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Sequence of Atlantic cod (*Gadus morhua*) GLUT4, GLUT2 and GPDH: developmental stage expression, tissue expression and relationship to starvation-induced changes in blood glucose

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Summary

cDNAs of putative glucose transporters, GLUT4 and GLUT2, were cloned from Atlantic cod (*Gadus morhua*). The GLUT4 cDNA encodes a 503 amino acid and the GLUT2 cDNA a 506 amino acid protein. Phylogenetic analysis, amino acid sequence alignment, and tissue distribution support categorizing them as homologues of mammalian GLUT4 and 2. GLUT4 clusters with GLUT4s from fish and other vertebrates. It shows 84% amino acid identity to GLUT4 from coho salmon and brown trout and 65% identity with other vertebrates. It is most highly expressed in heart, strongly expressed in red and white skeletal muscle and present at lower levels in gill, gonad, intestine, and kidney. GLUT2 clusters with GLUT2 from rainbow trout and other vertebrates. It shows 75% amino acid identity with rainbow trout and 62% identity with chicken GLUT2. In Atlantic cod, GLUT2 is most highly expressed in liver with lower levels noted in intestine and kidney.

Food deprivation for 2 months was used as a vehicle to monitor GLUT expression at different blood glucose levels. Starvation resulted in a decrease in blood glucose and liver glycogen that recovered following 20 days of re-feeding. GLUT4 expression in heart was decreased with starvation and increased with re-feeding. GLUT4 mRNA level in heart correlated with blood glucose. It is suggested that this relationship is related to insulin responsiveness. GLUT4 expression in white muscle increased with starvation and decreased with re-feeding. It is proposed that this is due to the necessity to maintain high levels of the glucose transporter protein in the face of starvation-associated proteolysis. GLUT2 expression in liver correlated with blood glucose, consistent with higher rates of glucose transport from liver to blood in the fed state than in the food-deprived state.

Glycerol-3-phosphate dehydrogenase (GPDH) cDNA was also cloned. It encodes a 351 amino acid protein, which is 73–90% identical to GPDH from numerous other fish species. GPDH is ubiquitously expressed. Expression in heart decreased with starvation and increased with re-feeding, whereas expression in liver did not change with starvation.

In other studies, gene expression was monitored at nine time points from fertilization of eggs to larval development. GLUT4 is detectable in fertilized eggs and is fully expressed by the halfway to hatching point. GLUT2 is not evident at fertilization, is detectable at halfway to hatching, and fully expressed at hatching. GPDH expression was evident from fertilization.

Key words: Atlantic cod, *Gadus morhua*, glucose transporter, GLUT4, GLUT2, insulin responsiveness, starvation, GPDH.

Introduction

Movement of glucose across plasma membranes is dependent upon either sodium-dependent or facilitative glucose transporters. Facilitative glucose transporters (GLUTs), which utilize the diffusion gradient across the plasma membrane, are ubiquitous. GLUTs are categorized into three classes dependent upon structure and transport characteristics. The class I facilitative transporters include GLUTs 1–4 and have been comprehensively described in mammals (Wood and Trayhurn, 2003). The study of glucose transport in fish is receiving considerable attention for a variety of reasons including species-specific differences in blood glucose levels that range from less than 0.5 mmol l^{-1} to in excess of 50 mmol l^{-1} (e.g. Chavin and Young, 1970; MacCormack et al., 2003), up to sixfold changes in blood glucose level within species as a function of hypoxia or osmotic challenge (Shoubridge and Hochachka, 1983; Sangiao-Alvarellos et al., 2005), use of dietary carbohydrate in an aquaculture context (Hemre et al., 2001), and the phenomenon of glucose intolerance (Moon, 2001). GLUT1 has been characterized in

common carp (Teerijoki et al., 2001b), rainbow trout (Teerijoki et al., 2000; Teerijoki et al., 2001a) and Atlantic cod (Hall et al., 2004). GLUT3 has been characterized in grass carp (Zhang et al., 2003) and Atlantic cod (Hall et al., 2005). Here we describe the sequence, developmental pattern, tissue expression, and relationship to plasma glucose levels of GLUTs 4 and 2 in Atlantic cod. Food deprivation in Atlantic cod leads to mobilization of liver lipid and glycogen, in association with decreases in plasma glucose and insulin (Black and Love, 1986; Hemre et al., 1990; Sundby et al., 1991). We capitalize upon this to assess the expression of GLUTs in association with altered plasma glucose.

GLUT4 in mammals is expressed primarily in heart, skeletal muscle and adipose tissue and is the only insulin-sensitive transporter within the class I GLUTs (Wood and Trayhurn, 2003). A mammalian-like GLUT4 was cloned and sequenced from brown trout (Planas et al., 2000) and coho salmon (Capilla et al., 2002). In brown trout GLUT4 was expressed primarily in red and white skeletal muscle, gill, kidney and adipose tissue but only to a limited extent in heart (Planas et al., 2000). Also, GLUT4 is insulin sensitive in adipocytes from coho salmon (Capilla et al., 2004). In salmonids, red muscle shows the hallmarks of insulin responsiveness but white muscle does not. Starvation of brown trout, leading to decreases in plasma insulin and glucose, was associated with decreased GLUT4 mRNA levels in red muscle but not in white muscle, and rainbow trout injected with porcine insulin showed an increase in GLUT4 mRNA in red but not white muscle (Capilla et al., 2002). Given the weak expression of GLUT4 mRNA in brown trout heart and the apparent lack of responsiveness in white muscle, despite presumed changes in glucose usage, it was considered of interest to extend these findings to other species.

GLUT2 in mammals occurs primarily in liver, pancreas, intestine and kidney. In liver, this glucose transporter serves in the bi-directional movement of glucose, which is dependent upon dietary/hormonal status (Wood and Trayhurn, 2003). In rainbow trout, GLUT2 is expressed in liver, kidney and intestine (Krasnov et al., 2001; Panserat et al., 2001). There was no change in expression found following 4 days of starvation (Panserat et al., 2001).

The current study also provides an analysis of glycerol-3-phosphate dehydrogenase (GPDH). The expression of the gene is regulated in liver of rainbow smelt in accordance with rates of glycerol 3-phosphate (glycerol 3-*P*) and subsequent glycerol production (Ewart et al., 2001; Liebscher et al., 2006). Glycerol 3-*P* is a precursor of triglyceride synthesis and glycerol is produced from the breakdown of triglycerides. As such, we considered this protein to be a potential candidate for change during alterations in triglyceride management.

