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Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*)

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Abstract

In this study, we tested for the presence of sexual dimorphism in the hepatic transcriptome of the adult zebrafish and examined the effect of long term manipulation of dietary carbohydrate on gene expression in both sexes. Zebrafish were fed diets comprised of 0%, 15%, 25%, or 35% carbohydrate from the larval stage through sexual maturity, then sampled for hepatic tissue, growth, proximate body composition, and retention efficiencies. Using Affymetrix microarrays and qRT-PCR, we observed substantial sexual dimorphism in the hepatic transcriptome. Males up-regulated genes associated with oxidative metabolism, carbohydrate metabolism, energy production, and amelioration of oxidative stress, while females had higher expression levels of genes associated with translation. Restriction of dietary carbohydrate (0% diet) significantly affected hepatic gene expression, growth performance, retention efficiencies of protein and energy, and percentages of moisture, lipid, and ash. The response of some genes to dietary manipulation varied by sex; with increased dietary carbohydrate, males up-regulated genes associated with oxidative metabolism (e.g. *hadhβ*) while females up-regulated genes associated with glucose phosphorylation (e.g. glucokinase). Our data support the use of the zebrafish model for the study of fish nutritional genomics, but highlight the importance of accounting for sexual dimorphism in these studies.

Keywords

Carbohydrate; Gene expression; Glucose homeostasis; Liver; Sexual dimorphism; Microarray; Nutritional genomics; Zebrafish

1. Introduction

The vertebrate liver performs a number of complex functions, including nutrient and vitamin metabolism, synthesis of plasma proteins, inactivation of various substances, and immunity. More specifically, in conjunction with the gastrointestinal tract, the liver plays a major role in glucose metabolism, including gluconeogenesis and glycogen storage. Glucose homeostasis and carbohydrate metabolism are fundamental biological processes that significantly influence growth and development, and define numerous pathological conditions. Most of our knowledge of these processes comes from studies in mammals. Our understanding of these processes in fish species is limited in a number of key areas, including the chronic effects of long term dietary manipulations (i.e. lasting from the larval stage to sexually mature adult) and the potential role of sexual dimorphism in the regulation of nutrient partitioning. These data are important in a variety of contexts, including improving our fundamental understanding of hepatic function and adaptability in fishes, design of alternative diets for aquaculture species (Trushenski et al., 2006) and the use of fish models in biomedical research (Papasani et al., 2006; Elo et al., 2007).

In fish, the genes involved in glucose homeostasis and carbohydrate metabolism respond rapidly to changes in dietary carbohydrate intake (Hemre et al., 2002), although there appears to be considerable interspecific variation in the capacity to utilize dietary carbohydrate (Panserat and Kaushik, 2002; Panserat et al., 2002). For example, carnivorous fish such as the rainbow trout are considered to be glucose-intolerant (Hemre et al., 2002). Rainbow trout suffer prolonged postprandial hyperglycemia, poor utilization of dietary glucose, and reduced growth when dietary carbohydrate levels are above 25–30% (Panserat et al., 2001a; Panserat and Kaushik, 2002). The persistence of high levels of hepatic fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) gene expression suggests that rainbow trout have reduced transcriptional regulation of gluconeogenic genes by dietary carbohydrates (Panserat et al., 2001a; Panserat and Kaushik, 2002). In contrast, expression of gluconeogenic enzymes is more tightly regulated by dietary carbohydrates in two other fish species, the common carp (*Cyprinus carpio*) which are relatively tolerant of dietary carbohydrate, and gilthead seabream (*Sparus aurata*) (Panserat et al., 2002).

While several studies of acute regulation of carbohydrate metabolism have been performed, less work has examined the persistent effect (i.e. in the post-absorptive state) of long term dietary manipulations on hepatic gene expression. Current research indicates striking interspecific variation in metabolic phenotypes. For example, rainbow trout glucokinase activity remains elevated 24 h after a meal, while in common carp glucokinase activity returns to levels similar to those observed in fish fed a diet devoid of carbohydrate (Panserat et al., 2000). In contrast, short term manipulation of dietary carbohydrate in rainbow trout had no effect on the persistent (24 h) expression levels of glucose-6-phosphatase expression (Panserat et al., 1999). While these studies have been important, they have focused on a few genes known to be acutely regulated by carbohydrates, and a detailed genomic approach has not yet been taken in any fish species. Are expression levels of glycolytic and gluconeogenic genes influenced by long term dietary manipulation during post-absorptive periods, or is regulation of these genes only associated with acute, postprandial events? Are genes outside the gluconeogenic and glycolytic pathways affected by long term dietary manipulation?

In addition, most studies conducting nutritional manipulations (particularly those involving carbohydrates) were performed using sexually immature fish (see Hemre et al., 2002). This likely reflects the fact that market sizes of most aquaculture species are dependent on age of maturation, and the cost effective growth phase occurs prior to sexual maturation. While experiments using immature fish have been vital in establishing how key glycolytic and gluconeogenic genes are acutely regulated in response to carbohydrate availability, few data

on nutritional effects in sexually mature adults are available in any fish species. This imposes unnecessary limitations on our understanding of genetic, biochemical, and physiological mechanisms that determine hepatic function, growth performance, and overall fish health.

When considering the effects of dietary carbohydrate manipulation in mature fish, one must account for potential sexual dimorphism in the response to dietary manipulation. In many taxa, there is substantial sexual dimorphism in a wide range of physiological phenotypes; however, the extent to which sex differences exist in glucose homeostasis remains unclear. The liver is one of the most sexually dimorphic organs in mammals with respect to patterns of gene expression; thousands of genes show sexual dimorphism in the mouse liver (Yang et al., 2007), and the expression of several commonly measured metabolic genes varies between male and female rats (Verma and Shapiro, 2006). In rats it has been postulated that sex-dependent secretion of steroids (Pankiewicz et al., 2003) and growth hormone (Clodfelter et al., 2006) either directly or indirectly play an important role in the regulation of liver gene expression and enzyme activity. As a result, sexual dimorphism may affect different hepatic functions related to glucose metabolism and metabolic phenotypes, and contribute to sex differences in physiology, homeostasis, and energy metabolism. Most studies of sexually dimorphic gene expression have occurred in mammals, and studies of sexual dimorphism in fish transcriptomes are rare [but see (Aubin-Horth et al., 2005; Wen et al., 2005; Santos et al., 2007)]. The extent of sexual dimorphism in the hepatic transcriptome and its resultant influence on physiological phenotypes are unknown in any fish species.

