of blood glucose in organismal processes is not limited to energy production, as glucose plays a key role as an intermediate in many molecular pathways.

Nutrients, such as glucose, are not used as just metabolic fuels, but they are also utilized in cell signaling by way of nutrient receptors. The level of blood glucose is used as the main control for the central metabolic hormone, insulin. Insulin is the primary regulatory signal of homeostasis in animals and when present, insulin causes cells to take up glucose from circulation. In addition, in some cell types, insulin regulates the storage of glucose internally in the form of glycogen, the uptake and storage of lipids, cellular electrolyte balance, and amino acid uptake (Akedo and Christensen, 1962). The level of circulating blood glucose is the most important signal to the insulin-producing cells of the pancreas and therefore circulating glucose levels serves as an internal signal on metabolic status. Because the level of circulatory glucose is

largely determined by the intake of dietary carbohydrates, diet controls major aspects of metabolism through the direct regulation of insulin levels. Other hormones that regulate blood glucose levels include somatostatin (Mazziotti et al., 2009), glucagon (Unger et al., 1962), epinephrine (Porte et al., 1966), cortisol (Chan and Woo, 1978), growth hormone (Johannsson et al., 1997), and adrenocorticotropic hormone (Heald et al., 1965). Many of these hormones are important for growth and are either regulated by or they regulate glucose levels in circulating blood. These can be specific to a particular nutrient and will relay information on physiological status to downstream targets to maintain homeostatic regulation of body processes (Craig and Moon, 2011). The depletion of circulating blood glucose has been correlated to a reduction in anabolic processes such as protein synthesis (Fulks et al., 1975; Li et al., 1979; Weekers et al., 2003; Vanhorebeek et al., 2005; Craig and Moon, 2011).

Glucose regulation of physiological pathways has been observed in relation to somatostatin, which is also known for its inhibitory regulation of many physiological processes by inhibiting growth hormone, thyroid-stimulating hormone, and several gastrointestinal hormones (Siler et al., 1973; Arimura et al., 1975; Ahren et al., 1978). Glucose has been shown to directly stimulate the expression of preprosomatostatin in a dose dependent manner (Ehrman et al., 2000), suggesting that glucose may be involved as an intermediate in physiological processes that regulate growth because somatostatin negatively regulates the main growth-promoting hormone, growth hormone. Collectively, the roles of glucose in metabolic and physiological homeostasis suggest that a reduction in dietary intake due to fasting could impact these metabolic and physiological responses greatly.

1.1.2. Glucose regulation in mammals

One physiological mechanism that allows mammals to regulate glucose levels in times of dietary restriction involves homeostatic hormones like insulin, glucagon, and growth hormone (Hansen and Johansen, 1970). Mammals have evolved the ability to regulate blood glucose levels with a combination of insulin and glucagon secretion (Lam et al., 2009). Insulin is secreted in response to high blood glucose levels to lower the risk of hyperglycemia and the negative impacts associated with this such as ketoacidosis kidney damage, and neurological conditions. When blood glucose levels rise, insulin is released from beta cells found in the Islets of Langerhans in the pancreas. Insulin causes cells in liver, muscle, and fat tissues to take up glucose from the blood, storing it in the liver and muscle as glycogen. Insulin stops the use of fat as an energy source by inhibiting the release of glucagon. When the glucose level falls below the basal level, insulin release from the β-cells slows and glucagon secretion from α-cells increases. In a postprandial (fed) state hepatocytes of the liver take in more glucose from the blood than they release, thereby increasing storage as glycogen. However, after a meal has been digested and glucose levels begin to decline, insulin levels are reduced and glycogen synthesis ceases. Conversely, glucagon is released in response to low blood glucose levels to induce the conversion of stored glycogen in the liver into glucose for release into the bloodstream in order to balance and maintain basal levels of glucose in circulation. Studies in fish have determined that there are differences in how glucose metabolism is regulated when compared to mammals.

1.1.3. Glucose regulation in fish

While the fundamental biochemical process of glucose metabolism has been well characterized in mammals, there is still debate as to the importance of glucose in fish metabolism. An increasing amount of research focused identifying the regulatory mechanisms of

glucose metabolism in fish models is surfacing (Elo et al., 2007; Eames et al., 2010). Research has suggested that carbohydrates are the key to metabolism of all vertebrates, including fishes (Moon and Foster, 1995); however, elasmobranch fishes show a much more lipid- and protein-centered metabolism which deemphasizes the importance of glucose (Ballantyne, 1997). In spiny dogfish (*Squalus acanthias*) insulin has been shown to decreased blood glucose levels to undetectable levels, without any apparent ill effects on the organisms (Deroos et al., 1985). Low blood glucose levels following exposure to mammalian insulin have been reported in trout (Cowey et al., 1977) and toadfish (Tashima and Cahill, 1968), without adverse effects of insulin shock in the fishes. In addition, evidence suggests that rainbow trout (*Oncorhynchus mykiss*) utilize carbohydrates poorly as prolonged postprandial hyperglycemia is detected following increased carbohydrate intake (Palmer and Ryman, 1972; Bergot, 1979). In addition, when dietary carbohydrate intake exceeds 25%, overall growth rates decrease (Palmer and Ryman, 1972; Bergot, 1979). Conversely, more recent evidence has suggested that this generalization about fish glucose utilization may not be accurate (Moon, 2001). Glucose tolerance tests using both oral and intraperitoneal glucose challenges demonstrate that glucose is utilized by all fish, but at a much slower rate among carnivorous fishes (Moon, 2001). Recent evidence demonstrates that in rainbow trout hepatic glucose phosphorylation is closely regulated by dietary carbohydrate levels, while hepatic gluconeogenesis is not (Panserat et al., 2001). Warm water omnivorous fish utilize glucose to a much greater extent than do cold-water carnivorous fish (Hemre et al., 1993; Wilson, 1994; Stone, 2003), as they have a diet that contains much higher levels of carbohydrates. Zebrafish, which are omnivorous, possess the ability to dynamically regulate glucose metabolism (Eames et al., 2010) in a manner that is similar to what is observed in mammals, as they respond to fasting with decreased blood glucose levels with a

return back to basal levels rapidly after being fed. These data suggest that zebrafish are suitable models for investigating glucose metabolism in response to a fast.

1.1.4. Glucose response to fasting

Fasting is associated with a series of physiological responses that protect body tissues from degradation by efficiently using expendable energy reserves while sparing protein (Champagne et al., 2006). In mammals, fasting causes a shift in fuel utilization from carbohydrates and fat in the fed state to almost exclusively fat in the fasting state. This is typically activated by a decrease in blood glucose levels with a subsequent rise in glucagon levels. Glucagon is secreted by the α-cells of the pancreas and causes glycogen stores in the liver to be converted to glucose that can be secreted into circulation to increase blood glucose levels. Recent studies have shown that a group of nuclear hormone receptors, the peroxisome proliferator–activated receptors (PPARs), play an important role in fatty acid and glucose metabolism (Desvergne et al., 1998; Picard and Auwerx, 2002; Lee et al., 2006; Liao et al., 2007). PPARα is a key part of a network of signaling pathways in the liver that is active during fasting to stimulate fatty acid oxidation to form substrates that can be metabolized by other tissues (Kersten et al., 1999). Long-term fasting in mammals is generally not a normal life history trait, except when it is accompanied by a reduction in metabolic rate as is observed in torpor or hibernation.

In many fish species however, long-term fasting bouts are normal activities undertaken during periods of overwintering, migration, or spawning. Mechanisms regulating metabolic homeostasis are therefore thought to be somewhat complex compared to non-hibernating mammals. Long-term responses to food deprivation in fish have been well studied, and evidence suggests that life history plays a huge role in defining a fish's ability to regulate glucose levels in

response to fasting. Fish with a primarily predatory lifestyle do not exhibit a dynamic regulation of blood glucose levels. For example, no changes in blood glucose levels were observed after several weeks of fasting in the hagfish (*Myxine glutinosa*) (Falkmer and Matty, 1966), or even months in species such as the American eel (*Anguilla rostrata*) (Suarez and Mommsen, 1987) or African catfish (*Clarias lazera*) (Navarro and Gutierrez, 1995). Investigations into fasting in omnivorous species have shown that there is much more variation in blood glucose levels than was previously reported among carnivorous species (Figueiredo-Garutti et al., 2002). This variation suggests that when a fish moves from a postprandial state to a fasting state there is an energy crunch not observed in carnivorous fish, which lack this reliance on blood glucose. This is overcome in the long term as energy reserves are mobilized to ease the detrimental effects of fasting, which is not observed in carnivorous fishes. While the omnivorous fish had more dramatic changes in blood glucose levels in response to a fast than was observed in other fish, they also exhibited a better ability to cope with dietary restriction by quickly adjusting their metabolic usage to make up for glucose deprivation.

