Abstract

Background: Many fish species experience long periods of fasting in nature often associated with seasonal reductions in water temperature and prey availability or spawning migrations. During periods of nutrient restriction, changes in metabolism occur to provide cellular energy via catabolic processes. Muscle is particularly affected by prolonged fasting as myofibrillar proteins act as a major energy source. To investigate the mechanisms of metabolic reorganisation with fasting and refeeding in a saltwater stage of Atlantic salmon (Salmo salar L.) we analysed the expression of genes involved in myogenesis, growth signalling, lipid biosynthesis and myofibrillar protein degradation and synthesis pathways using qPCR.

Results: Hierarchical clustering of gene expression data revealed three clusters. The first cluster comprised genes involved in lipid metabolism and triacylglycerol synthesis (ALDOB, DGAT1 and LPL) which had peak expression 3-14d after refeeding. The second cluster comprised ADIPOQ, MLC2, IGF-I and TALDO1, with peak expression 14-32d after refeeding. Cluster III contained genes strongly down regulated as an initial response to feeding and included the ubiquitin ligases MuRF1 and MAFbx, myogenic regulatory factors and some metabolic genes.

Conclusion: Early responses to refeeding in fasted salmon included the synthesis of triacylglycerols and activation of the adipogenic differentiation program. Inhibition of MuRF1 and MAFbx respectively may result in decreased degradation and concomitant increased production of myofibrillar proteins. Both of these processes preceded any increase in expression of myogenic regulatory factors and IGF-I. These responses could be a necessary strategy for an animal adapted to long periods of food deprivation whereby energy reserves are replenished prior to the resumption of myogenesis.

Background

Fasting is a natural phenomenon in high latitude fish and is associated with low winter temperatures, short-days which restrict feeding opportunities and/or reduced availability of prey items. Atlantic salmon (Salmo salar L.) experience periods of fasting during the completion of their lifecycle, when adult fish return to freshwater to spawn. Reproduction and migration in anadromous salmonids requires substantial energy input, which is often mobilised from stores in visceral and somatic tissues [1]. These energy stores are mostly acquired in the ocean [2], since salmon cease feeding prior to and during their upstream
migrate to spawning grounds in freshwater [3,4]. Considerable energy is also used for gonadal development, nest construction, courtship and intrasexual competition [1,5-8]. Kelt (previously spawned adult salmon), which overwinter in fresh water pools and descend in spring [9], rely entirely on stored energy reserves to survive and are often in an emaciated condition [10]. Juvenile salmon may also experience fasting, as juveniles which show delayed levels of activity, delay their migration to the sea and overwinter in freshwater [11]. To survive the winter months, these fish rely heavily on lipid reserves accumulated during autumn [12-14].

Fasting in fish is associated with a reduction in metabolic rate at the whole animal level [15]. Reductions in protein synthesis with extended fasting are undoubtedly an important component of the observed metabolic depression [16]. Net changes in tissue mass are a function of the balance between protein synthesis and degradation. In rainbow trout liver prolonged starvation resulted in no change in fractional protein synthesis rates, a large decrease in absolute protein synthesis rates and increased protein degradation rates [17]. The amino acids produced from the net mobilisation of proteins from the myotomal muscles represent a major energy source for other tissues [18,19]. Fish myotomes contain slow and fast muscle fibre types primarily used in sustained and burst swimming respectively [20,21]. Biochemical and ultrastructural studies have shown that slow muscle is relatively spared from the effects of fasting, presumably because it is needed for continuous swimming at all speeds [22,23]. Atrophy in fast muscle follows depletion of glycogen and lipid stores and is associated with a decrease in myofibrilar diameter, and a preferential degradation of peripheral myofilaments [23]. Feeding fish to appetite following fasting or a period of growth restriction results in faster growth relative to continuously fed control groups, a phenomenon referred to as compensatory or catch-up growth [24,25]. The primary mechanism underlying compensatory growth with food restriction in Atlantic salmon is an increase in feeding intensity [25).

Fasting-refeeding protocols have become one of the main manipulative tools used to investigate the molecular and genetic mechanisms regulating growth [26-29]. As in mammals, insulin-like growth factor-I (IGF-I) and IGF-II are the key hormones which stimulate protein synthesis in fish muscle [26,29,30]. IGF-I from the liver is delivered to the muscle by the circulation and in addition both IGF-I and IGF-II are locally synthesized in response to environmental and nutritional stimuli [31]. The complete IGF-system in fish comprises 4 splice variants, several membrane receptors and six IGF-binding proteins which act on the PI3K/AKT/mTOR pathway [31,29]. Rescan et al., [28] used a cDNA microarray containing 9023 rainbow trout (Oncorhynchus mykiss) sequences to provide a general description of some of the changes in muscle gene expression that accompanied recovery growth following fasting. Fasting was associated with an upregulation of cysteine protease cathepsins and components of the ubiquitin-proteosome involved in protein degradation as well as tuberous sclerosis component 2 (TSC2) an inhibitor of mTOR function and the translational repressor 4E-BP1 [28,32]. 4 to 11 days after refeding there was a downregulation of genes involved in protein catabolism and an upregulation of genes involved in translation, protein folding and maturation and ribosome formation [28].

