Ploidy effects on genes regulating growth mechanisms during fasting and refeeding in juvenile rainbow trout (Oncorhynchus mykiss)

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Abstract

Diploid and triploid rainbow trout weighing approximately 3 g were either fed for five weeks, or feed deprived for one week, followed by refeeding. During feed deprivation gastrointestinal somatic index decreased in diploids, but not triploids, and during refeeding, carcass growth rate recovered more quickly in triploids. Although not affected by ploidy, liver ghr2 and igfbp2b expression increased and igfpt1b decreased in fasted fish. Effects of ploidy on gene expression indicate potential mechanisms associated with improved recovery growth in triploids, which include decreased hepatic igfbp expression, which could influence IGF-I bioavailability, differences in tissue sensitivity to TGFbeta ligands due to altered tgb and smad expression, and differences in expression of muscle regulatory genes (myf5, mstn1a, and mstn1b). These data suggest that polyplody influences the expression of genes critical to muscle development and general growth regulation, which may explain why triploid fish recover from nutritional insult better than diploid fish.

1. Introduction

The production of triploid animals is becoming more prevalent in the aquaculture industry. Triploid fish lack the ability to reproduce, but only the triploid females exhibit a near absence of gonad development. This inability to develop functional gonads provides several advantages that make triploid production desirable (Pifferer et al., 2009). From a production standpoint, impaired triploid ovarian growth prevents the negative effects of sexual maturation on growth performance and fillet quality (Aussanasuwannakul et al., 2011; Salem et al., 2006), allowing for improved production of larger female fish (Sumpter et al., 1991). Furthermore, producing sterile individuals is advantageous in situations where native species and conspecific populations require protection from stocked or escaped fish, including those with enhanced phenotypes, or when proprietary germplasm requires protection from propagation.

Although some studies report similar growth performance among triploid and diploid salmonids, including rainbow trout, diploids have been shown to grow faster than triploids when both ploidy types are reared in cohabitation, while triploid females grow faster than sexually maturing diploid females (Maxime, 2008; Pifferer et al., 2009). Although most cells in triploid tissues display hypertrophy, gigantism is not observed in triploid fish due in large part to hyperstromy being offset by hypoplasia (Benley, 1999). However, this compensation is not as clear in muscle as differences in muscle fiber size distributions with ploidy, which diminishes with age, has been observed in both rainbow trout (Suresh and Sheehan, 1998) and Atlantic salmon (Johnston et al., 1999). These differences derive from reduced rates of fiber recruitment as a result of lower satellite cell density in triploids compared with diploids, which is offset in triploids by a compensatory increase in muscle fiber hypertrophy (Johnston et al., 1999; Suresh and Sheehan, 1998). As hyperplasia is a predominant mechanism of muscle growth in rainbow trout between 1 g and 25 g size (Johansen and Overturf, 2005; Rowlerson and Veggetti, 2001), differences in satellite cell densities, rate of fiber recruitment, and extent of hypertrophy suggest differences in the regulation of muscle growth between diploids and triploids during this phase of muscle growth.

Feed deprivation followed by refeeding is an experimental design that results in distinct growth phases: (1) basal or “normal” growth in pre-fasting or pair-fed control animals, (2) weight loss characterized by catabolic metabolism during feed deprivation, and (3) recovery or compensatory growth during refeeding characterized by hyperphagia and fast growth rates (Ali et al., 2003). Therefore, this experimental design is useful as a model for characterizing the effects of ploidy on physiological and endocrine mechanisms responsible for regulating growth and nutrient utilization. We reported previously that juvenile triploid rainbow trout (∼3 g) are better able to recover from feed deprivation and accelerate elongation and growth of nonvisceral tissues earlier into refeeding than diploids (Cleveland and Weber, 2013). In contrast, diploids mobilize visceral nutrient stores and replace them upon...
refeeding to a greater extent than triploids (Cleveland and Weber, 2013). Additionally, during refeeding expression of autophagy-related genes in diploids overshoot those of continually fed controls, while expression in triploids returns only to baseline, suggesting that ploidy effects on proteolytic mechanisms contribute to differences in recovery growth. However, the GH/IGF-I axis, myostatins (MSTN), and other regulatory mechanisms may also be affected by ploidy as these mechanisms are affected by feed deprivation and refeeding in rainbow trout (Chauvigne et al., 2003; Gabillard et al., 2006; Johansen and Overturf, 2006; Montserrat et al., 2007), and IGF-I signaling is affected by ploidy in other fish species (Radaelli et al., 2010; Zhong et al., 2012).