The primary hormone responsible for regulating blood glucose in response to glucose deprivation is glucagon. The pancreatic α-cells secrete glucagon when blood glucose levels fall below basal level to initiate the conversion of stored glycogen into glucose. The structure of glucagon is highly conserved between fish and mammals (Plisetskaya and Mommsen, 1996), and as such was believed to be useful as an indicator of nutritional status in fish. However, in a study in rainbow trout, experimentally increased glucagon levels resulted in no significant changes in key metabolic pathways (Mommsen et al., 2001). These findings suggest that the use of glucagon as an indicator of nutritional status in fish should be used with particular awareness of the dietary habits and energy mobilization of the model organism used. In addition, these

findings indicate that baseline glucose levels are a more reliable metabolic marker to observe nutritional status.

1.1.5. Overview of myostatin

Myostatin is a member of the transforming growth factor beta (TGF-β) superfamily that was characterized in 1997 as a muscle growth inhibitor (McPherron et al., 1997; McPherron and Lee, 1997). In mammals, mstn is predominately expressed in skeletal muscle tissue (McPherron et al., 1997), which is also the primary target of mstn action in mammals. A lack of functional mstn in cattle, sheep, mice, domestic dogs, and humans dramatically increases muscle mass and produces a phenotype referred to as "double muscling' (Rodgers and Garikipati, 2008). The increased muscle mass in these animals is due to both hyperplastic (increased cellular number) and hypertrophic (increased cellular size) growth. In addition to increased overall muscle mass, mstn-null mice have reduced fat mass and are resistant to high fat-induced insulin resistance, indicating that inhibition of mstn may be favorable to whole body metabolism (Hocquette et al., 1999; McPherron and Lee, 2002; Carvan et al., 2004). Recent investigations have also demonstrated an effect of dietary intake on local mstn regulation in the spleen tissue of mice (Lyons et al., 2010). These findings suggest an intricate relationship between mstn and metabolism that has yet to be elucidated.

In contrast to mammalian species, mstn expression in piscine species is ubiquitous with the highest expression levels observed in skeletal muscle, brain, and heart (Ostbye et al., 2001; Rescan et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001; Roberts and Goetz, 2003; Garikipati et al., 2006). In addition, the developmental expression of mstn in fishes is dynamic (Maccatrozzo et al., 2001; Garikipati et al., 2007; Helterline et al., 2007), suggesting that the role of mstn in fish may be even more extensive than negative regulation of muscle growth. In teleost

fish, a genome duplication event resulted in two mstn paralogs, mstn-1 and mstn-2 (Garikipati et al., 2007), which was followed by another duplication event that further separated the mstn paralogs, mstn-1a, -1b, -2a, and -2b (Kerr et al., 2005; Garikipati et al., 2006; Garikipati et al., 2007; Ostbye et al., 2007). Rainbow trout and Atlantic salmon mstn-2b paralogs both contain in-frame stop codons in their first exons and are lacking a 51-bp cassette common to all fish mstn genes. In all fish that contain a mstn-2b paralog, it is known to be a pseudogene that is considered non-functional. The other mstn paralogs (-1a, -1b, and -2a) are differentially expressed and regulated in a variety of tissue types underlying many possible functions (Ostbye et al., 2001; Garikipati et al., 2007; Helterline et al., 2007; De Santis and Jerry, 2011). These genome duplication events have made possible potential redundancy allowing for one of the mstn paralog protein products to potentially exhibit functions that encompass an even more diverse role in muscle and metabolic regulation.

1.1.6. Myostatin response to fasting

As a regulator of growth and metabolic processes, mstn regulation is tightly coordinated by energy intake and thereby affected by a reduction of energy by way of fasting. Investigations focused on mstn expression responses to fasting in mammals have been inconclusive. An early study in juvenile pigs demonstrated no change in *mstn* levels in response to fasting (Ji et al., 1998). While this study did not show a response to fasting, the fasting event itself was only 3 days long and may not have provided an adequate insult to nutrition to elicit a physiological response with regard to mstn. Interestingly, an increase in *mstn* expression has been shown to be associated with every type of muscle atrophy examined to date (Carlson et al., 1999; Lang et al., 2001; Dasarathy et al., 2007; Chen et al., 2009; Plant et al., 2010), and it is well documented that food deprivation in mammals results in muscle atrophy (Orngreen et al., 2003). However, no

clear mechanistic role of mstn in regulating compensatory metabolic shifting in response to food deprivation has been identified. Both increases (Jeanplong et al., 2003; Allen et al., 2010) and decreases (Rodgers et al., 2003; Terova et al., 2006) in *mstn* expression have been reported in response to food deprivation scenarios, with variations depending on duration of deprivation and species tested. Much of the research conducted with mammalian organisms in regard to mstn expression and function has focused on different atrophic pathologies or differential dietary intake and not necessarily fasting per se. However, the data in the literature does suggest that mstn action would be increased in mammalian muscle in response to significant fasting as a method of energy conservation.

In many fish species, prolonged periods of food deprivation is a natural part of their life histories and is therefore utilized as a common strategy for evaluating metabolic responsiveness in regards to endocrine and growth factor function in many fish. In addition, fasting is a known stressor that influences the expression levels of *mstn* genes in teleost fish (Rodgers et al., 2003; Terova et al., 2006). As previously mentioned, *mstn-1* and *-2*, and respective paralogs, are differentially regulated in fish depending on the type of tissue and the age of the fish, suggesting that the response of *mstn-1* and *-2* to fasting could be different not only between the two genes, but within the paralogs depending on spatial location. This differential response to fasting was demonstrated in *Lates calcarifer* tissues, where *mstn-1* was increased in liver and muscle but decreased in brain tissue, while *mstn-2* expression was only upregulated in gill and liver tissue in response to fasting (De Santis and Jerry, 2011).

Many fish are able to tolerate extended periods of fasting with little physiological strain. This may stem from variation in life history that requires a fish to travel great distances in a migration, a reduction in food availability in response to temperature, or a reproductive event.

Research into how fish respond to fasting needs to be suitably in length for species and life stage to provide a sufficient metabolic effect. Even within species, discrepancies in *mstn* expression levels have been reported. In tilapia (*Oreochromis mossambicus*), larval *mstn* mRNA levels were sometimes elevated after a short-term fast and were consistently reduced with prolonged fasting (Rodgers et al., 2003). These results suggest that *mstn* mRNA levels may initially rise during a fast but ultimately fall after an extended period of fasting. This same study observed little change in adult *mstn* mRNA levels when fasted for 30 days. The discrepancy between adult and larval tilapia suggests that fish with strict dietary intakes are more likely to exhibit sensitivity in *mstn* regulation in response to fasting. Larval tilapia rapidly grow to aid in a greater success of survival, while the life history for tilapia suggests that as adults they are capable of dealing with extended periods of time with limited nutritional resources by 'riding them out'. Long-term studies in sea bass (*Dicentrarchus labrax*) demonstrated an increase in *mstn* levels in response to fasting and decreased levels upon refeeding (Terova et al., 2006). With these studies in mind it is expected that zebrafish should show an increased level of *mstn* in response to a sufficiently long fast. Zebrafish do not generally fast for extended periods of time, suggesting the time from the start of fasting to the metabolic response will be limited.

1.1.7. Myostatin and glucose

Fasting has been shown to reduce blood glucose levels while mstn levels generally increase soon after caloric restriction. This paired trend suggests that there may be a scenario in which glucose and mstn are dependent on each other in regards to regulation and potentially action. Over time, as metabolic status is maintained during the fast, initial levels of blood glucose rise with the mobilization of glycogen stores. As was previously discussed in larval tilapia, there

was a decrease in mstn levels as the duration of the fasting progressed, suggesting a correlation with glucose increases.