In mammals, the signals that regulate muscle atrophy and hypertrophy are linked through the PI3K/AKT/mTOR pathway [33]. Activation of the PI3K/AKT/mTOR pathway by IGF-I causes a phosphorylation cascade that leads to an increase in translation and therefore protein synthesis, resulting in skeletal muscle hypertrophy [34]. Phosphorylation of AKT also results in inhibition of key regulators of skeletal muscle atrophy, the muscle specific ubiquitin ligases MAFbx and MuRF1, through phosphorylation of the FOXO transcription factors [35]. Conversely, dephosphorylation of FOXO transcription factors, for example during nutrient restriction, results in increased expression of MuRF1 and MAFbx. MAFbx and MuRF1 are E3 ubiquitin ligases involved in the targeting of proteins, such as myosin light chain 2, for degradation via the ubiquitin pathway [35,36]. MAFbx targets elongation initiation factor 3-f (eif3-f) for degradation, a key regulator of mTOR mediated translation of muscle structural proteins including myosin heavy chain and desmin [37,38]. MuRF1 has also recently been shown to depress energy metabolism in mammalian muscle via effects on pyruvate dehydrogenase and creatine kinase [39]. Furthermore, MyoD, a master transcriptional factor for myogenesis is a target of MAFbx [40], indicating a widespread role of ubiquitin ligases in regulating muscle growth in mammals.

Quantitative PCR (qPCR), if performed with appropriate normalisation, statistical analysis and under standardised operating procedures such as those described in the Minimum Information for publication of Quantitative real-time Experiments guidelines (MIQE), is the method of choice for reliably quantifying changes in gene expression [41]. In the present study, qPCR was used to investigate the phasing of gene expression in the fast muscle of Atlantic salmon during fasting-induced recovery growth. Since the response of both MuRF1 and MAFbx expression to fasting-refeeding has not been investigated in Atlantic salmon we particularly wanted to determine the expression of MuRF1 and MAFbx in relation to IGF-I and marker genes for myogenesis, glucose homeostasis and lipid metabolism.
Methods
The methods described in this paper for qPCR analysis of gene expression are compatible with the MIQE guidelines [41].

Fish and experimental design
All experiments were conducted at EWOS Innovation, Dirdal, Norway and were approved by the local animal welfare committee. Atlantic salmon (Salmo salar. L 1327 g ± 336.1 g, Mean ± SD, n = 55) were individually passive induced transponder (PIT) tagged (Fish Eagle, Lechlade, Gloucestershire England) so that growth rate could be calculated. Fish were fasted for 32 days then fed to satiation with a commercial feed (EWOS Innovation, 52% fish meal, 13% rape seed oil, 12% wheat protein, 10% fish oil, 5% pea protein, 5% soy protein, 3% krill meal, vitamin C-35 0.29%, vitamin mix 0.15%, vitamin E-50 0.03%, methionine 0.02%) for 32 days. The average daily temperature was 7.8°C, oxygen was 13.96 mg L⁻¹, and average daily salinity was 28.9 ppm. Samples were taken at 0 d, 3 d, 7 d, 14 d and 32 d following refeeding with 8 fish sampled at each time point. Fish were humanely killed by anesthetics at each time point. Oxygen was 13.96 mg L⁻¹, and average daily salinity was 28.9 ppm. Analyses were conducted using a real-time PCR with Power SYBR green master mix (Applied Biosystems, Foster City, CA, USA). cDNA used in qPCR assays was first diluted 80-fold with nuclease free water to a final volume of 15 μl. Amplification was performed in 96 well plates (Stratagene, La Jolla, CA, USA) with the following thermal cycling conditions: initial activation 95°C 10 minutes, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Dissociation analysis of the PCR products was performed by running a gradient from 60 to 95°C to confirm the presence of a single PCR product. Products were also sequenced to confirm identity. A dilution series made from known concentrations of plasmid containing the PCR inserts was used to calculate absolute copy numbers for each of the genes examined.

