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RESEARCH ARTICLE

Insulin-like growth factor (IGF) signalling and genome-wide transcriptional regulation in fast muscle of zebrafish following a single-satiating meal

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SUMMARY

Male zebrafish (*Danio rerio*) were fasted for 7 days and fed to satiation over 3 h to investigate the transcriptional responses to a single meal. The intestinal content at satiety (6.3% body mass) decreased by 50% at 3 h and 95% at 9 h following food withdrawal. Phosphorylation of the insulin-like growth factor (IGF) signalling protein Akt peaked within 3 h of feeding and was highly correlated with gut fullness. Retained paralogues of IGF hormones genes were regulated with feeding, with *igf1a* showing a pronounced peak in expression after 3 h and *igf2b* after 6 h. Igf-I receptor transcripts were markedly elevated with fasting, and decreased to their lowest levels 45 min after feeding. *igf1rb* transcripts increased more quickly than *igf1ra* transcripts as the gut emptied. Paralogues of the insulin-like growth factor binding proteins (IGFBPs) were constitutively expressed, except for *igfbp1a* and *igfbp1b* transcripts, which were significantly elevated with fasting. Genome-wide transcriptional responses were analysed using the Agilent 44K oligonucleotide microarray and selected genes validated by qPCR. Fasting was associated with the upregulation of genes for the ubiquitin-proteasome degradation pathway, anti-proliferative and pro-apoptotic genes. Protein chaperones (*unc45b*, *hspd1*, *hspa5*, *hsp90a.1*, *hsp90a.2*) and chaperone interacting proteins (*ahsa1* and *stip1*) were upregulated 3 h after feeding along with genes for the initiation of protein synthesis and mRNA processing. Transcripts for the enzyme ornithine decarboxylase 1 showed the largest increase with feeding (11.5-fold) and were positively correlated with gut fullness. This study demonstrates the fast nature of the transcriptional responses to a meal and provides evidence for differential regulation of retained paralogues of IGF signalling pathway genes.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/13/2125/DC1

Key words: teleost, myotomal muscle, fish nutrition, growth, transcriptomics.

INTRODUCTION

Growth hormone is synthesized, stored and secreted by specialized cells in the anterior pituitary and plays a central role in controlling feeding behaviour, cell growth, osmoregulation and reproduction in teleosts (Kawauchi and Sower, 2006). Growth hormone acts directly on muscle through sarcolemmal receptors and indirectly *via* the production of insulin-like growth factors (IGFs) in the liver and peripheral tissues, which are released into the circulation (Wood et al., 2005). IGFs are also produced by paracrine pathways and are stimulated by amino acid influx into the muscle (Bower and Johnston, 2010a). In mammals, the IGF system comprises ten components: two hormones (IGF-I, IGF-II), two receptors (IGF-IR, IGF-IIR) and six binding proteins (IGFBP1–6) (Duan et al., 2010). IGFBPs have distinct physiological roles in development and regulate IGF release to tissues in association with specific proteases (Duan et al., 2010). Binding of IGF-I to its receptor activates several downstream signalling cascades including the P13K–AKT–TOR and MAP kinase pathways that are well conserved in fish and mammals (Engert et al., 1996; Duan et al., 2010). Activation of P13K–AKT–TOR stimulates a phosphorylation cascade that increases translation and protein synthesis (Engert et al., 1996; Duan et al., 2010) and inhibits protein degradation by the 26S proteasome system (Witt et al., 2005). In the zebrafish (*Danio rerio*), no fewer than 16 components of the IGF system have been described (Maures et al., 2002; Maures and Duan, 2002; Chen et al., 2004; Zhou et al., 2008; Wang et al., 2009; Zou et al., 2009; Dai et al., 2010). The larger number of IGF components in zebrafish compared with mammals reflects a whole genome duplication (WGD) that occurred at the base of teleost evolution (Jaillon et al., 2004). It is thought 15% of the duplicated genes or paralogues from this basal WGD have been retained in extant species (Jaillon et al., 2004). The distinct patterns of tissue expression and transcriptional regulation of many IGF system paralogues observed in zebrafish (Maures et al., 2002; Maures and Duan, 2002; Chen et al., 2004; Zhou et al., 2008; Wang et al., 2009; Zou et al., 2009; Dai et al., 2010) is consistent with either subfunctionalization or neofunctionalization of these genes.

Fasting-refeeding protocols are commonly used to investigate transcriptional regulation in the IGF-system in teleosts following the transition from catabolic to anabolic states (Chauvigne et al., 2003; Salem et al., 2005; Gabillard et al., 2006; Rescan et al., 2007; Bower et al., 2008). Feeding to satiation after a prolonged fast results in increased feeding intensity relative to continuously fed controls and a period of compensatory or catch-up growth (Nicieza and Metcalfe, 1997). The transcriptional responses observed in such experiments are dependent on the nutritional state of the fish prior to fasting, particularly the extent of fat stores, the duration of the fast, body size and temperature (Johnston et al., 2011). Fish show diurnal rhythms in feeding behaviour and activity driven by central oscillators in the brain that are synchronised by environmental cycles and co-ordinated with peripheral clock genes regulating metabolism

(Davie et al., 2009). In aquaculture, meal times entrain biological rhythms and ready physiological systems in anticipation for processing the food (Sanchez et al., 2009). As a consequence, great care should be taken in designing fasting-feeding experiments in order to define all experimental variables including the frequency and timing of feeding in relation to diurnal cycles.

Following the digestion and assimilation of a meal, metabolism changes from an overall catabolic to an anabolic state, utilizing the nutrients from the meal to produce energy and synthesize new molecules, which is characteristic of the postprandial period. To our knowledge there is no study describing the transcriptional changes during and following a postprandial period in fish, with most studies focusing on the changes in metabolic rate (Clark et al., 2010; Vanella et al., 2010) and the plasma level of metabolites following feeding (Eames et al., 2010; Eliason et al., 2010; Wood et al., 2010). We have investigated transcriptional regulation in the fast myotomal muscle of male zebrafish in response to a single satiating meal delivered at first light. Expression of all 16 genes of the IGF system was investigated by quantitative polymerase chain reaction (qPCR) and supplemented with a genome-wide survey of transcript abundance using the Agilent 44K oligonucleotide microarray. Transcript abundance and the phosphorylation of the signalling protein Akt were determined in relation to the presence of food in the gut as a reference point. The single-meal experimental design potentially provides greater temporal resolution for studying transcriptional responses compared with continuous refeeding where early and late events quickly become confounded. The aims of the study were to test the hypothesis that paralogues of IGF-system genes were differentially regulated with feeding, and to discover novel genes associated with the fasting and fed states in skeletal muscle.

MATERIALS AND METHODS

Fish and water quality

The F5 generation of a wild-caught population of zebrafish [*Danio rerio* (Hamilton 1822)] from Mymensingh, Bangladesh, was used in this study. All fish were adult males aged 9 months. Prior to the single meal experiment the fish were maintained in a single 50 litre tank at 27.6±0.4°C in a 12 h:12 h light:dark photoperiod. The fish were fed bloodworms (Ocean Nutrition™, Essen, Belgium) to satiety twice daily for 1 week. Nitrite (0 p.p.m.), nitrate (10–20 p.p.m.), ammonia (0 p.p.m.) and pH (7.6±0.2) were tested during acclimation and experimental periods using a Freshwater Master Test Kit (Aquarium Pharmaceuticals Inc., Chalfont, PA, USA).

The single meal experiment

Two replicate experiments were carried out, 3 months apart, with identical environmental conditions and food to account for any tank-to-tank variation in the feeding response. The experimental protocol involved fasting fish for 7 days and then feeding a single meal of bloodworms delivered over a 3 h period, after which any uneaten food was removed from the tank by siphoning. In the first replicate experiment seven fish per time-point were sampled and in the second replicate experiment six fish per time-point were sampled at the following times: –156, –24, 0 h (before the meal) and 0.75, 3, 6, 7.5, 9, 11, 24 and 36 h (after the meal). The average body mass in the first replicate experiment was 0.46±0.02 g and the standard length (from tip of snout to last vertebra) of the fish was 29.8±0.4 mm (N=77), and in the second replicate experiment was 0.53±0.016 g and 32.6±0.3 mm (N=66), respectively (means ± s.e.m.). Fish were killed humanely by an overdose of ethyl 3-aminobenzoate methanesulphonate salt (MS-222; Fluka, St Louis, MO, USA).

Fast skeletal muscle was dissected from the dorsal epaxial myotomes, flash frozen in liquid nitrogen and stored at –80°C prior to total RNA and protein extraction. The digestive tract was dissected and fixed in 4% (m/v) paraformaldehyde for later quantification of intestine content to the nearest milligram. Fixation was necessary to prevent tissue loss during dissection and to achieve an accurate quantification of intestine content. Because the nature of the tissue and food were the same for all samples, any shrinking caused by fixation should be proportional to the amount of material and was not considered in the interpretation of the results. All experiments and animal handling were approved by the Animal Welfare and Ethics Committee, University of St Andrews and conformed to UK Home Office guidelines.

Protein extraction

Total protein was extracted from fast skeletal muscle from five randomly selected fish per time-point in each of the independent experiments. Tissue (30 mg) was homogenized in Lysing Matrix D (MP Biomedicals, Qbiogene, Irvine, CA, USA) in a FastPrep® machine (Qbiogene) using 350 µl of 25 mmol l^{-1} MES (2-morpholino-ethanesulfonic acid monohydrate) pH 6.0 containing 1 mol l^{-1} NaCl, 0.25% (m/v) CHAPS, DNA/RNA nuclease (Invitrogen, Carlsbad, CA, USA) and protease inhibitor cocktail (Invitrogen).

Western blotting

Samples (20 µl, containing 20 µg of protein) were added to 6 µl of a solution containing 5 µl of 5× protein loading buffer and 1 µl 20× reducing agent (Fermentas, Vilnius, Lithuania), heated for 5 min at 95°C, loaded into NuPAGE® Novex 4–12% Bis-Tris gels (Invitrogen) and electrophoresed at 120 V. A protein ladder ranging from 10 to 250 kDa (Fermentas) and a reference sample were loaded in all gels to estimate the molecular mass of proteins of interest and to serve as a normalization sample, respectively. Proteins separated by electrophoresis were transferred to a PVDF Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA) at 25 V for 105 min. Successful protein separation and transfer were confirmed by Ponceau S staining (Sigma, St Louis, MO, USA). PVDF membranes were blocked overnight at 10°C using 5% (m/v) non-fat milk (AppliChem, Darmstadt, Germany) prepared in PBS (Sigma) containing 0.1% (v/v) Tween 20 (Sigma). Blocked membranes were incubated overnight at 10°C with the following primary antibodies (IgGs): P-Akt [1:1000 dilution (v/v), Cell Signaling #4060, Danvers, MA, USA], Akt [1:1000 (v/v), Cell Signaling #2966], actin [1:20,000 (v/v), Sigma A2066] and GAPDH [1:30,000 (v/v), Sigma G9545]. Probed membranes were incubated at 20°C for 1 h with the secondary antibody against mouse or rabbit IgG conjugated to horseradish peroxidase [both from Sigma and used at 1:60,000 (v/v)]. After incubation for 1 min with ECL Western Blotting Detection Reagents (GE Healthcare, Amersham, Buckinghamshire, UK) at room temperature, membranes were exposed to Hyperfilm ECL (GE Healthcare). Experimental variations in the electrophoresis and transfer were normalized using a reference sample common to all membranes. The fold-change in phosphorylation of Akt at each time-point was compared with the samples from –159 h. Further information on preliminary optimisation experiments, the normalisation strategy and tests of P-Akt antibody specificity are given in the Appendix Fig. A1.