Removal of functional mstn has generated genetic mouse models of obesity and diabetes as these mice lacking functional mstn exhibit improved glucose metabolism (McPherron and Lee, 2002). In fact, *mstn* null mice are resistant to high-fat diet induced obesity (Zhao et al., 2005; Hamrick et al., 2006; Yang and Zhao, 2006; Guo et al., 2009; Wilkes et al., 2009), suggesting a predominate role of mstn in energy metabolism regulation. Furthermore, recent studies have also provided direct evidence that loss of mstn function increases glucose utilization and improves insulin sensitivity (Guo et al., 2009; Wilkes et al., 2009). Inhibition of mstn function can cause major metabolic alterations (Lin et al., 2002; McPherron and Lee, 2002; Zimmers et al., 2002), suggesting that mstn may potentially act in maintaining energy homeostasis. Recently, research done in mice has shown that mstn can directly regulate glucose metabolism by promoting glycolysis and glucose uptake as well as reducing glycogen stores in C2C12 myotubes (Chen et al., 2010). Therefore mstn is a key target for understanding metabolic regulation, and this suggests an area of study which may help shed light on how it is involved in regulating compensatory metabolic shifting.

1.2. Zebrafish as a Model Organism

Zebrafish are becoming a useful model organism due to their rapid growth, readily observable embryo development (Kimmel et al., 1995), and fully sequenced genome which shares many important homologies with mammalian model organisms (Vascotto et al., 1997). Another key factor that contributes to the widespread use of the zebrafish is the availability of mutant strains developed by chemical screens (Granato and Nusslein-Volhard, 1996) and uniparental, gynogenetic screens (Cheng and Moore, 1997). Zebrafish are relatively easy to care

for and to house in large numbers as well as generally being prolific breeders which can help to maintain a colony long-term. Zebrafish pancreas specification and morphogenesis have been well studied making this organism attractive for further development into a physiological model of pancreas function (Kinkel and Prince, 2009; Tiso et al., 2009). The zebrafish islet is similar in structure to human islets making them a reasonable research model for metabolism related research (Li et al., 2009).

Because of their widespread use in scientific research, many strains of zebrafish have been developed and are maintained in a stock center. The Zebrafish International Research Center is a clearing house for many inbred and outbred strains of zebrafish, as well as many transgenic zebrafish that have been generated for specific research purposes. Within the scientific literature there is a wide diversity in zebrafish strains that are utilized, and often times, the origin or genetic background of the specific zebrafish used is not listed. When details of the strains that were utilized have been mentioned, they are typically described as outbred and genetically heterogeneous (Gerhard et al., 2002; Maack et al., 2003). However, the majority of studies have given little attention to the genetic variability among these fairly diverse strains. In addition, many studies have also utilized zebrafish purchased from commercial dealers like local pet stores where little to no genetic background is known.

A recent study in zebrafish demonstrated a significantly lower level of genetic variation in inbred zebrafish strains when compared to wild-type or outbred strains (Coe et al., 2009). It has generally been observed that there is a negative impact on reduction in genetic diversity on population fitness (Reed and Frankham, 2003). Correlations between genetic diversity and population fitness have been drawn from studies focusing on traits such as growth, survival, and reproductive success in birds (Bellinger et al., 2003), mammals (Charpentier et al., 2005;

Fritzsche et al., 2006) and fish (Shikano and Taniguchi, 2002; Primmer et al., 2003). In all of these studies, a negative influence was associated with loss of genetic diversity. With this in mind, it is important that genetic variation be considered when assessing physiological effects in different strains of inbred laboratory zebrafish as well as outbred or wild-type strains. Reduction in genetic diversity and the subsequent negative influence could lead to misleading results when the information gained is used to characterize the species as a whole when it only truly applies to a small subset of the greater whole.

To date, no studies have been performed to examine the variations in glucose metabolism or muscle physiology present between strains of zebrafish used as model organisms for studies of physiology and disease. Understanding the strain-to-strain differences in glucose metabolism will enable researchers to make informed decisions regarding strain selection for future studies. Lack of attention to the genetic background of the model organism negatively impacts the scientific landscape through gaps in the knowledge of the scientific community with regard to potential artifacts in strains that can alter experimental results. The research proposed herein will contribute to the scientific landscape through evaluating strain-to-strain variation in glucose metabolism and muscle physiology in commonly used zebrafish lines.

CHAPTER 2. INBRED STRAINS OF ZEBRAFISH EXHIBIT VARIATION IN GROWTH PERFORMANCE AND MYOSTATIN EXPRESSION FOLLOWING FASTING

2.1. Introduction

In recent decades zebrafish (*Danio rerio*) have become one of the most widely used organisms for research in developmental biology and molecular physiology. Zebrafish exhibit several characteristics, including rapid embryonic development and readily observable embryos, that make them excellent model organisms for effective experimental use (Kimmel et al., 1995). The zebrafish has also been used successfully as a model organism because of the availability of mutant strains developed by chemical screens and the genetic information available from the their sequenced genome (Granato and Nusslein-Volhard, 1996) and uniparental, gynogenetic screens (Cheng and Moore, 1997). Due to their vast use in research, several inbred strains of zebrafish are available through a genetic stock center, the Zebrafish International Research Center (ZIRC, www.zebrafish.org). ZIRC maintains eight inbred wild-type lines, numerous mutant strains, and several outbred strains. These strains are widely utilized by the research community, however many research groups often use zebrafish purchased from local pet stores or fish that are gathered in the wild and brought into the laboratory setting. There is limited information on the physiological variation among different strains of zebrafish. The potential for variation in strain background and the availability of many strains begs the question: are there artifacts related to strain selection in growth performance of zebrafish?

Phenotypic variation among inbred strains of organisms has been well documented in many laboratory model organisms from prokaryotes and viruses to vertebrates such as birds and mice. Several studies have reported genetic variability in laboratory mice (*Mus musculus*) with respect to growth, hormone sensitivity (Spearow, 1988a; Spearow, 1988b; Spearow et al., 1999),

genetic variation in susceptibility to endocrine disruption by estrogen in mice, fecundity (Silver, 1995), and immune cell populations (Petkova et al., 2008). Genetic variability has also been studied extensively in other laboratory model organisms such as *Drosophila*, many species of bacteria, and viruses (Lewontin and Hubby, 1966; Phillips et al., 1991; Robertson and Meyer, 1992). Studies focusing on strain variation in zebrafish are more limited. Previous studies in zebrafish larval development have reported variation between strains when they are exposed to a teratogen such as ethanol (Loucks and Carvan, 2004). Inter-strain differences in zebrafish wild-type strains can be readily observed with simple visual inspection in many cases when appropriate strains are chosen. Previous research on behavioral traits between domesticated laboratory strains and wild-derived strains has also demonstrated phenotypic differences between a wild-derived line and the AB line in boldness and shoaling behaviors (Wright et al., 2006). Interestingly, the majority of inter-strain behavioral differences are maintained when different strains are reared together indicating a genetic basis for the phenotypic variation between zebrafish strains (Robison and Rowland, 2005).

Strain variation in growth and metabolic performance has been previously documented in murine models (West et al., 1992; Lewis et al., 2007). A recent study in inbred strains of mice demonstrated that dietary composition affects growth performance and growth-related gene expression in a strain-specific manner (Lyons et al., 2010). Lyons and colleagues demonstrated that myostatin (mstn) was regulated differentially based upon genetic background. Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily, and is known to specifically inhibit skeletal muscle development in mammals (McPherron and Lee, 1997). Myostatin is therefore considered a useful indicator of potential muscle regulation and can be used to observe variation in somatic growth and muscle development. There are multiple isoforms of mstn

present in fish due to multiple genome duplication events and these isoforms are differentially regulated (Ostbye et al., 2001; Rescan et al., 2001; Roberts and Goetz, 2001; Biga et al., 2005; Kerr et al., 2005; Ostbye et al., 2007). This suggests that rather than having a redundant mstn gene, the duplicated copies of mstn has developed a different function in fish beyond simply inhibiting muscle growth. Previous studies in teleost fish have shown that *mstn* mRNA expression increases with fasting and subsequently decreases after re-feeding (Terova et al., 2006). This pattern would be consistent with the mammalian model of energy conservation following a fast with muscle growth being limited during depressed nutrient intake and muscle growth promoted to higher levels following re-feeding. Myostatin expression is a good indicator of growth because of its role in energy partitioning, but a more comprehensive look would require examining mstn in conjunction with a metabolite responsible for energy in the body, namely glucose.