Here we report the complete sequence for GLUT4, GLUT2 and GPDH cDNAs, the timing of expression of the genes from fertilization to the larval stage, tissue distribution in juvenile fish, and the response to starvation. The most important finding is that heart GLUT4 and liver GLUT2 expression correlate with plasma glucose, whereas, white muscle GLUT4 does not.

Materials and methods

Animals

Atlantic cod (*Gadus morhua*, Linnaeus) were cultured and raised in the Aquaculture Research and Development Facility at the Ocean Sciences Centre, Memorial University of Newfoundland. Animals were killed by a sharp blow to the head and the tissues removed quickly. For biochemical analysis, tissues were snap frozen in liquid nitrogen and stored at –80°C. For RNA preparation, tissues were stored in RNA*later* (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's protocol.

cDNA cloning

Full length cDNAs for Atlantic cod GLUT4, GLUT2 and GPDH were cloned using a combination of RT–PCR, RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM–RACE) and genome walking. The sequences of all primers used in cDNA cloning are presented in Table 1.

The three partial cDNAs were cloned using the following RT–PCR methodology. Total RNA was extracted using Trizol Reagent (Invitrogen, Burlington, ON, Canada) from either red muscle (GLUT4) or liver (GLUT2, GPDH) and then treated with amplification grade DnaseI (Invitrogen). RNA was reverse-transcribed with an oligo(dT) primer using M-MLV reverse transcriptase (Invitrogen). PCR amplification was performed using DyNAzyme EXT (MJ Research, Waltham, MA, USA). Touchdown PCR was used with 40 cycles of 94°C for 30 s, 65°C↓0.5°C per cycle for 30 s and 72°C for 1–2 min. PCR products were subcloned into pGEM-T Easy (Promega, Madison, WI, USA) and triplicate clones sequenced on both strands at MOBIX, McMaster University.

The 5′ and 3′ ends of the three partial cDNAs were cloned using a commercial kit for RLM–RACE, GeneRacer Kit (Invitrogen) and poly(A)$^+$ RNA isolated from total RNA using the Oligotex mRNA Mini Kit (Qiagen Inc.). PCR amplification was performed at 94°C at 30 s, 70°C↓0.3°C per cycle for 30 s and 72°C for 1–2 min for 40 cycles using DyNAzyme EXT (MJ Research) with the exception of GLUT2 3′RACE, which was performed at 94°C for 10 s, 72°C for 1.5 min for 7 cycles followed by 94°C for 10 s, 67°C for 30 s, 68°C for 1 min for 32 cycles, using Elongase Enzyme Mix (Invitrogen).

Where genome walking was performed, genomic DNA was extracted from liver using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. GenomeWalker libraries were constructed using the Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. PCR amplification was performed at 94°C for 10 s, 70°C↓0.3°C per cycle for 30 s and 68°C for 3 min for 40 cycles using DyNAzyme EXT (MJ Research).

GLUT4

For GLUT4, a partial cDNA was cloned using RT–PCR. Primers were designed based upon consensus sequences from conserved areas of aligned vertebrate GLUT4s. Primers 1 and

Table 1. *Sequences of oligonucleotides used in cDNA cloning*

Primer number	cDNA	DNA sequence[a]	Direction[b]	Position of 5′ end in cDNA
1	GLUT4	5′-ATGCC**GT**C**K**GGN**N**TT**Y**CA**R**CA-3′	F	73
2	GLUT4	5′-CCCTGGGAGAA**G**A**G**CTC**I**GCC-3′	R	1277
3	GLUT4	5′-A**T**GAACCAGGGGATGGG**I**CC-3′	R	1253
4	GLUT4	5′-TGGCTGCGGACGATGTAGAGGA-3′	R	749
5	GLUT4	5′-AGAACCATCATCTCTATGGAGCGG-3′	R	461
6	GLUT4	5′-ATGGGCCGACGGACCCTCCAT-3′	F	1081
7	GLUT4	5′-ATTGGCACTCGCCTTATCGGACA-3′	F	1146
8	GLUT4	5′-TTGTCGCCACATGTGATGAACCT-3′	F	Intronic
9	GLUT4	5′-TGGGCGCGTCAACCTAGATGTATG-3′	F	Intronic
10	GLUT4	5′-GAGACAAGGTGTAGAGCGATGGAA-3′	F	1671
11	GLUT4	5′-ACTCGTACTCGAGACTGCGTTCAA-3′	F	1694
12	GLUT2	5′-ATGTATTGGTC**T**CTGTCTGTG**T**C-3′	F	258
13	GLUT2	5′-CCCTGG**GA**GAA**G**AGCTC**I**GCC-3′	R	1264
14	GLUT2	5′-CTC**I**GCCAC**I**ATGAACCA**G**GG**G**-3′	R	1250
15	GLUT2	5′-CTTAGAAGAATACCAACGACGATGG-3′	R	583
16	GLUT2	5′-CAGTAGAAGCCCATGACAGCGCG-3′	R	469
17	GLUT2	5′-ATGGACTACTCATGGATGAGCTAC-3′	F	1146
18	GLUT2	5′-GTCGGCTGTGTTCCTCTTTGTGT-3′	F	1178
19	GPDH	5′-CTGGG**N**CTGATGGAGATGATC-3′	F	750
20	GPDH	5′-ACATGTG**T**TC**N**GGGTG**R**TTCTGCA-3′	R	1113
21	GPDH	5′-TTATGAACTCTGCGACAGGGTGG-3′	R	1083
22	GPDH	5′-CCCGGCGGTGCAGAAGATGCG-3′	R	800
23	GPDH	5′-CTTCGCACGCATCTTCTGCACC-3′	F	773
24	GPDH	5′-GACGGGCAAGTCCATCGAGGA-3′	F	902

[a]Nucleotides in bold differ from the actual cDNA sequence.
[b]F, forward and R, reverse direction.