The current study examines expression of components of GH/IGF-I axis, including IGFBPs, components of the TGF-β/MSTN family regulatory system, muscle regulatory factors (myogenin, MyoD, Myf5) in muscle, and estradiol receptors in liver and muscle in fasting and refeeding diploid and triploid rainbow trout. Determining how these genes are regulated will provide insight into how physiological and endocrine mechanisms are affected by ploidy, thereby improving our understanding of how mechanisms regulating growth and nutrient partitioning differ between diploid and triploid fish.

2. Materials and methods

2.1. Experimental design

Experimental procedures adhered to animal care and use committee (ACUC) guidelines and received approval from the institutional (NCCCW) ACUC (protocol #078). Details regarding the experimental design have been previously reported (Cleveland and Weber, 2013). Briefly, diploid and triploid rainbow trout were generated from each of 4 full sibling families to produce a total of 8 experimental families (4 families × 2 ploidy). At approximately 2 months of age, 50 fish from each family were stocked into two 10 liter tanks (n = 25 fish per tank, n = 16 tanks) and allowed to acclimate for one week prior to the initiation of the study. One tank per family was fed to satiation for two weeks, then feed deprived for one week (wk 1), followed by a four week recovery period (wk 2 through wk 5) during which they were fed to satiation. The second tank per family was fed to satiation during the entire experimental period.

All fish were weighed (to 0.01 g) at the beginning of the study and fork lengths (to 0.1 cm) and weights were recorded at the end of wk 0 and weekly thereafter. Immediately prior to feed deprivation (wk 0) fish averaged 2.89 g and 6.0 cm. Fish were anesthetized with 50 mg tricaine methanesulfonate (MS-222) per liter water during each weighing event. Additionally, after week 0, 1, 2, 3, and 5, two fish per tank were sampled by recording fork length and body, liver, and eviscerated body weights. Liver and white muscle samples were frozen in liquid nitrogen for gene expression analysis. To determine short-term effects of refeeding, two fish per tank on the scheduled feeding were sampled 4 h and 24 h after the initial reinitiation of feed following the feed deprivation period. Two fish in each control tank were removed during these sampling periods to maintain similar densities across tanks.

Specific growth rates, on either a whole body weight (SGR<sub>WBW</sub>) or eviscerated body weight (SGR<sub>EVBW</sub>) basis, are presented as percent gained per day and were calculated by the following equation: SGR = ln(\text{value}_{t+1}/\text{value}_{t})/(t_{t+1} - t_{t}) * 100. Average EVBW for each tank was calculated by multiplying the average fish weight by the average percent EVBW for each treatment by ploidy combination. Gastrointestinal somatic index (GtSI) was calculated using values from fish harvested during each sampling period. The following equation was used to calculate GtSI:

\[ \text{GtSI} = \left( \frac{\text{WBW} - \text{EVBW}}{\text{WBW}} \right) * 100, \]

therefore GtSI represents the weight of all components of the viscera and alimentary canal, including the liver and digesta.

2.2. Gene expression analysis

To isolate RNA, 50 – 100 mg of white muscle was homogenized in 1 ml TRIZol (Invitrogen, Carlsbad, CA) per manufacturer’s suggested protocol using a 5 mm steel bead and a multi-tube shaker. The RNA pellet was washed twice with 75% ethanol, and resuspended in nuclease-free water. RNA quality and quantity was determined by measuring absorbance at 260 nm and 280 nm. The GenomeLab GeXP genetic analysis system (Beckman Coulter Inc.) was used to simultaneously analyze gene expression in liver and muscle tissue. Gene symbols, protein names, and gene accession numbers are categorized and reported in Table 1. Primer sequences and amplicon sizes are presented in supplementary Table 1. Optimization of the multiplex, standard curve, reverse transcriptase (RT) and PCR reactions, and capillary electrophoresis were performed as recommended by the manufacturer (GeXP Chemistry protocol A29143AC; February, 2009) with reagents provided in the GeXP Start Kit (Beckman Coulter Inc.).

For the RT reaction, 100 ng of DNase treated RNA was diluted 1:5 with nuclease-free water, and 2.5 µl was used in a 10 µl RT reaction that included 2 µl 5X RT buffer, 1 µl gene-specific reverse primer mix, 0.5 µl RT, and 1.25 µl kanamycin resistance gene RNA (internal control, 1:4 dilution). The RT was incubated according to kit instructions. An aliquot (4.65 µl) of the resultant cDNA was used in a PCR reaction that included 2 µl 25 mM MgCl<sub>2</sub>, 2 µl 5X PCR buffer, 1 µl forward primer mix, and 0.35 µl DNA Taq polymerase. The PCR was incubated according to kit instructions. The PCR products (1 µl) were combined with 38.5 µl sample loading solution and 0.5 µl size standard 400. The PCR products were separated by capillary electrophoresis in the GeXP Genetic Analysis System using the Frag-3 protocol.