Glucose is the primary metabolic energy source for most vertebrates. Zebrafish can serve as a model of vertebrate glucose metabolism due to their fast glucose clearance and dynamic regulation of this important metabolite (Eames et al., 2010). Glucose homeostasis has a significant influence on growth and development (Robison et al., 2008) and, as such, can be used as a good analytical tool for investigating inter-strain metabolic variation in the zebrafish model.

A better understanding of strain-specific growth variation would be of immense help to the zebrafish research community. Without it, evaluating differential population responses or laboratory-to-laboratory variation may be confounded. To fully evaluate the significance and implications of research performed on select strains of a model organism, it is important for the scientific community to be cognizant of the variations among strains and the potential influence of those variations on experimental results. A great effort has been made to characterize the

strain differences in many more established model organisms. The relatively recent emergence of zebrafish as a model organism within the last few decades has led to great strides in developmental studies; however, there has been minimal effort, to date, on differences in growth and glucose metabolism manifested between the wild-type strains that are commonly used for developmental and physiological research.

For this study, we utilized the five most commonly used inbred strains of zebrafish, along with a common pet store variety, to examine variations in growth performance among strains in response to a fasting event as a metabolic insult (fasting). Following fasting, variation among strains was quantified using molecular and physiological end-points. We evaluated differential expression of mstn genes as well as compared growth attenuation by changes in overall fish length and metabolic status by differences in blood glucose values. We have identified several variations in growth performance among these select zebrafish strains, highlighting the need for careful strain selection during the development of an experiment.

2.2. Materials and Methods

2.2.1. Animal care

Male and female zebrafish (*Danio rerio*) were obtained from the genetic stock center or from a Mid-western pet distributor (Apet). Zebrafish embryos from the Tuebingen (TU), Tupfel long fin (TL), AB, SJA, and WIK strains were obtained from the Zebrafish International Resource Center located at the University of Oregon (Portland, OR). Embryos were raised to maturity and maintained as described in *The Zebrafish Book* at 28°C and a 14:10 light:dark cycle (Westerfield, 2000). All juvenile and adult fish were housed in 10-liter tanks in a recirculating freshwater system (Marine Biotechnologies, Aquatic EcoSystems) maintained at 28°C under a 12 h light: 12 h dark photoperiod. Zebrafish were fed twice daily to apparent satiation with a

semi-moist pellet food (Zeigler® Adult Zebrafish Diet) until experiment was started. Prior to sampling, fish were overanesthetized with buffered tricaine methylsulfonate (MS-222, Sigma). All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at North Dakota State University, Fargo.

2.2.2. Metabolic insult

Mature zebrafish (0.2 – 0.35 g) were randomly assigned to one of eight groups (n=6 fish per group): 0 hour, 3 day, 5 day, 7 day fasting and fed groups, and a 7 day postprandial group. This experimental setup was modeled after previously described fasting experiment in zebrafish demonstrating effects of this fasting regime on key metabolic and food intake regulating pathways (Amole and Unniappan, 2009). Zebrafish in the fed treatment group were fed to satiation daily with Zeigler® Adult Zebrafish Diet (55% crude protein, 15% crude fat, 1.5% crude fiber, 12% moisture), while the fasted groups were not given any food. The 7-day postprandial group was fasted for 7 days and fed to apparent satiation the morning of day 7. The postprandial group was sampled 4 hours following the refeeding period.

2.2.3. Measurements and tissue sampling

To assess overall physiological effects of fasting on zebrafish, whole body weight (g) and length (mm) measurements were collected on days 3, 5, and 7 of the experimental regime. Fish length was measured from the anterior-most region of the caudal peduncle, using digital calipers. To evaluate the effects of fasting on metabolic status, whole blood was collected in a heparinized capillary tube after severing the caudal peduncle. Whole blood glucose values were analyzed using a commercial Accu-Chek® Advantage (Roche Diagnostics) blood glucose meter with Comfort Curve test strips as previously described (Eames, Philipson et al. 2010). For analysis of *myostatin* gene expression, tissue samples (muscle and spleen) were collected and immediately

frozen at -80°C. Total RNA was isolated using RNAzol®RT (Molecular Research Center) according to manufacturer's instructions. Total RNA concentration was determined using a NanoDrop Spectrophotometer (ThermoFisher). Total RNA samples were aliquoted and stored at -80°C for subsequent analysis.

2.2.4. Quantitative real-time PCR

Real-time quantitative PCR (qPCR) was used to evaluate *mstn* mRNA expression differences in muscle and spleen tissues between zebrafish strains following fasting. Briefly, total RNA samples were reverse transcribed into cDNA using oligo dT and the ImProm-II™ Reverse Transcription System (Promega, Madison, WI). The cDNA strands were used as a template for qPCR analysis using an Applied Biosystems 7500 Fast Real-Time PCR machine and the following cycling parameters: 95°C for 2 min, 40 cycles of 95°C for 15 s, 52°C for 15 s, and 72°C for 30 s. Conditions were similar for all PCR assays, no template controls were run for all primer pairs, dissociation curves were run to ensure single product detection, and all samples were tested for genomic DNA contamination by running no RT controls. All assays were normalized to starting input cDNA quantity and primer PCR efficiencies were calculated for each primer:tissue pair. Absolute quantification was conducted using standard curves generated for each *mstn* isoform. The following primers were used for specific analysis of *myostatin* isoform mRNA expression: *mstn-1*, forward primer (5'-GTCCGGTGCGTGGTGAGGTTC-3'), reverse primer (5'-AGCTCGCTTTCCTCCGTGGC-3'); *mstn-2*, forward primer (5'-ACAAGGCCAACCCAAGGGGC-3'), reverse primer (5'-AACCGCAAAGGTCTACCACCATCG-3'). These primers were previously validated (Biga and Meyer, 2009).

2.2.5. Statistical analysis

All normalized qPCR data were analyzed using the standard curve method where cycle threshold values were compared to standard curves generated and validated for each primer pair to result in a relative starting amount of mRNA (copy number). Two-way analysis of variance (ANOVA) was performed to analyze morphologic, metabolic, and gene expression data, with factors being treatment and time. Post-hoc multiple comparison tests were conducted using Bonferroni's post-tests. T-tests were utilized to compare baseline control levels to day 7 re-fed levels for each measure to ensure that the experimental setup was valid and re-feeding could restore levels to baseline. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All results were considered significant at $P<0.05$.

2.3. Results

2.3.1. Morphological analysis

Following a 7-day fasting regime, dry body weight and length measurements were collected to ascertain the whole body physiological responses to nutritional insult in 6 strains of zebrafish. Fasting had no effect on whole body weight (Figure 2a) in PET, SJA, TL, or WIK zebrafish strains. Overall, fasting decreased body weight compared to fed control animals in both AB (0.218±0.010 vs. 0.271±0.021 g, respectively) and TU (0.269±0.014 vs. 0.343±0.017 g, respectively) zebrafish strains. Fasting had no effect on body length (Figure 2b) in AB, PET, SJA, TL, or WIK zebrafish strains. Overall, fasting decreased body length in TU zebrafish compared to fed control animals (28.78±0.70 vs. 30.72±0.35 mm, respectively). In addition, refeeding after a 7-d fast had no effect on body weight or length compared to the control period.

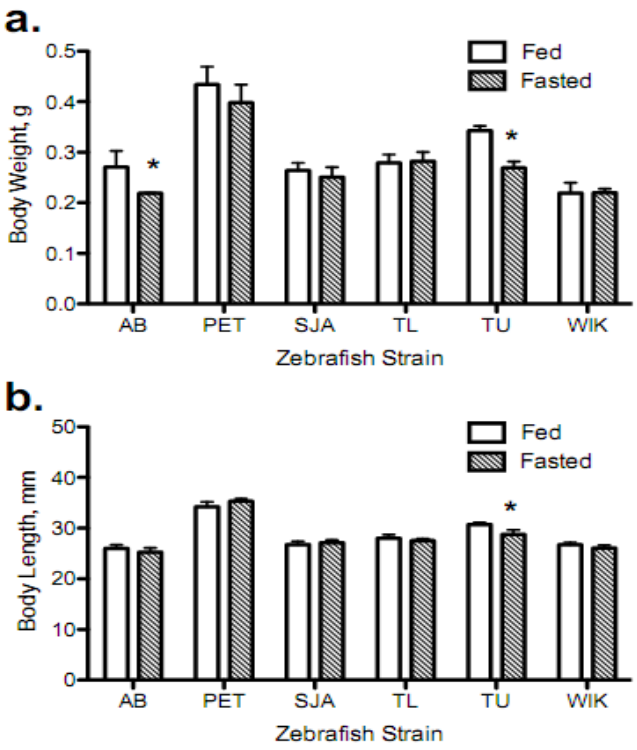

Figure 2: Morphological analysis following a 7-day fasting regime. Whole body weight (a) and body length (b) of fed controls (open bars) compared to the fasted group (shaded bars). Data are shown as mean ± SEM (n=6: *p<0.05).