Total RNA extraction from skeletal muscle and first strand cDNA synthesis

Total RNA was extracted by homogenization in Lysing Matrix D (Qbiogene) using 1 ml of TRI reagent (Sigma) in a FastPrep®

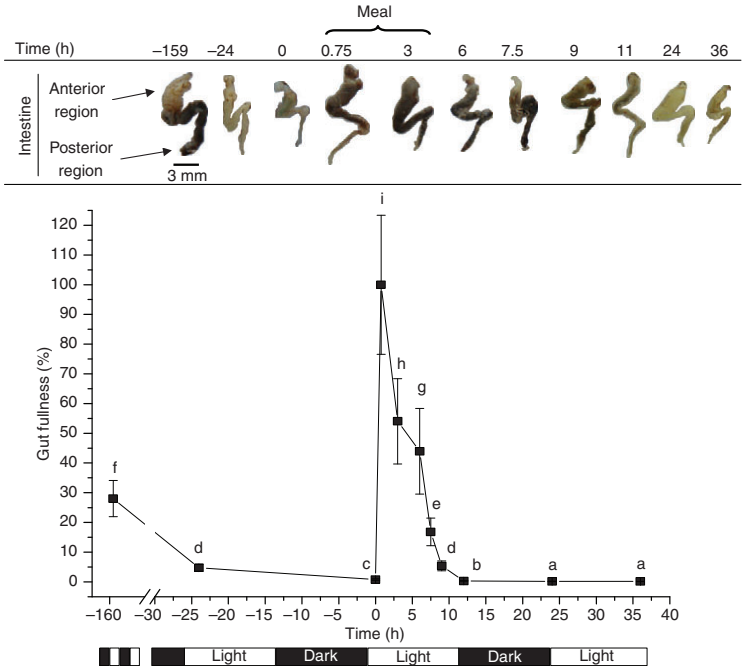

Fig. 1. The feeding response of male zebrafish during the course of the single meal experiment. Fish that had been fed twice daily were sampled after 9, 144 and 168 h of fasting (–156, –24 and 0 h prior to the single meal) and then fed an excess of bloodworms. Further samples were taken, 45 min, 3, 6, 7.5, 9, 11, 24 and 36 h after the initiation of feeding. (Upper panel) The micro-dissection of a representative intestine for each sample point. Gut fullness reached a maximum after 45 min, indicating satiety.
(Lower panel) The relative intestinal content (percentage maximum fullness) throughout the experiment, together with the light:dark cycle. Values are means ± s.e.m., N=13 fish per sample. Different letters signify statistically different means (P<0.05; see text for details).

machine (Qbiogene). The RNA concentration 260/280 and 260/230 ratios were measured using a NanoDrop® spectrophotometer (ThermoFisher Scientific, Loughborough, Leicestershire, UK) and were 1.5–2.0 and >2, respectively. RNA integrity was also checked by agarose gel electrophoresis. A Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany) was used to produce first-strand cDNA from 0.6 µg of total RNA following the manufacturer's instructions.

Microarray experiments

Microarray experiments were carried out by an Agilent-certified microarray service provider (Microarray Centre, University Health Network, Toronto, Canada) using the Dual-Mode Gene Expression Analysis Platform (Agilent Technologies Inc., Santa Clara, CA, USA) in a 4×44K slide format [Zebrafish (v2) Gene Expression Microarray]. RNA from the 3 h and 6 h time-points were hybridized with the RNA from the 0 h sample to identify differentially regulated genes in 7-day fasted fish following a single satiating meal. The 3 h sample coincided with 50% of maximum gut fullness (Fig. 1). Six phenotypic replicates from each group were used. R, version 2.9n.0, with arrayQualityMetrics_2.2.0 and limma_2.18.0 was used for quality analysis of microarray data. Microarray results were also analysed using GeneSpring®, v7. The intensities of spots among arrays was normalised using the AQuantile method and intensities were log transformed before performing a t-test using the Benjamini and Hochberg method for multiple testing correction (Benjamini and Hochberg, 1995). A list of differentially regulated genes was produced by screening against the following criteria: >twofold change in expression, B-value statistic >0 and an adjusted P-value <0.05. Blast2GO (www.blast2go.org) software was used to analyse the gene ontology (GO) terms of the differentially expressed genes. GO enrichment analysis of the annotated genes was performed with the GOSSIP tool using Blast2GO software. The European Bioinformatics Institute Miame ArrayExpress accession number for the microarray experiment is E-MEXP-2887.

Primer design and cloning

Transcript sequences from the Ensembl database (release 55; www.ensembl.org) were used to design primer pairs for each gene listed in supplementary material Table S1. Primers were designed using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html; Premier BioSoft, Palo Alto, CA, USA). Where possible the primer pairs were designed so that at least one primer spanned an exon–exon boundary (otherwise primers pairs were in different exons), with a T_m close to 60°C. First-strand cDNA from muscle was used as a template to synthesise PCR products for cloning. The PCR reaction mixture contained 1.5 mmol l^{-1} $MgCl_2$, 1× NH_4 buffer, 0.25 µl BioTaq™ DNA polymerase (Bioline, London, UK), 0.2 mmol l^{-1} dNTP (Promega, Madison, WI, USA), 1 µl cDNA and 0.5 µmol l^{-1} of each primer (Invitrogen) and the following thermal cycling conditions were employed: denaturation for 5 min at 95°C followed by 36 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, final elongation for 5 min at 72°C. After agarose gel electrophoresis the products of expected size were extracted and purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned using a StrataClone™ PCR Cloning Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Positive clones were picked after 16 h of growth at 37°C in LB-agar plates containing ampicillin (0.1 mg ml^{-1}) and transferred to 96-wells plates containing LB broth and ampicillin (0.1 mg ml^{-1}). After 16 h of growth at 37°C colony-PCR was performed to confirm the sequence of the insert using Big Dye terminator sequencing (Applied Biosystems, Foster City, CA, USA) at the University of Oxford (T3 and T7 primers were used to confirm the sequence in both directions). The clones bearing plasmids with the expected insert were grown in 5 ml LB broth and plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen). The plasmid concentration, 260/280 and 260/230 ratios were measured using a NanoDrop® instrument and were higher than 1.8 and 2.0, respectively.

qPCR

All protocols and reporting of qPCR assays adhered to 'Minimum Information for Publication of Quantitative Real-Time PCR

Experiments' guidelines (Bustin et al., 2009). The qPCR reaction mixture contained 7.5 µl 2× Brilliant II SYBR® QPCR Low ROX Master Mix (Stratagene), 6 µl 40-fold diluted cDNA, 0.25 µmol l^{-1} each primer and nuclease-free water (Qiagen) to a final volume of 15 µl in 96-well plates (Stratagene). The reactions were performed in a Stratagene MX3005P machine (initial activation at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C) and the fluorescence results, collected after the elongation step (72°C), were recorded using the MxPro software v4.10 (Stratagene). Negative controls were included and run in duplicate, and contained either all components of the reverse transcription mixture, except reverse transcriptase (no reverse transcriptase control) or water instead of a cDNA template (no template control). After the qPCR, a dissociation curve (from 55 to 95°C) was produced to verify the presence of a single peak. The specificity of each qPCR assay was also validated by sequencing transformants for each qPCR product. Absolute copy number of each gene was calculated based on a standard curve of at least six orders of magnitude, prepared with the plasmids. This data was also used to analyse the efficiency of each primer pair (supplementary material Table S1). The threshold of fluorescence (for dRn values) used for determination of the quantification cycle (C_q) was set to 1.0 for all plates to allow for comparison between plates. Comparison between plates was possible after normalization to six samples loaded on all plates.

Six reference genes selected from the literature (*ef1a*, *rpl13a*, *bactin2*, *b2m*, *usp5*, *tbp* and *cyp1a*) and four selected from the microarray experiment (*tomm20b*, *rpl7a*, *lman2* and *dis3l2*) were analysed using Genorm v3.5 (Vandesompele et al., 2002) with M set to <1.5. The two genes with the most stable level of expression across the experimental conditions were *ef1a* and *lman2* (M=0.4). The expression of genes of interest was normalized to the geometric average of the two most stable genes and gene expression was reported as arbitrary units (a.u.).

Data analysis and statistics

All data were analysed for normal distribution and equality of variance. Normally distributed data were analysed using ANOVA followed by Tukey's *post hoc* tests using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). Kruskal–Wallis non-parametric tests followed by Conover *post hoc* tests were used for the data that were not normally distributed using BrightStat software (Stricker, 2008). There was no statistically significant difference in the standard length, body mass, intestine content or normalized gene expression between the two replicate experiments (P>0.05). Therefore, the results from both experiments were combined to facilitate their interpretation, resulting in N=13 per time-point (i.e. seven fish from the first replicate experiment plus six from the second replicate). In order to combine the mRNA expression data from both experiments, the results of gene expression from the second replicate experiment were normalized to the average value of seven samples from the first replicate experiment, which were included from the cDNA synthesis step onwards. Correlation of gene expression from both qPCR and microarray experiments was analysed by Spearman's correlation test using PASW Statistics 18 (SPSS Inc.). Clustering of gene expression was performed using PermutMatrix (http://www.lirmm.fr/~caraux/PermutMatrix/EN/index.html).

RESULTS

Feeding response during the single meal experiment

Fish were continuously fed to satiation and then fasted for 7 days prior to feeding a single meal over 3 h. Three samples were taken during the fast: –156, –24 and 0 h, corresponding to 9 h, 144 h and 168 h after the last food. Only traces of food remained in the gut after 9 h of fasting (–156 h time-point), corresponding to 0.1% of body mass, with only bile observed with more prolonged fasting (–24 and 0 h time-points). The maximum average gut fullness, equivalent to 6.3% of body mass, was observed at 0.75 h (45 min) after food became available, indicating satiety had been reached. Intestinal contents had decreased by 50% 3 h after food was withdrawn and 95% of the food ingested had been assimilated or excreted by 9 h (Fig. 1).

Phosphorylation of the IGF signalling protein Akt

In fast myotomal muscle the protein Akt showed a marked increase in phosphorylation to peak levels within 3 h of the start of feeding and became steadily dephosphorylated as the intestine emptied (Fig. 2). The level of the protein load controls GADPH and actin and the dephosphorylated Akt did not change significantly over the course of the experiment.

Transcriptional regulation of the IGF system

The expression of all the paralogues of IGF-system genes, including those of Igf1, Igf2, Igf receptors and Igf binding proteins, was determined by qPCR. In many cases retained paralogues of IGF system components showed distinct patterns of transcriptional regulation over the course of the experiment. Changes in transcript levels could be directly attributed to feeding because marked responses were largely present in only the first of two light:dark cycles following the meal.

IGF hormone genes

igf1a expression was correlated with, but lagged behind gut fullness showing a distinct peak 3 h after the start of feeding (P<0.05;

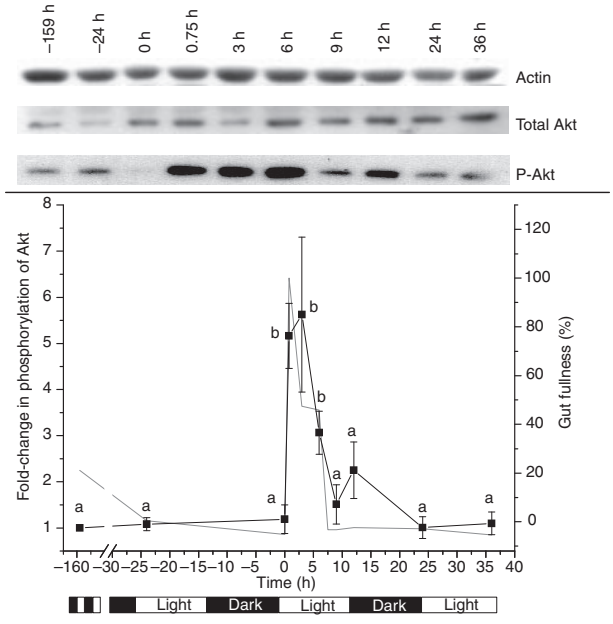

Fig. 2. Phosphorylation of the insulin-like growth factor (IGF) signalling protein Akt in the fast myotomal muscle of male zebrafish during the course of the single meal experiment. (Top) A representative western blot with the phosho-Akt antibody. (Bottom) Quantification of the phosphorylation of Akt (solid squares and black line). The grey line represents the average gut fullness illustrated in Fig. 1. Values are means ± s.e.m., N=5 fish per sample. Different letters signify statistically different means (P<0.05; see text for details).

