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Inbred strains of zebrafish exhibit variation in growth performance and myostatin expression following fasting

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Abstract

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

Keywords

zebrafish; glucose; fasting; myostatin

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 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

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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: is there intraspecific variation in physiology and the response to fasting in 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, 1988; Spearow et al., 1999), 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 (Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; MacGowan et al., 2009; Robertson and Reeve, 1952). Studies focusing on strain variation in zebrafish are more limited, however studies have reported variation between strains when they are exposed to a teratogen such as ethanol in developing zebrafish (Loucks and Carvan, 2004). Inter-strain differences in zebrafish wild-type strains can be readily observed with simple visual inspection in many cases; for example Tupfel long fin zebrafish exhibit longer fins than other strains. Behavioral analyses between domesticated laboratory strains and wild-derived strains demonstrated phenotypic differences in boldness and shoaling behaviors (Wright et al., 2006). A recent study demonstrated inter-strain behavioral variability in zebrafish in response to nutritional manipulations (Benner et al., 2010). 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 (Lewis et al., 2007; West et al., 1992). 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 et al., 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 (Biga et al., 2005; Kerr et al., 2005; Ostbye et al., 2001; Ostbye et al., 2007; Rescan et al., 2001; Roberts and Goetz, 2001). Previous studies in teleost fish have shown that *mstn* mRNA expression increases with fasting and subsequently decreases after re-feeding (Terova et al., 2006), consistent with mammalian data demonstrating 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.

A better understanding of strain-specific growth variation in zebrafish can aid in evaluating differential population responses or laboratory-to-laboratory variation. 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, to define strain variation among zebrafish. Therefore, this study was designed to evaluate differences in growth regulation manifested between the wild-type strains that are commonly used for developmental and physiological research. Using fasting as a metabolic challenge, we identified several variations in growth performance among the tested select zebrafish strains, highlighting the need for careful strain selection during the development of an experiment.

2. Materials and Methods

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 for 6 months and maintained as described in *The Zebrafish Book* at 28 °C and a 14:10 light:dark cycle (Westerfield, 2000). All fish were housed in 10-liter tanks at a density of 6 fish/L 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. All strains of zebrafish were raised from embryos (except for the PET strain) under identical conditions. The PET strain of zebrafish were obtained from a pet distributor (Apet) and arrived as mature adults (estimated age 10 months; 0.35 ± 0.07 g) were housed under conditions identical to those of the other mature zebrafish strains. PET zebrafish were allowed to acclimate to the rearing conditions for 2 months prior to experimental procedures. 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, ND, USA.

2.2 Metabolic Insult

Mature zebrafish (*Danio rerio*) (0.2 – 0.40 g, 6–12 months) were randomly assigned to one of eight groups (n=6 fish per group): 0 h, 3 day, 7 day fasting and fed groups, and a 7 day refeeding group. During the experimental procedures, all fish were held at a density of 2 fish/L. 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 h following the refeeding period. No mortalities were recorded during the experimental period.

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 and 7 of the experimental regime. All sampling was conducted at the same time (10:00 h) on each sampling day. 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 et al., 2010). For analysis of *myostatin* gene expression, tissue samples (muscle and spleen) were collected and immediately frozen at -80°C . Muscle samples (10 mg) were collected from the epaxial quadrant rostral the dorsal fin beginning. Total RNA was isolated using RNeasy® 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.