2.3.2. Metabolic analysis

2.3.2.1. Whole blood glucose analysis. Whole blood glucose was measured following 3, 5, or 7 days of fasting or feeding using a commercially available handheld glucose meter. Overall, fasting decreased whole blood glucose levels in PET, TL, and TU zebrafish strains (Figure 3a). Whole blood glucose levels were decreased 47.0% in PET (77.61±5.85 to 40.94±3.38 mg/dL), 31.7% in TL (63.00±7.58 to 43.50±6.11 mg/dL), and 46.2% in TU (78.28±6.36 to 42.06±5.74 mg/dL) zebrafish strains. Whole blood glucose levels were not affected by fasting in AB, SJA, or WIK strains of zebrafish in response to food deprivation (Figure 3a).

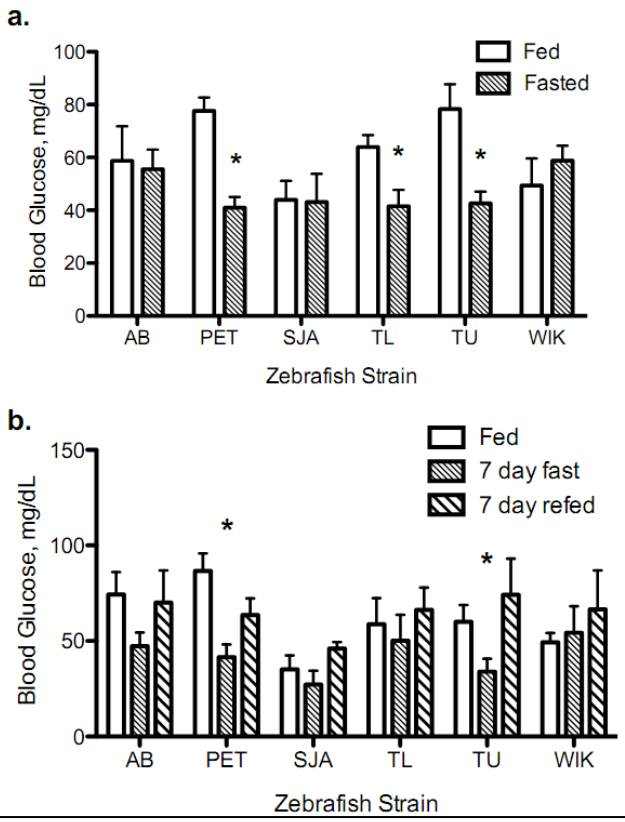

Figure 3: Metabolic analysis of overall whole blood glucose levels of fed controls and fasted zebrafish. (a) Whole blood glucose levels from six different strains of zebrafish were fed (open bars) or fasted (shaded bars) for 7 days. (b) Whole blood glucose levels following 7-day fast with a subsequent 7-day fast/refed group. Results are mean glucose mg/dL ± SEM (n=6: *$p<0.05$).

Glucose levels on individual days following fasting (3, 5, and 7 d) were decreased in PET, TL, and TU zebrafish (data not shown) as seen with overall changes in glucose levels (Figure 3a). Refeeding after 7 days of fasting returned whole blood glucose levels to control fed levels in all strains measured (Figure 3b). Following 7 days of fasting, refeeding to apparent satiation in PET and TU zebrafish returned whole blood glucose levels to control (fed) levels, while no effect was detected in zebrafish strains that fasting resulted in no change in glucose levels (Figure 3b).

2.3.2.2. Myostatin gene expression analysis. Using quantitative real-time PCR, we evaluated the effects of fasting on *mstn* mRNA expression in six strains of zebrafish. Overall, fasting significantly decreased *mstn-1* and *-2* mRNA levels in muscle tissue from SJA zebrafish compared to controls (Figures 4 and 5).

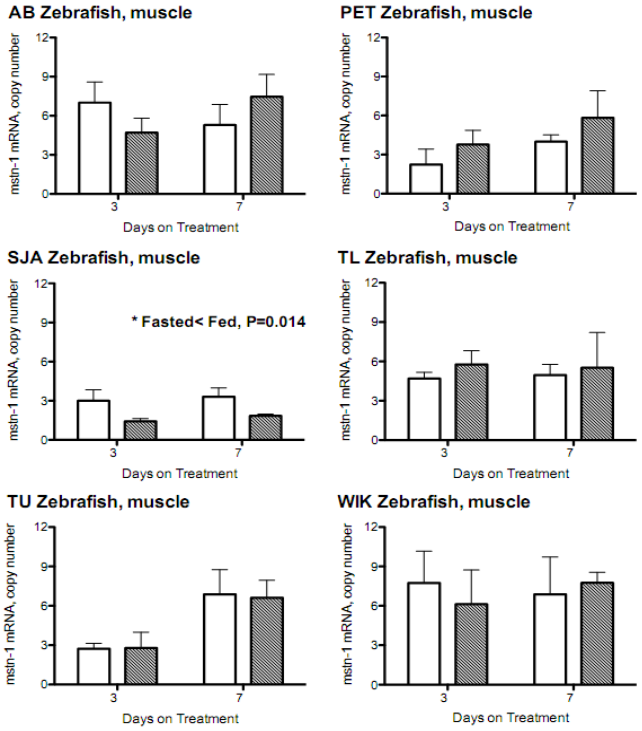

Figure 4: Muscle myostatin-1 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bar: fed controls; Shaded bars: fasted animals. Data are shown as mean myostatin-1 mRNA copy number ±SEM (n=6: *p<0.05).

Alternatively, fasting for 3 days increased *mstn-2* mRNA levels in muscle from TL zebrafish (Figure 5). Fasting had no effect on *mstn-1* and *-2* mRNA in muscle tissue from AB, PET, TU or WIK zebrafish (Figures 4 and 5). In addition, fasting had no effect on *mstn-1* or *-2* mRNA levels in spleen tissue in any of the strains (Figures 6 and 7).

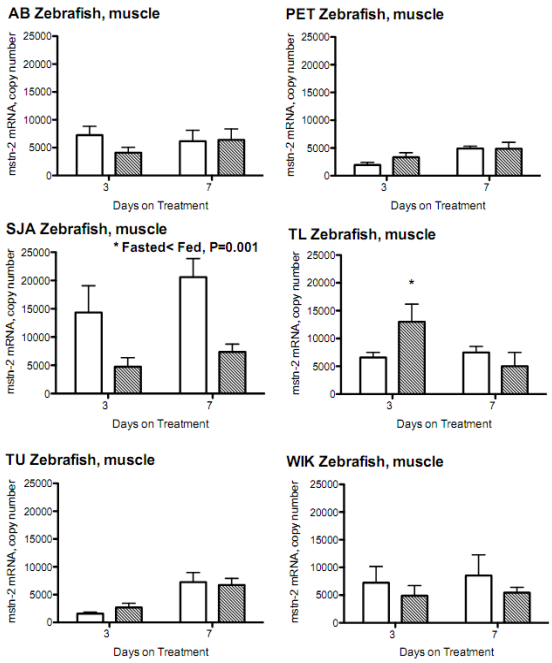

Figure 5: Muscle myostatin-2 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bar: fed controls; Shaded bars: fasted animals. Data are shown as mean myostatin-2 mRNA copy number ±SEM (n=6: *p<0.05).