In this paper, we tested for the presence of sexual dimorphism in the hepatic transcriptome in a model teleost species, the zebrafish (*Danio rerio*). We then considered the potential effects of this dimorphism on the post-absorptive response to a long term manipulation of dietary carbohydrate. The zebrafish is a well recognized biomedical model system with an emerging new role in the study of glucose metabolism and its associated diseases (Gnugge et al., 2004; Elo et al., 2007). Although the zebrafish is an important biomedical research model, we know surprisingly little about its physiology, metabolic phenotype, and nutritional requirements. These data are important in extending the utility of the zebrafish beyond its traditional role as a model for developmental biology. The goals of this study were therefore to 1) test for sexual dimorphism in the zebrafish hepatic transcriptome, and 2) identify the persistent effects of long term manipulation of dietary carbohydrate on the zebrafish hepatic transcriptome in both males and females.

2. Materials and methods

2.1. Zebrafish rearing conditions

Larval zebrafish ($N=2400$, mean initial mass 49.7 mg) were obtained from a commercial breeder (Seacrest Farms, FL, USA), randomly assigned to groups of 200 fish, bulk-weighed, and placed into twelve 40-L rectangular glass tanks. Each tank was supplied with constant temperature (28.0 °C) spring water at the Hagerman Fish Culture Experiment Station (University of Idaho). Photoperiod was fixed at 14 h light/10 h dark for the duration of the study. Tanks were randomly assigned to one of four dietary treatments (described below), with three replicate tanks ($n=200$ fish per tank) per treatment. Fish were fed to apparent satiation three times daily, six days per week, for 12 weeks. The total weight of food delivered to each tank was recorded weekly. Feeding of experimental diets continued until the termination of the experiment, at which point the fish were sexually mature adults. Fish in each tank were bulk-weighed and counted at the end of weeks 4, 8 and 12.

2.2. Experimental diets

Four experimental diets were produced at the United States Department of Agriculture, Agriculture Research Service, Bozeman Fish Technology Center (Bozeman, MT, USA). A high protein, low carbohydrate basal diet (the 0% carbohydrate diet of our study) was formulated to contain 35% of inert filler mixture by weight (Table 1). Energy levels in the other three diets were increased progressively by replacing the inert filler mixture with wheat starch (15%, 25% and 35% diets). The four experimental diets were *iso*-nitrogenous and *iso*-lipidic. To ensure gelatinization of starch, the diets were produced by extrusion at 130 °C (Buhler DNDL-44, Minneapolis, MN, USA). Diets were produced as 2.5 mm diameter pellets, which were subsequently crumbled and screened to appropriate particle sizes for the fish.

2.3. Sampling

Fish were sampled at the commencement of the experiment and at four week intervals during the 12-week feeding trial. Fish were fasted for 24 h prior to each sampling event. At each sampling point, 10 fish were removed from each tank and rapidly euthanized with an overdose of MS222 in accordance with the experimental protocol approved by the University of Idaho's Institutional Animal Care and Use Committee. The fish were then measured for both standard length and body weight. Condition index (CI) was then calculated from these data as $CI = \text{body weight (mg)} / \text{length (mm)}^3 \times 100$. At the termination of the experiment (12 weeks), the animals were large enough to collect samples of individual tissues. At this point, 10 fish were removed from each tank, euthanized as described above, and the liver of each fish was placed in a sterilized 1.5-ml poly-propylene tube containing TRIzol (Invitrogen, Carlsbad, CA, USA). Samples were immediately homogenized and then stored at -80 °C until RNA extraction following the manufacturer's protocol. Fish were sexed by examination of external secondary sexual characteristics, including coloration of the anal fin and ventral body surface (yellow in males), and body shape (rounding of the belly in females).

In some cases, phenotypic sex indicated by body color and shape remained ambiguous. Therefore, we measured vitellogenin mRNA (see below) in each liver sample to confirm sex since vitellogenin expression can be used as a reliable indicator of sex in zebrafish (Wen et al., 2005).

2.4. Growth and body composition data

After sampling of individual fish for gene expression studies, we collected body composition data using bulk samples of the remaining fish from each tank. To determine proximate composition, fish were euthanized as described above, then dried and analyzed using AOAC (1990) methods for moisture and ash. A LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI, USA) and a Soxhlet extraction apparatus with methylene chloride as the solvent (Soxtec System HT, Foss Tecator AB, Hoganas, Sweden) were used to determine protein (N $\times$ 6.25) and crude lipid, respectively. Energy content of each sample was determined using a bomb calorimeter (Parr Instrument Co., Moline, IL, USA). Samples of feed were analyzed using the same methods to confirm composition and to allow calculations of dietary nutrient retention by the fish.

Concentrations of moisture, crude protein and energy in fish at the beginning and end of the study, and the quantities of each fed during the study, were used to calculate apparent nutrient retention values over the 12 week feeding trial. Indices were calculated on a per-tank basis for each dietary treatment group as follows: Specific growth rate (Sgr)=[ln(final weight) - ln(initial wt)] / duration of experiment (days), apparent feed conversion ratio (FCR)=feed intake/body weight gain, apparent protein retention efficiency (PRE%)=protein

gain in fish (g) / protein intake in feed (g)×100%, apparent energy retention efficiency (ERE %)=energy gain in fish (g) / energy intake in feed (g)×100%.

Average tank values were used as the experimental unit of observation for statistical analysis of growth, proximate compositions, and retention efficiencies. Percentage values for proximate composition were arc-sin transformed before statistical analysis. Analyses were conducted using the GLM procedure within SAS (version 9.0, Cary, NC, USA), and significant differences among dietary treatment means were calculated using Fisher's protected LSD.

2.5. Microarray analyses

We used Affymetrix GeneChip Zebrafish Genome Arrays (Affymetrix, Santa Clara, CA, USA) to characterize both the response of the hepatic transcriptome to manipulation of dietary carbohydrate and the level of sexual dimorphism in hepatic gene expression. We hybridized a total of 18 microarrays, including 10 females (5 for each of the 0% and 25% diets) and 8 males (4 for each of the 0% and 25% diets). We restricted our microarray analysis to the 0% and 25% carbohydrate treatments because of cost considerations. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO), under accession number GSE8856.