2.4 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 treated with DNase I (Promega, Madison, WI, USA). Total RNA purity ($A_{260}/A_{280} = 1.9$) was verified by spectrophotometry (NanoDrop, ThermoFisher). Samples were then reverse transcribed into cDNA using oligo dT and the ImProm-II™ Reverse Transcription System (Promega). 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 utilized a comparative baseline strategy using the ΔC_q method that standardized to starting input cDNA quantity (Bustin et al., 2009; De Santis et al., 2011). Primer PCR efficiencies were calculated for each primer:tissue pair using $\log_{10}$ dilution series of input cDNA as well as generated standards. Efficiency (E) was determined by the equation $[E = 10^{(-1/\text{slope})}]$ and ranged from 90.3% to 94.6%. Reproducibility was represented by the R^2 value of the standard curve and was always greater than 0.98. Quantification was conducted using standard curves generated for each *mstn* isoform. Standards were generated using pGEM-T Easy Vector system (Promega). The coefficient of variation (CV, %) of C_q values was calculated ($\text{CV} < 0.45$). To avoid confounding technical variation between runs, all treatment and control samples from each strain were analyzed on the same plate for each gene and standard curves were included on each plate. Relative starting copy number was calculated for each triplicate sample using the generated standard curves. The following primers were used for specific analysis of *myostatin* isoform mRNA expression: *mstn-1*, forward primer (5'-GTCCGGTGCGTGAGGTTTC-3'), reverse primer (5'-AGCTCGCTTTCCTCCGTGGC-3'); *mstn-2*, forward primer (5'-ACAAGGCCAACCAAGGGGC-3'), reverse primer (5'-AACCGCAAAGGTCTACCACCATCG-3'). These primers were previously validated (Biga and Meyer, 2009).

2.5 Statistical analysis

All standardized qPCR data were analyzed using the ΔC_q method where cycle threshold values were compared to standard curves generated and validated for each primer pair to result in a relative starting copy number of target mRNA (Bustin et al., 2009; De Santis et al., 2011). 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. Normality of sample distribution was tested by Normal Quantile Plot (Q-Q Plot), and residuals were plotted against predicted values to evaluate dependency between the means and the variances and test the assumption of homogeneity of variances. All assumptions were met for two-way ANOVA. T-tests were utilized to compare baseline control levels to day 7 refed 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 CA, USA; www.graphpad.com). All results were considered significant at $P < 0.05$.

3. Results

3.1 Morphological analysis

Following a 7-day fasting regime, dry body mass and length measurements were collected to ascertain whole body physiological responses to nutritional insult in six strains of zebrafish. Fasting for 3 days had no effect on whole body mass in AB, PET, SJA, TL, or WIK strains (Figure 1). Body mass was decreased 23.2 and 23.1% in TU following 3 and 7 days of fasting, respectively (Figure 1e). Additionally, fasting for 7 days decreased body mass in AB (26.6%) and PET (25.7%) strains (Figure 1a and b). Re-feeding after a 7-d fast restored body mass to control fed levels as no differences were detected between refed and 7d fed animals in any strain (Figure 1). Fasting had no effect on body length (data not shown).

3.2 Glucose analysis

Whole blood glucose was measured following 3 or 7 days of fasting or feeding using a commercially available handheld glucose meter. Following 3 days of fasting, whole blood glucose levels decreased 37.9% in PET, 59.8% in TL, and 47.1% in TU strains (Figure 2b, d, and e). After 7 days of fasting, whole blood glucose levels remained decreased in PET (52.2%) and TU (43.6%) strains (Figure 2b and e). Fasting for 7 days reduced whole blood glucose levels 36.5% in AB zebrafish (Figure 2a). Refeeding after 7 days of fasting restored whole blood glucose levels in AB, PET, and TU strains (Figure 2c). Whole blood glucose levels were not affected by fasting in SJA or WIK strains in response to food deprivation at any sampling day (Figure 2c and f). Overall whole blood glucose levels in fed zebrafish varied by strain (Figure 3). Basal whole blood glucose levels were lower in SJA and WIK strains compared to PET and TU basal levels.

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 in SJA fish significantly decreased *mstn-1* and *-2* mRNA levels in muscle tissue compared to control animals (Figures 4c and 5c). *Mstn-1* mRNA levels were decreased 52.6% after 3 and 45.3% after 7 days of fasting, while refeeding after the 7-day fast returned *mstn-1* levels to fed control levels in muscle tissue from SJA (Figure 4c). Additionally, *mstn-2* mRNA levels in muscle tissue from SJA were decreased 66.9% after 3 and 64.2% after 7 days of fasting with a return to fed control levels following refeeding (Figure 5c). Alternatively, fasting for 3 days increased *mstn-2* mRNA levels 97.3% in muscle from TL (Figure 5d). Fasting had no effect on *mstn-1* and *-2* mRNA in muscle tissue from AB, PET, TU or WIK strains (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 (data not shown). Interestingly, refeeding decreased *mstn-1* mRNA levels 45.5% in muscle tissue of WIK, even though there was no effect of fasting on *mstn-1* mRNA levels (Figure 4f). Refeeding had no effect on *mstn-1* or *-2* mRNA levels in spleen tissue from any zebrafish strain evaluated (data not shown).