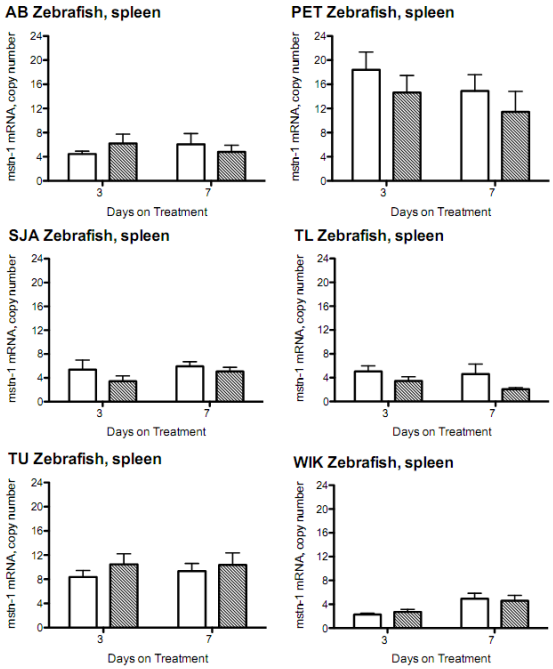

Figure 6: Spleen myostatin-1 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bar: fed controls; Shaded bars: fasted animals. Data are shown as mean myostatin-1 mRNA copy number ±SEM (n=6: *p<0.05).

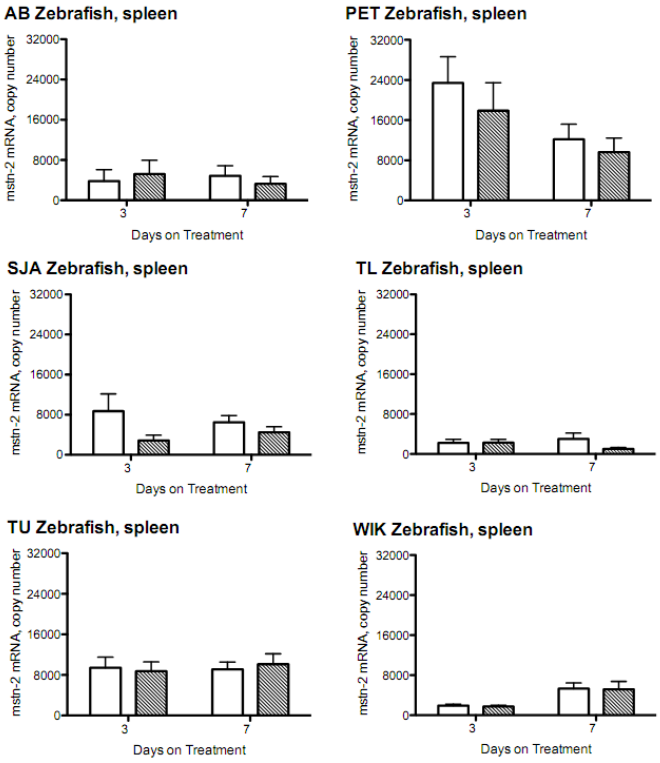

Figure 7: Spleen myostatin-2 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bar: fed controls; Shaded bars: fasted animals. Data are shown as mean myostatin-2 mRNA copy number ±SEM (n=6: *p<0.05).

Interestingly, variation was detected in *mstn* expression between strains in both muscle and spleen tissue. In muscle and spleen tissues, *mstn-2* mRNA levels were consistently higher compared to *mstn-1* mRNA levels in all strains measured (Figure 8a, b). In muscle tissue, *mstn-2* mRNA levels were not different between AB, TL, and TU zebrafish strains. However, PET and WIK *mstn-2* steady-state levels were lower while SJA *mstn-2* mRNA levels were higher compared levels detected in AB, TL, and TU zebrafish strains (Figure 8a). In addition, *mstn-2* mRNA steady state levels detected in spleen tissue from PET and TU zebrafish were higher than levels detected in all other strains (Figure 8b).

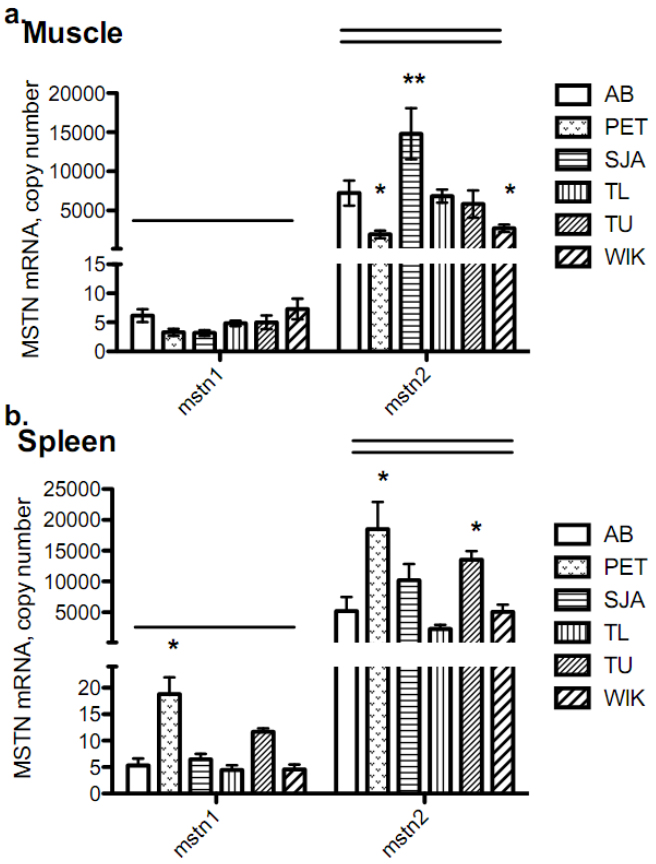

Figure 8: Steady-state myostatin-1 and -2 mRNA levels from different zebrafish strains. Levels were analyzed in muscle (a) and spleen (b) tissue. Data are shown as mean relative mRNA copy number ±SEM (n=6: *p<0.05).

Refeeding after a 7 day fasting period resulted in significant changes in *mstn* levels in muscle tissue of SJA and WIK zebrafish strains. The fasting-induced decrease in *mstn-1* mRNA levels in muscle from SJA zebrafish was removed following refeeding on day 7 (Figure 9a). Interestingly, refeeding decreased *mstn-1* mRNA levels in muscle tissue of WIK zebrafish, even though there was no effect of fasting on *mstn-1* mRNA levels. In addition, the fasting induced decrease in *mstn-2* mRNA levels in muscle tissue from SJA zebrafish was removed following refeeding and *mstn-2* mRNA levels returned to fed control levels (Figure 9b). Refeeding had no effect on *mstn-1* or *-2* mRNA levels in spleen tissue from any zebrafish strain evaluated (Figure 9c and 9d).

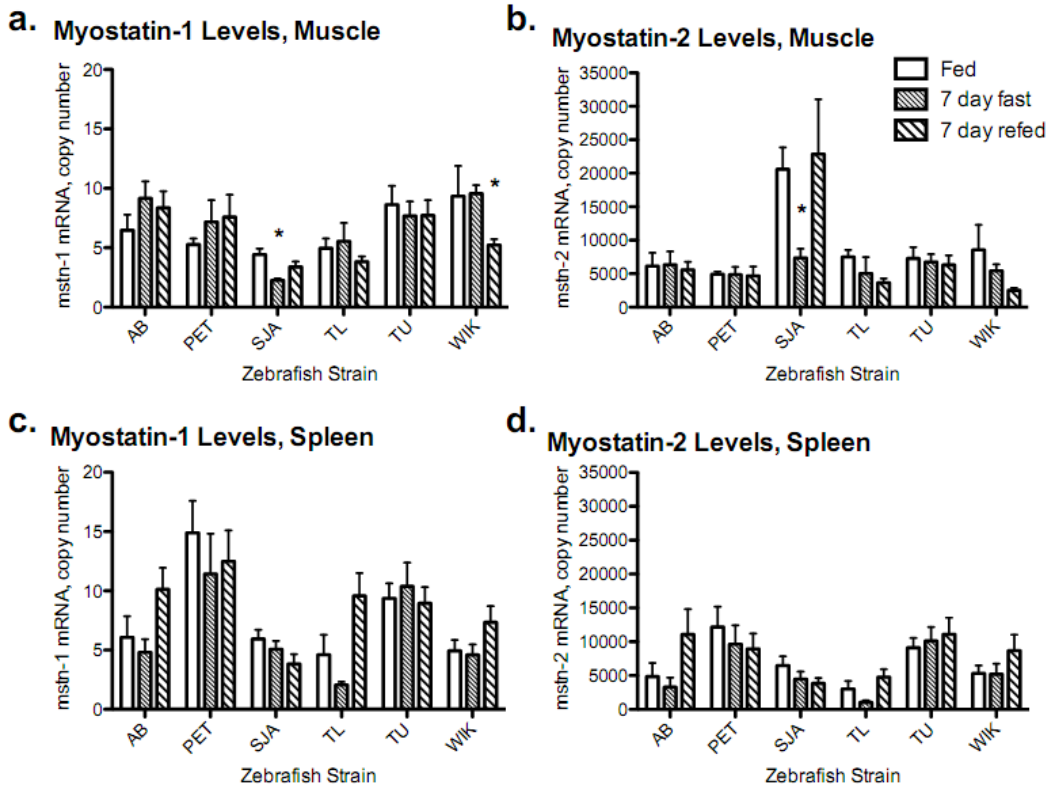

Figure 9: Myostatin gene expression following refeeding in different zebrafish strains. Figures represent a comparison among strains with regards to muscle myostatin-1 (a), muscle myostatin-2 (b), spleen myostatin-1 (c), and spleen myostatin-2 mRNA expression. Data are shown as mean myostatin mRNA copy number ±SEM (n=6: *$p<0.05$).