RNA extraction and cDNA synthesis
Total RNA was extracted by addition of 100 μg of muscle to Lysing matrix D (Qbiogene, Irvine, California) with 1 ml Tri Reagent (Sigma, Gillingham, Dorset, UK) and homogenised using a Fast Prep instrument (Qbiogene, Irvine, California). Total RNA was quantified based on absorbance at 260 nm. Only samples with a A260/280 ratio between 1.8 and 2.1, and a an A260/230 ratio above 1.8 were used for reverse transcription. Genomic DNA contamination was removed by treatment with Turbo DNA-free (Ambion, Austin, Texas, USA), and the integrity of purified RNA confirmed by agarose gel electrophoresis. First strand cDNA was synthesised from 1 μg total RNA using Superscript III (Invitrogen, Carlsbad, CA, USA) as per manufacturer’s guidelines

Primer design
Previously published primers were used for LPL [42]. Primers were designed using Net primer (Premier BioSoft) to have Tm of 60°C, and where possible, were designed to cross an exon-exon junction to avoid amplification of contaminating genomic DNA. The primers, amplicon size, amplicon melting temperature and accession numbers of genes used for qPCR are listed in table 1. The following genes were studied: Insulin-like growth factor I (IGF-I), myogenin (MYOG), myoblast determination factor 1 (myoD1a), myocyte enhancer factor 2A (MEF2A), muscle ring finger protein 1 (MuRF1), muscle-specific X box protein (MAFbx), myosin heavy chain (MHC), myosin light chain 2 (MLC2), pyruvate kinase (PKM), phosphoglycerate kinase (PGK), transaldolase (TALDO1), fructose 1,6 bisphosphatase (FBP1), Aldolase B (ALDOB), cyclic AMP response element binding protein (Creba), lipoprotein lipase (LPL), diacylglycerol O-acyltransferase homolog 1 (DGAT1), and adiponectin (ADIPOQ).

Quantitative PCR
qPCR was performed using a Stratagene MX3005P QPCR system (Stratagene, La Jolla, CA, USA) with SYBR green chemistry (Power SYBR, Applied Biosystems, Foster City, CA, USA). cDNA used in qPCR assays was first diluted 80-fold with nuclease free water. Each qPCR reaction mixture contained 7.5 μl 1 × Power SYBR green master mix, 6 μl cDNA (80-fold dilution), 500 nM each primer and RNase free water to a final volume of 15 μl. Amplification was performed in 96 well plates (Stratagene, La Jolla, CA, USA) with the following thermal cycling conditions: initial activation 95°C 10 minutes, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Dissociation analysis of the PCR products was performed by running a gradient from 60 to 95°C to confirm the presence of a single PCR product. Products were also sequenced to confirm identity. A dilution series made from known concentrations of plasmid containing the PCR inserts was used to calculate absolute copy numbers for each of the genes examined.

Data analysis
Genorm [43] was used to analyse the stability of several reference genes including 18S Ribosomal RNA, hypoxanthine phosphoribosyltransferase 1 (HPRT1), -actin, RNA polymerase II and Elongation factor 1 (EF1- ). Analysis revealed HPRT1, RNA polymerase II and -actin to be the
most stable genes in this experimental system (M = 0.41), thus the geometric average of these genes was used for normalisation of qPCR data. Statistical analysis was performed using minitab (Minitab Inc). Significant differences in expression between time points were calculated by ANOVA using Fisher’s individual error rate post hoc tests. Correlations in gene expression were calculated using linear regression and Pearson’s correlation. Hierarchical clustering was performed using Cluster3 software [44].

### Results

#### Fish Growth characteristics
Prior to fasting, fish had an average mass of 1327 ± 336.1 g (Mean ± SD, n = 55), which after fasting for 32 days, had reduced by an average of 91.2 ± 23.1 g (Fig. 1), with an average TGC of -1.0 ± 0.14. After feeding, weight gains were 58.4 ± 18.3 g, 100.6 ± 38.7 g, 125.4 ± 66.6 g and 387.6 ± 114.3 g for days 3, 7, 14 and 32 respectively (n = 10). Early TGC calculations are unreliable as the food present in the gut gives a false indication of growth.
whereas the later time points provide a more reliable estimate. The TGC for fish (when calculated from weight at the start of feeding, day 0) was mean 3.1 ± 1.1, n = 8 at 14 d and mean 3.7 ± 0.8, n = 8 at 32 d. At day 3, there was an initial increase in weight of 58.4 ± 18.3 g, contributed by the presence of food in the gut. If this value is subtracted from the later time points, then actual weight gain for days 7, 14 and 32 are 42.2 ± 24.9 g, 67.0 ± 48.1 g and 329.2 ± 114.2 g.