2 were used for the initial PCR reaction and 1 μl of this was used as template for a semi-nested PCR using Primer 1 and Primer 3. The 1180-nucleotide partial cDNA sequence was aligned with sequences from three other glucose transporters cloned from Atlantic cod and gene-specific PCR primers were designed in areas to specifically amplify GLUT4. 5′RACE was performed using Primer 4 and Primer 5 (nested) with the GeneRacer 5′ Primer and the GeneRacer 5′ Nested Primer, respectively. The 461-nucleotide PCR product contained the 72-nucleotide 5′UTR and the 20-nucleotides of unverified 5′coding sequence. The 3′ end of GLUT4 was obtained using a combination of genome walking and 3′ RLM–RACE. Genome walking was initially chosen over 3′ RACE to break up the remaining 3′ sequence into smaller fragments based upon the presence of restriction enzymes within. The first walk was performed using Primer 6 and Primer 7 (nested) with the GenomeWalker Adaptor Primer 1 (AP1) and the GenomeWalker Nested Adaptor Primer 2 (AP2), respectively. A 575 bp PCR product was amplified from the *Ssp*I library, which contained an additional 110 bp of ORF sequence. To obtain additional 3′ sequence, a second genome walk was performed using the intronic primers, Primers 8 and 9 (nested). A 676 bp PCR product was amplified from the *Msl*I library, which contained the final 222 nucleotides of the ORF and 181 nucleotides of the 3′UTR. The remaining 3′ sequence was obtained by RLM–RACE. Primer 10 and Primer 11 (nested) were used with the GeneRacer 3′ Primer and the GeneRacer 3′ Nested Primer, respectively. A 806 bp band was generated that contained the remaining 735 nucleotides of the 3′UTR.

GLUT2

For GLUT2, a partial cDNA was cloned by RT–PCR. Primer 12 was used with Primer 13 for the primary PCR. 1 μl of this PCR reaction was used as template for a semi-nested PCR using Primer 12 and Primer 14. A 993-nucleotide PCR product was amplified. The 5′ end of GLUT2 was cloned by RLM–RACE. Primer 15 and Primer 16 (nested) were used to amplify a 469-nucleotide PCR product that contained the 26-nucleotide 5′UTR and the remaining 231 nucleotides of 5′ coding sequence. The 3′ end of GLUT2 was also cloned by RLM–RACE. Primer 17 and Primer 18 (nested) were used to amplify two bands (475 bp and 539 bp) that contained the remaining 319 bp of the coding sequence (CDS) and 3′UTR sequences of 106 bp and 170 bp, respectively. The size difference is due to the presence of two polyadenylation signals at position 1635 and 1695, respectively.

GPDH

For GPDH, a partial cDNA clone was amplified by RT–PCR. Primer sets 19 and 20 amplified a 363 bp product.

The 5′ end of GPDH was cloned by RLM–RACE. Primers 21 and 22 (nested) were used to amplify an 800 bp PCR product, which contained a 59 bp 5′ UTR and an additional 711 bp of the CDS. The 3′ end of GPDH was also cloned by RLM–RACE. Primers 23 and 24 (nested) were used to amplify a 790 bp PCR product that contained the final 2 bp of the CDS and the 577 bp 3′UTR. It should be noted that a GPDH-like clone the CDS of which shared 70% sequence identity with the GPDH described here was also amplified during the cloning process. However, this clone contained a 37 bp frameshift deletion and would therefore code for a nonsense protein.

Sequence analysis

Sequence data was compiled and analyzed using Vector NTI v. 6.0 (Informax Inc., Bethesda, MD, USA). Alignments were performed using AlignX (Informax Inc.), which uses the CLUSTAL W algorithm (Thompson et al., 1994). For phylogenetic and molecular evolutionary analyses, alignments were imported into *MEGA* version 2.1 (Kumar et al., 2001). Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) with Poisson correction. Bootstrap analysis was performed with 1000 replicates. Exon/intron boundaries from genome walking sequences were analyzed using GENSCAN (http://genes.mit.edu/GENSCAN.html). Transmembrane helices were predicted using HMMTOP (http://www.enzim.hu/hmmtop) (Tusnady and Simon, 1998; Tusnady and Simon, 2001).

Gene expression analysis by real-time reverse transcription PCR

Levels of GLUT4, GLUT2 and GPDH mRNA were quantified by real-time reverse transcription PCR (qRT–PCR), using TaqMan® probe-based chemistry and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probes used in gene expression analysis are presented in Table 2. Primers and probes for GLUT4 and GLUT2 were designed in areas to specifically amplify their respective GLUT, when aligned with other GLUTs from Atlantic cod. Primers and probes for GPDH were designed in areas to specifically amplify GPDH and not the nonsense transcript that had been detected during the cloning process. TaqMan® probes were designed to span intron-exon boundaries (identified by genome walking) (data not shown) to eliminate amplification from any contaminating genomic DNA that may remain following DNaseI treatment.

First-strand cDNA was synthesized from 1 μg of DnaseI-treated total RNA using random primers and M-MLV Reverse Transcriptase (Invitrogen). PCR amplification for the target genes was performed in a 25 μl reaction using 1 μl of cDNA, 900 nmol l^{-1} each of forward and reverse primer, 250 nmol l^{-1} TaqMan® probe and 1× TaqMan® Universal PCR Master Mix, with AmpErase® UNG (Applied Biosystems). Expression levels of the target genes were normalized to 18S ribosomal RNA, using the Eukaryotic 18S rRNA Endogenous Control (VIC/MGB Probe, Primer Limited) (Applied Biosystems). PCR amplification for the endogenous control was performed in a separate 25 μl reaction using 1 μl of a 1:10 dilution of the cDNA, 1× probe/primer mix and 1× TaqMan® Universal PCR Master Mix, with AmpErase® UNG (Applied Biosystems). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. On each plate, for every sample, the target gene and endogenous control were tested in triplicate along with no RT controls. The fluorescence threshold cycle (C_T) was determined using the 7300 PCR Detection System SDS Software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems). The relative starting quantity of each transcript was determined using the comparative C_T method for relative quantification (Livak and Schmittgen, 2001).