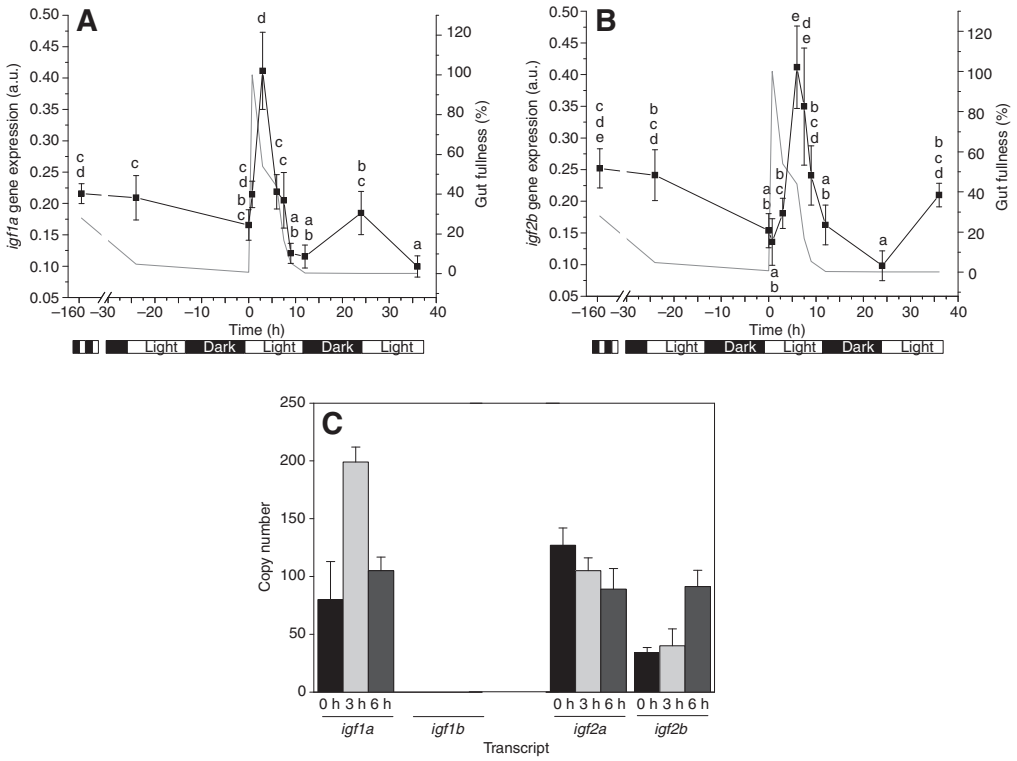

Fig. 3. Transcriptional responses of the IGF system genes in the fast myotomal muscle of male zebrafish during the course of the single meal experiment, determined by qPCR. (A,B) Hormone transcripts (solid squares and black lines) of *Igf1a* (A) and *Igf2b* (B). The grey line represents the average gut fullness illustrated in Fig. 1. (C) Copy number of *Igf1a*, *Igf1b*, *Igf2a* and *Igf2b*. Values are means ± s.e.m., N=13 fish per sample. Different letters signify statistically different means (P<0.05; see text for details).

Fig. 3A). The *igf1b* paralogue was not detected in fast myotomal muscle after 35 cycles of PCR (Fig. 3C). *igf2b* expression was also increased following feeding, showing peak expression 3 h later than *igf1a* (Fig. 3B). The *igf2a* paralogue was 3.7 times more abundant than the *igf2b* transcripts at 0 h (Fig. 3C) and was constitutively expressed during the experiment with no discernible pattern in relation to feeding (not shown).

IGF receptor genes

The expression of the IGF receptor gene *igf1ra* increased more than 2.7-fold between 9 h and 168 h of fasting, decreased to its lowest levels 45 min after feeding and then showed variable, though still depressed, expression over the next 36 h (Fig. 4A). *igf1rb* paralogue expression was inversely related to gut fullness showing a more than 3.3-fold decrease within 45 min of feeding, returning to levels not significantly different from those after fasting for only 12 h (Fig. 4B). *igf1rb* transcripts were more abundant than *igf1ra* transcripts over the single meal experiment, with 7.4 more copies at the start of feeding (Fig. 4D). Transcripts of the single *igf2r* gene showed no consistent changes in expression over the fasting–feeding–fasting cycle (not shown).

IGF binding proteins genes

The two retained paralogues of the IGF binding protein 1 gene (*igfbp1a* and *igfbp1b*) showed similar changes in expression over the experiment, with high levels during prolonged fasting (144–168 h), a marked reduction in transcript abundance within 45 min of the start of feeding and variable, but generally low levels over the following 36 h (Fig. 4C). Expression of *igfbp1a* was considerably higher than *igfbp1b* over the experiment, with a 12.4 times greater copy number at 0 h (Fig. 4D). The remaining IGF binding protein genes (*igfbp2a*, *igfbp2b*, *igfbp3*, *igfbp5a*, *igfbp5b*, *igfbp6a* and *igfbp6b*) showed no consistent change in expression in response to a single satiating meal (not shown).

Genome-wide changes in gene expression with feeding

In order to identify some nutritionally responsive candidate genes for further investigation, whole genome microarray analysis was performed. Total RNA from maximally fasted fish (0 h) was hybridized with RNA from fish sampled 3 h and 6 h after the initiation of feeding. Genes were considered differentially regulated if they showed a more than twofold change in expression, a B value >0, and the difference in expression was significant at P<0.05, following correction for multiple comparisons. The hybridizations of fasting and 6 h fed samples produced a relatively short list of genes that were also represented in the hybridization of fasting and 3 h fed samples and so were not considered further. Fast skeletal muscle from zebrafish fasted for 7 days had 56 upregulated genes (Table 1) and 91 downregulated genes (Table 2) compared with fish fed to satiation over 3 h. 45 of the upregulated genes in fasting and 79 of the upregulated genes with feeding had associated gene ontology (GO) terms. For the genes upregulated with fasting GO term analysis revealed a significant enrichment of terms associated with catabolic processes, ubiquitin ligase activity and positive regulation of endothelial cell differentiation, including the genes *btg1* and *btg2* that have anti-proliferative properties (Winkler, 2010) (full listing in Table 3). Analysis of the genes upregulated with feeding showed enrichment for GO terms such as unfolded protein binding, protein folding, endoplasmic reticulum lumen, protein maturation, chaperone binding, sarcomerogenesis, myosin filament assembly, collagen biosynthesis and regulation of the JAK-STAT cascade (full listing in Table 3). The gene showing the largest fold change of 32.2 with fasting (Table 1) coded for a novel protein with ~23% identity to the mammalian orthologue of *harbinger transposase derived 1* (*harbi1*), which is thought to have nuclease activity (Kapitonov and Jurka, 2004). The list of genes upregulated with feeding included many chaperone genes (*unc45b*, *ptges3* and *serpinh1*), heat shock protein (*hsp90a.1*, *hsp90a.2*, *hspd1* and *hspa5*) and heat shock protein-associated (*ahsa1*, *calrl* and *stip1*)

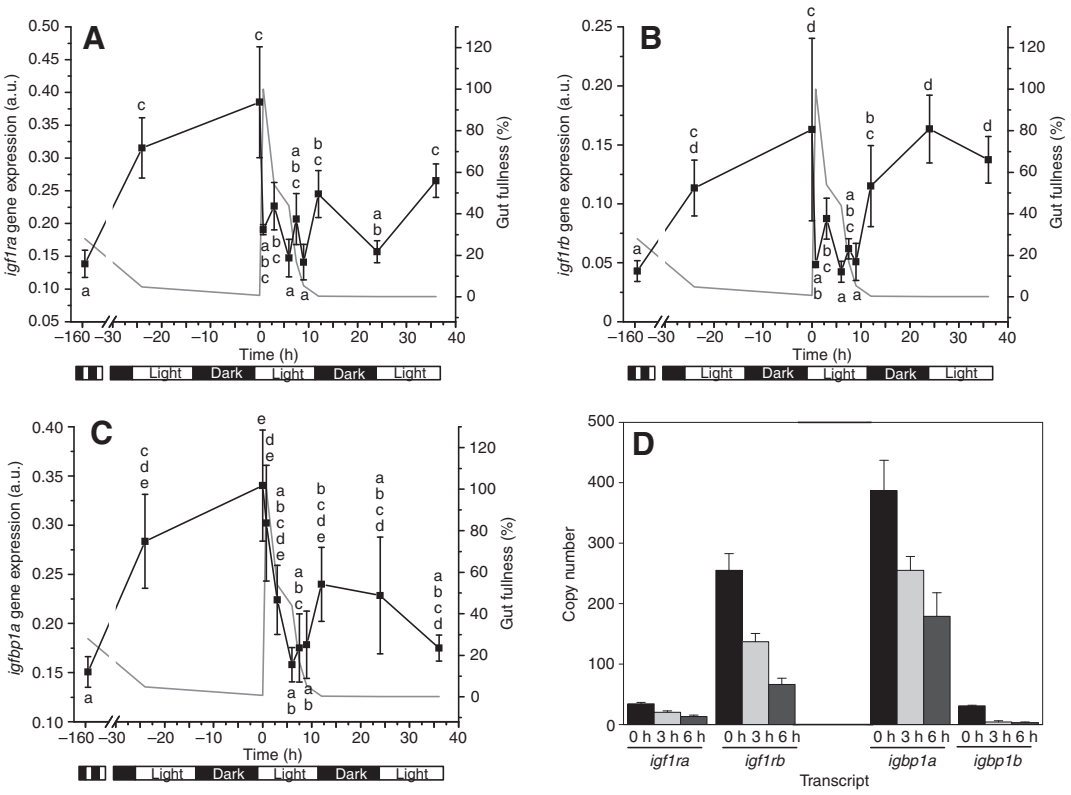

Fig. 4. Transcriptional responses of Insulin-like growth factor (IGF) system genes in the fast myotomal muscle of male zebrafish during the course of the single meal experiment determined by qPCR. (A–C) IGF receptors *igf1ra* (A) and *igf1rb* (B) and IGF binding protein, *igfbp1a* (C; solid squares and black lines) The grey lines represent the average gut fullness illustrated in Fig. 1. (D) Copy number of *igf1ra*, *igf1rb*, *igfbp1a* and *igfbp1b* . Values are means ± s.e.m., *N*=13 fish per sample. Different letters signify statistically different means ($P<0.05$; see text for details).

genes (Table 2). Feeding was also associated with enrichment of the interleukin-20 receptor binding GO term (Table 3) and increased *il34* expression (Table 2).

Expression and clustering of candidate nutritionally regulated genes

A selection of candidate nutritionally regulated genes comprising eight genes upregulated with fasting (*fbox32*, *zgc:86757*, *klf11b*, *nr1d1*, *cited2*, *bbc3*, *znf653* and *hsf2*) and six genes upregulated with feeding (*odc1*, *fkbp5*, *sae1*, *foxo1a*, *hsp90a.1* and *hsp90a.2*) plus some contractile protein genes (*mylz2* and *tnni2a.4*) was further investigated using qPCR. The log fold-change in expression of all the genes assayed by qPCR showed a good correlation with the microarray experiment ($R=0.79$; $P<0.001$; $N=76$; supplementary material Fig. S1). Genes from the IGF pathway and those selected from the microarray experiment formed five major clusters (Fig. 5). Cluster I contained genes upregulated with feeding, with low levels of expression during prolonged fasting (–24 and 0 h) and intermediate levels of expression from 9 to 36 h (*Igf1a*, *hsp90a.1*, *odc1*, *foxo1a*, *igfbp6a*, *Igf2b*, *sae1* and *fkbp5*). Cluster II comprised genes that were downregulated at –159 h and from 0.75 to 9 h, with upregulation during prolonged (–24 and 0 h) and early fasting (12–36 h) *(igf1ra*, *igf2r*, *igf1rb*, *fbxo32*, *zgc:86757*, *klf11b*, *bb3* and *znf653*). Cluster III contained genes that were only upregulated with prolonged fasting (144 and 168 h) and just after the beginning of feeding (45 min), with low expression at other time-points (*tnni2a.4*, *hsp90a.2*, *nr1d1*, *igfbp1a*, *hsf2* and *igfbp1b*). Cluster IV comprised genes with high expression during prolonged fasting, low expression while the intestine was full (0.75–6 h) and intermediate levels of expression from 7.5 to 36 h (*Igf2a*, *igfbp3* and *cited2*). Cluster V comprised genes with high expression with prolonged fasting, but with intermediate level of expression during feeding and early fasting (*mylz2*, *igfbp5b*, *igfbp2b*, *igfbp5a*, *igfbp2a* and *igfbp6b*). Genes pairs that showed strong significant correlations ($R>0.7$, $P<0.05$, $N=126$) were considered candidates for coregulated expression. Eight strong positive correlations were found and included *hsf2 versus nr1d1* ($R=0.83$; $P<0.001$) and *fbxo32* ($R=0.78$; $P<0.001$); *bbc3 versus znf653* ($R=0.76$; $P<0.001$) and *klf11b* ($R=0.75$; $P<0.001$); *fbxo32 versus MURF1* ($R=0.73$; $P<0.001$); and *bbc3 versus cited2* ($R= 0.73$; $P<0.001$) and *fbxo32* ($R=0.72$; $P<0.001$). The only strong negative correlation found was between *odc1* and *bbc3* (cluster II).