Heights for each peak within the multiplex were exported to eXpress Profiler software (Beckman Coulter, Inc.) for analysis and normalization to the internal kanamycin control. Concentrations were interpolated from the standard curves for each gene of interest. Data were input into GeNorm software to determine which reference genes were most stable through calculation of an M-value, which describes the variation of a gene compared to all other candidate genes. The most stable reference genes were actb, rplp2, and eef1a, with average M-values in liver of 0.423, 0.445, and 0.422, respectively, and in muscle of 0.424, 0.541, and 0.429 respectively, therefore their geometric mean was used to generate a normalization factor for each sample. Thus, the normalized expression of each gene transcript is reported as the quantity relative to the geometric mean of the selected reference genes.

2.3. Statistical analysis

For growth performance, carcass characteristics, and gene expression values, main effects of feeding treatment, ploidy, family, and interactions between these variables were determined using the general linear models (GLM) procedure for analysis of variance with PC-SAS software (V9.2). Although main effects of family were observed, there were no notable interactions (P > 0.10) between family and other independent variables, therefore only main effects of feeding treatment and ploidy, and their interaction are presented. When main effects or interactions were significant (P < 0.05), a pairwise comparison between lsmeans was used to identify differences between treatments within each week. All values are presented as lsmeans ± SEM.

Gene expression values are transformed and reported as log<sub>2</sub>(fold change), where fold change is calculated relative to the
average expression level of the continuously fed fish within each ploidy. Therefore, the average value for control treatments was zero; log2(1.0) = 0.0, and a negative and positive value for the schedule feeding treatments indicates lower and higher expression levels, respectively, relative to control. The 4 h and 24 h post-feed deprivation values are expressed as fold change relative to the 1 wk control. Main effects of week, ploidy, family and their interaction were determined for gene expression values for fish on the schedule treatment. Although main effects of family were observed, there no notable interactions (P > 0.10) between family and gene expression.

3. Results

3.1. Growth response

Effects of ploidy and feeding treatment on feed intake and the growth response have been previously reported (Cleveland and Weber, 2013). The percent loss in whole body weight (WBW), eviscerated body weight (EvBW), and gastrointestinal somatic index (GtSI) are shown in Fig. 1a. In both diploids and triploids one week of feed deprivation resulted in a loss of approximately 12% WBW and 8% EvBW. In diploids GtSI was reduced 19% during feed deprivation, while the 9% reduction in GtSI in triploids was not statistically significant.

Regardless of ploidy, specific growth rate (SGR) as a function of WBW was between 110% and 140% greater than continually fed controls during the refeeding period (Fig. 1b). These data, in combination with the previously reported hyperphagia indicate that refeeding fish were exhibiting recovery growth, although a true compensatory growth response was not observed as the body weight of refeeding fish did not catch up to that of continuously fed controls (Cleveland and Weber, 2013). However, during the first week of refeeding SGRWBW in triploids was 135% of controls, while only 124% in diploids controls, indicating that triploids at this age or size can better accelerate WBW gain during refeeding. Additionally, during the first week of refeeding triploid SGR as a function of EvBW (SGREvBW) was significantly greater than continually fed controls while SGREvBW in diploids did not exceed controls until two weeks into refeeding (Fig. 1c). In contrast, recovery of GtSI occurred rapidly in diploids (122% of controls), while GtSI in triploid fish was not affected by fasting or refeeding (Fig. 1d).

3.2. Main effect of feed deprivation and refeeding

Gene expression values are pooled across ploidies to present main effects of feed deprivation and refeeding on gene expression within heat maps in Fig. 2, with numerical fold change values and associated statistics presented within Supplementary Fig. 1 (liver) and Supplementary Fig. 2 (muscle).

### Table 1

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3.2.1. Expression of GH/IGF system genes