In vivo experiments

Developmental expression patterns

GLUT4, GLUT2 and GPDH expression was monitored throughout the developmental period of Atlantic cod from egg to larval fish at intervals corresponding to developmental stages and changes in diet. Temperature was maintained at 5–6°C. Eggs were sampled at fertilization (day 0), 45 degree days (HH – halfway to hatching) and at 105 degree days (hatching). Post-hatch samples were then taken at day 2 (feeding solely from yolk sac), day 19 (rotifers), day 47 (rotifers/*Artemia*), day 52 (*Artemia*), day 54 [*Artemia*/dry food (GEMMA micro diet; Skretting, NB, Canada)], and day 60 (dry food). Typical body mass of day 60 larvae was about 100 mg. At each interval,

Table 2. *Sequences of primers and probes used in gene expression analysis*

cDNA	DNA sequence	Direction/type	Position of 5′ end in cDNA
GLUT4	5′-CCAACTGGACCGCCAACTT-3′	Sense primer	1313
GLUT4	5′-GAGCCCGGCGAAGATCA-3′	Antisense primer	1404
GLUT4	5′-6FAM-CAGTCCTTGGCTGACCT-MGBNFQ-3′	Sense probe	1351
GLUT2	5′-GGCATGGTCTCCTCCTTTCTG-3′	Sense primer	297
GLUT2	5′-TTTTGCACAGGCCCATCAG-3′	Antisense primer	414
GLUT2	5′-6FAM-CTTCACCCTTCCTCGCAG-MGBNFQ-3′	Antisense probe	353
GPDH	5′-GGTCGCAACCGCAAGGT-3′	Sense primer	867
GPDH	5′-TGACCGTTGAGCATTTCCTTCT-3′	Antisense primer	949
GPDH	5′-6FAM-CGGGCAAGTCCATCGA-MGBNFQ-3′	Sense probe	904

approximately 100 mg of biomass was used in the RNA extraction. Individuals were pooled to achieve the required amount of biomass.

Fasting/re-feeding

Fasting/re-feeding was used as a means to alter blood glucose levels. Atlantic cod, less than 1 year old, were divided into two groups and held in identical aerated, flow-through seawater 2000 l tanks at 8°C and natural photoperiod. One group was fed a commercial diet (Shur Gain, Truro, NS, Canada) while the other was deprived of food. Experiments were initiated on March 1, 2005. Fed and starved fish were sampled after 1 and 2 months. The food-deprived fish were then re-fed and sampled after 20 days. The Canadian Council on Animal Care policy with respect to experiments requiring withholding of food is that fish should not be permitted to lose more than 15% of body mass during periods of food restriction. The current study falls within these guidelines (see Results). Body mass, length, liver mass, blood glucose and liver glycogen were determined for all fish. Total lipid level and triglyceride levels were assessed in liver from fed and starved fish only. Heart and white muscle GLUT4, liver GLUT2, and heart and liver GPDH expression were assessed in fish starved for 2 months, fed fish sampled at the same time and fish re-fed for 20 days following starvation.

Biochemical analysis

Glycogen and glucose were assayed as described elsewhere (Clow et al., 2004) with the exception that absorbance was determined with a DTX 880 microplate reader (Beckman Coulter, Mississauga, ON, Canada). Lipids were extracted in chloroform/methanol using a Folch procedure (Folch et al., 1957) modified according to Parrish (Parrish, 1998). Lipid classes were separated using a MARK V Iatroscan (Iatroscan Laboratory, Tokyo, Japan) analyzer as described elsewhere (Parrish, 1987).

Data analysis

For qRT–PCR analysis, normalized transcript levels are expressed relative to a calibrator sample (assigned a value=1) in all studies. In the developmental and tissue distribution experiments, the condition/tissue that expressed the particular transcript at the lowest detectable level was the calibrator. In the fasting/re-feeding experiments, the first sample analyzed was the calibrator. Statistical analysis of data was performed using a one-way ANOVA followed by Tukey's HSD *post-hoc* test. In all cases, $P<0.05$ was considered to be statistically significant. Values are expressed as means ± s.e.m.

Results

cDNA cloning

RT–PCR, RLM–RACE and genome walking were used to clone two members of the class I family (sodium-independent) of facilitated glucose transporters, namely the insulin sensitive transporter, GLUT4 (GenBank accession number DQ109810), and the hepatic transporter, GLUT2 (GenBank accession number AY795481), as well as GPDH (GenBank accession number AY635584) from Atlantic cod. The 2500 bp GLUT4 cDNA contains a 72 bp 5′UTR, a 1512 bp open reading frame (ORF) and a 916 bp 3′UTR. The 1653/1716 nucleotide GLUT2 cDNA contains a 26 bp 5′UTR, a 1521 bp ORF and a 106/170 bp 3′UTR. The 1692 bp GPDH cDNA contains a 59 bp 5′UTR, a 1056 bp ORF and a 577 bp 3′UTR.

Deduced amino acid sequence analysis

Atlantic cod GLUT4 encodes a 503 aa protein, which has a predicted molecular mass of 54.7 kDa and an isoelectric point of 7.09. Atlantic cod GLUT2 encodes a 506 aa protein, with a predicted molecular mass of 55.1 kDa and an isoelectric point of 6.73.

Phylogenetic analysis (Fig. 1) was performed to determine the relatedness of Atlantic cod GLUT4 and GLUT2 to class I GLUTs (1–4) from other vertebrates, including all those reported in fish. Atlantic cod GLUT4 clusters with GLUT4s from coho salmon and brown trout, as well as, GLUT4s from other vertebrates. Atlantic cod GLUT2 clusters with the GLUT2 from rainbow trout and with GLUT2s from other vertebrates. They do not cluster with other fish class I GLUTs including GLUT1s from Atlantic cod, rainbow trout and common carp nor with GLUT3s from Atlantic cod and grass carp nor with a GLUT from pacific hagfish.

At the amino acid level, Atlantic cod GLUT4 shows the highest sequence identity to GLUT4s from coho salmon and brown trout (84%). It shows about 65% identity with other vertebrate GLUT4s. When compared to human class I GLUTs, Atlantic cod GLUT4 shows 65%, 61%, 58% and 49% sequence identity to GLUT4, 1, 3 and 2, respectively. Atlantic cod GLUT2 shows the highest sequence identity with rainbow trout GLUT2 (75%) and 56–62% sequence identity with GLUT2 from other vertebrates. When compared to human class I GLUTs, Atlantic cod GLUT2 shows 56%, 54%, 53% and 51% sequence identity to GLUT2, 4, 1 and 3, respectively. To summarize the cloning of the class I GLUTs from Atlantic cod and highlight their sequence motifs characteristic of glucose transporters, protein sequences for all members were aligned (Fig. 2A). GLUT4 shows 60.8% identity to GLUT1, 52.2% identity to GLUT2 and 57.3% identity to GLUT3. GLUT2 shows 50.6% identity to GLUT1 and 48.4% identity to GLUT3.