Microarrays were hybridized at the UI-WSU Center for Reproductive Biology Genomics Core Facility (Pullman, WA, USA). For each array, up to 10 µg of total RNA were converted to cDNA. Biotinylated cRNA was then produced *in vitro* using the GeneChip expression 3' amplification one-cycle target labeling kit (Affymetrix). Microarrays were hybridized with fragmented biotinylated cRNA for 16 h at 45 °C with constant rotation (45 rpm), and processed using the Affymetrix GeneChip Fluidic Station 450. Streptavidin-conjugated phycoerythrin (SAPE) was used for staining, followed by amplification using a biotinylated anti-streptavidin antibody. This was followed by another round of SAPE prior to scanning using a GeneChip Scanner 3000 (Affymetrix).

All analyses of microarray data were conducted within the Bioconductor package of R (www.R-project.org). Analysis of RNA degradation using AffyRNAdeg from Bioconductor's Affy package revealed that one of the arrays hybridized with RNA from a male in the 0% carbohydrate treatment had some RNA degradation. This array was therefore removed from the analysis. After hybridization and quality control, probesets were filtered based on the following criteria: First, probesets were eliminated that were not classified as present in all arrays of any one combination of sex and treatment using PMA calls. Second, four separate normalizations of the data (MAS 5, RMA, GCRMA, and PLIER) were performed, and only those probesets that were above the 95th percentile of the background for all four normalizations were retained. The distribution of background intensity values in each case was determined using the negative control probes on the microarray. Ultimately, 6165 probesets passed the filter criteria and were used in subsequent analyses. Arrays were then clustered based on expression levels of all expressed genes in our filtered set to test for the presence of outlier arrays. In this case, one sample (a male from the 25% carbohydrate treatment) did not cluster with the other arrays and was removed from the analysis.

We performed a stringent analysis of differential expression using a factorial model with Sex, Diet, and Sex * Diet interaction terms. The significance threshold for differential expression was set at $\alpha=0.05$ after correcting for a false discovery rate of 0.05 according to the method of Benjamini and Hochberg (2000). For differential expression analyses, we used only the RMA normalized data set (Irizarry et al., 2003) after application of the filters described above.

We then identified gene ontologies that were significantly over-represented in the lists of differentially expressed genes for each model term. In this analysis, the consequences of rare Type II errors (false positives) were less dire because we do not specifically identify individual genes. We therefore used the same factorial model of sex and diet, but included genes with p -values of less than $p=0.005$, unadjusted for multiple comparisons. We only retained genes for ontology analysis that were listed as significant in at least one term in the model using all four commonly used normalization routines (MAS5, RMA, GCRMA, PLIER). We then used these genes to test for over-representation of specific gene ontologies using a hypergeo-metric test (HyperG within Bioconductor).

2.6. Quantitative Real-Time PCR analyses

Expression levels of 12 genes were quantified using quantitative Real-Time PCR (qRT-PCR) (Table 2). This allowed us to validate results observed in the microarray experiment for several genes, including vitellogenin (*vtg1*), carnitine palmitoyl transferase 1 (*cpt1*), hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein) beta subunit (*hadhb*), uncoupling protein 2 (*ucp2*), hepatic nuclear factor 4 alpha (*hnf4a*), and glucokinase (*gck*). These analyses also allowed us to examine expression patterns of key genes that were not present on the Affymetrix Zebrafish microarray: glucocorticoid receptor (*gr*), hexokinase 1 and 2 (*hex1* and *hex2*), hepatic nuclear factor 4 gamma (*hnf4γ*), and glycogen synthase 1 and 2 (*gys1* and *gys2*). Finally, qRT-PCR analyses provided an effective way to expand our analysis of gene expression to include the entire range of dietary treatments (0%, 15%, 25%, and 35% dietary carbohydrates). We sought to test the hypotheses that expression of these 12 genes was sexually dimorphic, and that they were sensitive to chronic dietary carbohydrate manipulation.

We performed qRT-PCR using a random subset of 68 fish spanning all four dietary treatments. cDNA was synthesized for each sample using 400 ng of total RNA, 100 ng random primers (Invitrogen) and 50 U Superscript II (Invitrogen), following the manufacturer's protocol. qRT-PCR was performed on an ABI 7900HT Fast Real-Time PCR System using SYBR®-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. cDNAs were diluted 1:5 with TE (pH 8) and used as template in the reactions. Specificity of the reactions was verified using melting curve analysis. Because not all samples would fit on a single 96-well plate, samples were randomly divided into two groups (Sample Blocks) for qRT-PCR analysis. Two technical replicates were performed for each sample, each on separate plates. Each plate contained standard curves for both the target gene and the endogenous control to allow estimation of amplification efficiencies and to permit comparison among plates.

We chose 18S rRNA as our endogenous control gene after evaluating several potential genes, including β actin, eukaryotic elongation factor 1 α , and cyclophilin. Although 18S rRNA transcripts were very abundant compared to target genes, its expression was the least variable in technical replicates and showed no significant response to Diet or Sex. Primer sequences for all target genes and 18S rRNA are reported in Table 2.

2.7. qRT-PCR data analysis

Traditionally qRT-PCR data CT values for both target genes and endogenous controls are averaged across technical replicates before the target gene is normalized against the endogenous control prior to statistical analysis. However, analyses in this manner disregard variation among technical replicates and compound the error variances of the target and reference genes. Instead, we used a repeated measures split plot Analysis of Covariance (ANCOVA). Tanks were treated as whole plots with Diet as the whole plot treatment.

Individuals were treated as subplots with Sex as the subplot treatment. Plate was included as a blocking factor to account for possible covariance of expression measures from the same 96-well plate and Sample Block was used to account for covariance of expression for groups of samples that were assayed together throughout the study. Finally, expression (CT) of the endogenous control was included as a covariate. Tank and Individual were treated as random effects while Diet, Sex, Diet×Sex interaction, Plate, and Sample Block were treated as fixed effects. Means of the primary factors, adjusted (normalized) for expression of the endogenous control, were estimated using least squares. These analyses were performed on raw CT values for each target gene; identical analyses on log-transformed quantities estimated using standard curves yielded similar results (data not shown). All analyses were performed using the MIXED procedure within the SAS statistical software (version 9.13, Cary, NC). Assumptions of normality were evaluated by examining the distributions of the residuals of the analysis of each gene, and no notable violations were detected.