Gene expression

Overview

A summary heat map and hierarchical clustering of gene expression patterns during fasting-induced recovery growth is shown in Fig. 2. Three main clusters of gene expression were identified. Cluster I comprised ALDOB, DGAT1 and LPL which had peak expression at 3-14 d after refeeding (Fig. 2). Cluster II comprised ADIPOQ, MLC2, IGF-I and TALDO1 which were later responding showing peak expression 14-32 d after refeeding (Fig. 2). In this cluster, MLC2 also showed high transcript levels in fasted fish (Fig. 2). Cluster III contained the largest number of genes (MuRF1, MAFbx, CrebA, MYOG, MEF2A, FBP1, PGK, PKM, MHC and MyoD1a) which were mostly up-regulated in fasted fish and generally down regulated with refeeding (Fig. 2).

Muscle-specific ubiquitin ligases

Both the muscle specific E3 ubiquitin ligases, MuRF1 and MAFbx were significantly down regulated at all time points in fed relative to the fasted fish (P < 0.01, Fig. 3A, B). MAFbx transcript levels were down regulated up to 98% (Fig. 3B).

IGF-I

Transcripts of IGF-I in fast muscle were similar in fasted fish and 3 d, 7 d and 32 d after feeding, but were significantly upregulated by 142% at 14 d (P < 0.05) compared to the 0 d sample (Fig. 3C).

Myogenic regulatory factors

The myoD family of transcription factors regulate the commitment of myoblasts to the myogenic lineage (myoD) and muscle differentiation (MYOG) [45]. MYOG expression decreased significantly between the 0 and 7d
Figure 3
Expression profiles for MuRF1 (A), MAFbx (B), IGF-I (C), MYOG (D), myoD1a (E), MEF2A (F), MHC (G) and MLC2 (H) in fast muscle of Atlantic salmon from fasted fish (0 d), fed to satiation at 3 d, 7 d, 14 d, 32 d. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis), see text for details. Values represent mean ± S.E., 8 fish per sample point. Significant differences between means are indicated by different letters.
samples (P < 0.01) and then remained relatively constant with refeeding (Fig. 3D). There are three retained paralogues of myod1 in Atlantic salmon each with distinct expression patterns during development and in different fibre types [46]. Myod1a is the paralogue predominantly expressed in fast skeletal muscle [47]. Myod1a was upregulated with fasting and showed only minor differences between the fed samples (Fig. 3E). The myocyte-enhancer 2 gene family (MEF2) proteins act co-operatively with myoD proteins to regulate myogenesis [48]. MEF2 transcripts were upregulated in starved individuals and tended to decrease with feeding, producing a statistically significant difference after 32 d (p < 0.05, Fig. 3F).

**Myofibrillar proteins**

The primers to myosin heavy chain were designed to conserved regions of the protein and could potentially amplify multiple isoforms of the MHC transcripts in fast muscle. Expression of this gene(s) was relatively high in fasted fish and significantly decreased 0-14d (P < 0.05) after feeding before increasing to 84% of fasted levels after 32 d (Fig 3G). Myosin light chain 2 significantly decreased (P < 0.01) an average 66% between fasted fish and the 3 d feeding sample before increasing to reach 117% of day 0 levels in the 32 d sample (Fig. 3H), although this increase was not statistically significant.

**Metabolic genes**

Transcripts for PKM (Fig. 4A) and PGK (Fig. 4B) decreased by 50% between the fasted and 3 d sample (p < 0.05) and showed no further change with feeding. In contrast, levels of TALDO1 transcripts were similar in the fasted, 3 d and 7 d samples before increasing 141% at 14 d and 32 d (P < 0.05) (Fig. 4C). FBP1 mRNA was down regulated 69% at 3 d and 78% at 7 d (p < 0.05) relative to the fasted sample before partially recovering 14d and 32 d after refeeding (Fig. 4D). ALDOB transcripts increased with feeding reaching a peak at 14d which was significantly higher than in fasted fish (P = 0.05) (Fig. 4E). CrebA was down regulated 38% between the fasted and 3 d fed sample (p < 0.05) and showed a further 69% decline by the 32d sample (Fig. 4F). PKM expression was positively correlated with expression of MuRF1 (r² = 0.69, P < 0.0001, r = 0.71; Figure 5).