All eight of the candidate genes that were found to be upregulated with fasting in the microarray experiments were validated by qPCR. The expression of the muscle-specific ubiquitin ligases, *fbxo32* (also known as MAFbx/*Atrogin-1*) and *MURF1* (annotated as *zgc:86757* in the zebrafish genome assembly) was highly sensitive to nutritional status. Transcripts for *fbxo32* and *MURF1* increased 13.3 and 2.7-fold, respectively, between 9 h and 144 h of fasting and were downregulated by 55% (*fbxo32*) and 77% (*MURF1*) in the 45 min sample after feeding, with lowest levels observed after 6 h (Fig. 6A,B). Expression of both genes started to increase soon after food was cleared from the intestine and mRNA levels were at the 168-h fasting levels by 36 h after the meal (Fig. 6A,B).

Four genes (*bbc3*, *cited2*, *znf653* and *klf11b*) had expression patterns that were inversely correlated with gut fullness. Transcript abundances were lowest 45 min to 3 h after feeding and rapidly increased as the gut emptied (Fig. 7A–D). *nr1d1* and *hsf2* mRNA levels increased ~130- and ~21-fold, respectively, between 9 h and 144 h of fasting, and were strongly downregulated with feeding and showed a transient increase in levels 15 h after the intestine was empty (Fig. 7E,F). Three of the selected genes upregulated with feeding in the microarray experiment showed expression patterns related to gut fullness. *fkbp5* and *odc1* showed a peak of expression co-incident with the presence of food in the intestine, corresponding to a 5.6- and 17.2-fold increase in transcript abundance relative to

Table 1. Filtered list of genes from the microarray experiment showing transcripts upregulated with fasting in the zebrafish single meal experiment

	Gene symbol	Gene description	Fold change*	Adjusted *P*-value
1	*Novel gene*	Novel gene	32.2±10.4	0.048
2	*zgc:86757*	*MURF1* (*muscle-specific RING finger protein 1*)	25.4±7.6	0.022
3	*fbxo32*	(*MAFbx*) *F-box protein 32*	17.9±5.7	0.040
4	*pdk2*	*pyruvate dehydrogenase kinase 2*	16.9±3.8	0.022
5	*zp2*	*zona pellucida glycoprotein 2.3*	13.8±5.6	0.049
6	*h1m*	*linker histone H1M*	12.8±4.2	0.049
7	*si:ch211-284a13.1*	Novel protein (Si:ch211-284a13.1)	12.5±2.6	0.022
8	*klf11b*	*Kruppel-like factor 11b*	11.0±0.7	0.020
9	*zgc:162945*	*Hypothetical protein LOC560936*	9.8±2.9	0.032
10	*bbc3*	*BCL2 binding component 3*	9.4±3.1	0.038
11	*si:ch211-63o20.5*	Hypothetical protein LOC566703	9.3±1.6	0.022
12	*ypel3*	*yippee-like 3*	8.7±2.8	0.037
13	*cited2*	*Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2*	8.6±1.8	0.022
14	*nr1d1*	*nuclear receptor subfamily 1, group D, member 1*	8.5±1.7	0.023
15	*ccng2*	*cyclin G2*	7.6±1.2	0.022
16	*gbp*	*glycogen synthase kinase binding protein*	7.6±1.6	0.049
17	*gpr137ba*	*G protein-coupled receptor 137ba*	7.2±1.8	0.048
18	*si:dkey-42i9.4*	*B-cell translocation gene 1-like*	6.9±1.1	0.025
19	*zgc:100920*	Hypothetical protein LOC445241	6.6±2.2	0.049
20	*LOC566363*	*mucin 3-like*	6.3±2.1	0.049
21	*zgc:55582*	*myomegalin*	5.9±1.0	0.028
22	*slc16a9a*	*solute carrier family 16 (monocarboxylic acid transporters), member 9a*	5.6±1.2	0.032
23	*rab40b*	*RAB40B, member RAS oncogene family*	5.4±0.6	0.025
24	*TCEANC*	*transcription elongation factor A (SII) N-terminal and central domain containing*	5.4±1.0	0.040
25	*zgc:77714*	*carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 2*	5.3±0.9	0.040
26	*stk11ip*	*serine/threonine kinase 11 interacting protein*	5.3±0.6	0.025
27	*btg2*	*B-cell translocation gene 2*	5.2±1.2	0.049
28	*LOC794083*	*pyruvate dehydrogenase kinase 2-like*	5.2±1.1	0.040
29	*vgll2b*	*vestigial like 2b*	5.2±1.4	0.047
30	*hsf2*	*heat shock factor 2*	5.1±0.6	0.023
31	*znf653*	*zinc finger protein 653*	5.1±0.8	0.025
32	*heca*	*headcase homolog*	4.8±0.8	0.036
33	*per1b*	*period homolog 1b*	4.5±0.8	0.048
34	*zgc:92851*	*Jun dimerization protein 2*	4.4±1.1	0.049
35	*pcmtd2*	*protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2*	4.3±0.6	0.036
36	*LOC559993*	Similar to THAP domain containing, apoptosis associated protein 1	4.3±0.7	0.042
37	*brms1l*	*breast cancer metastasis-suppressor 1-like protein*	4.0±0.7	0.048
38	*usf1*	*upstream transcription factor 1*	3.9±0.7	0.042
39	*ubr1*	*ubiquitin protein ligase E3 component n-recognin 1*	3.8±0.5	0.040
40	*vsg1*	*vessel-specific 1*	3.7±0.7	0.048
41	*si:dkey-86e18.1*	Hypothetical protein LOC557342	3.6±0.4	0.040
42	*slc25a1*	*solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1*	3.6±0.6	0.048
43	*si:ch73-138e16.8*	Hypothetical protein LOC100006084	3.4±0.5	0.049
44	*n4bp2*	*NEDD4 binding protein 2*	3.4±0.4	0.040
45	*fbxo25*	*F-box only protein 25*	3.4±0.4	0.042
46	*ccdc149*	*coiled-coil domain containing 149*	3.2±0.4	0.050
47	*npl*	*N-acetylneuraminate lyase* (NALase; EC 4.1.3.3) (*N-acetylneuraminic acid aldolase*; *N-acetylneuraminate pyruvate-lyase*; *sialic acid lyase*; *sialate lyase*; *sialate-pyruvate lyase*; *sialic acid aldolase*)	3.2±0.4	0.050
48	*zgc:110708*	Hypothetical protein LOC553793	3.1±0.4	0.048
49	*mtus1a*	*mitochondrial tumor suppressor 1 homolog A*	3.1±0.4	0.050
50	*zgc:171727*	Hypothetical protein LOC799470	3.1±0.2	0.040
51	*id2b*	*inhibitor of DNA binding 2, dominant negative helix-loop-helix protein, b*	3.0±0.3	0.048
52	*nbr1*	*neighbor of BRCA1 gene 1*	3.0±0.4	0.048
53	*gmcl1*	*germ cell-less homolog 1 (Drosophila)*	3.0±0.3	0.049
54	*LOC799552*	Hypothetical protein	2.9±0.2	0.045
55	*zgc:163003*	*inactive Ufm1-specific protease 1*	2.9±0.2	0.047
56	*spns1*	*spinster homolog 1* (*spinster-like protein*)	2.8±0.2	0.047

*Values are means ± s.e.m., *N*=6. Names in parentheses are alternative names.

maximal fasting values, respectively (Fig. 8A,B). *sae1* showed a ~6.5-fold increase in expression 6 to 9 h after the start of the meal and a steady decline to levels not significantly different from fasting values by 36 h (Fig. 8C).

DISCUSSION

Transcriptional regulation of the IGF system

In the absence of information on plasma hormone and amino acid levels in small tropical fishes, transit of food through the

Table 2. Filtered list of genes from the microarray experiment showing transcripts upregulated with feeding in the zebrafish single meal experiment

	Gene symbol	Gene description	Fold change*	Adjusted *P*-value
1	*odc1*	*ornithine decarboxylase 1*	11.5±2.8	0.022
2	*pptc7*	*PTC7 protein phosphatase homolog*	10.5±1.8	0.022
3	*ctsll*	*cathepsin L, like*	9.9±3.2	0.027
4	*ddx5*	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 5*	9.8±3.4	0.038
5	*pdip5*	*protein disulfide isomerase-related protein*	9.5±1.9	0.025
6	*si:ch211-76m11.7*	si:ch211-76m11.7	9.0±2.5	0.038
7	*mylk4*	*myosin light chain kinase family, member 4*	8.9±3.1	0.040
8	*mfsd2b*	*major facilitator superfamily domain-containing protein 2-B*	8.0±1.1	0.022
9	*zgc:110154*	*eukaryotic translation initiation factor 4E-like*	7.9±2.0	0.038
10	*fkbp5*	*FK506 binding protein 5*	7.7±2.2	0.040
11	*rcn3*	*reticulocalbin 3, EF-hand calcium binding domain*	7.5±2.3	0.048
12	*dyrk2*	*dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2*	7.5±2.1	0.028
13	*calrl*	*calreticulin-like*	7.2±2.2	0.049
14	*ahsa1*	*AHA1, activator of heat shock protein ATPase homolog 1*	7.0±1.2	0.025
15	*eif4a1a*	*eukaryotic translation initiation factor 4A, isoform 1A*	6.8±1.3	0.025
16	*klf13l*	*Kruppel-like factor 13 like*	6.8±1.9	0.049
17	*LOC792864*	Similar to Dual specificity protein phosphatase 13 (Testis- and skeletal-muscle-specific DSP) (Dual specificity phosphatase SKRP4)	6.6±1.5	0.049
18	*slc25a25*	*calcium-binding mitochondrial carrier protein SCaMC-2-A* (*small calcium-binding mitochondrial carrier protein 2-A*; *solute carrier family 25 member 25-A*)	6.5±1.5	0.049
19	*LOC100332265*	Adaptor-related protein complex 1 associated regulatory protein-like	6.3±1.0	0.025
20	*mid1ip1*	*MID1 interacting protein 1*	5.9±0.7	0.025
21	*zgc:73230*	Hypothetical protein LOC406311	5.8±0.9	0.032
22	*hsp90a.1*	*Heat shock protein HSP 90-alpha 1*	5.7±0.6	0.022
23	*ctrl*	*chymotrypsin-like*	5.7±1.3	0.045
24	*hsp90a.2*	*heat shock protein 90-alpha 2*	5.6±0.7	0.025
25	*foxo1a*	*forkhead box O1 a*	5.4±0.7	0.025
26	*zgc:110801*	*protein phosphatase 5, catalytic subunit*	5.3±1.6	0.049
27	*zgc:158222*	*adenosylhomocysteinase*	5.3±1.0	0.040
28	*syncripl*	*synaptotagmin binding, cytoplasmic RNA interacting protein, like*	5.1±1.0	0.045
29	*dnaja4*	*DnaJ (Hsp40) homolog, subfamily A, member 4*	5.1±0.6	0.025
30	*fam69b*	*family with sequence similarity 69, member B*	5.0±0.9	0.048
31	*s1pr2*	*sphingosine 1-phosphate receptor 2* (*S1P receptor 2*; *S1P2*; *sphingosine 1-phosphate receptor Edg-5*; *S1P receptor Edg-5*)	5.0±1.1	0.048
32	*pias4*	*protein inhibitor of activated STAT, 4*	5.0±0.9	0.042
33	*lmnb1*	*lamin B1*	5.0±1.2	0.049
34	*ehmt1b*	*euchromatic histone-lysine N-methyltransferase 1b fragment*	4.9±0.3	0.022
35	*smyd1b*	*SET and MYND domain containing 1b*	4.8±0.8	0.032
36	*LOC100006303*	*novel protein similar to glutaminase (Gls)*	4.8±1.2	0.048
37	*hspd1*	*heat shock 60 kD protein 1*	4.7±1.0	0.042
38	*slmo2*	*slowmo homolog 2*	4.6±1.0	0.048
39	*abcf2*	*ATP-binding cassette, sub-family F*	4.6±0.9	0.042
40	*zgc:92429*	Hypothetical protein LOC445063	4.6±0.7	0.040
41	*kctd20*	*potassium channel tetramerisation domain containing 20*	4.5±0.5	0.026
42	*ppig*	*peptidylprolyl isomerase G*	4.5±0.9	0.049
43	*g3bp1*	*ras-GTPase-activating protein SH3-domain-binding protein*	4.4±0.9	0.042
44	*slc4a2a*	*solute carrier family 4, anion exchanger, member 2a*	4.4±0.4	0.025
45	*LOC100150539*	Novel protein similar to vertbrate adenylate cyclase 9 (ADCY9) Fragment	4.3±0.9	0.050
46	*sulf1*	*sulfatase 1*	4.3±0.9	0.048
47	*sae1*	*SUMO-activating enzyme subunit 1* (*ubiquitin-like 1-activating enzyme E1A*)	4.3±0.5	0.028
48	*zgc:85702*	Hypothetical protein LOC321718	4.2±0.7	0.042
49	*abcb10*	*atp-binding cassette, sub-family B (MDR/TAP), member 10*	4.2±0.9	0.048
50	*ranbp1*	*ran binding protein 1*	4.1±0.4	0.028
51	*dazap1*	*dazap1 protein fragment*	4.0±0.7	0.049
52	*il34*	*interleukin 34*	4.0±0.6	0.040
53	*frzb*	*frizzled-related protein*	3.8±0.4	0.037
54	*zgc:113183*	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	3.8±0.5	0.041
55	*smarca5*	*SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5*	3.8±0.7	0.049
56	*agr2*	*anterior gradient 2 homolog*	3.7±0.5	0.047
57	*zgc:136374*	*2′-phosphodiesterase*	3.7±0.5	0.040
58	*stip1*	*stress-induced-phosphoprotein 1* (*Hsp70/Hsp90-organizing protein*)	3.7±0.6	0.048