Atlantic cod GPDH encodes a 351 aa protein, with a predicted molecular mass of 38.0 kDa and an isoelectric point of 5.47. Protein sequences for all known GPDH cDNAs from fish were aligned (Fig. 2B). Atlantic cod GPDH shows 90%, 85%, 84% and 73% sequence identity with GPDH from puffer fish, rainbow smelt, Atlantic salmon and zebrafish, respectively.

Developmental and tissue expression patterns for Atlantic cod GLUT4, GLUT2 and GPDH

Atlantic cod were monitored from eggs to larval fish to determine at which point expression of GLUT4, GLUT2 and GPDH becomes detectable. Gene expression was analyzed in

one sample from each time point by qRT–PCR. GLUT4 is weakly expressed in the day 0 eggs (Fig. 3) but by halfway to hatching, is well expressed. GLUT2 is not expressed at all in the day 0 eggs but is weakly expressed at halfway to hatching. Upon hatching, GLUT2 is expressed at high levels. GPDH is expressed in the day 0 eggs, with expression increasing threefold by halfway to hatching.

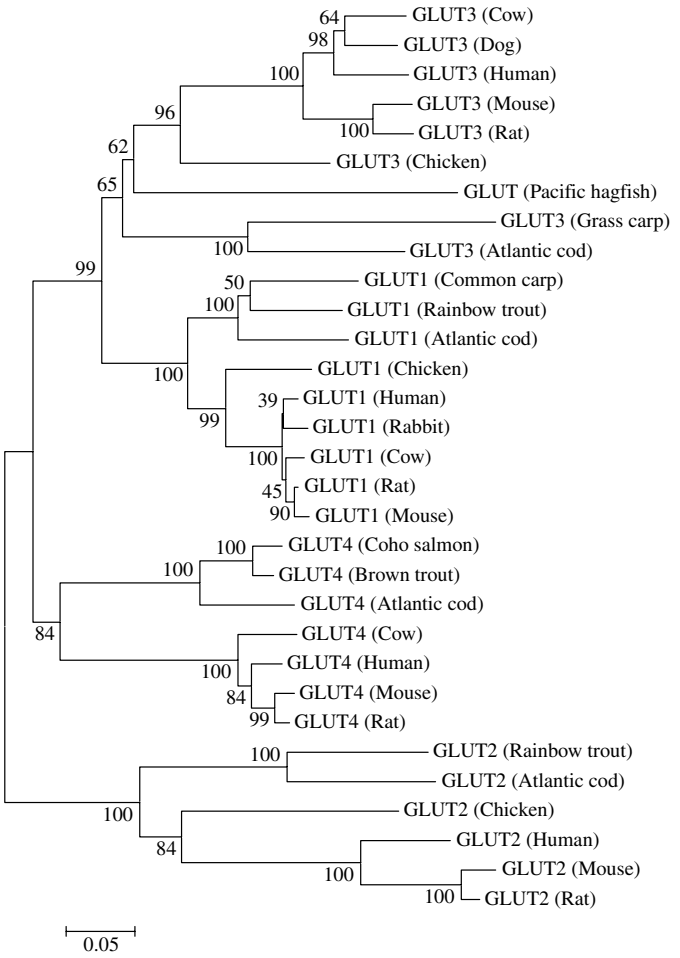


Fig. 1. Phylogenetic analysis of Atlantic cod GLUT4 and GLUT2. A phylogenetic tree of protein sequences of Atlantic cod GLUT4 and GLUT2 and GLUT1–GLUT4 from other vertebrates. SwissProt accession numbers are as follows: GLUT1 common carp (AAF75683), GLUT1 rainbow trout (AAF75681), GLUT1 Atlantic cod (AAS17880), GLUT1 chicken (AAB02037), GLUT1 human (AAA52571), GLUT1 rabbit (P13355), GLUT1 cow (P27674), GLUT1 rat (P11167), GLUT1 mouse (AAA37752), GLUT2 chicken (Q90592), GLUT2 rainbow trout (AAK09377), GLUT2 human (AAA59514), GLUT2 mouse (P14246), GLUT2 rat (P12336), GLUT2 Atlantic cod (AAV63984), GLUT3 cow (AAK70222), GLUT3 dog (P47842), GLUT3 human (AAB61083), GLUT3 mouse (AAH34122), GLUT3 rat (Q07647), GLUT3 chicken (AAA48662), GLUT3 grass carp (AAP03065), GLUT3 Atlantic cod (AAT67456), GLUT4 cow (Q27994), GLUT4 human (AAA59189), GLUT4 mouse (P14142), GLUT4 rat (P19357), GLUT4 coho salmon (AAM22227), GLUT4 brown trout (AAG12191), GLUT4 Atlantic cod (AAZ15731), Pacific hagfish (AAL27090). The tree was constructed as described in the text. The scale bar represents the number of substitutions per amino acid site.

Tissues were examined for expression of GLUT4, GLUT2 and GPDH by qRT–PCR from a single juvenile Atlantic cod (body mass approximately 45 g) maintained at 8°C and fed a commercial diet (Shur Gain, Truro, NS, Canada) daily (Fig. 4). The calibrator sample for GLUT4 was intestine, for GLUT2, kidney and for GPDH, white muscle. GLUT4 is highly expressed in heart, red muscle and white muscle with lower levels in gill, gonad, kidney and intestine. GLUT2 is highly expressed in liver, intestine and kidney. GPDH is ubiquitously expressed, with highest levels in gonad, kidney, intestine, liver, brain and heart. Lower levels were detected in other tissues sampled.