3. Results

3.1. Chronic effects of dietary carbohydrate on growth and whole body composition

Using the bulk weight data at the 12 week time point, we observed a significant effect of dietary carbohydrate level on specific growth rate ($F_{3,8}=4.23$, $p=0.046$), total body mass ($F_{3,8}=4.68$, $p=0.036$), and percent weight gain ($F_{3,8}=4.71$, $p=0.035$) at the end of the experiment. For each variable, the pattern of response was the same; growth in the 0% carbohydrate diet was significantly lower than in the other three treatments (Fig. 1). Feed conversion ratio followed the same trend, but the effect of dietary carbohydrate was only marginally significant ($F_{3,8}=3.56$, $p=0.067$). Fish fed the 0% carbohydrate diet exhibited reductions in relative retention efficiencies of both protein ($F_{3,8}=9.28$, $p=0.006$) and energy ($F_{3,8}=9.58$, $p=0.005$) (Fig. 1).

Proximate analysis revealed significant dietary effects on percentage of moisture ($F_{3,8}=240.2$, $p<0.0001$), lipid ($F_{3,8}=237.2$, $p<0.0001$), and ash ($F_{3,8}=10.16$, $p=0.004$) in the whole body of the fish. However, percentage protein did not differ among the dietary treatment groups ($F_{3,8}=0.94$, $p=0.467$). With the exception of the percentage of crude protein, these data followed the same pattern as growth and retention efficiency data, in which fish fed the 0% carbohydrate diet grew less relative to those fed the other diets (Fig. 2). Unlike the growth and efficiency data, there were some subtle differences among the treatment groups receiving dietary carbohydrate. Specifically, increases in dietary carbohydrate level reduced moisture and crude ash content and elevated fat content.

With the individual samples collected at the end of the study, we observed a significant interaction effect of Diet and Sex on both body mass ($F_{3,52}=3.80$, $p=0.015$) and condition index ($F_{3,52}=4.05$, $p=0.012$) (Fig. 3). As expected, we also observed a significant main effect of sex on these variables; males weighed less than females ($F_{1,52}=25.87$, $p<0.0001$) and had a lower condition index ($F_{1,52}=18.24$, $p<0.0001$). Unlike our results using bulk tank weights, diet did not have a significant effect on either body mass ($F_{3,52}=2.77$, $p=0.108$) or condition index ($F_{3,52}=0.58$, $p=0.640$). Neither Diet ($F_{3,52}=2.27$, $p=0.156$), Sex ($F_{1,52}=2.02$, $p=0.161$), or their interaction ($F_{3,52}=0.93$, $p=0.435$) had a significant effect on body length; however there was a non-significant trend for the males to have the largest body size (length) when fed the 15% carbohydrate diet, whereas female body length did not vary among treatments in which carbohydrate was available (Fig. 3).

3.2. Microarray analysis of gene expression

3.2.1. Effect of Sex—We observed 1249 genes (20.3% out of 6165 classified as expressed in the liver) that differed in hepatic expression levels between males and females regardless

of carbohydrate level. An additional 194 genes had significant Sex effects along with significant Diet or interaction effects (Fig. 4). Differentially expressed genes and their expression differences and FDR corrected *p*-values for each term in the statistical model are listed in Supplementary Table S1. Notable among the genes with significant Sex effects are several that would be expected to vary substantially between males and females, including vitellogenin genes (*vtg1*, *vtg3*), zona pellucida glycoprotein (*zp3*), and estrogen receptor (*esr1*). However, we also observed many other genes that have not previously been described as sexually dimorphic in fish, including genes involved in oxidative metabolism, insulin signaling, synthesis of macromolecules, and cell cycle regulation.

In males, we observed an up-regulation of genes associated with oxidative metabolism (including carbohydrate metabolism) and amelioration of oxidative stress. These included *hadhβ*, thioredoxin, prostaglandin synthase, and hexokinase (Table S1). This finding was also supported by our ontology analysis. We identified 57 biological process gene ontologies that were significantly over-represented in our list of genes up-regulated in males. Of these ontologies, many were related to carbohydrate metabolism and the production of energy (Supplementary Table S2).

Among genes up-regulated in females, we observed a variety of over-represented gene ontologies, many of which were associated with translation, synthesis of macromolecules, and cell cycle regulation (Supplementary Table S2). Up-regulation of translation-related genes in females seemed to be primarily associated with increased expression of 65 ribosomal protein genes, as well as three genes encoding translation elongation factor components and four genes encoding translation initiation factors (Table S1).

We also observed sexually dimorphic expression in some aspects of insulin signaling, although the pattern of expression (up-regulation in either males or females) varied by gene. For example, we observed higher levels of insulin induced gene 1 (*insig1*) and lower levels of phosphoenolpyruvate carboxykinase (*pepck*) in females than in males. There were also significant sex differences in key molecules of the insulin-like growth factor axis. Expression of insulin-like growth factors (*igf-1* and *igf-2*) and insulin-like growth factor binding protein 5 (*igfbp-5*) mRNA levels were greater in males than in females, as were levels of mitogenic genes, such as *fos* and *junb*, that are transcriptionally regulated by IGF.

3.2.2. Effect of Diet—One hundred sixty-one (161) genes indicated a significant response to Diet alone, and another 184 genes responded to Diet and had significant effects of Sex or Sex×Diet interaction (Fig. 4). All differentially expressed genes are listed in Supplementary Table S1. More genes (273) were up-regulated in response to increasing dietary carbohydrate level than were down-regulated (71). Considering only genes that were up-regulated in the 25% carbohydrate treatment, we observed a total of 17 gene ontologies that were over-represented, most of which were associated with the synthesis of ribosomes and fatty acids (Table S2). A total of 21 biological process ontologies were over-represented in the genes that were significantly down-regulated in the 25% carbohydrate treatment. These ontologies were primarily associated with regulation of transcription (Table S2).

3.2.3. Interaction between Diet and Sex—One hundred twenty-eight (128) genes were differentially expressed between males and females in a treatment dependent manner (Fig. 4). All of these genes also significantly responded to Sex and several also responded to Diet. We observed a more limited response to diet in the males compared to females, although this may be a reflection of the reduced statistical power in male samples. In males, 21 genes were significantly differentially expressed between the 0% and 25% treatments (3 down-regulated in the 25% diet and 18 up-regulated). In contrast, we observed 255 genes that differed in expression between females fed the 0% and 25% carbohydrate diets. Of these

genes, the vast majority (232) were up-regulated in the 25% diet, with only 23 down-regulated.

3.3. Quantitative RT-PCR analyses of gene expression

qRT-PCR analysis of *vtg*, *cpt1*, *hadh β* , and *hnf4a* validated differences between sexes or diets (0% and 25%) detected by the microarray analysis. However, by including all four diets in our analysis we were able to construct a more complete picture of the response to carbohydrate levels and the effect of sex. Below, we describe these effects for genes that were present on the microarray as well as those that were not.