Table 2. Continued on next page

Table 2. Continued

	Gene symbol	Gene description	Fold change*	Adjusted *P*-value
59	*si:ch211-132p20.4*	*sodium-coupled neutral amino acid transporter 2* (*amino acid transporter A2*; *system A amino acid transporter* 2; *system N amino acid transporter 2*; system A transporter 1; *solute carrier family 38 member 2*)	3.7±0.4	0.038
60	*zgc:172028*	*ADP-ribosylation factor 6-like*	3.6±0.6	0.048
61	*zgc:153972*	*transmembrane and coiled-coil domain family 1*	3.5±0.6	0.049
62	*zgc:153290*	*coiled-coil domain containing 51*	3.5±0.5	0.045
63	*zgc:114204*	*Golgi transport 1 homolog B*	3.5±0.2	0.032
64	*zgc:86751*	Hypothetical protein LOC415227	3.4±0.5	0.048
65	*ppp4cb*	*serine/threonine-protein phosphatase 4 catalytic subunit B* (PP4C-B)(EC 3.1.3.16)	3.3±0.5	0.048
66	*LOC558153*	*adaptor-related protein complex 2, alpha 1 subunit-like*	3.3±0.4	0.048
67	*cmpk*	*ump-cmp kinase* (EC 2.7.4.14) (*cytidylate kinase*; *deoxycytidylate kinase*; *cytidine monophosphate kinase*; *uridine monophosphate kinase*; *uridine monophosphate/cytidine monophosphate kinase*; *UMP/CMP kinase*; *UMP/CMPK)*	3.3±0.5	0.049
68	*si:ch211-253h3.1*	*si:ch211-253h3.1*	3.3±0.5	0.049
69	*hspa5*	*heat shock 70kDa protein 5*	3.3±0.3	0.045
70	*slc38a4*	*solute carrier family 38, member 4*	3.3±0.2	0.039
71	*ncl1*	*nicalin-1 precursor* (*nicastrin-like protein 1*)	3.2±0.2	0.039
72	*ssrp1a*	*structure specific recognition protein 1a*	3.2±0.4	0.044
73	*zgc:153327*	*arginine-rich, mutated in early stage tumors*	3.2±0.3	0.041
74	*lamb1*	*laminin, beta 1*	3.2±0.3	0.041
75	*zgc:77244*	*potassium channel tetramerisation domain containing 5*	3.2±0.5	0.050
76	*zgc:158393*	Hypothetical protein LOC564849	3.2±0.4	0.048
77	*zgc:171630*	*serine (or cysteine) proteinase inhibitor, clade H, member 1*	3.2±0.4	0.047
78	*unc45b*	*unc-45 (C. elegans) related*	3.1±0.2	0.040
79	*zgc:153981*	*muscle-restricted dual specificity phosphatase*	3.1±0.2	0.041
80	*sf3b4*	*splicing factor 3b, subunit 4*	3.1±0.4	0.048
81	*thoc6*	*THO complex 6 homolog (Drosophila)*	3.0±0.4	0.050
82	*pl10*	*pl10*	3.0±0.3	0.047
83	*tram1*	*translocating chain-associating membrane protein 1*	3.0±0.3	0.048
84	*zgc:56005*	*oRELI domain containing 1*	3.0±0.2	0.044
86	*per2*	*period homolog 2*	3.0±0.3	0.048
87	*si:ch211-59d15.5*	*YLP motif containing 1*	2.8±0.1	0.042
88	*snrnp40*	*small nuclear ribonucleoprotein 40 (U5)*	2.8±0.1	0.042
89	*ehd1*	*EH-domain containing 1*	2.8±0.3	0.048
90	*si:ch211-286m4.4*	*exportin-T* [*tRNA exportin*; *exportin(tRNA)*]	2.8±0.2	0.049
91	*alg9*	*asparagine-linked glycosylation 9 protein*	2.7±0.3	0.050

*Values are means ± s.e.m., *N*=6. Names in parentheses are alternative names.

gastrointestinal system and phosphorylation of the IGF-pathway signalling protein Akt provide a means of interpreting the transcriptional response to a single satiating meal. In a previous fasting–refeeding study using rainbow trout, *Oncorhynchus mykiss*, maximum plasma insulin and amino acid levels were recorded 30 min and 2.5 h after feeding and were quickly followed by phosphorylation of several kinases, indicating activation of the TOR signalling pathway (Seiliez et al., 2008). Amino acids and insulin

Table 3. Enrichment analysis of gene ontology terms for biological processes associated with genes differentially regulated in response to a single-satiating meal, using the 44K Agilent zebrafish microarray V2

GO identifier	GO term	Number of genes differentially expressed	FDR*
Enriched with fasting			
GO:0043632	Modification-dependent macromolecule catabolic process	8	0.031
GO:0045603	Positive regulation of endothelial cell differentiation	2	0.052
Enriched with feeding			
GO:0042026	Protein refolding	3	0.004
GO:0051604	Protein maturation	3	0.035
GO:0030241	Skeletal muscle thick filament assembly	2	0.035
GO:0034619	Cellular chaperone-mediated protein complex assembly	2	0.035
GO:0042517	Positive regulation of tyrosine phosphorylation of Stat3 protein	2	0.035
GO:0048769	Sarcomerogenesis	2	0.035
GO:0046425	Regulation of JAK-STAT cascade	3	0.042
GO:0045618	Positive regulation of keratinocyte differentiation	2	0.045
GO:0045606	Positive regulation of epidermal cell differentiation	2	0.054
GO:0070096	Mitochondrial outer membrane translocase complex assembly	2	0.068

*FDR, false discovery rate.
Only the most-specific terms are shown.

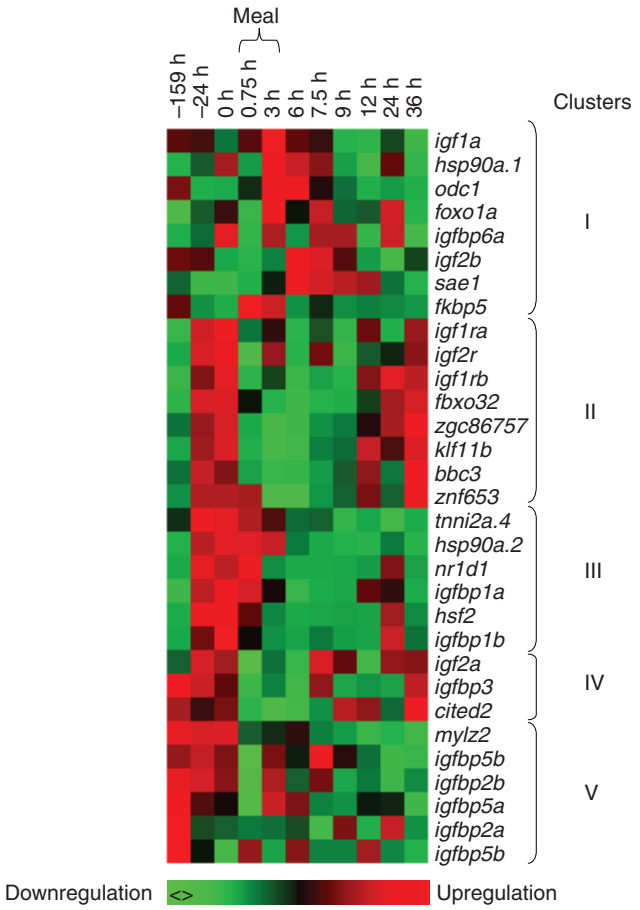

Fig. 5. Hierarchical clustering and heat map of IGF system gene transcripts and candidate nutritionally regulated genes identified from microarray experiments over the time course of the single meal experiment. The Roman numerals indicate clusters discussed in the text. Rows (mRNA transcripts) in the heat map are standardized to have mean of zero and standard deviation of one (i.e. standard score normalization). Red and green shading, respectively, indicates the highest and lowest expression levels, as indicated in the scale bar at the bottom of the figure. Each block represents the average standard-score normalization for the 13 fish sampled at each time-point in the experiment.

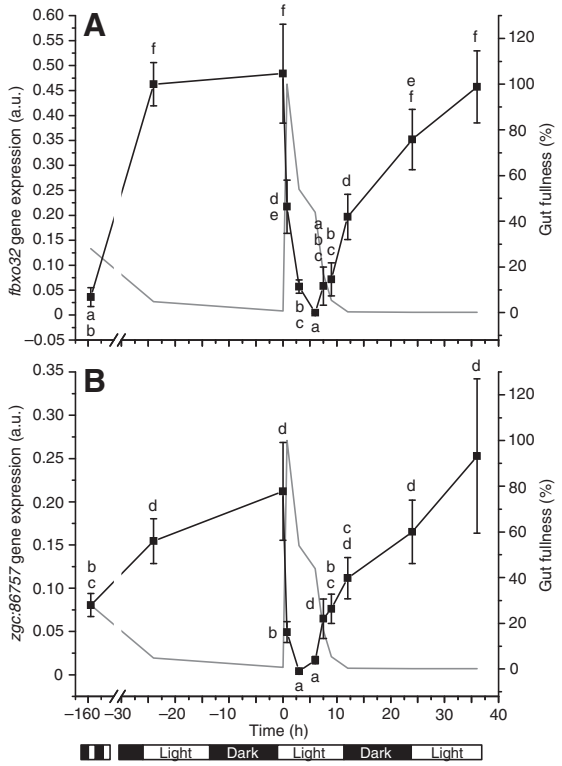

Fig. 6. Expression profiles of candidate nutritionally regulated genes in male zebrafish identified from microarray experiments over the time course of the single meal experiment as determined by qPCR. Genes upregulated during fasting (solid squares and black lines): (A) *fbxo32* and (B) *muscle-specific RING finger protein 1–MURF1* (*zgc:86757*). The grey lines represent the average gut fullness illustrated in Fig. 1. Values are means ± s.e.m., *N*=13 fish per sample. Different letters signify statistically different means ($P<0.05$; see text for details).

also rapidly increase the level of circulating IGFI in brown trout (Banos et al., 1999). In our experiments with zebrafish, 50% of the food ingested had been processed and eliminated within 3 h after the start of feeding and the gut was empty after 9 h (Fig. 1). There was a significant increase in phosphorylation of Akt within 45 min, with peak levels at 3 h after feeding and dephosphorylation over a similar time course to the elimination of food from the gut (Fig. 2). Changes in the levels of muscle mRNAs also mostly took place with a time course of hours and were generally quicker than described in the literature for larger temperate fish species maintained at lower temperatures (Chauvigne et al., 2003; Bower et al., 2008).