While there was no main effect of fasting or refeeding on expression of ghrl in liver, expression of ghrl in liver and ghrl and ghrl in muscle were increased after feed deprivation before returning to control levels by 1 week of refeeding, indicating an increase in GH sensitivity in both tissues during fasting that returns to control levels upon long-term (>24 h) refeeding (Fig. 2, Supp. Figs. 1a and 2a). In contrast, an increase in tissue sensitivity to IGF signaling occurred only during short-term refeeding (4, 24 h) in both liver and muscle, as expression of igf1r1 was up-regulated only during this period (Fig. 2, Supp. Figs. 1a and 2a). Expression of igf1 decreased in liver during feed deprivation, whereas expression in muscle increased only during refeeding (Fig. 2, Supp. Figs. 1a and 2a). Expression of igf2 decreased in muscle during feed deprivation, and increased in liver and muscle during short-term refeeding (4 and/or 24 h) before decreasing during wk 2 (Fig. 2, Supp. Figs. 1a and 2a). Regulation of igfbp genes occurred during fasting and refeeding in both liver and muscle (Fig. 2, Supp. Figs. 1b and 2b). In liver, expression of igfbp genes did not display a consistent pattern, with two genes decreasing only during feed deprivation (igfbp1b and igfbp4), others increasing during refeeding (igfbp2 and igfbp6), while some were not affected by feeding treatment (igfbp2b and mac25/igfbp-rp1). In contrast, regulation of igfbp genes in muscle was more consistent, with overall decreased expression during fasting, although decreases were not always significant. During short-term refeeding expression increased to or beyond control levels, and several igfbps exhibited an overshoot response, with expression levels decreasing below controls one week (wk 2) into refeeding.

3.2.2. Expression of TGFbeta system genes

Expression of the MSTNs, mstn1a and mstn1b showed little change in expression, both reduced only at mid-reefeding (Fig. 2, Supp. Fig. 2c). Increased liver and muscle expression of igfbp1 with fasting (Fig. 2, Supp. Figs. 1c and 2d), and increased expression of liver and muscle acvr1b during refeeding (Fig. 2, Supp. Figs. 1f and 2e), supports increased sensitivity to TGFbeta superfamily ligands in response to changes in nutrient supply. Expression of smads responsible for TGFbeta and MSTN signaling; smad2a, smad2b, and smad3, and expression of the co-smads, smad4a and smad4b varied with feeding treatment and tissue (Fig. 2, Supp. Figs. 1c and 2d). The inhibitory smad, smad7, was affected by feeding treatment only in liver, with increased expression early into refeeding followed by decreased expression by one week into refeeding (wk 2). Hepatic expression of bambi and fst, inhibitors of TGFbeta superfamily activity, was increased upon fasting and refeeding, respectively (Fig. 2, Supp. Fig. 1e). In contrast, in muscle, fst expression was increased with fasting and bambi was only increased 24hrs into refeeding (Fig. 2, Supp. Fig. 2f).

3.2.3. Expression of muscle specific genes

Expression of mlc was increased during early refeeding then reduced at 1 week of refeeding supporting a transient increase in muscle fiber growth with refeeding (Fig. 2, Supp. Fig. 2c). Expression of myog and pax7a were reduced during feed deprivation and early refeeding, whereas myf5 and myod expression were increased during early and mid refeeding respectively (Fig. 2, Supp. Fig. 2c). Changes in expression of muscle regulatory factors during feed deprivation and refeeding indicates regulation of myogenesis via effects on proliferation and differentiation mechanisms. During fasting, reductions in expression of pax7a and myog suggests the transition of myosatellite into a quiescent state and decreased cell differentiation (Fig. 2, Supp. Fig. 2c).

3.2.4. Expression of estrogen receptors

Hepatic expression of estrogen receptors responded differently to feed deprivation, with expression of era1 and erb1 decreasing and expression of era2 and erb2 increasing with feed deprivation (Fig. 2, Supp. Fig. 1d). Furthermore, era2 and erb2 demonstrated an overshoot response during refeeding (wk 2). In muscle, expression of all estrogen receptors, except era2, increased during feed
deprivation, which was sustained at least 24 h into refeeding for era1 and erb2 (Fig. 2, Supp. Fig. 2e).

3.3. Effect of ploidy on gene expression in response to fasting and refeeding

Only those genes exhibiting a main effect of ploidy or an interaction between ploidy and sampling period are presented in Figs. 3 and 4 (liver) and Fig. 5 (muscle). There were consistent effects of ploidy on the regulation of genes in hepatic tissue (Figs. 3 and 4), which were more prevalent during feed deprivation and throughout the early part of refeeding (4 h, 24 h, wk 2) than during the latter part of refeeding (wk 5) when gene expression had returned to control levels. For genes that were reduced during feed deprivation (i.e.: smad2a, smad2b, smad3, jst, igfbp4, igfbp5), down-regulation was often significant in triploids but not diploids (Figs. 3 and 4). Furthermore, when down-regulation was significant in both ploids (i.e.: era1, igf1), gene expression returned to control levels more quickly in diploids during refeeding (Figs. 3 and 4). In terms of up-regulation, diploid fish consistently demonstrated greater fold changes during feed deprivation or refeeding than triploids (i.e.: bambi, tgfbr1, smad4b, smad7, lbfr1b, igfbp6) (Figs. 3 and 4). Furthermore, when up-regulation occurred, triploids demonstrated a tendency to return transcript abundance to control levels more quickly than diploids, and often demonstrated an overshoot response during wk 2 or wk 3 (i.e.: erb2, ghr2, igfbp2), a response not present in diploids (Figs. 3 and 4). In contrast, in muscle there were no distinct differences in patterns of regulation between diploids and triploids (Fig. 5).