**Genes involved in lipid metabolism and adipocyte differentiation**

Expression of DGAT1 showed a small increase over fasting levels after 3 d of feeding and remained at this level throughout the experiment, although this increase was not statistically significant (Fig. 6A). Expression of LPL was significantly upregulated at 3 d, expression then decreased at 7 d and 14 d and returned to near day 0 values at 32 d (Fig. 6B). ADIPOQ transcript levels were significantly increased at 7 d, 14 d and 32 d compared to fasted fish (Fig. 6C). ADIPOQ was positively correlated with expression of MLC2 at all time points (r² = 0.35 p = 0.003, r = 0.60, Fig. 7A) and even higher correlation at fed time points (3 d, 7 d, 14 d, 32 d) (r² = 0.48 p < 0.0001, r = 0.69, Fig. 7B)

**Discussion**

Atlantic salmon continue to grow throughout their life cycle. It is known that muscle fibres are continuously produced until ~2 kg body mass, after which growth only involves fibre hypertrophy and associated nuclear accretion [49]. Thus salmon of the size studied show active myogenesis. A 32 d fast at 8°C, resulted in negative growth (TGC = -1.0) corresponding to a 6.9% decrease in body mass (Fig. 1). Following satiation refeeding, there was an average weight gain of 5.4% and 26.7% after 14d and 32d respectively. The myofibrillar proteins, principally actin and myosin, are a major source of the amino acids mobilised during fasting and migration in salmonids and comprise two thirds of the total protein content of fast muscle [50]. In the present study, mRNA transcripts for MHC and MLC2 decreased as an early response to feeding as reported earlier for Atlantic salmon undergoing a transition from zero to fast growth [29]. Expression of both these genes increased markedly by 32 d refeeding, with MLC2 expression exceeding that at 0 d (Fig. 3H).

Mammals respond to muscle wasting conditions, such as starvation, with the transcriptional upregulation of the ubiquitin ligases MuRF1 and MAFbx [51]. These proteins function in the ubiquitination of contractile proteins including troponin I [52] and myosin [53,54]. Furthermore, decreased MAFbx leads to an increase in the translation regulator eif3-f [38]. In the present study, we found that expression of the E3 ubiquitin ligases MAFbx and MuRF1 were also elevated in fasted Atlantic salmon and that both were significantly and strongly down regulated in response to feeding (Fig. 3A, B). MAFbx formed a cluster with MuRF1, MYOG and CrebA, which had strong down regulation at all time points following refeeding and with myod1a, MHC, PKM, PGK, FBP1 and MEF2A, which were also down regulated but not as strongly, following refeeding (Fig. 2). A reduction in protein degradation through down regulation of MuRF1, together with a concomitant increase in translational efficiency mediated by MAFbx may well allow the replacement of myosin mobilised during fasting much earlier than indicated by changes in transcript levels.

During fasting in mammals, glucagon release causes an increase in cAMP levels, activating the transcription factor CrebA [55], which induces the expression of genes involved in gluconeogenesis [56]. Due to the presence of cyclic AMP response elements in the promoter of CrebA, expression of CrebA is induced in a positive feedback mechanism [57]. In the present study, expression of CrebA...
Expression profiles for PKM (A), PGK (B), TALDO1 (C), FBP1 (D), ALDOB (E) and CrebA (F) in fast muscle of Atlantic salmon from fasted fish (0 d), fed to satiation at 3 d, 7 d, 14 d, 32 d. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis), see text for details. Values represent mean ± S.E., 8 fish per sample point. Significant differences between means are indicated by different letters.
was significantly down regulated in response to feeding as was FBP1 consistent with relatively high levels of gluconeogenesis occurring with fasting and a reduction in gluconeogenic activity with feeding.

In mammals, MuRF1 has been shown to decrease the abundance of certain glycolytic enzymes [39]. Thus in fasted fish elevated levels of MuRF1 might be expected to lead to a reduction in the flux through the glycolytic pathway. However, we found that the mRNAs for PKM and PGK were highest in fasted fish and decreased with feeding (Fig. 4). Interestingly, expression of PKM and MuRF1 were correlated ($r^2 = 0.48$, $P < 0.0001$, $r = 0.71$; Fig 5). It is possible that the elevated levels of PKM and PGK transcripts in fasted fish represent a response to decreased flux through the glycolytic pathway due to MuRF1 inhibition. Decreased flux through the glycolytic pathway could result in elevated levels of glucose which is a potent stimulator of PKM expression [58]. Johansen and Overturf [59] also found that expression of PKM increased during starvation and suggested that this increase was a requirement for the catabolisation of certain amino acids.