The first aim of this study was to test the hypothesis that retained paralogues of IGF-system genes were differentially regulated following transition from a catabolic to an anabolic state. Fasting has been shown to result in an upregulation of muscle IGF receptors in a number of teleost species and is probably a response to a decrease in circulating IGF hormone levels (Chauvigne et al., 2003; Gabillard et al., 2006; Bower et al., 2008; Bower and Johnston, 2010b). In rainbow trout, prolonged fasting resulted in an upregulation of *IGFR-Ia*, but not *IGFR-Ib* (Chauvigne et al., 2003) whereas in zebrafish both IGFR paralogues were upregulated (Fig. 4A,B). However, zebrafish *igf1rb* increased more rapidly following gut emptying than *igf1ra* reaching fasting levels >36 h and <25 h, respectively (Fig. 4A,B), indicating differential regulation of IGFI receptor paralogues. Functional characterisation studies of IGFIIR in fish are scarce and its role in the response to nutrient levels is not yet clear. In Atlantic salmon, *IGFIIR* transcripts were significantly upregulated with prolonged fasting and downregulated after 7 days of refeeding (Bower et al., 2008). In this study there was no discernible pattern of transcriptional regulation of *igf2r* over the experiment. Muscle IGF hormone transcript levels showed distinct peaks within a few hours of feeding (Fig. 3A,B). In the case of *Igf1a* peak values were found within 3 h of feeding and had declined to fasting levels before the gut was emptied (<5 h; Fig. 3A). In contrast, *Igf2b* transcripts reached a peak 6 h after feeding and were not significantly different to fasting levels by 9 h (Fig. 3B). *Igf1b* transcripts were not detected in fast muscle whereas the *Igf2a* paralogue was constitutively expressed and showed no consistent change in expression over the fasting-feeding-fasting cycle associated with a single meal. *In vitro* studies with Atlantic salmon (*Salmo salar*) myocytes have shown synergistic affects of insulin, IGF-I and amino acids on muscle IGF-I transcript abundance, indicating multiple pathways leading to IGF transcription (Bower

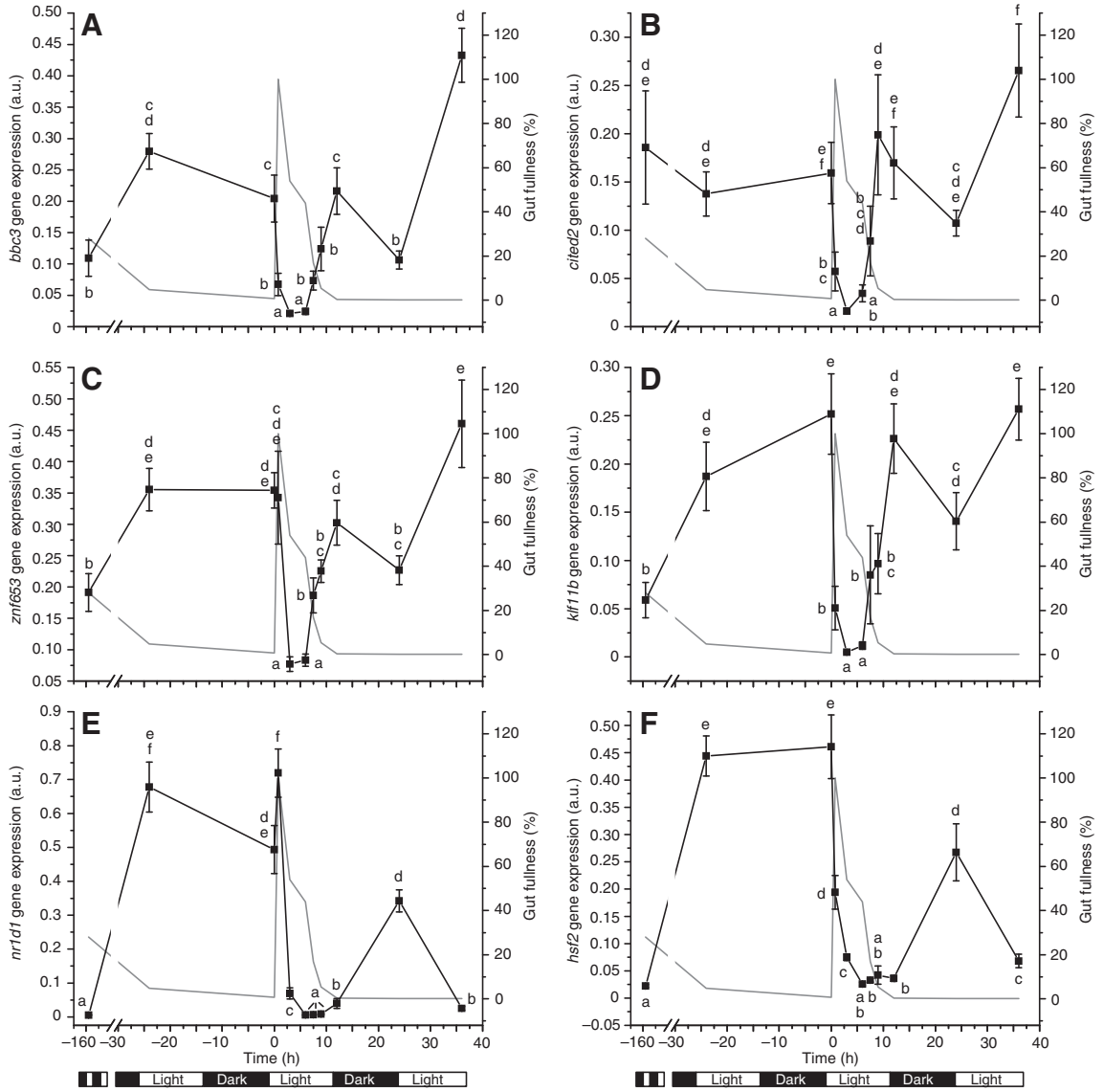

Fig. 7. Expression profiles of candidate nutritionally regulated genes in male zebrafish identified from microarray experiments over the time course of the single meal experiment as determined by qPCR. Genes upregulated during fasting (solid squares and black lines): (A) *BCL2 binding component 3* (*bbc3*), (B) *Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain*, 2 (*cited2*), (C) *zinc finger protein 653* (znf653), (D) *Kruppel-like factor 11b* (*klf11b*), (E) *nuclear receptor subfamily 1, group d, member 1* (*nrld1*), and (F) *heat shock factor 2* (*hsf2*). The grey lines represent the average gut fullness illustrated in Fig. 1. Values are means ± s.e.m., *N*=13 fish per sample. Different letters signify statistically different means ($P<0.05$; see text for details).

and Johnston, 2010a). In mammals, the binding of IGF to the IGF-IR induces its auto-phosphorylation resulting in the activation of several down-stream signal transduction cascades *via* adaptor molecules such as the insulin receptor substrate 1 (IRS-1), which has multiple tyrosine phosphorylation sites (Duan et al., 2010). IGF-I stimulates growth *via* effects on protein synthesis (Rommel et al., 2001), myoblast proliferation and differentiation acting through distinct signalling pathways (Ren et al., 2010). The effective concentration of IGF in the muscle is regulated by six IGFBPs, which are degraded by specific proteases to release the hormone to target sites (Wood et al., 2005). Evidence, largely from mammals, indicates that IGFBPs can inhibit and/or potentiate IGF actions depending on the cellular context and/or environmental conditions (Duan et al., 2010). In Atlantic salmon, the transition from maintenance to fast growth was associated with a constitutive upregulation of *IGFBP-4*, a transient increase in *IGFBP-5.1* and a downregulation of *IGFBP-2.1* (Bower et al., 2008). The two retained zebrafish paralogues of *igfbp1* had similar expression in the single meal experiment with high transcript abundance during fasting, a marked reduction in mRNA levels within 45 min of feeding and variable, but generally low level of expression over the following 36 h (Fig. 4C). Mammalian studies indicate that in addition to its role as a modulator of IGF1 availability, IGFBP-1 has putative IGF-independent biological activities through interaction with cell-surface integrins, with putative direct effects on the P13K–AKT pathway (Wheatcroft and Kearney, 2009). There was no evidence for the transcriptional regulation of *igfbp2a*, *igfbp2b*, *igfbp3*, *igfbp5a*, *igfbp5b*, *igfbp6a* and *igfbp6b* paralogues with feeding in our experiments. Although experimental context may explain some of these differences in IGFBP expression it is clear that there are

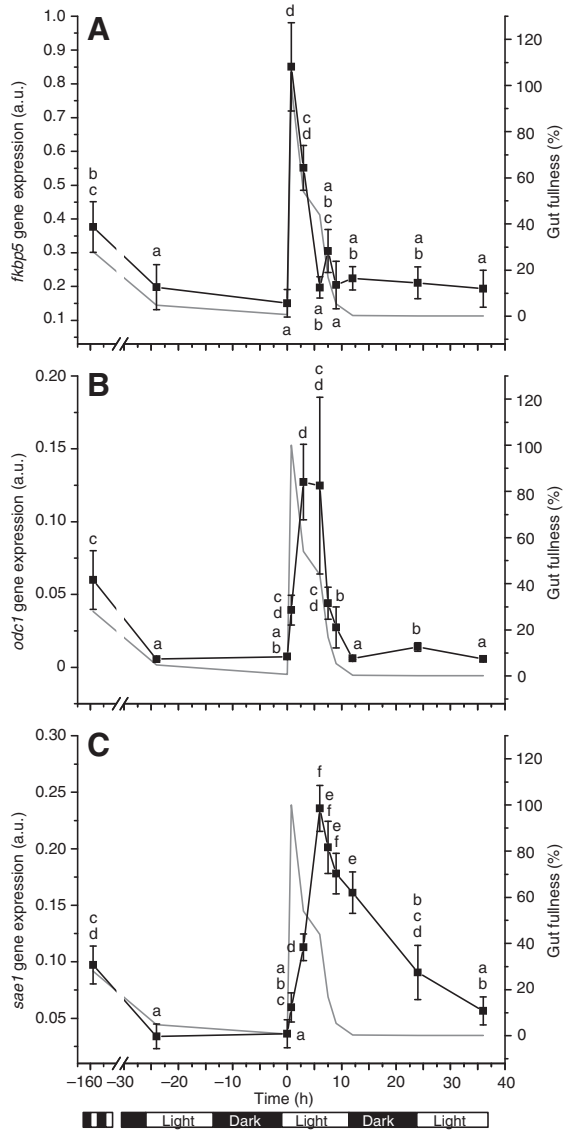

Fig. 8. Expression profiles of candidate nutritionally regulated genes in male zebrafish identified from microarray experiments over the time course of the single meal experiment as determined by qPCR. Genes upregulated with feeding (solid squares and black lines): (A) *FK506 binding protein 5* (*fkbp5*) (B) *ornithine decarboxylase 1* (*odc1*) and (C) SUMO-activating enzyme subunit 1 (*sae1*). The grey lines represent the average gut fullness illustrated in Fig. 1. Values are means ± s.e.m., *N*=13 fish per sample. Different letters signify statistically different means ($P<0.05$; see text for details).

lineage-specific differences in IGFBP function and regulation within the teleosts. For example, *igfbp4* is not represented in the current *Danio rerio* genome assembly (http://www.sanger.ac.uk/Projects/D_rerio/) and is probably absent from the zebrafish lineage. Our results at least indicate that caution is needed in inferring similar functions of IGFBPs between teleost lineages and certainly between teleosts and mammals. Overall we conclude that following WGD some of the retained paralogues of IGF-system genes show differential transcriptional regulation with fasting and refeeding in skeletal muscle, but that complex patterns of regulation have evolved between and within lineages.