Expanding our analysis of gene expression to all four dietary carbohydrate treatments through the use of qRT-PCR, we observed significant main effects of carbohydrate on the expression of glucokinase (*gck*) and *hadh β* , and a marginally significant effect on *cpt1* (Table 3). In each case, gene expression increased with dietary carbohydrate, although the exact pattern was specific to each gene. For example, we observed a trend towards increased *gck* expression in response to increasing dietary carbohydrate, while elevation of *cpt1* expression in both sexes occurred only in the 35% treatment (Fig. 5).

In contrast to the relatively limited numbers of genes responding to long term manipulation of dietary carbohydrate, eight of the 12 genes tested showed a significant main effect of sex (Table 3). For the majority of these genes, males had elevated expression relative to females (Figs. 6 and 7). The exceptions to this pattern were vitellogenin 1 (Fig. 6) and glucokinase (Fig. 4). The extensive sexual dimorphism in hepatic gene expression detected using qRT-PCR is similar to our microarray results, in which the bulk of the differentially expressed genes were also associated with differences among sexes.

Three genes (*pfk*, *gr*, *hadh β*) also had a significant Diet $\times$ Sex interaction, indicating that the sexes responded differently to manipulation of dietary carbohydrate. In particular, we observed elevated expression of *pfk* in the 35% treatment in males but not females, and increased expression of glucocorticoid receptor in males in the 15% treatment (Fig. 5). There was a similar pattern in *cpt1* (high in the males receiving 15% carbohydrate), although the interaction term was not significant. In the case of *hadh β* , we observed a sharp increase in the males with increased dietary carbohydrate, similar to our observations in the microarray data. Females increased expression of *hadh β* more gradually as levels of dietary carbohydrate increased (Fig. 7). We also observed a non-significant trend indicative of an interaction in *gck*, in which the females experience a higher up-regulation of the gene in response to increasing dietary carbohydrates relative to males (Fig. 5).

There were a number of similarities between the sex-specific patterns of growth and hepatic gene expression. Fish fed the 0% diet showed relatively little sexual dimorphism in gene expression, a pattern also observed in the growth data. Also, in males, effects of dietary carbohydrate level had a similar pattern for expression of *cpt1*, *gr*, and growth (Fig. 5). For these genes, we observed a spike in gene expression at the 15% diet level in males only, which is also the diet in which males perform best with respect to growth (Fig. 3).

4. Discussion

The goals of our study were to 1) test for sexual dimorphism in hepatic gene expression in zebrafish and 2) identify the effects of long term manipulation of dietary carbohydrate on the zebrafish hepatic transcriptome in sexually mature males and females. We detected substantial sexual dimorphism in the zebrafish hepatic transcriptome. Long term manipulation of dietary carbohydrate influenced growth, body composition, and hepatic gene expression patterns 24 h after a meal. In most cases, these differences were between the

0% diet and the diets containing carbohydrate. The transcriptional response of some genes to dietary manipulation varied by sex.

4.1. Sexual dimorphism of the hepatic transcriptome

Both microarray and qRT-PCR analyses of gene expression in the zebrafish indicated a high degree of sexual dimorphism in the hepatic transcriptome, regardless of the level of dietary carbohydrate. In mammals, the liver is one of the most sexually dimorphic organs in terms of gene expression (Yang et al., 2007). Widespread sexual dimorphism in gene expression has previously been identified in analyses of brains in Atlantic salmon (Aubin-Horth et al., 2005) and whole zebrafish (Wen et al., 2005), and comparisons between ovaries and testes in mature zebrafish (Santos et al., 2007); however, to the best of our knowledge, extensive measures of hepatic sexual dimorphism have not been documented in any fish species.

Analysis of gene ontologies from the microarray data indicated that male zebrafish have higher hepatic expression of many genes associated with carbohydrate metabolism, aerobic respiration, and production of energy compared with females. This is supported by our qRT-PCR results. With respect to genes associated with energy production, we observed expression levels of *hadh β* , a marker of mitochondrial energetics, to be increased markedly in males. In addition, we observed consistently higher expression in males of hexokinase and un-coupling protein 2, which are also associated with increased cellular metabolism. The next logical step in this line of research will be to determine if these differences in expression coincide with marked sexual dimorphism in liver metabolism.

The microarray data also indicate that males had increased expression of at least two genes involved with inflammation; prostaglandin synthase and *nfkbiab*, an inhibitor of the proinflammatory nf Kappa b (see Table S1). Expression levels of several genes associated with amelioration of oxidative stress were also elevated in male livers. A potential explanation for these observations is that energy production and oxidative metabolism is increased in male livers and may be inducing oxidative stress and inflammation. Thus, in males, increased expression of genes associated with mitochondrial energy production was accompanied by increased expression of genes associated with reduction of reactive oxygen species and inflammation. One of the potential negative effects of hyperglycemia associated with diabetes is damage to cells from oxidative stress (Brownlee 2005; Meugnier et al., 2007), indicating that hepatic sexual dimorphism should be considered when using the zebrafish as a model in studies of abnormal states of glucose homeostasis.

In females, we observed up-regulation of genes associated with translation and protein synthesis, particularly ribosomal proteins. Expression levels of three genes encoding translation elongation factor components and four genes encoding translation initiation factors were also increased in females. In all, sixty-five ribosomal protein genes or genes highly similar to them were more highly expressed in females. The increased expression of ribosomal protein genes in females might be indicative of higher protein biosynthesis and hence growth. Alternatively, up-regulation of these genes may also reflect an increased investment in reproduction and production of plasmatic molecules, consistent with our observation of increased expression of genes such as vitellogenin and zona pellucida glycoprotein.

Although the ontology analysis of our microarray data indicated male specific up-regulation of genes associated with carbohydrate metabolism, qRT-PCR analysis revealed that females tended to have higher levels of glucokinase expression when carbohydrates were present in the diet (Fig. 4). Glucokinase is a critical component of carbohydrate metabolism (Kim et al., 2004); however, to our knowledge, this pattern of sexual dimorphism in glucokinase expression has not previously been described in any vertebrate species. While there was a

highly significant effect of sex on *gck* expression, there was effectively no difference in expression levels between males and females fed the 0% carbohydrate treatment, a scenario where increased glucokinase expression and intracellular phosphorylation of glucose would serve little purpose. Expression of glucokinase is known to be acutely regulated by glucose treatment in other species of fish, including sea bream, trout, and carp (Panserat et al., 2000; Panserat et al., 2001a). Our data indicate a persistent up-regulation in females 24 h after a meal, raising the question as to whether expression of glucokinase is also sexually dimorphic during the acute phase of regulation immediately after a meal.