3.4. Effect of ploidy in continually fed diploid and triploid controls

Differences in gene expression in continually fed diploid and triploid fish are shown in Supplementary Fig. 3 (liver) and 4 (muscle). Only those genes for which a main effect of ploidy or an interaction between ploidy and week is significant are presented. While several genes displayed a trend (P < 0.10) towards differential expression in liver in diploids and triploid fish, only two genes, smad2a and smad7, displayed a significant (P < 0.05) main effect of ploidy, with triploids displaying overall increased expression compared to diploids (Supp. Fig. 3). Similarly, in muscle tissue only mlc and mstn1b were affected by ploidy, with increased overall higher expression in diploids versus triploids (Supp. Fig. 4). However, for tgf2, triploids exhibited increased expression compared to diploids only during experimental week 3 (Supp. Fig. 4).

4. Discussion

Juvenile diploid and triploid rainbow trout exhibited distinct responses to feed deprivation and refeeding, in terms of nutrient partitioning, growth performance, and gene expression response as...
we previously reported for these same fish (Cleveland and Weber, 2013).

The observed differences in gene expression between the ploidy during fasting and refeeding indicate that regulation of physiological mechanisms are affected by ploidy and likely contribute to observed differences in nutrient partitioning and growth. As this study is correlative in nature, it identifies mechanisms associated with a ploidy-specific response, thus laying the foundation for subsequent causative studies that isolate and determine the physiological relevance of those mechanisms. Additionally, understanding of the basic physiological differences between 2N and 3N fish is lacking, therefore the identification of genes and tissues susceptible to effects of polyploidy provides specific targets for those studies.

![Graphs showing fold change in gene expression for GH/IGF-I axis genes in liver from fish on the schedule feeding treatment. Values within all graphs are expressed as log2 (fold change), where fold change is relative to the average of the control tanks, set at 1.0 (log2 1.0 = 0), harvested the same week. The 4 h and 24 h samples are expressed relative to the control tanks harvested on wk 1. Different letters indicate significant differences within each time period, P < 0.05. Asterisks indicate a significant difference from the control treatment, P < 0.05.](image)

Fig. 3. Effects of ploidy on expression of GH/IGF-I axis genes in liver from fish on the schedule feeding treatment. Values within all graphs are expressed as log2 (fold change), where fold change is relative to the average of the control tanks, set at 1.0 (log2 1.0 = 0), harvested the same week. The 4 h and 24 h samples are expressed relative to the control tanks harvested on wk 1. Different letters indicate significant differences within each time period, P < 0.05. Asterisks indicate a significant difference from the control treatment, P < 0.05.
Measurements of GtSI during fasting and refeeding are suggestive of mobilization and replenishment, respectively, of visceral nutrient stores, that are predominantly composed of visceral adipose tissue. Therefore, the significant reduction in GtSI in diploids (2N, 20%), which was not present in triploids, is suggestive that diploids mobilize visceral nutrient stores during feed deprivation to a greater extent than triploids. However, the diploids were rapidly able to replenish these stores within two weeks of refeeding. Potentially due to the metabolic effort the diploids put into replacing visceral nutrient stores, the rate of non-visceral tissue growth (EvBW) did not exceed controls until after the first week of refeeding. In contrast, the loss of visceral nutrient stores during feed deprivation in triploids was not significant, suggesting that triploids mobilize less visceral energy stores during feed deprivation. Furthermore, while early recovery growth was primarily rebuilding of visceral tissues in diploids, in triploids early recovery growth

Fig. 4. Effects of ploidy on expression of TGFbeta superfamily and estrogen receptor genes in liver from fish on the schedule feeding treatment. Values within all graphs are expressed as log2(fold change), where fold change is relative to the average of the control tanks, set at 1.0 (log2 1.0 = 0), harvested the same week. The 4 h and 24 h samples are expressed relative to the control tanks harvested on wk 1. Different letters indicate significant differences within each time period, \(P < 0.05\). Asterisks indicate a significant difference from the control treatment, \(P < 0.05\).
was driven by carcass weight gain, which exceeded controls as early as the first week of refeeding. In contrast, carcass growth in diploids did not exceed controls until the second week of refeeding, potentially due to the metabolic effort placed on first replenishing visceral stores.