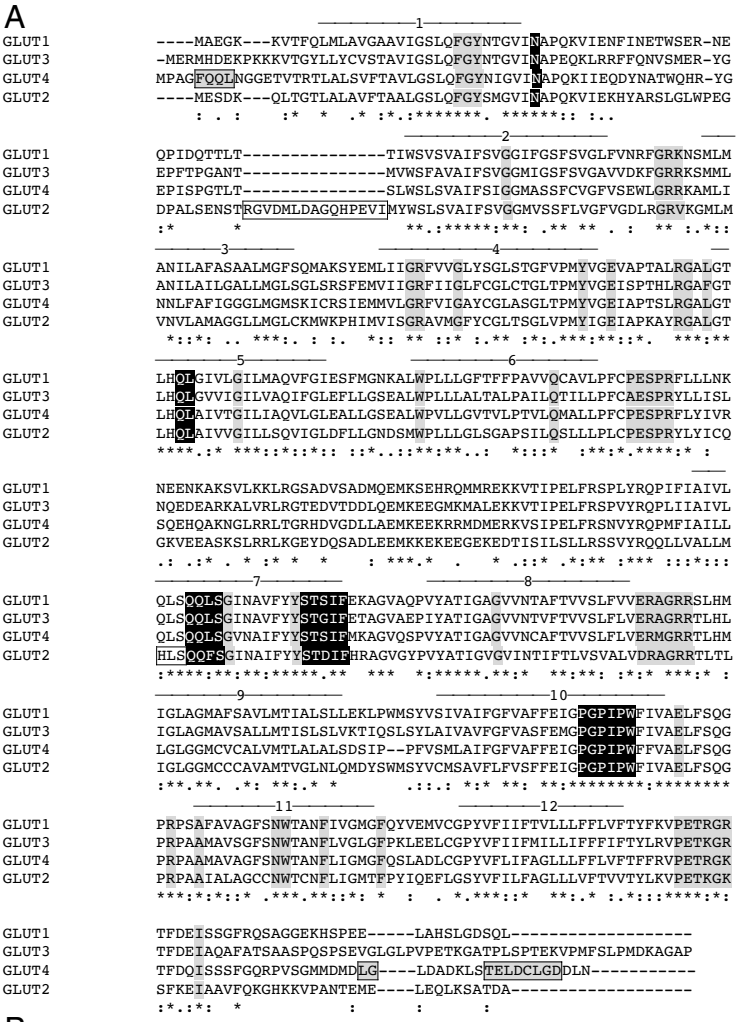
The effects of fasting/re-feeding

Atlantic cod were deprived of food for 1 or 2 months and thereafter re-fed for 20 days. Control (i.e. fed) fish were sampled at the same time as the 1 or 2 month food-deprived fish. There was no significant difference in fish length amongst the five groups (Fig. 5). After 2 months the starved fish weighed significantly less than the time matched controls (P=0.014). Re-feeding, following 2 months of starvation, did not lead to a change in body mass. There was no significant difference in condition factor amongst the five groups. Overall, the hepatosomatic index tended to change during the course of the study (P=0.06) with the starved groups having lower mean values than the time matched fed fish.

As there were no significant differences in blood glucose, liver glycogen or liver lipids between the two groups of fed fish these values were pooled in the following analysis. Blood glucose levels decreased from 9.4 μmol ml^{-1} to approximately 2.3 μmol ml^{-1} following 1 month starvation (Fig. 6). There was no further change during the second month of food deprivation. With re-feeding, blood glucose levels increased to 6.7 μmol ml^{-1}, a value not significantly different from the control group. Liver glycogen levels decreased from 100 μmol glucosyl units g^{-1} to 38 μmol glucosyl units g^{-1} after 1 month and to 5.1 μmol glucosyl units g^{-1} following 2 months of starvation. Twenty days of re-feeding led to increases in glycogen levels to 172 μmol glucosyl units g^{-1}, a value significantly higher than all the other groups. When values for blood glucose and liver glycogen from all individuals were considered, there was a strong correlation between the two variables. There were two fish in the control group with blood glucose levels of 239 and 202 μmol ml^{-1}. As these values were so far removed from the norm they were not included in the analysis.

Neither total lipid nor triglyceride level in liver changed even with 2 months of starvation (Fig. 7). Total lipid level was approximately 500 mg g^{-1}, equivalent to 50% of the liver mass. Triglyceride accounted for approximately 90% of the total lipid pool.

Expression of GLUT4, GLUT2 and GPDH was assessed by qRT–PCR in fish that had been continuously fed, starved for 2 months, and re-fed for 20 days (Fig. 8). GLUT4 expression



was analyzed in two tissues that highly express GLUT4, heart and white muscle. In heart, GLUT4 expression changed significantly (P=0.016) and was twofold higher in the fed fish than in the starved fish. When the food-deprived fish were re-fed, GLUT4 levels doubled with respect to starved levels returning to those seen in the control fed fish. In white muscle, GLUT4 expression also showed a significant change (P=0.021) but in an opposite pattern to that seen in heart. GLUT4 levels were 2.5-fold higher in white muscle of starved fish than in control fed fish. When food-deprived fish were re-fed, GLUT4 levels dropped 1.8-fold, with respect to levels seen in the starved fish, returning to values that were not significantly different from those in the control fed fish. GPDH expression was analyzed in heart and liver and GLUT2 in liver only. The pattern of GPDH expression in heart showed significant change, similar to that of GLUT4, in that GPDH level was 1.7-fold lower in starved than in control fed fish. GPDH expression in heart then increased upon re-feeding to a value not significantly different from the control fed fish. In liver, there was no statistically significant difference in GLUT2 and GPDH expression amongst fed, food-deprived and re-fed groups.

When values for GLUT expression, from all individuals, were plotted against blood glucose, there was a significant correlation between heart GLUT4 and blood glucose and between liver GLUT2 and blood glucose (Fig. 9). There was no correlation between white muscle GLUT4 and blood glucose.

Discussion

Amino acid sequences

Amino acid sequence comparisons, phylogenetic analysis, and tissue distribution patterns (discussed below) support categorizing the Atlantic cod GLUT4 and GLUT2 cDNAs cloned here as type 4 and type 2 members, respectively of the class I family of glucose transporters.

Fig. 2. (A) Alignment of protein sequences for class I GLUTs from Atlantic cod. The single letter code is used for the amino acids. Gaps in the amino acid sequences are indicated with a dash (–). Positions where there is a single, fully conserved residue are indicated with an asterisk (*), positions with a 'strong' group fully conserved are indicated with a colon (:), and positions with a 'weaker' group fully conserved are indicated with a dot (.). Numbered lines above the sequences indicate the approximate positions of the predicted membrane spanning helices. Residues that are highly conserved in all glucose transporters are highlighted in grey, and those that are specific to class I glucose transporters are highlighted in black. Residues discussed with respect to GLUT4 are indicated by a grey box and those to GLUT2 with a white box. (B) Alignment of GPDH protein sequences from Atlantic cod and other fish species.