There are indications in our data that some components of insulin signaling are also sexually dimorphic. Two genes that are transcriptionally regulated by insulin, *insig-1* and *pepck* (Diamond et al., 1993), were up- and down-regulated respectively in female zebrafish. *insig1* is implicated in cholesterol homeostasis and lipogenesis (Flury et al., 2005; Nakagawa et al., 2006), whereas *PEPCK* is a key regulatory enzyme implicated in gluconeogenesis (Burgess et al., 2007). Higher expression of *insig1* and lower expression of *pepck* in females suggests an increase in lipogenesis (perhaps for egg production), and a decrease in gluconeogenesis. As *pepck* is transcriptionally regulated by several other hormones in addition to insulin, interpretation of its regulation and its relative response in male and female zebrafish requires careful consideration. The IGF axis genes, *igf-1*, *igf-2* and *igfbp-5* were up-regulated in males, suggesting increased mitogenic activity similar to previous results in rainbow trout (Gabillard et al., 2003). It is possible that increased expression of components of the IGF axis in males is associated with protein economy, as one mechanism of IGF is to decrease protein degradation (Hill et al., 1999; Bell and McDermott, 2000). While our expression data suggest dimorphism in insulin signaling, interpretation of this result is limited by the fact that insulinemia or glycemia data are not available.

The large degree of sexual dimorphism in hepatic gene expression in the zebrafish is similar to that observed in rodent models (Clodfelter et al., 2007; Yang et al., 2007). The mechanisms governing this dimorphism in mammals are not fully understood, but appear to be associated with both hormone-dependent and hormone-independent mechanisms (Rinn and Snyder, 2005). Hepatic nuclear factor 4 alpha (*hnf4a*) is known to contribute to hepatic carbohydrate sensing pathways and activation of lipogenesis (Adamson et al., 2006). In addition, *hnf4a* regulates expression of sexually dimorphic genes in the mouse liver through its interaction with *stat5* (Park et al., 2006). In zebrafish, we observed higher expression levels of both *hnf4a* and *hnf4γ* in male livers. Previous work also indicates that exposure to ethyl estradiol results in a dose dependent decrease in expression of *hnf4a* in female zebrafish liver (Hoffmann et al., 2006). Collectively these data indicate that the HNF4α mechanism that regulates mammalian hepatic sexual dimorphism could potentially be conserved between mammals and fish (Gong et al., 2004).

Although we observed widespread sexual dimorphism in hepatic gene expression, there was less dimorphism in the fish fed the 0% carbohydrate treatment. This pattern extended to both growth data (e.g. Fig. 3), and expression of some genes (e.g. *gck*). Reduced carbohydrate availability in the 0% diet likely resulted in hypoglycemia, compromised hepatic energy metabolism, and reduced investment in reproductive functions associated with the liver, potentially retarding sexual maturation. In mammals, sufficient dietary energy intake is a prerequisite for progression through puberty to sexual maturity (Cunningham et al., 1999; Margetic et al., 2002). However, it is not currently known how reduced energy intake influences the maturation process in zebrafish.

4.2. Response to dietary carbohydrate manipulation

Long term (12 weeks) manipulation of dietary carbohydrate impacted somatic growth, energy partitioning, and patterns of hepatic gene expression in the zebrafish. Fish fed the 0% carbohydrate diet grew significantly less relative to the other diets, as indicated by differences in specific growth rate, proximate body composition, and nutrient retention efficiencies. We were not able to separate males and females for measures because a relatively large amount of tissue was required for these assays. Given the differential hepatic response of males and females to carbohydrate manipulation, it is possible that proximate composition and retention efficiencies may also vary with sex.

It is important to note that the goal of our study was to determine the persistent effects (i.e. in the post-absorptive state) of long term dietary manipulation rather than acute responses associated with recent feeding. Fish were fasted for 24 h prior to sampling, and thus expression changes of genes that are acutely regulated in response to recent glucose and other nutrient intake would likely not be observed. Previous studies have considered responses to acute and chronic dietary carbohydrate manipulation in cultured fish species, including salmonids, sea bream, and the common carp, but few have carried out long term dietary manipulations from the larval stage to adulthood (see Hemre et al., 2002). Of these, most use fish that are not yet sexually mature. Our knowledge of nutrient utilization in sexually mature fish, and the potential for sexual dimorphism in metabolic phenotypes, is therefore limited.

One of the key findings from examination of glucose metabolism in aquaculture species is that, while there are conserved mechanisms shared between fish and mammals, there is also extensive variation among species in their capacity to utilize carbohydrates. Carp have a high capacity to use carbohydrates (Panserat et al., 2001b; Panserat et al., 2002), and several key glycolytic genes are inducible by a 10 week exposure to increased dietary glucose (Panserat et al., 2000; Panserat et al., 2002). In contrast to cyprinids, salmonids are relatively carbohydrate intolerant (Panserat and Kaushik, 2002). While it was once thought that this intolerance resulted from a lack of induction of hepatic glucokinase, more recent work has shown induction of hepatic glucokinase expression in trout (Panserat et al., 2000). The ability of zebrafish (which, like carp, belong to the family Cyprinidae) to utilize dietary carbohydrates has not been studied. While it is tempting to assume that all cyprinids have similar patterns of carbohydrate metabolism, the cyprinids are the largest family of freshwater fish, containing approximately 2420 species among which there is striking variation in life histories. The extent to which data on metabolic phenotypes in carp can be generalized across cyprinids, or specifically to zebrafish, has yet to be determined.

In our study, expression of glucokinase, a key regulatory gene of liver glycolysis, was significantly higher in fish fed the diets containing carbohydrate than those fed the 0% carbohydrate diet. This increase was detectable 24 h after the last meal. Our data therefore suggest that regulation of glucokinase in zebrafish may be affected by the duration of the dietary treatment. A previous study found that expression and enzymatic activity of glucokinase increased postprandially in carp and sea bream fed a 20% carbohydrate diet for 10 weeks but returned to pre-meal levels within 24 h (33). In the same study, rainbow trout had detectable increases in glucokinase expression 24 h after the last meal, but at 6 fold lower levels than those observed only hours after a meal. Somewhat unexpectedly, we found that glucokinase expression in zebrafish was still elevated 24 h after a meal containing carbohydrates. This elevation was most pronounced in the 35% diet in our study but was also seen in response to diets with carbohydrate levels similar to the previous study (15% and 25%), and was especially prominent in females. These data highlight the potential for large degrees of interspecific variation in patterns of carbohydrate metabolism, even within cyprinids.