A principal mechanism regulating growth in fish is the GH/IGF-I axis, which is predominantly regulated at the level of the liver, although regulation of its components at the local level is becoming increasingly recognized as physiologically relevant. In the current study increased expression of ghr2 in liver during feed deprivation is opposite of what has been previously reported in long-term (6 wk) fasted rainbow trout (Norbeck et al., 2007). However, fish in the two studies differed in age (3 mon vs 1 yr) and weight (3 g vs 250 g) and therefore regulation of the GH/IGF system could also differ. As GH stimulates hepatic IGF-I production, reduced hepatic ghr2 and igf1 expression in triploids as main effects of ploidy suggests that triploids are less sensitive to GH signaling than diploids, and that this may contribute to lower levels of IGF-I in these fish. However, reduced hepatic IGF-I production would suggest the triploids exhibit greater weight loss and a reduced capacity for recovery growth, neither of which occurred.

Furthermore, studies in rainbow trout and tilapia indicate plasma IGF-I concentrations return to, but do not exceed, control levels during refeeding (Fox et al., 2010; Gabillard et al., 2006; Rahimi et al., 2010). While the increased local production of IGF in white muscle during short-term refeeding likely contributes to recovery...
growth (Eppler et al., 2007; Fox et al., 2010; Fuentes et al., 2012), in the current study increased expression of igf1 and igf2 in muscle during refeeding did not differ between diploids and triploids. Therefore, additional mechanisms beyond that of IGF production, whether in liver or muscle, are likely responsible for the differences in recovery growth response between the two ploidies. These mechanisms may include tissue sensitivity to IGF binding via IGF receptors or regulation of IGF binding proteins, both of which are regulated by fasting and refeeding (Gabillard et al., 2006; Montserrat et al., 2007).

Although expression of the IGF receptor igfr1b increased during short-term refeeding in both liver and muscle, only expression in liver exhibited a main effect of ploidy, with overall reduced expression levels in triploids compared to diploids. Therefore, IGF receptor abundance may affect liver sensitivity to IGF and contribute to ploidy differences in the recovery growth response, especially in terms of nutrient partitioning. However, faster rates of muscle growth during early refeeding in triploids is likely not driven directly by improved sensitivity to IGF via increased receptor number.

Approximately 1% of plasma IGF-I is free, while the remaining is bound to IGF binding proteins (Shimizu et al., 1999). In salmonid plasma there are measurable concentrations of three species of IGF binding proteins, each with a unique molecular weight, that have been identified as IGFBP1a, IGFBP1b, and IGFBP2b (previously misannotated as igfbp3), the latter of which is a functional homolog of mammalian IGFBP3 (Rodgers et al., 2008; Shimizu et al., 2011a,b) and responsible for 80% of the IGFBP in plasma (Shimizu et al., 1999). Therefore, hepatic expression of these binding proteins largely regulate the stability and amount of IGF-I free in plasma whereas local production of IGF binding proteins likely have greater implications on actions of IGF-I signaling in specific tissues (Duan, 2002). In salmon, expression of igfbp genes during short-term refeeding is more dynamic than regulation of igf1, suggesting that regulation of plasma IGF-I levels is primarily driven by changes in IGF binding proteins rather than changes in IGF-I production (Shimizu et al., 2009). The common response among fish is for fasting to increase hepatic expression of igfbp1 and decrease that of igfbp2b, while refeeding decreases expression of igfbp1 and increases that of igfbp2b (Pierce et al., 2005; Reinld and Sheridan, 2012; Shimizu et al., 2009). However, the opposite occurred in the current study: fasting decreased expression of igfbp1b, regardless of ploidy, and increased expression of igfbp2b in diploids. Previous studies in salmonids and tilapia indicate GH decreases igfbp1 expression and increases igfbp2b or igfbp3 expression in liver (Cheng et al., 2002; Pierce et al., 2006; Shepherd et al., 2007). Therefore, in the current study fasting-induced increases in ghre2 expression may have promoted GH actions, leading to decreased igfbp1b and increased igfbp2b expression. Therefore, although the effects of fasting on liver ghre2, igfbp1b, and igfbp2b were opposite of the expected fasting response, the relative changes in expression are consistent with the established effects of GH on IGFBPs and suggest that the fasting response in small fish is different from that of larger juveniles. However, expression of the igfbps returned to control values during refeeding earlier than expression of ghre2, suggesting additional GH-independent mechanisms are involved in regulation of igfbp. Additionally, the abundance of the GH receptor-1 subtype, for which expression was affected by ploidy in liver, may contribute to ploidy differences in nutrient partitioning during fasting and refeeding, although there was no main effect of feeding schedule on ghre1 expression.