Genome-wide transcriptional regulation with catabolic to anabolic transition

Microarray experiments provided a snapshot of the fast muscle transcriptome during fasting and at the point IGF transcripts reached their maximum abundance following feeding. The screening criteria used to build lists of differentially regulated genes were apparently robust because all 14 genes tested were validated by qPCR and were well correlated (R=0.79; supplementary material Fig. S1). Fasting in zebrafish (Fig. 6A,B) and Atlantic salmon (Bower et al., 2008; Bower and Johnston, 2010b) is associated with a large increase in the abundance of E3 ubiquitin ligases *MURF1* and *fbxo32* transcripts. In mammals, the ubiquitin substrate recognition system has been implicated in specific degradation of myoD (Tintignac et al., 2005; Finn and Dice, 2006) and other promyogenic transcription factors (Finn and Dice, 2006). Two substrate recognition components of the ubiquitin ligase system, *F-box only protein 25* (*fbxo25*) and *RAB40B, member RAS oncogene family* (*rab40b*), were also highly upregulated with fasting in zebrafish fast muscle (Table 1). Two genes with putative roles in autophagy were found to be upregulated during food deprivation [*microtubule-associated protein 1 light chain 3 beta* (*map1lc3b*) and *neighbour of BRCA1 gene 1* (*nbr1*)] (Table 1). It is known that myofibrillar proteins are degraded to provide a source of amino acids for energy metabolism during prolonged fasting in teleosts (Johnston and Goldspink, 1973). However, the relative importance of the ubiquitin-proteasome degradation pathway, autophagy and other classes of proteases in mediating protein breakdown during normal protein turnover and short periods of fasting remains to be established. Cell cycle arrest and apoptosis also seems to be an important response in adapting to periods of limited energy supply in zebrafish, as evidenced by the upregulation of anti-proliferative protein gene transcripts [*B-cell translocation gene 1* and *2* (*btg1* and *btg12*), *THAP domain-containing protein 1* (*thap1*) and pro-apoptotic genes (*bbc3* and *klf11b*)] (Table 1).

Transition to an anabolic state 3 h after the meal resulted in major changes in the muscle transcriptome. Feeding was associated with the upregulation of transcripts for chaperone proteins (*unc45b*, *ppig*, *pdip5*, *dnaja* and *stip1*) including various heat shock proteins and associated proteins (*hsp90a.1*, *hsp90a.2*, *hsdp1* and *hspa5*; Table 2). Molecular chaperones are essential for both the folding and maintenance of newly translated proteins and the degradation of misfolded and destabilized proteins (Zhao and Houry, 2007). In zebrafish, the chaperones Hsp90a and Unc45 are coregulated and involved in the folding of the globular head of myosin during myofibrillargenesis, associating with the Z-line once myofibrillar assembly is completed (Etard et al., 2008). The sequencing of subtractive cDNA libraries from fast skeletal muscle of Atlantic salmon fed either maintenance or satiating rations also revealed that expression of chaperone proteins indicative of unfolded protein response (UPR) pathways such as *DNAJ4*, *HSPA1B*, *HSP90A* and *CHAC1* was an early response to increased food intake and growth (Bower and Johnston, 2010b). Accumulation of unfolded proteins can occur when the amounts of newly synthesized proteins exceeds that of the protein folding capacity of the endoplasmic reticulum (Okada et al., 2002). Taken together these results indicate that an increase in protein chaperone gene expression and activation of UPR pathways is a general response of teleost skeletal muscle following the transition from a catabolic to anabolic state.

The largest increase in transcript abundance found with feeding was for the gene encoding the enzyme ornithine decarboxylase 1 (*odc1*; 11.5-fold). Ornithine decarboxylase, a key metabolic enzyme of the polyamine biosynthesis pathway, also showed an increase in

activity after feeding in fasted rat tissues (Moore and Swendseid, 1983). Polyamines have a number of biological functions including cell growth and apoptosis and act in a concentration-dependent manner (Larqué et al., 2007). The anabolic state was associated with enrichment of GO terms for mitochondrial translocase activity, initiation of protein synthesis [e.g. *eukaryotic translation initiation factor 4A isoform 1A* (*eif4a1a*), *dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2* (*dyrk2*)] and mRNA processing, maturation and export protein genes [e.g. *DEAD (Asp-Glu-Ala-Asp) box polypeptide 5* (*ddx5*) and *small nuclear ribonucleoprotein 40 (U5)* (*snrnp40*)]. Furthermore, *pias4* and *sae1*, which are involved in the post-translational conjugation of SUMO (small ubiquitin-like modifier) to target proteins, were upregulated with feeding. Sumoylation of proteins may affect their stability, localization and activity (Geiss-Friedlander and Melchior, 2007). In contrast to the ubiquitin-conjugated proteins, sumo-conjugated proteins are not targeted for degradation and indeed sumoylation may function as an antagonist pathway to the ubiquitin-proteasome pathway (Desterro et al., 1998; Hay, 2005).

Feeding was also associated with significant changes in the expression of genes that may function as epigenetic switches coding for proteins that modify chromatin structure and alter the expression of suites of other genes. For example, *SET and MYND domain containing 1b* (*smyd1b*) transcripts increased 4.8-fold between the fed and fasted states (Tables 1 and 2). Smyd1 functions as a transcriptional repressor in mouse cardiac muscle (Gottlieb et al., 2002) and is required for normal skeletal muscle development in zebrafish (Tan et al., 2006). In the present study feeding also resulted in a 4.9-fold increase in transcript abundance for the *euchromatic histone-lysine N-methyltransferase 1b* (*ehmt1b*). The human orthologue of Ehmt1b was found to be part of the E2F6 complex and is probably involved in the silencing of MYC- and E2F-responsive genes (Ogawa et al., 2002), and is thought to play a role in G0–G1 cell-cycle transition.

The recruitment and hypertrophy of fast myotomal muscles in zebrafish was shown to be typical of teleosts (Johnston et al., 2009). The present study also demonstrates the utility of the zebrafish for mechanistic studies on the regulation of growth signalling pathways in teleost muscle, providing the advantages of a sequenced genome, commercially available molecular resources and low husbandry costs because of the small body size. However, caution should be applied in extrapolating all results from model to aquaculture fish species in the light of evidence for lineage-specific patterns of paralogue retention (Macqueen and Johnston, 2008a; Macqueen and Johnston, 2008b) and IGFBP gene expression (this study). The single meal paradigm also provides an interesting alternative to the use of continuous feeding regimes to investigate transcriptional regulation during the transition from a catabolic to an anabolic state.

APPENDIX

Detailed information on protein extraction and western-blotting detection experimental protocols.

Protein extraction

Skeletal muscle protein from five random replicates for each time-point was extracted by homogenization of 30 mg of tissue in 350 µl of 25 mmol l^{-1} MES pH 6.0 containing 1 mol l^{-1} NaCl, 0.25% (m/v) CHAPS, DNA/RNA nuclease (Invitrogen) and protease inhibitor cocktail (Invitrogen) using Lysing Matrix D (Qbiogene) in a FastPrep® machine (Qbiogene).

Optimal protein loading for SDS separation and transfer

The optimal protein amount to be used for electrophoresis separation and transfer was empirically determined by applying from 10 to 60 µg of protein of a reference sample in duplicates and analysing the density of the Ponceau S staining (Fig. A1A,B). TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, Tyne and Wear, UK) was used to analyse the density of bands from Ponceau S staining and western blots. Protein saturation was observed when more than 30 µg of protein was loaded in the gel. The optimal amount was 20 µg considering both Ponceau-S-staining linearity (Fig. A1B) and total protein availability for the study.

Loading protein control and P-AKT antibody specificity

The membranes used for optimal protein loading determination included a reference sample treated with calf intestinal alkaline phosphatase (A2356; Sigma) to confirm that the antibody targeted the phosphorylated moiety of the protein of interest (Fig. A1C). Actin intensity was better correlated with Ponceau S staining when compared with GAPDH (Fig. A1C) and was used to normalize differences in protein loading. Dephosphorylation of P-AKT significantly decreased its detection by P-AKT specific antibody (Fig. A1C) whereas no change in detection was observed for actin antibody in the dephosphorylated sample (Fig. A1C), confirming the specificity of the P-AKT antibody to the phosphorylated moiety of AKT.

Western-blotting of target proteins

The samples (20 µl, containing 20 µg of protein) were added to 6 µl of a solution containing 5 µl of 5× protein loading buffer and 1 µl 20× reducing agent (Fermentas), heated for 5 min at 95°C and loaded in NuPAGE® Novex 4–12% Bis-Tris gels (Invitrogen) and run at 120 V. A protein ladder of 10–250 kDa (Fermentas) and a reference sample were loaded in all gels to estimate the molecular mass of proteins of interest and serve as a normalization sample, respectively. Proteins separated by electrophoresis were transferred to a PVDF Immobilon-P Transfer Membrane (Millipore) at 25 V for 105 min. Successful protein separation and transfer were confirmed by Ponceau S staining (Sigma). PVDF membranes were blocked overnight at 10°C using 5% (m/v) nonfat milk (AppliChem) prepared in PBS (Sigma) containing 0.1% (v/v) Tween 20 (Sigma). Blocked membranes were incubated overnight at 10°C with the following primary antibodies (IgGs): P-AKT (1:1000 dilution; Cell Signaling, #4060), AKT (1:1000; Cell Signaling, #2966), actin (1:20,000; Sigma A2066) and GAPDH (1:30,000; Sigma, G9545). Probed membranes were incubated at 20°C for 1 h with the secondary antibody against mouse or rabbit IgG conjugated to horseradish peroxidase (both from Sigma and used at 1:60,000 dilution). Positive reactions were recorded by exposing Hyperfilm ECL (GE Healthcare) to the membranes after incubation for 1 min with ECL Western Blotting Detection Reagents (GE Healthcare) at room temperature. Experimental variations in the electrophoresis and transfer were normalized using a reference sample common to all membranes. The fold-change in phosphorylation of AKT in each time point was compared with the samples from –159 h.

GLOSSARY

Myotome
: Tissues within the embryonic somites that form the trunk muscles.

Neofunctionalization
: Gain of a new function by one paralogue, while the other retains the original function performed by the ancestral gene.

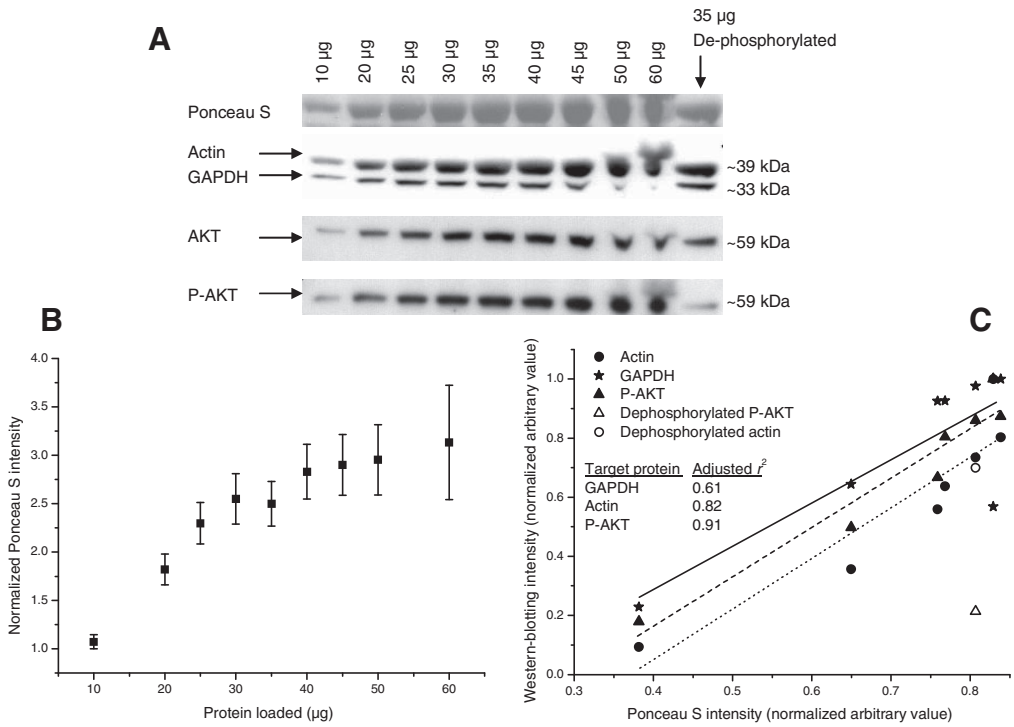

Fig. A1. Determination of the optimal amount of protein for SDS-PAGE separation and antibody detection linearity by Ponceau S staining (A,B) and comparisons of Ponceau S staining with intensity of the signals in western blots. (C). A considerably lower signal was observed for P-AKT after submitting the sample to de-phosphorylation(unfilled symbols) in comparison to the untreated sample (filled symbols).