2.4. Discussion

Zebrafish have become an important model organism for many areas of scientific inquiry, including developmental biology and physiology. Due to their importance and wide use in research, several inbred strains of zebrafish are available from the Zebrafish International Research Center (ZIRC). ZIRC maintains eight inbred wild-type lines, numerous mutant strains, and several outbred strains. In addition to these available genetic strains, many research groups often use zebrafish purchased from local or national pet suppliers. Previous work in zebrafish larval development demonstrated that variation among strains exists in response to ethanol exposure (Loucks and Carvan, 2004). Inter-strain differences in zebrafish wild-type strains can

be readily observed with simple visual inspection in many cases when appropriate strains are chosen (e.g., TL with long fins versus AB with normal fin lengths). Previous research on behavioral traits demonstrated boldness and shoaling behavior differences between zebrafish strains (Wright et al., 2006). Interestingly, the majority of observed inter-strain behavioral differences are maintained when different strains are reared together, suggesting a genetic basis for the phenotypic variation between zebrafish strains (Robison and Rowland, 2005).

2.4.1. Variation in physiological characteristics exists between strains of zebrafish in response to fasting

For this study, 6 wild-type strains of zebrafish used, including AB (AB), pet store-purchased (PET), Tuebingen (TU), Tupfel long fin (TL), WIK (WIK), and SJA (SJA). These strains, minus the PET strain, are maintained by the Zebrafish International Research Center. Figure 10 depicts how these strains are related to one another. Briefly, the AB strain was derived from the original A and B lines purchased by George Streisinger from a pet store. This line has been the primary background for most of the transgenic and mutant lines that are currently available. The TU laboratory strain at the Max Planck Institute was screened to remove embryonic lethal mutations for subsequent use for mutagenesis screenings and the Zebrafish Sequencing Project (Nusslein-Volhard, 2002). The Tupfel long fin line (TL) is a pet store favorite that was derived from a cross between an AB with a spotted phenotype and a TU with a long finned phenotype. This strain can be useful for animal screening in that visual inspection may be used to discriminate TL zebrafish from other strains. The WIK line was derived from a wild caught line in India and is highly polymorphic in comparison to the AB and TU lines. The SJA line, on the other hand, is an inbred *AB line produced to reduce polymorphisms in the population making it a useful research tool. The pet store variety (PET) was derived from an

unknown and presumably heterogeneous background. Therefore, genetically distinct laboratory strains include AB, TU, and WIK, while the other three strains exhibit some genetic overlap. The TU and WIK lines are so genetically distinct that they differ at the nucleotide level to such an extent that WIK/TU crosses are routinely used to map gene locations by bulk-segregrant analysis (Nechiporuk et al., 1999).

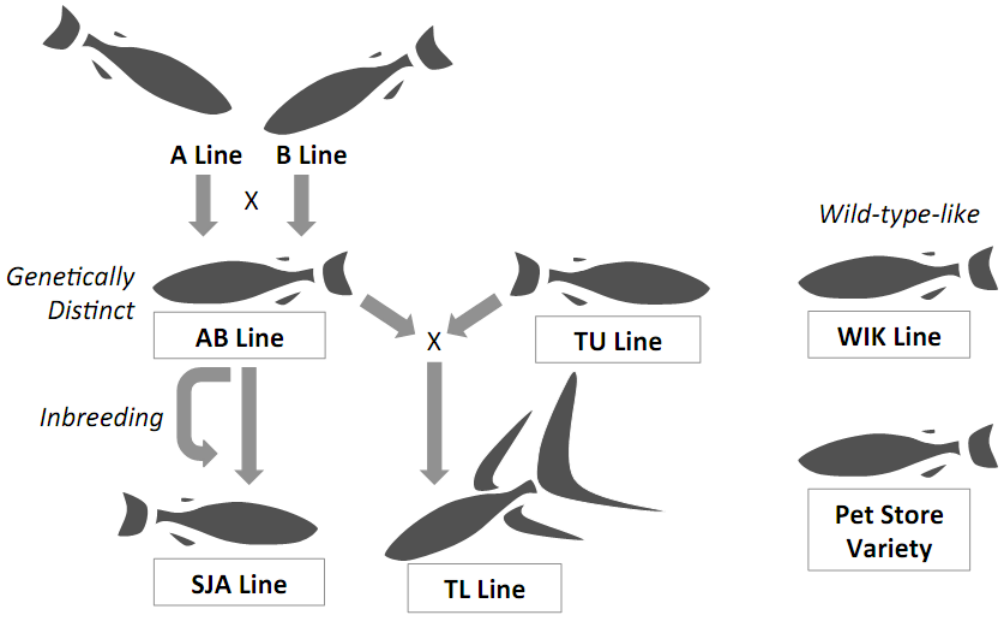

Figure 10: Zebrafish strain relationships. Relationships between the six zebrafish strains used in this study showing origins in genetic similarities coming from the original A and B zebrafish strains that George Streisinger purchased from an Oregon pet store.

This study utilized these six strains to evaluate whether nutritional insult would elicit different effects on basic physiological parameters in a strain-dependent manner. Overall, seven days of fasting had little effect on body weight and length in most strains evaluated. Only the AB and TU zebrafish exhibited a decrease in body weight following fasting, and only TU zebrafish exhibited a decrease in overall body length. Previous research has demonstrated that the same fasting regime (7 days) was effective at increasing the expression of an orexigenic factor, ghrelin, in zebrafish (Amole and Unniappan, 2009) suggesting that this fasting period is adequate

in eliciting a response to drive food intake. Additionally, fasting produces an 'excitatory' response in activity levels for 2 days following food removal, after which a decline in activity is detected for the remainder of the fasting period (Novak et al., 2005). The decrease in activity levels following fasting plateaued after 7 days of fasting (Novak et al., 2005), suggesting that a short-term fasting regime consisting of 7 days of food withdrawal is sufficient in eliciting a nutritional insult capable of altering physiological effects. The present study demonstrated that only the TU line was physiologically affected by fasting, as body weight, length, and glucose values were decreased overall by a 7-day fasting period. AB zebrafish exhibited a body weight decrease, while PET and TL lines exhibited decreased glucose levels following fasting. These results suggest that TU zebrafish are more metabolically sensitive to short term food restriction that the other strains evaluated. Conversely, of the genetically separate strains, the WIK strain appears to be the most resilient to nutritional insult and is the most 'wild-like' of all of the strains evaluated.

Glucose metabolism is an important source of energy for most organisms, as the breakdown of glucose supplies energy by way of glycolysis. As fasting occurs, glucose is utilized but not replaced, which induces a decrease in whole blood glucose levels the regulation of which has been observed in a variety of vertebrates such as fish (Wilson, 1994), birds (Belo et al., 1976), and mammals (Katz et al., 1976). Glucose is primarily obtained in the diet from ingestion of carbohydrates, and studies on the dietary importance of carbohydrates to fish have shown that warm water omnivorous fish utilize glucose to a much greater extent than do cold water carnivorous fish (Wilson, 1994; Stone, 2003). Zebrafish are therefore likely to use glucose to a large extent, as they are warm water omnivores. Recent studies have demonstrated that the amount of carbohydrate in a zebrafish diet is positively correlated to growth rate with decreased

amounts of carbohydrates ingested leading to a reduced rate of growth (Robison et al., 2008). The aforementioned importance of glucose metabolism in vertebrate physiology necessitated our evaluation of glucose metabolism as a potentially important variable in the physiology of different zebrafish strains.