In mammals IGFBP-3 has an established role as a regulator of metabolism and muscle growth, therefore the differential regulation of the homologous igfbp2b gene in liver in diploid and triploid rainbow trout may contribute to ploidy-specific effects on nutrient partitioning during feed deprivation and refeeding. In mammals, IGFBP-3 attenuates insulin-stimulated glucose uptake in adipocytes (Chan et al., 2005) and skeletal muscle (Silha et al., 2002), and inhibits adipocyte differentiation (Chan et al., 2009), indicating that IGFBP-3 is important for regulation of glucose homeostasis and energy metabolism. Additionally igfbp3-/ mice demonstrate faster growth rates, improved feed efficiency, increased fasting glucose levels, and impaired glucose tolerance (Yamada et al., 2010) compared to wild type. Fasting hyperglycemia, impaired glucose tolerance, and insulin resistance are also a phenotypes observed in mice transgenic for IGFBP3 (Silha et al., 2002), further supporting a role of IGFBP3 in carbohydrate metabolic and regulation of energy balance. Research in mice also suggests growth is enhanced with increases in the IGF-I:IGFBP-3 ratio (Williams et al., 2011), which can occur from increased IGF-I or decreased IGFBP-3. Therefore, consistent with these concepts is the observation that fastest rates of EvBW growth occurred in triploids concurrently with reduced igfbp2b expression, suggesting ploidy effects on igfbp2b expression contributes to their distinct responses to fasting and refeeding. Furthermore, triple knockout mice lacking IGFBP3, -4, and -5, or combinations thereof, exhibit reduced growth, effects on glucose clearance rates, metabolic abnormalities, and reduced muscle Erk signaling (Ning et al., 2006). Therefore, in the current study ploidy effects on igfbp2b, igfbp4, and igfbp5 expression in both liver and muscle likely contribute to ploidy differences in the fasting and refeeding growth response.

White muscle growth is a main contributor of eviscerated body weight gain, and muscle regulatory factors are predominant in control of muscle fiber hypertrophy. With the exception of pax7a, there was a significant effect (P < 0.05) or a trend (P = 0.10) for ploidy to affect expression of the muscle regulatory factor genes during refeeding. Rates of eviscerated body weight gain in refeeding triploids, but not diploids, exceeded controls during the first week of refeeding (wk 2). Ploidy effects on muscle-specific gene expression were also observed during this time period, and include mlic and myf5, for which triploids exhibit lower expression, and mstn1a and mstn1b, for which triploids exhibit higher expression. In vitro studies in rainbow trout primary myocyte cultures indicate that myostatin inhibits proliferation but stimulates cell differentiation via up-regulation of genes required for cell differentiation and maturation, such as myod, myog, and myf5 (Garikipati and Rodgers, 2012a,b; Seiliez et al., 2012). Therefore, while triploids may have an enhanced capacity for muscle cell differentiation based on mstn expression levels, this does not also translate into increased expression of the aforementioned differentiation-related genes. Furthermore, throughout the extended refeeding period (wk 3–5), mstn expression was not different from control fish, suggesting that effects of myostatin on cell differentiation mechanisms are not a driving force for recovery growth. Furthermore up-regulation of myod and myog in triploids during wk 3, although suggestive of an increased capacity for muscle growth, was concurrent with the slowest rates of EvBW gain compared to control fish. Therefore, whereas expression of muscle regulatory factors indicate regulation of mechanisms affecting cell differentiation and growth, their relevance to increased growth rates during refeeding, and effects of ploidy on myostatin-related signaling warrant additional investigation.

Effects of myostatin on target tissues, as well as effects of other TGFbeta superfamily ligands, are dependent upon TGFbeta receptors, intracellular signaling SMADs, and TGFbeta ligand inhibitors like follistatin, and the pseudoreceptor BAMBI. Actions of MSTN and follistatin in regulating muscle growth and responding to nutritional state have been described in rainbow trout (Medeiros et al., 2009; Rodgers and Garikipati, 2008) and a novel TGFbeta6 has also been shown to respond to nutritional state and GH in gilthead sea bream (Sparus aurata) (Funkenstein et al., 2010). However, roles of the TGFbeta/SMAD family in liver in regulation
of growth and nutritional state have received little attention in fish. Nevertheless, MSTN has been shown to have systemic actions regulating the IGF and IGF binding protein axis in mice (Williams et al., 2011), and in the current study hepatic up-regulation of the type I receptor for myostatin, acvr1b, during refeeding is consistent with a role for systemic MSTN in the refeeding response. Also in mammals, follistatin and TGFbeta have positive effects on liver size and regeneration after injury (Braun et al., 1988; Endo et al., 2006; Takabe et al., 2003) and in our study hepatosomatic index (HSI) decreased by 50% during feed deprivation but rebounded to levels greater than continuously fed controls within one week (Cleveland and Weber, 2013). Therefore, increased fst expression in liver during early refeeding may contribute to the regeneration of liver mass in rainbow trout as well.