Paralogue
: One of a pair of genes that arose from a common ancestral gene *via* an inherited mutation involving duplication of a region in the genome.

Promyogenic transcription factors
: A family of four basic-helix-loop-helix proteins (myoD, myog, myf5 and myf6) that control the expression of hundreds of muscle-specific genes, acting as master regulators of myogenesis.

Subfunctionalization
: The function previously performed by an ancestral gene is partitioned between the paralogues.

Sumoylation
: Post-translational conjugation of proteins with a small ubiquitin-related modifier (SUMO).

Unfolded protein response
: Cellular response to prevent accumulation of unfolded proteins. It mainly involves the processes leading to protein folding, protein export into the endoplasmic reticulum, degradation of misfolded proteins and apoptosis.

Whole genome duplication
: An inheritable mutation involving a doubling of all sets of chromosomes and usually followed by massive loss of genes duplicates through evolutionary time. Occurred twice in a common ancestor of vertebrates, again in a common teleost ancestor and again in several other lineages; many paralogues generated from these events are still retained.

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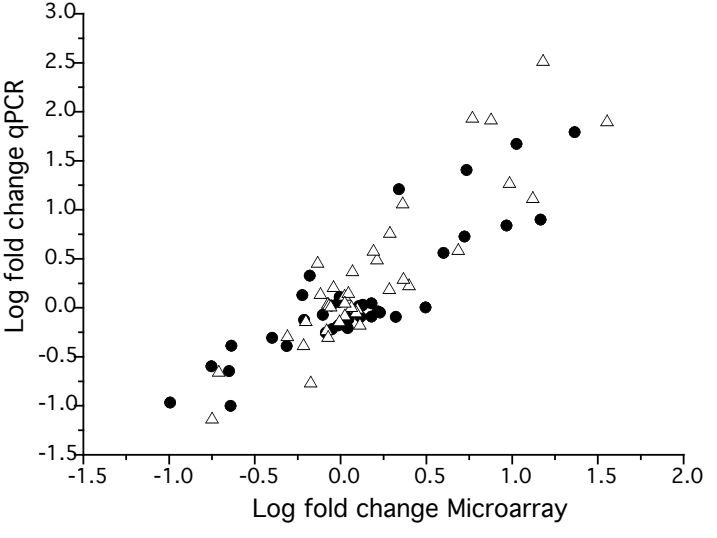
Log fold change qPCR
Log fold change Microarray
3.0
2.5
2.0
1.5
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Table S1. Primers used in this study. Linearity of standard curve and Ensembl gene ID are shown

Ensembl gene	f/r	Primer 5′–3′ sequence	Product size (bp)	T_m (°C)	E (%)	R^2	Ensembl gene ID
Reference genes selected from the microarray experiment							
tomm20b	f:	GCTGCTGGCTCAGGGAGACTATG	170	86.2	103.8	0.999	ENSDARG00000044636
	r:	CGCTGACGATACGCTGGCTG					
rpl7a	f:	CCCATTGAGCTGGTGGTGTTCT	216	84.8	96.2	0.999	ENSDARG00000019230
	r:	ACGGATCTCCTCATATCTGTCATTGTA					
lman2	f:	GGATCGCTCCTTTCCATACATTTC	176	81.7	104.7	0.999	ENSDARG00000061854
	r:	CCACCATTATCGTGAGTCTGCCT					
*si:ch211-273k1.4**	f:	GCTGTTTTTGTGAAGGAGTGTGGTC	193	82.4	98.5	0.998	ENSDARG00000033259
	r:	TTTCCCAAACAAGCGTCATCTCTG					
Genes upregulated during fasting selected from the microarray experiment							
odc1	f:	CGACTGTGCCAGCAAAACGG	201	85.7	103.5	1.000	ENSDARG00000007377
	r:	CGGAGAACCAGCTTGGCATTT					
hsp90a.1	f:	TGGCGAACTCAGCGTTTGTG	259	83.3	100.6	0.997	ENSDARG00000010478
	r:	ACGGTGACCTTCTCAATCTTTTTG					
fkbp5	f:	GACACAGTATTTCAAGGCAGGACG	215	84.3	99.2	0.999	ENSDARG00000028396
	r:	CCAGCTCCATTACCTTGTTGCAG					
sae1	f:	GCAAGTGCTTCTGAAGTTTCGC	232	83.6	106.3	0.999	ENSDARG00000010487
	r:	CTGAGACAGCGCCTTGACAATC					
hsp90a.2	f:	CTGGAGAAGAAAGTGGAGAAGGTCA	367	85.4	102.0	0.998	ENSDARG00000024746
	r:	CCTCATCAATGCCTAAGCCCAG					
foxo1a	f:	GCGGGCTGGAAGAACTCAATCA	219	85.1	101.7	0.999	ENSDARG00000063540
	r:	CACCCTGAAGAGCCAGCTTTTTCT					
Genes downregulated during fasting selected from the microarray experiment							
klf11b	f:	GCCCCAGTCGCCAGTATCTTC	240	86.8	97.8	0.999	ENSDARG00000013794
	r:	GGTTTCTCTCCTGTATGGGTTCTGA					
nr1d1	f:	GAAGGCTGGAACATTTGAGGTC	228	83.3	101.2	0.999	ENSDARG00000033160
	r:	GCAGACACCAGGACGACCG					
cited2	f:	AGCGGAGAGGGGAATGGTAGAC	264	84.8	103.4	0.998	ENSDARG00000030905
	r:	CGGGCAGGCAAGTTTCCATT					
bbc3	f:	GGGACAATTCAGGAACAGAACAGGA	222	86.8	95.2	0.999	ENSDARG00000069282
	r:	GCGGGACGGCATTCCTCTG					
znf653	f:	GCCATCAGCAGTTTCCAGAATCAT	255	81.9	100.7	0.999	ENSDARG00000093469
	r:	CTGATACCCACATATCTCACATTGTAATG					
hsf2	f:	CCTTCTGGGCAAAGTTGAGCTG	194	80.3	100.2	0.997	ENSDARG00000053097
	r:	GCTGCTTGTCTGTGTTTTCTGAATC					
Reference genes selected from the literature							
ef1a	f:	GAGGAGTGATCTCTCAATCTTGAAAC	191	83.8	101.0	0.999	ENSDARG00000020850
	r:	CCCTTGCCCATCTCAGCG					
rpl13a	f:	AAAATTGTGGTGGTGAGGTGTG	183	81.5	100.3	0.998	ENSDARG00000044093
	r:	GGTTTTGTGTGGAAGCATACCTCT					
Bactin2	f:	CCCAAACCCAAGTTCAGCC	112	83.0	100.5	0.999	ENSDARG00000037870
	r:	GAAGACAGCACGGGGAGCA					
b2m	f:	GGGGAAAGTCTCCACTCCGAAA	166	81.7	102.7	0.997	ENSDARG00000053136
	r:	CAGGTCGGTCTGCTTGGTGTCC					
usp5	f:	GACCCGGAAAACACAGAAGGAG	101	79.5	102.9	0.999	ENSDARG00000014517
	r:	CAAACCCTCCCTCAATACCAATG					
tbp	f:	CCTGCGAATTATCGTTTACGTCTTTT	151	81.7	98.4	0.999	ENSDARG00000014994
	r:	CCCTGTGGAGATGCCAGACCT					
cyp1a	f:	GACCTATTCGGAGCCGGTTTCG	120	82.8	103.7	1.000	ENSDARG00000026039
	r:	CCCGATCTTTTCATCCAATTCTCTTTG					
IGF pathway components							
IGF-Ia†	f:	GCATTGGTGTGATGTCTTTAAGTGTA	188	87.1	95.9	1.000	ENSDARG00000094132
	r:	GTTTGCTGAAATAAAAGCCCCT					
IGF-Ib[2]	f:	GGCTTTTACATAGGCAAACCTGGAG	166	84.8	NA	NA	ENSDARG00000058058
	r:	GCAGCACAGATGCAGGGACAT					

IGF-IRa	f:	GCCCGTGGAGAAGTCTGTGG	154	81.3	98.0	0.999	ENSDARG00000027423
	r:	GTGTGCGAAAGTGTTCCTGGTT					
IGF-IRb	f:	CACAACTACTGCTCCAAAGAACTGA	238	83.8	94.5	0.999	ENSDARG00000034434
	r:	GCCTGTCTGGAGGTCTGGGA					
IGF-IIa	f:	GAAACACGAACAACGATGCG	346	82.8	91.8	1.000	ENSDARG00000018643
	r:	AGTACTTCACATTTATGGTGTCCTTG					
IGF-IIb	f:	ACAGACAGTTTCGTAAATAAGGTCATAA	236	85.5	97.0	0.999	ENSDARG00000033307
	r:	CAACACTCCTCCACAATCCCAC					
IGF-IIR	f:	ACCCCTGTCCTCAAGTAACAGAT	176	80.1	99.4	0.998	ENSDARG00000006094
	r:	TTGCACACCGTCAGTACAAAAG					
IGFBP-1a	f:	ACTGGTGGAACAGGGTCCCT	158	82.1	98.0	1.000	ENSDARG00000014947
	r:	CTAGAGATGATTCGCACTGTTTGATT					
IGFBP-1b	f:	GCTCATCCAGCAGGGTCCG	151	83.5	101.2	0.999	ENSDARG00000038666
	r:	CGACACACACTGTTTGGCCTTG					
IGFBP-2a	f:	GGGAAGTCAGCGGTGAGGTG	196	83.0	100.8	0.999	ENSDARG00000052470
	r:	TGCTGGCACTGGCTCTGTTTA					
IGFBP-2b	f:	GTCAGCAGCACACAGTGGAGAAGTA	188	82.5	99.7	0997	ENSDARG00000031422
	r:	GCTCCTGTTGACACTGGCTCTG					
IGFBP-3	f:	AATGAATATGGCCCATGTCGT	146	80.9	101.2	0.999	ENSDARG00000014859
	r:	CCTTTGGATGGACTGCACTGT					
IGFBP-5a	f:	ACAACAAGCTAAGCTCGGTCCA	209	81.0	100.5	0.998	ENSDARG00000039264
	r:	TAGAGGGCTTACACTGTTTGCG					
IGFBP-5b	f:	GCACCCACCCATTGATCGT	241	82.8	95.3	0.999	ENSDARG00000025348
	r:	CCTTCTGCACGGACCAAATTC					
IGFBP-6a	f:	CCCTCCGCCTACAGACTATGA	180	83.1	100.7	0.997	ENSDARG00000070941
	r:	GACGAGCGACACTGCTTCCT					
IGFBP-6b	f:	CGTTGGGGGAGCCCTGCG	201	82.3	96.9	0.998	ENSDARG00000090833
	r:	GAGCCTTTTCCATTTCACCACTGT					
Muscle-specific ubiquitin ligases							
fbxo32	f:	GAGCACCAAAGAGCGTCAT	154	82.8	105.6	0.999	ENSDARG00000040277
	r:	CACTCCACTCAGAGAAGGCAG					
zgc:86757‡	f:	CCTGGCTTTGAGAGTATGGACC	225	80.1	102.5	0.999	ENSDARG00000028027
	r:	GCCCCTTGCCTCACAGTTAT					
Muscle structural proteins							
tnni2a.4	f:	GCAGACAAGGAGATTGAGGATCTG	199	83.8	101.7	0.998	ENSDARG00000029069
	r:	GTTCTACAGACTCCTCCTTGACCTCC					
mylz2	f:	GGAGAGAAGTTGAAGGGTGCTGAC	154	83.8	103.9	1.000	ENSDARG00000053254
	r:	GATTCTTCATCTCCTCTGCGGTG					

Ensembl gene symbols: f and r, forward and reverse primer sequences; T_m, product melting temperature; E, amplification efficiency;

*Orthologue of the gene *dis3l2* in various species.

†According to Zou et al. (Zou et al., 2009) the genes *igf1* and *igf3* annotated in the zebrafish ensembl database V58 are two paralogues of the *igf1* gene and should be named *igf1a* and *igf1b*, respectively.

‡Orthologue of the gene *trim63* (*murf1*) in various species.