An alternative fate for the increased glucose present with feeding is to enter the pentose phosphate pathway [60]. TALDO1 is a key regulator of the pentose phosphate pathway, responsible for the generation of NADPH required for fatty acid synthesis, and ribose-5-phosphate required for nucleotide and nucleic acid synthesis [61,62]. Increased expression of TALDO1 is indicative of activation of the pentose phosphate pathway. Increased flux through this pathway could provide ribose-5-phosphate required for nucleotide production during the period of rapid growth with the NADPH used for fatty acid synthetase.

Figure 5
Correlation between MuRF1 and PKM mRNA ($r^2 = 0.50$, $P < 0.0001$, $n = 32$).

Figure 6
Expression profiles for DGAT1 (A), LPL (B) and ADIPOQ (C) in fast muscle of Atlantic salmon from fasted fish (0 d), fed to satiation at 3 d, 7 d, 14 d, 32 d. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis), see text for details. Values represent mean ± S.E., 8 fish per sample point. Significant differences between means are indicated by different letters.
sis to replace the fatty acids used during the period of nutrient restriction. Generation of NADPH is also required to maintain glutathione in a reduced state and protect cellular integrity from reactive oxygen species [63]. The production of reactive oxygen species has been demonstrated to be lower in caloric restricted rats [64]. Increased expression of transaldolase may be required to protect cells from the elevated production of reactive oxygen species with refeeding.

In salmonids, muscle is a major site for fat storage [65]. In Atlantic salmon fast muscle, the fat content makes up 9.6% of the wet weight, with triacylglycerols contributing 93.3% of the total lipid content [66]. These reserves are likely to be exhausted during fasting, and must be replenished during periods of feeding. ALDOB is involved in the synthesis of triacylglycerols via the phosphatidic acid pathway [67]. Johanssen and Overturf [59] found that levels of ALDOB were 10 times higher during refeeding than in normal feeding, implying that during fasting-induced recovery growth, higher rates of fatty acid deposition are occurring. Also, Witt et al. [36] identified aldolase as a target for MuRF1, so the elevated levels of MuRF1 could further lead to reduced levels of aldolase in fasted fish.

Analysis of gene expression patterns reveals a cluster of genes (cluster I, Fig. 2) corresponding to triacylglycerol synthesis and adipogenic differentiation, which increase prior to any increases in MRFs or IGF-I (Fig. 2). LPL hydrolyses triglycerides, with the free fatty acids produced available as a direct energy source or used for storage in adipocytes [68]. Together, with the increased aldolase expression, increased LPL expression in the first few days of feeding, suggests TAGs are resynthesised in skeletal muscle to replace those used during the period of nutrient restriction. In Atlantic salmon, high densities of adipocytes are present in the myosepta of white muscle where the majority of lipids are stored as triacylglycerols [69]. We also examined the expression of adiponectin (ADIPOQ), an adipocytokine which increases in expression during differentiation of adipocytes and thus may serve as a marker of adipocyte differentiation [70,71]. Expression of this gene was significantly upregulated from 7 d after refeeding, indicating that adipogenic differentiation is occurring during these times. The expression of Diacylglycerol:acyl CoA acyltransferase (DGAT1), an enzyme involved in TAG synthesis, was also examined. Expression of DGAT1 increased early in response to feeding and remained elevated. DGAT1 mRNA expression has been shown to increase in differentiating 3T3-L1 adipocytes, with even greater increases in protein activity observed, suggesting that DGAT1 is also post transcriptionally regulated [72]. Although LPL and ALDOB gene expression decreased at day 32, DGAT1 remained elevated indicating that TAG synthesis is still occurring.

The decreased expression ALDOB and LPL after 32 d refeeding coincides with the increased expression of MLC2 and MHC (relative to 3 d, 7 d and 14 d). We found a positive correlation between MLC2 and ADIPOQ mRNA at all time points (A) ($r^2 = 0.36, p = 0.003, r = 0.59, n = 40$), and at fed time points (B) (3 d, 7 d, 14 d, 32 d; $r^2 = 0.48, p < 0.001, r = 0.69, n = 32$).

![Figure 7](http://www.frontiersinzoology.com/content/6/1/18)

**Figure 7**

Correlation between MLC2 mRNA and ADIPOQ mRNA at all time points (A) ($r^2 = 0.36, p = 0.003, r = 0.59, n = 40$), and at fed time points (B) (3 d, 7 d, 14 d, 32 d; $r^2 = 0.48, p < 0.001, r = 0.69, n = 32$).
tant regulators maintaining the ratio of skeletal muscle to adipose tissue [75]. Receptors for ADIPOQ have been found to be expressed in zebrafish muscle [76]. Furthermore, myostatin has been shown to inhibit myogenesis and promote adipogenesis in multipotent mesenchymal cells [77]. Additional experiments in Atlantic salmon are required to determine if crosstalk between adipocytes and skeletal muscle is occurring and if this plays any role in regulating the myogenic program following refeeding.