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 6a, b). In muscle tissue, *mstn-2* mRNA levels were not different between AB, TL, and TU strains. However, in muscle tissue PET and WIK *mstn-2* steady-state levels were lower while SJA *mstn-2* mRNA levels were higher compared to levels detected in AB, TL, and TU strains (Figure 6a). In addition, *mstn-2* mRNA steady state levels detected in spleen tissue from PET and TU were

higher than levels detected in all other strains (Figure 6b). In PET zebrafish, *mstn-1* and *-2* mRNA levels were higher than the other strains in both spleen and muscle tissue.

4. Discussion

The results of this study demonstrate that selection of zebrafish strain is an important component of experimental designs. Here we report that short-term fasting elicits varied effects on morphometric, metabolic, and molecular indices in a strain-dependent manner. In addition, we report that basal levels of circulating blood glucose vary among zebrafish strains. This is an important finding as many recent studies utilizing zebrafish as models have focused on diet, growth, and metabolic regulation (Biga and Goetz, 2006; Biga and Meyer, 2009; Eames et al., 2010; Novak et al., 2005; Palstra et al., 2010; Robison et al., 2008).

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

For this study, six wild-type strains of zebrafish used, including AB (AB), pet store-purchased (PET), Tuebingen (TU), Tüpfel long fin (TL), WIK (WIK), and SJA (SJA). Figure 7 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 and has been the primary background for most of the transgenic and mutant lines that are currently available. The TU 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 Tüpfel long fin line (TL) was derived from a cross between an AB with a spotted phenotype and a TU resulting in 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 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 (Figure 7). 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-segregant analysis (Nechiporuk et al., 1999). This study utilized these six strains to evaluate whether nutritional insult would elicit different effects on basic physiological parameters in a strain-dependent manner. Only the AB, PET, and TU strains exhibited a decrease in body weight following fasting, while no changes were observed 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 levels plateaued after 7 days of fasting (Novak et al., 2005). Also, previous work demonstrated that blood glucose levels rose significantly after a 2-day fasting period, but subsequently decreased to baseline levels by day 4 in outbred wild type zebrafish (Eames et al., 2010). These data suggest 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 AB, PET, and TU strains were physiologically affected by fasting, as body weight and glucose values were decreased overall by a 7-day fasting period. TL fish exhibited decreased glucose levels following only 3 days of fasting, but exhibited no change after 7 days of fasting, while TU exhibited decreased body weight and whole body glucose

following 3 days of fasting that was maintained for the duration of fasting. These results suggest that TU zebrafish are more metabolically sensitive to short term food restriction than 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. The lack of response in SJA and WIK 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 AB, PET, TL, and TU strains. Refeeding returned blood glucose levels to baseline in AB, PET, and TU (Figure 2), consistent with previous reports, where a 2-day refeeding following a 4-day fast returned glucose levels to steady-state levels in outbred wild-type zebrafish (Eames et al., 2010). The PET strain is likely genetically closer to the outbred wild-type strain used by Eames and co-workers (2010), suggesting that our results are consistent with their report. The PET strain has a heterogeneous breeding background with no breeding guidelines for maintenance of the line, as it is not an official genetically utilized strain. The PET zebrafish used in this study were older and larger (~0.10 g) than the other 5 strains and it is likely that differences detected between PET and the other strains is an artifact of these differences. 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 was created from a cross between the AB and TU, potentially shedding light on the similarities between PET, TU, and TL. Similarly, AB and SJA lines are related from an earlier *AB line. These data suggest that genotype plays a significant role in metabolic shift and certain zebrafish strains, namely AB, PET, and TU, can serve as good models for short-term metabolic analysis studies. Conversely, other strains, namely 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.