Zebrafish fed a diet devoid of carbohydrate had significantly reduced levels of hepatic glucokinase expression, similar to the pattern observed in the growth and proximate composition measurements. Consistent with mammalian research (Rencurel and Girard, 1998; Mitro et al., 2007), it is possible that circulating D-glucose, its intracellular glycolytic derivatives, and/or insulin levels (Kim et al., 2004) serve as a metabolic regulator and directly modulate hepatic gene expression in zebrafish. To test the relative contributions of these mechanisms in regulation of liver glucokinase expression during chronic manipulations of dietary carbohydrate in zebrafish, future studies of transcriptional factors and transactivation activity will be required.

Expression of *hadhb* in the liver increased with dietary carbohydrate, suggesting the capacity for an increase in beta oxidation of fatty acids and mitochondrial production of ATP. *hadhb* is known to be an indicator of mitochondria number as established by the constant proportion enzyme principle, and as such is associated with cellular metabolic rate (Pette and Hofer, 1979). In general, we also observed up-regulation of multiple genes related to mitochondrial energy production in fish fed the 25% carbohydrate diet relative to fish that were carbohydrate restricted (see Table S1). In mammals, thioredoxin interacting protein is sensitive to carbohydrates and its promoter region contains carbohydrate response elements (Ma et al., 2006). Expression of thioredoxin interacting protein (*txnip*) increased in our study, reaching levels 2.3 times higher in the 25% dietary carbohydrate treatment. Together, these observations suggest restriction of carbohydrates has a profound effect on hepatocyte energy metabolism in zebrafish.

4.3. Summary

Our data show substantial sexual dimorphism in the zebrafish hepatic transcriptome and represent the first detailed description of a sexually dimorphic hepatic transcriptome in any fish species. Sexual dimorphism in the hepatic transcriptome has the potential to alter nutrient partitioning and utilization in a sex-specific manner in mature fish, as indicated by the patterns we detected in expression of genes involved with carbohydrate metabolism. We also showed that long term manipulation of dietary carbohydrate levels had a significant effect on body weight gain, condition factor, body composition, and hepatic gene expression in sexually mature adults. Male and female zebrafish responded differently to the dietary carbohydrate manipulation, both in terms of growth and hepatic gene expression. Many of the genes that showed a sex-dependent response to carbohydrate manipulation were involved in metabolism, oxidative stress, and translation. The next logical steps in studying the sexual dimorphism in zebrafish physiology are biochemical analyses of the dimorphic genes to determine whether dimorphism in the transcriptome is reflected at the protein level. As these genes are likely to influence the physiological parameters associated with normal and disease states, sexual dimorphism should be considered when using fish models to study hepatic function and glucose homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.cbd.2008.01.001.

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Fig. 1.

Specific growth rate (SGR), weight per fish, feed conversion ratio (FCR), percent weight gain, percent energy retention efficiency (ERE), and percent protein retention efficiency (PRE) for zebrafish fed one of four levels of dietary carbohydrate. All data are from the terminal time point of the study (90 days), and are expressed as means $\pm$ SE. Bars with different letters are significantly different as indicated by Fisher's protected LSD.

Fig. 2.

Percentage body composition for moisture, lipid, ash, and crude protein of zebrafish fed one of our levels of dietary carbohydrate. All data are from the terminal time point of the study (90 days), and are expressed as means $\pm$ SE. Bars with different letters are significantly different as indicated by Fisher's protected LSD. In each graph, the body composition data from the initial time point of the experiment are indicated on the far left. Note that these data are included for comparison purposes only, and are not included in the statistical analysis of dietary effect.

Fig. 3.

Final body mass, standard length, and condition index of the zebrafish used for hepatic gene expression analyses. Data are separated by sex (filled squares = males, open circles = females). All data are from the terminal time point of the study, and are expressed as means $\pm$ SE. There are significant interactions between Diet and Sex for both weight and condition index, but not standard length.

Fig. 4.

Venn diagram indicating the number of genes from the Affymetrix microarray that were significantly differentially expressed for each term in the factorial model of dietary carbohydrate treatment (Treatment) and sex. Of the 6165 genes classified as expressed in the liver, 4561 were not significant for any of the model terms (bottom right).

Fig. 5.

Patterns of expression of glucokinase (*gck*), phosphofructokinase (*pfk*), carnitine palmitoyl transferase (*cpt1*), and glucocorticoid receptor (*gr*) in male (filled squares) and female (open circles) zebrafish fed different levels of dietary carbohydrate. Values are normalized to the lowest mean expression value for that gene, and are plotted on a log₂ basis (a difference of 1 on the y axis represents a doubling of expression level). Data are plotted as least square means (+/-SE) generated from the mixed model ANOVA.

Fig. 6.

Patterns of expression of vitellogenin 1 (*vtg1*), hexokinase 1 (*hex*), hepatic nuclear factor 4 γ (*hnf4\gamma*), and hepatic nuclear factor 4 α (*hnf4\alpha*) in male (filled squares) and female (open circles) zebrafish fed four levels of dietary carbohydrate. Values are normalized to the lowest mean expression value for that gene, and are plotted on a log₂ basis (a difference of 1 on the y axis represents a doubling of expression level). Data are plotted as least square means (+/-SE) generated from the mixed model ANOVA.

Fig. 7.

Patterns of expression of hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (*hadhβ*), uncoupling protein 2 (*ucp2*), glycogen synthase 1 (*gys1*), and glycogen synthase 2 (*gys2*) in male (filled squares) and female (open circles) zebrafish fed four levels of dietary carbohydrate. Values are normalized to the lowest mean expression value for that gene, and are plotted on a log₂ basis (a difference of 1 on the y axis represents a doubling of expression level). Data are plotted as least square means ($\pm$ SE) generated from the mixed model ANOVA.