**Conclusion**

In conclusion, after a period of fasting, refeeding Atlantic salmon results in changes in metabolism leading to the replacement of lost energy reserves through increased fatty acid deposition and replacement of myofibrillar proteins. Increased myofibrillar protein deposition likely occurs through the down-regulation of MuRF1 and MAFbx leading to decreased protein degradation and increased translation respectively. Both the replacement of myofibrillar proteins and activation of the adipogenic program precede an increase in transcripts for myofibrillar proteins and myogenic regulatory factors. For an animal which is adapted to long periods of food deprivation, such as Atlantic salmon, achieving a state where energy reserves have been replenished, before metabolic energy is directed towards production of new muscle fibres, could be a necessary strategy for long term survival.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

NB performed the experimental work and wrote the first draft of the manuscript. RT was responsible for fish maintenance. IJ contributed to study design and writing of the draft of the manuscript. RT was responsible for fish main-

**Acknowledgements**

All S. salar samples were provided by EWOS Innovation. This work was supported by a grant from the Biotechnology and Biological Research Council grant (BB/D015391/1), and grants from the Norwegian Research Council (174557 FUGE and 174222 HAVBRUK) to EWOS Innovation. The authors thank Dr Jorge Fernandes for EF1alpha, RNA pol II and β actin primers, and Dr Daniel MacQueen for MLC2 and myoD1a primers and standards.

**References**

1. Jonsson N, Jonsson B, Hansen LP: Changes in proximate composition and estimates of energetic costs during upstream migration and spawning in Atlantic salmon, *Salmo salar*. *J Anim Ecol* 1997, 66:425-436.

2. Brett JR: Production energetic of poulations of sockeye salmon, *Oncorhynchus nerka*. *Can J Zool* 1986, 64:555-564.

3. Jones JW: The salmon. London: Collins; 1959.

4. Kadri S, Metcalfe NB, Huntingford FA, Thorpe JE: What Controls the Onset of Anorexia in Maturing Adult Female Atlantic Salmon. *Funct Ecol* 1995, 9(5):790-797.

5. Semenchenko NN: Assessment of energy expenditure during spawning by male sockeye salmon, *Oncorhynchus nerka*, at various sex ratios in the spawning ground. *J Ichthyol* 1986, 26(1):89-97.

6. Jonsson N, Jonsson B, Hansen LP: Energetic Cost of Spawning in Male and Female Atlantic Salmon (Salmo-Salar L.). *J Fish Biol* 1991, 39(5):739-744.

7. Hendry AP, Berg OK, Quinn TP: Breeding location choice in salmon: causes (habitat, competition, body size, energy stores) and consequences (life span, energy stores). *Oikos* 93:407-418.

8. McVeigh BR, Healey MC, Wolfe F: Energy expenditures during spawning by chum salmon *Oncorhynchus keta* (Walbaum) in British Columbia. *J Fish Biol* 2007, 71(6):1696-1713.

9. Bardonnet A, Bagliniere JL: Freshwater habitat of Atlantic salmon (Salmo salar). *Can J Fish Aquatic Sci* 2000, 57(2):497-506.

10. Talbot C, Preston T, East BW: *Body-Composition of Atlantic Salmon (Salmo-Salar L.)*. (Salmo-Salar L.). Comp Biochem Physiol A Physiol 1986, 85(3):445-450.

11. Valdimarsson SK, Metcalfe NB, Thorpe JE: Huntingford FA: Seasonal changes in sheltering: effect of light and temperature on diel activity in juvenile salmon. *Anim Behav* 1997, 54:1405-1412.

12. Egglkaw HJ, Shackley PE: Growth, Survival and Production of Juvenile Salmon and Trout in a Scottish Stream, 196675. *J Fish Biol* 1977, 11(6):647-672.

13. Gardiner WR, Geddes P: The Influence of Body-Composition on the Survival of Juvenile Salmon. *Hydrobiologia* 1980, 69(12):67-72.

14. Rowe DK, Thorpe JE, Shanks AM: Role of Fat Stores in the Maturation of Male Atlantic Salmon (Salmo-Salar) Parr. *Can J Fish Aquatic Sci* 1991, 48(3):405-413.

15. Binner M, Kloas W, Hardewig J: Energy allocation in juvenile roach and burbot under different temperature and feeding regimes. *Fish Physiol Biochem* 2008, 34(2):103-116.

16. Houlahan DF, Waring CP, Mathers E, Gray C: Protein-synthesis and oxygen-consumption of the shore crab *Carcinus-Maenas* after a meal. *Physiol Zool* 1990, 63(4):735-756.