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

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 is further supported by studies conducted in fish species where fasting induced an increase in *mstn* mRNA levels in sea bass (*Dicentrarchus labrax*) (Terova et al., 2006) and barramundi (*Lates calcarifer*) (De Santis and Jerry, 2011; De Santis et al., 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 (Figures 4 and 5). Interestingly, SJA 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 for 3 days 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 AB, 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, which is similar to an increase in *mstn* expression detected in tilapia following a short term fast (Rodgers et al., 2003). An increase in *mstn* expression supports 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 the present study, the increase in *mstn* expression was only detected in TL muscle following 3 days of fasting, suggesting that another mechanism regulates muscle maintenance in zebrafish. In addition, this response is strain dependent as *mstn* levels were not affected by fasting in AB, PET, TU, or WIK strains. In addition, PET and TU zebrafish exhibited no change in *mstn* levels even though blood glucose levels were decreased, suggesting 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.

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 *mstn* plays alternative roles in metabolic regulation. In the present study, no changes in *mstn* expression were detected in spleen tissue from any zebrafish strain following short-term fasting (data not shown), suggesting 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 strains evaluated (Figure 6). Some strain variation was evident in steady-state *mstn-2* levels, where levels were highest in SJA muscle and PET spleen. Interestingly, *mstn-1* steady-state levels were highest in PET zebrafish spleen as well. In alignment with these data, steady-state blood glucose values were highest in PET and TU zebrafish (Figure 3). The PET zebrafish strain exhibited elevated steady-state glucose values and *mstn-2* levels in spleen tissue, while also demonstrated decreased body weight and glucose levels in response to fasting. However, no effects were observed in *mstn* expression in response to fasting in PET zebrafish. It is likely that age and/or size of PET zebrafish might have played a role in the differences detected in steady-state values as these fish were slightly older and larger than the other strains. 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. The large amount of variation documented in this study in response to fasting makes it apparent that more emphasis on strains utilized in studies focusing on growth and metabolism is imperative for a better understanding of these physiological processes.

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Figure 1.

Whole body mass (g) from zebrafish strains following a 7-day fasting regime. Experimental groups include: fed controls (open bars), fasted (narrow striped bars) for 3 or 7 days, and a refed group (wide striped bars). Following 7 days of fasting, the refed group was allowed to eat and then sampled 4 hours postprandially. Treatment and time effects were analyzed by two-way ANOVA. T-tests were used to test for differences between 7-day fasted and refed groups. Data are shown as mean $\pm$ SEM (n=6; *p<0.05).

Figure 2.

Metabolic analysis of overall whole blood glucose levels of fed controls (open bars), fasted zebrafish (narrow striped bars), and refed zebrafish (wide striped bars). Zebrafish strains were fasted or fed for 3 and 7 days, and then refed after 7 days of fasting. Treatment and time effects were analyzed by twoway ANOVA. T-tests were used to test for differences between 7-day fasted and refed groups. Results are mean glucose mMol/L $\pm$ SEM (n=6; *p<0.05).

Figure 3. Steady-state whole blood glucose levels from zebrafish strains. Data were analyzed by t-test and data are shown as mean glucose mMol/L $\pm$ SEM (n=18; *p<0.05).

Figure 4.

Muscle myostatin-1 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bars: fed controls; narrow striped bars: fasted animals; and wide striped bars: refed after 7 days. Treatment and time effects were analyzed by two-way ANOVA. T-tests were used to test for differences between 7-day fasted and refed groups. Data are shown as mean myostatin-1 mRNA relative copy number $\pm$ SEM (n=6; *p<0.05).

a. AB Zebrafish, muscle **b. PET Zebrafish, muscle** **c. SJA Zebrafish, muscle** **d. TL Zebrafish, muscle** **e. TU Zebrafish, muscle** **f. WIK Zebrafish, muscle** **Figure 5.**

Muscle myostatin-2 mRNA expression analysis in response to a 3-day or 7-day fast in six different zebrafish strains. Open bars: fed controls; narrow striped bars: fasted animals; and wide striped bars: refed after 7 days. Treatment and time effects were analyzed by two-way ANOVA. T-tests were used to test for differences between 7-day fasted and refed groups. Data are shown as mean myostatin-2 mRNA relative copy number $\pm$ SEM (n=6; *p<0.05).

Figure 6. Steady-state myostatin-1 and -2 mRNA levels in muscle (a) and spleen (b). Data were analyzed by t-tests and data are shown as mean relative mRNA copy number $\pm$ SEM (n=6; *p<0.05).

Figure 7.
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.