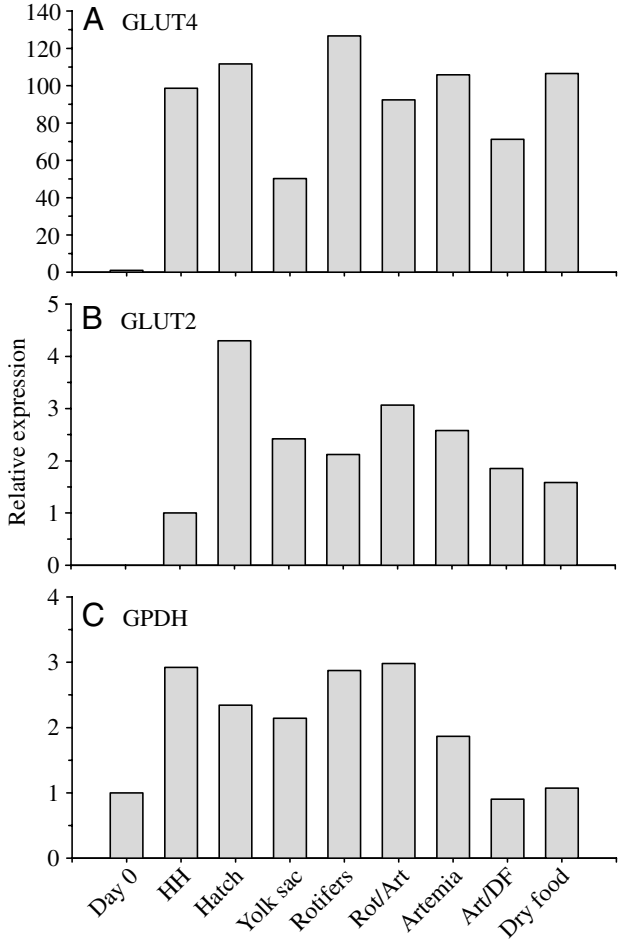


Fig. 3. Developmental expression patterns of (A) GLUT4, (B) GLUT2 and (C) GPDH in Atlantic cod. Atlantic cod were analyzed for GLUT4, GLUT2 and GPDH expression from eggs to larval fish by qRT–PCR. Each value represents a single analysis of a biomass of approximately 100 mg. Expression levels of the target gene were normalized to 18S rRNA. Units represent the relative expression of normalized target gene with respect to a calibrator sample, which in this case is the tissue that expresses the target gene at the lowest detectable level. Sampling times are based on developmental stages and changes in diet. Day 0, 45 degree days; HH, halfway to hatching; Hatch, 105 degree days. Post-hatch samples: Yolk sac, day 2; Rotifers, day 19; Rot/Atr (rotifers/*Artemia*), day 47; *Artemia*, day 52; Art/DF (*Artemia*/dry food), day 54; Dry food, day 60.

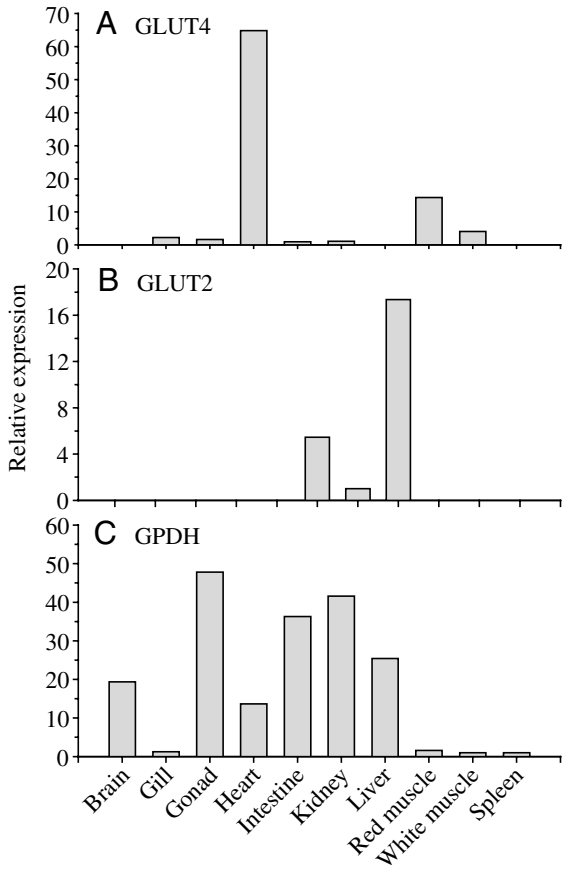


Fig. 4. Tissue distribution of (A) GLUT4, (B) GLUT2 and (C) GPDH in Atlantic cod. Tissues from a single fed juvenile Atlantic cod were analyzed by qRT–PCR for expression of GLUT4, GLUT2 and GPDH. Expression levels of the target gene were normalized to 18S rRNA. Units represent the relative expression of normalized target gene with respect to a calibrator sample, which in this case is the tissue that expresses the target gene at the lowest detectable level.

Glucose transporters have several amino acid sequences and transmembrane helices common to all classes, as well as class specific motifs (Joost and Thorens, 2001; Hruz and Mueckler, 2001). As highlighted in Fig. 2A, Atlantic cod GLUT4 and GLUT2 have all the features of glucose transporters in general and of class I GLUTs, with the exception of a small number of amino acid substitutions. However, the identification of structural features important in the function of individual members of class I GLUTs may be enhanced by comparison of species-specific sequences.

In the case of GLUT4, this is important when considering motifs that may play a role in trafficking/targeting. Mammalian GLUT4 isoforms are characterized by the presence of several unique motifs involved in protein trafficking, including the FQQI motif at the amino terminus, and the LL motif and the acidic cluster TELEYLGP located at the carboxyl terminus (Holman and Sandoval, 2001; Lalioti et al., 2001). In mammalian cells, the FQQI domain is important in the sorting process that separates GLUT4 from the endosome system (Holman and Sandoval, 2001; Lalioti et al., 2001). In Atlantic cod and coho salmon (Capilla et al., 2004) the FQQ motif is present, but the I is replaced with another hydrophobic amino acid, L. In brown trout, the motif is FQHL (Capilla et al., 2004). The LL domain appears to be involved in sorting between the *trans*-Golgi Network and GLUT4 storage vesicles; however, all three fish lack the LL motif, being LG in Atlantic cod. It has been suggested that these domains may be partially redundant in GLUT4 endocytosis (Holman and Sandoval, 2001; Lalioti et al., 2001), which may be correct, based upon amino acid data from fish which have an FQQL domain but are