Additional genes that respond to feed deprivation and refeeding, and were affected by ploidy include the family of estrogen receptors. This was an unexpected finding as the rainbow trout in the current study were juveniles so are not expected to be producing appreciable levels of the sex steroids that activate these receptors (Han et al., 2010; Schafhauser-Smith and Benfey, 2003). Therefore, without estrogen to activate its receptors, regulation in tissue sensitivity to estrogen signaling via er expression is not expected to be physiologically relevant. Estrogen produces catabolic effects on growth, in part via reductions in hepatic and peripheral IGF-I production (Norbeck and Sheridan, 2011) and increased protein catabolism in muscle (Cleveland and Weber, 2011), which is significant during sexual maturation when nutrients are mobilized to support vitelligenin production and gonad development. Estrogen itself regulates expression of estrogen receptors (Boyce-Derricott et al., 2009), and results from the current study indicate that changes in nutrient supply also regulate estrogen receptor expression. Furthermore, the estrogen receptor isoforms produced unique expression patterns, especially in liver, suggesting there are isoform-specific effects on nutrient partitioning and metabolism, as is the case for vitellogenin production (Nagler et al., 2010). The dynamic changes in estrogen receptor isoforms in response to changes in feed availability might also suggest potential for hepatic sensitivity to food-derived or environmental estrogens.

Hepatic gene expression patterns suggest that triploids show a greater capacity for down-regulation during fasting, and slower capacity for recovery during refeeding. In contrast, diploid fish exhibit a greater capacity to increase gene expression than triploids, although when up-regulation occurs, triploids demonstrate a tendency to return transcript abundance to control levels more quickly than diploids, and often demonstrate an overshoot response one week into refeeding, a response not present in diploids. Collectively, these observations suggest that the kinetics of regulation in liver differ between diploids and triploids, with an improved capacity for gene up-regulation in diploids, while triploids exhibit an enhanced capacity for gene down-regulation. Effects of altered chromosome copy number on gene expression is defined as the gene dosage effect or, if no differences are observed, the dosage compensation effect (Birchler et al., 2001). Plants, lower eukaryotic invertebrates, and lower vertebrates seem to cope well with changes in gene copy number, as both aneuploids or polyploids (Guo et al., 1996; Suzuki et al., 1999). The relatively similar aspects of growth performance, at least prior to sexual maturation, between diploid and triploid fish are evidence of the gene compensation phenomenon in fish (Benfey, 1999; Maxime, 2008). The current study supports this concept in that the relative levels of gene expression in the continuously fed fish were largely not affected by ploidy, as in both liver and muscle only a few genes weakly differed between the ploidy (0.05 < P > 0.03; supplementary Figs. 3 and 4). However, when the system is perturbed, like during fasting and refeeding, as the system deviates from its resting state the effects of gene dosage may overwhelm those of gene compensation.

5. Conclusion

Production of triploid fish is a valuable technology in the aquaculture industry, with sterility in triploid females being the trait of most value. During feed deprivation at this early age diploids mobilize visceral nutrient stores more than triploids, and during refeeding triploids increase carcass growth rates faster than diploids. Differences in kinetics of gene expression between triploids and diploids and between liver and muscle support that gene dosage effects contribute to improved recovery growth in triploids. Mechanisms affected by ploidy that are central to these effects include (1) reduced expression of IGF binding proteins in triploid liver, primarily that of igfbp2b which likely increases the amount of free IGF-I in plasma for anabolic signaling in peripheral tissues, (2) altered expression of muscle regulatory factors in muscle, leading to improved myogenesis and muscle growth, and (3) altered tissue responsiveness to TGFbeta superfamily ligands and smad-related signal transduction, which is central to regulation of multiple physiological and developmental processes. These results indicate that regulation of physiological mechanisms in response to feed deprivation and refeeding differ between early juvenile diploid and triploid rainbow trout, and that each ploidy may exhibit optimal growth and nutrient retention under different feeding strategies or diet formulations that exploit these mechanisms. Finally, several responses to fasting differed substantially from what is normally seen in adult animals in that liver gh2 was increased, and igfbp1 was decreased in both diploids and triploids, whereas igfbp2b was increased but only in diploids. The responses of igfbp1 and igfbp2b are consistent with increased GH sensitivity suggested by the increase in gh2 expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.09.024.

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