Table 1

Ingredient formulation and nutrient composition of extruded experimental diets

Component	Wheat starch inclusion level			
	Diet 1 (0%)	Diet 2 (15%)	Diet 3 (25%)	Diet 3 (35%)
<i>Ingredient formulation (wet basis)</i>				
Fish meal	36.0	36.0	36.0	36.0
Wheat starch	0.0	15.0	25.0	35.0
Alpha-cellulose (indigestible filler)	17.5	10.0	5.0	0.0
Silica (indigestible filler)	17.5	10.0	5.0	0.0
Wheat gluten meal	9.0	9.0	9.0	9.0
Soybean meal	4.0	4.0	4.0	4.0
Corn gluten, yellow	3.8	3.8	3.8	3.8
Fish oil	10.0	10.0	10.0	10.0
Choline chloride (60% active)	0.3	0.3	0.3	0.3
Vitamin/mineral premix ^a	1.9	1.9	1.9	1.9
<i>Calculated nutrient composition (dry basis)</i>				
Moisture (%)	7.3	7.3	7.3	7.3
Digestible protein (%)	38.0	38.0	38.0	38.0
Digestible fat (%)	14.8	14.8	14.8	14.8
Starch (%)	0.0	15.0	25.0	35.0
Ash (%)	27.4	19.4	14.0	8.6
Gross energy (cal/kg)	2927	3056	3141	3227

^aVitamin and mineral premix provided the following per kg diet (mg/kg of premix, unless otherwise listed): D calcium pantothenate, 26,840; pyridoxine (pyridoxine HCl), 7700; riboflavin, 13,200; niacinamide, 55,000; folic acid, 2200; thiamine (thiamine mononitrate), 8800; biotin, 88; vitamin B12, 5.5; menadione sodium bisulfite complex, 2.75; vitamin E (DL α -tocopherol acetate), 88,000 IU; vitamin D3 (stabilized), 110,000 IU; vitamin A (vitamin A palmitate, stabilized), 1,650,000 IU. Zn (as ZnSO₄·7H₂O), 75; Mn (as MnSO₄), 20; Cu (as CuSO₄·5H₂O), 1.54; I (as KIO₃), 10.

Primer sequences for qRT-PCR analysis of the effect of dietary carbohydrate level on gene expression in the livers of adult male and female zebrafish

Table 2

Gene	Accession #	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
<i>cpt1</i>	NM_001044854	GCAATTGATCGGCATCTCTTT	CAGTCTCCAAGGCTCTTGACA	98
<i>gck</i>	NM_001045385	GCTGTGAAGTCGGCATGATA	CTTCAACACAGCTCCACCTTAC	81
<i>gr</i>	DQ017615	CCAGCACTGTAAACGGAAACA	GACACTTGCAGTCGCACAAA	138
<i>gys1</i>	NM_201180	GCTCAGTGGATGAGTCTCTGTAA	ACGTAATACCGGCCACAGATA	145
<i>gys2</i>	NM_001018679	CGCGCAGGCAGAGAAATAAT	AATGTCTGGCGTGCAATGTAG	99
<i>hadhβ</i>	NM_200019	TTCTCTGTCCCTCGGTCATC	CTTCGATCACCATTGCATGA	146
<i>hex2</i>	NM_213066	CAACAAACGCCACCGTCAAAATG	GCCCAGATCCAATGCCAAGAAA	92
<i>hnf4a</i>	BC056550	ATTGTTTGGAAATGGCGAAGA	AGGTGTTCTTGGACCATG	117
<i>hnf4γ</i>	DQ017616	GCAGATGATTGAGCAGCTACAG	TGAGGGTGACCAATTGTGATG	129
<i>pfkf</i>	XM_695781 ^a	CGACAGGAACTTCGGCACTA	CCAATCACACATGCCGTATC	123
<i>ucp2</i>	NM_131176	GGGCCAAAGGCTTTTACA	CGTTTCAGCTGCTCGTAAGT	118
<i>vtg1</i>	AY034146	GCCAAAAAAGCTGGGTAAACA	AGTTCCGTCTGTGGATTGTGG	210
18S rRNA	NM_001089506/NM_001098396 ^b	GAACGCCACTTGTCCCTCTA	GTTGGTGGAGCGGATTGTCT	117

^aPredicted gene in zebrafish.

^bIdentified through high sequence similarity to *Cyprinus carpio* 18S rRNA sequence (accession# AF133089).

Table 3
Results of Analysis of Covariance (ANCOVA) of expression of metabolic genes in livers of adult zebrafish

Gene	Diet			Sex			Diet×Sex		
	F	df	p	F	df	p	F	df	p
<i>cpt1</i>	3.33	3, 8	0.0771	1.77	1, 49	0.1897	2.08	3, 49	0.1150
<i>gck</i>	4.44	3, 8	0.0407	15.98	1, 50	0.0002	1.17	3, 50	0.3289
<i>gr</i>	0.77	3, 8	0.5408	11.43	1, 50	0.0014	2.80	3, 50	0.0497
<i>gys1</i>	0.82	3, 8	0.5190	0.00	1, 48	0.9612	0.51	3, 48	0.6770
<i>gys2</i>	0.39	3, 8	0.7668	11.99	1, 50	0.0011	0.93	3, 50	0.4320
<i>hadhβ</i>	10.45	3, 8	0.0038	52.01	1, 49	<0.0001	2.39	3, 49	0.0796
<i>hex2</i>	0.75	3, 8	0.5538	6.17	1, 50	0.0164	0.38	3, 50	0.7659
<i>hnf4α</i>	1.08	3, 8	0.4102	20.97	1, 50	<0.0001	0.42	3, 50	0.7418
<i>hnf4γ</i>	0.38	3, 8	0.7686	12.84	1, 50	0.0008	0.49	3, 50	0.6924
<i>pfkf</i>	0.58	3, 8	0.6427	1.53	1, 49	0.2217	3.10	3, 49	0.0349
<i>ucp2</i>	0.18	3, 8	0.9094	34.22	1, 50	<0.0001	0.48	3, 50	0.7009
<i>vtg1</i>									
Females	2.00	3, 8	0.1928						
Males	1.96	3, 6	0.2220						

cpt1 = carnitine palmitoyl transferase 1; *gck* = glucokinase; *gr* = glucocorticoid receptor; *gys* = glycogen synthase; *hadhβ* = hydroxyacyl-Coenzyme A dehydrogenase/β-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit; *hex2* = hexokinase 2; *hnf4* = hepatocyte nuclear factor 4; *pfkf* = phosphofructokinase (liver); *ucp2* = uncoupling protein 2; *vtg1* = vitellogenin 1.

Cycle thresholds (CT) of target genes were analyzed using CTs of the reference gene (18S RNA) as a covariate.