The decrease in whole blood glucose concentration in response to short term fasting observed in PET, TL, and TU strains is consistent with previous reports of fasting in zebrafish where a 4-day fast was sufficient to decrease whole blood glucose levels (Eames et al., 2010). Following a 2-day fasting period, blood glucose levels rose significantly and then subsequently decreased to baseline levels by day 4 of the fasting regime in outbred wild type zebrafish (Eames et al., 2010). In the present study, AB, SJA, and WIK zebrafish strains did not exhibit a change in whole blood glucose levels in response to food deprivation. These results suggest that there is a "normal" response in glucose metabolism in PET, TU, and TL, making them a more ideal fit for metabolic research. The lack of a response to fasting in the AB, SJA, and WIK zebrafish strains makes them suspect for research into metabolic shift with regard to fasting; however a longer fasting period might elicit physiological changes similar to those detected in PET, TL, and TU strains.

Following a 7-day fasting regime, fish were refed 4 hours prior to sampling. After refeeding, PET and TU blood glucose levels returned to baseline levels (Figure 3), consistent with previous reports (Eames et al., 2010). Following a shorter term fasting (4 days) than the one in the present study (7 days), a 2-day refeeding period returned glucose levels to steady-state levels in outbred wild-type zebrafish (Eames et al., 2010). The PET zebrafish strain is likely genetically closer to the outbred wild-type strain used by Eames and co-workers (Eames et al., 2010), suggesting that our results are consistent with their report. The PET zebrafish have a

heterogeneous breeding background with no breeding guidelines for maintenance of the line, as it is not an official genetically utilized strain. The TU line was started from a pet store line in Germany and was bred to reduce lethal embryonic mutations, thus 'cleaning up' the line. This may give insight into the similarities in the glucose metabolism pattern between these strains. Furthermore, the TL line of zebrafish was created from a cross between the AB and TU line potentially shedding light on the similarities between PET, TU, and TL. The AB and SJA lines are related from an earlier *AB line suggesting a possible reason for their similarities. These data suggest that genotype plays a significant role in metabolic shift and certain zebrafish strains, namely PET, TL, and TU, can serve as good models for short-term metabolic analysis studies. Conversely, others zebrafish strains, namely AB, SJA, and WIK, might better serve as interesting comparative models for understanding shifts in glucose metabolism by comparing these to the more metabolically sensitive models to elucidate mechanistic actions for the inherent differences between how these strains regulate energy.

2.4.2. Myostatin levels were differentially regulated by short term fasting in a strain-dependent manner in zebrafish

Much work has been done in mammalian models to elucidate the mechanisms of mstn action, generally focusing on the regulation of skeletal muscle growth. It has been previously shown in mammals that fasting can induce an up-regulation of *mstn,* with a subsequent decrease during refeeding demonstrating a negative influence on muscle hypertrophy and hyperplasia (Lee and McPherron, 1999). The effect of fasting on *mstn* expression in muscle is further supported by studies conducted in fish species. In sea bass (*Dicentrarchus labrax*) fasting increased *mstn* mRNA levels followed by a subsequent refeeding-induced decrease in *mstn* expression (Terova et al., 2006). More recently, fasting was shown to increase *mstn-1* expression

in muscle tissue in barramundi (*Lates calcarifer*) (De Santis and Jerry, 2011). Interestingly, differential regulation of mstn isoforms was demonstrated in response to fasting in barramundi, as *mstn-2* expression remained stable in muscle but was upregulated in gill and liver following fasting (De Santis and Jerry, 2011). Additionally, short-term fasting increased *mstn* expression, while prolonged fasting decreased *mstn* expression in tilapia (*Oreochromis mossambicus*) larvae, suggesting a complex regulatory system controlling *mstn* mRNA levels (Rodgers et al., 2003). In the present study, a short-term fasting regime decreased both *mstn-1* and *-2* expression in muscle from SJA zebrafish, while *mstn-2* expression was increased in muscle of TL zebrafish (Figures 3 and 4). Interestingly, SJA zebrafish exhibited no changes in body weight, body length, or glucose levels in response to short-term fasting. A decrease in *myostatin* expression in muscle tissue of fish not exhibiting what are considered 'normal' metabolic changes during fasting (Jeanplong et al., 2003; Rodgers et al., 2003; Terova et al., 2006) might suggest a novel local mechanism of regulating energy utilization. However, TL zebrafish did exhibit a decrease in blood glucose levels in response to fasting and this corresponds to an increase in *mstn-2* expression in muscle. These results are consistent with other reports that suggest that mstn increases would decrease cell proliferation in a time of low nutrient availability to conserve energy. Most interestingly, this study demonstrated that short-term fasting in zebrafish is highly strain-dependent in terms of metabolic response to caloric restrictions.

It may be possible that the SJA strain of zebrafish has developed a mechanism for responding to the effects of a dietary restriction. A decrease in *mstn* levels at this point would allow for more muscle growth at a time when, as previous research suggests, animals should be trying to conserve energy by slowing growth and focusing on maintenance. Recent work on mstn energy conservation roles has observed in murine cell cultures that mstn can regulate glucose

metabolism by promoting glycolysis and glucose uptake in myotubes (Chen et al., 2010). This observation may help explain why *mstn* levels decreased in SJA zebrafish by suggesting the presence of a pathway in which a decrease in blood glucose levels affects local *mstn* expression. However, this response appears to be highly strain-dependent, as *mstn* levels were not affected in PET or TU zebrafish, as these strains exhibited decreased blood glucose levels following fasting.

Alternatively, fasting in the TL zebrafish strain resulted in an increase in *mstn-2* levels 3 days after fasting. This increase is consistent with previous research observations in which *mstn* levels are increased in response to a fast. This follows the hypothesis that fasting is a detriment to nutrition, which is compensated for by slowing muscle growth and maintaining current levels of muscle tissue. In this study, this effect is not observed after 6 days of fasting suggesting an acute response to the fasting stimulus. The shortness of this response makes it appear as if another mechanism is brought into play after a short period of lag. This delay could allow for time to find an alternate food source before a large-scale change in metabolism is activated. Again, this response is strain-dependent as *mstn* levels were not affected by fasting in AB, PET, TU, or WIK zebrafish. More interestingly, PET and TU zebrafish exhibited no change in *mstn* levels even though blood glucose levels were decreased. This suggests that various strains of zebrafish exhibit varying degrees of sensitivity to caloric restriction and that these data should be considered when evaluating fasting responses, or other nutritional alterations, in zebrafish in the future. As observed in previous studies (Rodgers et al., 2003; Terova et al., 2006), *mstn* levels were decreased back to fed levels in response to a refeeding event. In the present study, refeeding returned *mstn* levels back to control levels in SJA zebrafish muscle tissue, which exhibited decreased *mstn* levels in response to fasting (Figure 9).

Beyond the known role of mstn on muscle growth regulation, recent evidence from murine dietary experiments has demonstrated that *mstn* mRNA expression is altered in spleen tissue following increased dietary lipid intake (Lyons et al., 2010). Dynamic regulation of *mstn* outside of skeletal or cardiac muscle tissues may suggest that this protein plays alternative roles in metabolism. As mstn in zebrafish has been shown to be homologous to the previously identified teleost, avian, and mammalian orthologs (McPherron et al., 1997), spleen tissue was utilized in conjunction with muscle tissue to observe changes in *mstn* expression in response to caloric restriction. In the present study, no changes in *mstn* expression were detected in spleen tissue from any zebrafish strain following short-term fasting (Figures 6 and 7). This suggests that nutritional alterations that affect *mstn* expression may need to be sustained for a longer duration to affect a non-metabolic regulatory tissue. Interestingly, steady-state levels of *mstn-2* were relatively higher in both muscle and spleen tissue from all zebrafish strains evaluated. Some strain variation was evident in steady-state *mstn-2* levels, where levels were highest in SJA muscle and PET spleen (Figure 8). Interestingly, *mstn-1* steady-state levels were highest in PET zebrafish spleen as well. While these data are correlative at best, they do provide a starting platform for understanding metabolic and growth variation that exists among strains of zebrafish.

2.5. Conclusion

Variation occurs everywhere, and, as such, it needs to be considered when determining the appropriate model organism to use for research. This study investigated commonly used zebrafish strains as a measure of the model system. The large amount of variation documented in this study in response to fasting makes it apparent that more emphasis on zebrafish strains utilized in studies on growth and metabolism is imperative for a better understanding of these physiological processes.

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