17. Peragón J, Barroso JB, García-Salgueiro L, Aranda F, de la Higuera M, Lupianez JA: Selective changes in the protein-turnover rates and nature of growth induced in trout liver by long-term starvation followed by re-feeding. *Mol Cell Biochem* 1999, 201(12):1-10.

18. Mommsen TP, French CJ, Hochachka PW: Sides and patterns of protein and amino-acid utilization during the spawning migration of salmon. *Can J Zool* 1980, 58(10):1785-1799.

19. Moon TW: Metabolic reserves and enzyme-activities with food-deprivation in immature american eels, *Anguilla-Ros- trata* (Lesueur). *Can J Zool* 1983, 61(4):802-811.

20. Johnston IA, Davison W, Goldspink G: Energy-metabolism of carp swimming muscles. *J Comp Physiol* 1977, 114(2):203-216.

21. Bone Q, Kiceniuk J, Jones DR: Role of different fiber types in fish myometabolism at intermediate swimming speeds. *Fish Bull* 1978, 76(3):691-699.

22. Johnston IA, Goldspink G: Some effects of prolonged starvation on metabolism of red and white myotomal muscles of plaice *Pleuronectes-Platessa*. *Marine Biology* 1973, 19(4):348-353.

23. Beadall CH, Johnston IA: Lyosomal-enzyme activities in muscle following starvation and refeeding in the saithe *Pleuronectes-Platessa*. *Ophelia* 1978, 19(1):127-137.

24. Jobling M: Myoenergetics: feed intake and energy partitioning. In Fish Ecophysiology. Edited by: Rankin JC, Jensen FB. Chapman & Hall, London; 1993:1-44.

25. Nicieza AG, Metcalfe NB: Growth compensation in juvenile Atlantic salmon: Responses to depressed temperature and food availability. *Ecology* 1997, 78(8):2385-2400.

26. Chauvigne F, Gabbard JC, Weil C, Rescan PY: Effect of refeeding on IGF-I, IGFII, IGF receptors, FGFr, FGFr, and myostatin *mRNA* expression in rainbow trout myotomal muscle tissue. *Gen Comp Endocrinol* 2003, 132(2):209-215.

27. Gabbard JC, Kamangar BB, Montserrat N: Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol* 2006, 191(1):15-24.

28. Rescan PY, Montfort J, Ralliere C, Le Cam A, Esquerre D, Hugot K: Dynamic gene expression in fish muscle during recovery growth induced by a fasting-reefencing schedule. *Bmc Genomics* 2007, 8:438.
70. Fu YC, Luo NL, Klein RL, Garvey WT: Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J Lipid Res 2005, 46(7):1369-1379.

71. Korner A, Wabitsch M, Seidel B, Fischer-Posovszky P, Berthold A, Sturnwoll M, Bluher M, Kratzsch E, Kiess W: Adiponectin expression in humans is dependent on differentiation of adipocytes and down-regulated by humoral serum components of high molecular weight. Biochem Biophys Res Commun 2005, 337(2):540-550.

72. Yu YH, Zhang YY, Oelkers P, Sturley SL, Rader DJ, Ginsberg HN: Posttranscriptional control of the expression and function of diacylglycerol acyltransferase-1 in mouse adipocytes. J Biol Chem 2002, 277(52):50876-50884.

73. Wang CH, Mao XM, Wang LX, Liu ML, Wetzel MD, Guan KL, Dong LQ, Liu F: Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1. J Biol Chem 2007, 282(11):7991-7996.

74. Seillez I, Gabillard JC, Skiba-Cassy S, Garcia-Serrana D, Gutierrez J, Kaushik S, Panserat S, Tesseraud S: An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (Oncorhynchus mykiss). Am J Physiol Regul Integr Comp Physiol 2008, 295(1):R329-R335.

75. Argiles JM, Lopez-Soriano J, Almendro V, Busquets S, Lopez-Soriano FJ: Cross-talk between skeletal muscle and adipose tissue: A link with obesity? Med Res Rev 2005, 25(1):49-65.

76. Nishio SI, Gibert Y, Bernard L, Brunet F, Triqueneaux G, Laudec V: Adiponectin and adiponectin receptor genes are coexpressed during zebrafish embryogenesis and regulated by food deprivation. Dev Dyn 2008, 237(6):1682-1690.

77. Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen RQ, Groome NP, Fareez MM, Gonzalez-Cadavid NF: Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T1/2 mesenchymal multipotent cells. Endocrinology 2005, 146(8):3547-3557.