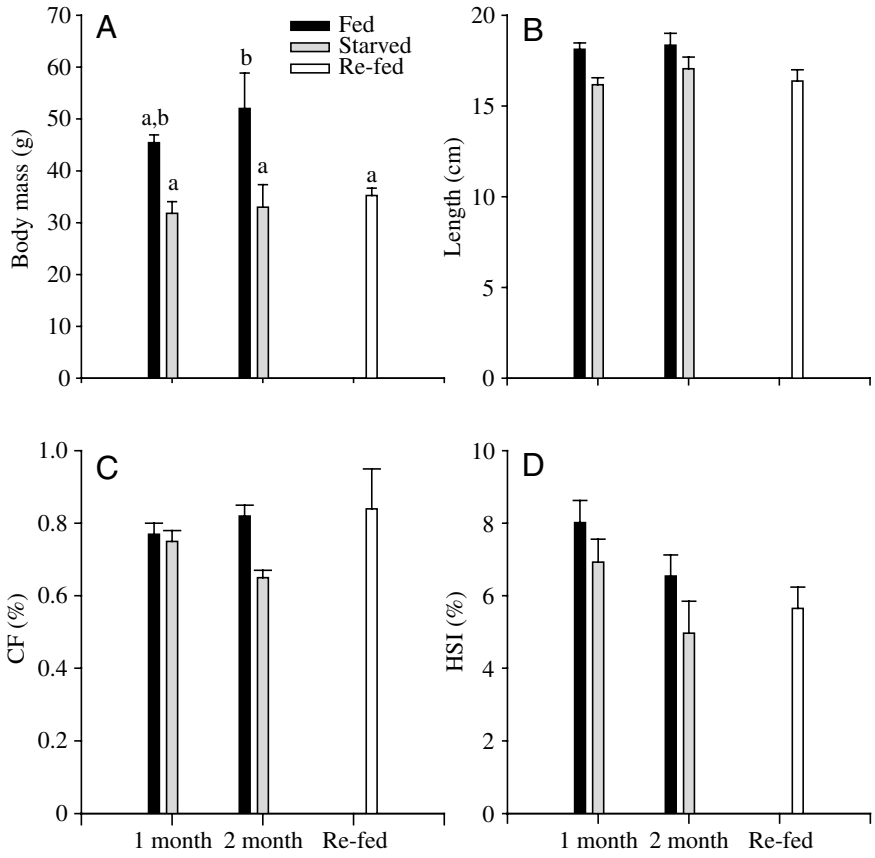


Fig. 5. The effects of fasting/re-feeding on morphometrics of Atlantic cod. (A) Body mass; (B) length; (C) condition factor (CF); (D) hepatosomatic index (HIS). Fish were deprived of food for 1 or 2 months with control fed fish being sampled at the same time. One group was re-fed for 3 weeks following 2 months of starvation. N=6 in all cases. Columns with different letters are significantly different (P<0.05).

lacking the LL domain. The acidic cluster is present in fish, albeit with minor amino acid substitutions.

In the case of GLUT2, all GLUT2s are characterized by the presence of an elongated extracellular loop between transmembrane segments 1 and 2. However, the size and sequence of this loop is highly variable, with an additional 32, 38, 14 and 15 amino acids in mammalian, chicken, rainbow trout and Atlantic cod GLUT2s, respectively. Furthermore, transmembrane 7 of mammalian GLUT2s contains an H(V/M)A motif that was thought to be needed for the transport of D-fructose (Wu et al., 1998). However, in chicken and both fish species, this motif is not conserved, with sequences of QIS and HLS, respectively.

Expression of GLUTs during development and tissue distribution

The energy requirements of the developing Atlantic cod are met by lipids and amino acids from fertilization to consumption of the yolk sac (Finn et al., 1995). Thereafter, carbohydrate becomes a component of the diet by virtue of the body composition of the prey species, rotifers and *Artemia*. The final diet of dry food contains 12% carbohydrate. GLUT4 is marginally detectable at fertilization but shows about a 100-fold increase in expression level by halfway to hatch. GLUT2 expression is not detectable at fertilization but is so at the halfway to hatch point with a further fourfold increase at hatching. Expression levels of GLUT 4 and 2 (reported here) and GLUT 1 (Hall et al., 2004) appear to be relatively stable between hatching and the 100 mg larval stage, suggesting that the fish are poised for carbohydrate transport and that the change in metabolic fuel associated with the transition from yolk sac dependence to dry food has little impact on relative gene expression of these glucose transporters.

The tissue distribution of the GLUT 4 and 2 transporters in Atlantic cod is similar to the mammalian paradigm (Wood and Trayhurn, 2003). GLUT4 expression is most prevalent in heart, followed by red muscle and white muscle, with lower levels in a number of other tissues; whereas, GLUT2 mRNA is most highly expressed in liver, at lower levels in intestine and kidney, and is undetectable in other tissues. Tissue-specific expression of GLUTs 1, 2, and 3 in Atlantic cod is generally consistent with that reported for GLUT1 and GLUT2 in rainbow trout (Teerijoki et al., 2000; Panserat et al., 2001) and GLUT3 in grass carp (Zhang et al., 2003). GLUT4 expression in heart appears to differ between Atlantic cod, where it is highly expressed, and brown trout, where it is expressed to only a limited extent (Planas et al., 2000). The functional significance of this difference in cardiac tissue remains to be resolved.

Impact of starvation on metabolic fuels and GLUT expression

The period of food deprivation was well within the tolerance limits of Atlantic cod as evidenced by maintenance of condition factor and levels of lipids in liver. However, the challenge resulted in decreases in blood glucose and liver glycogen, as previously reported (Black and Love, 1986; Hemre et al., 1990). Such starvation-induced decreases in blood glucose are associated with parallel decreases in plasma insulin (Hemre et al., 1990; Sunby et al., 1991). Although insulin was not measured in the current experiments, we assume that it decreased with starvation, given that the water temperature (5–8°C in previous studies; 8°C current experiment) and the length of starvation (3–4 weeks in previous studies; 4–8 weeks in current experiment) were in the same range as in the earlier work of Hemre et al. (Hemre et al., 1990) and Sunby et al. (Sunby et al., 1991). Re-feeding led to a recovery of blood glucose levels and an overshoot in liver glycogen, again as

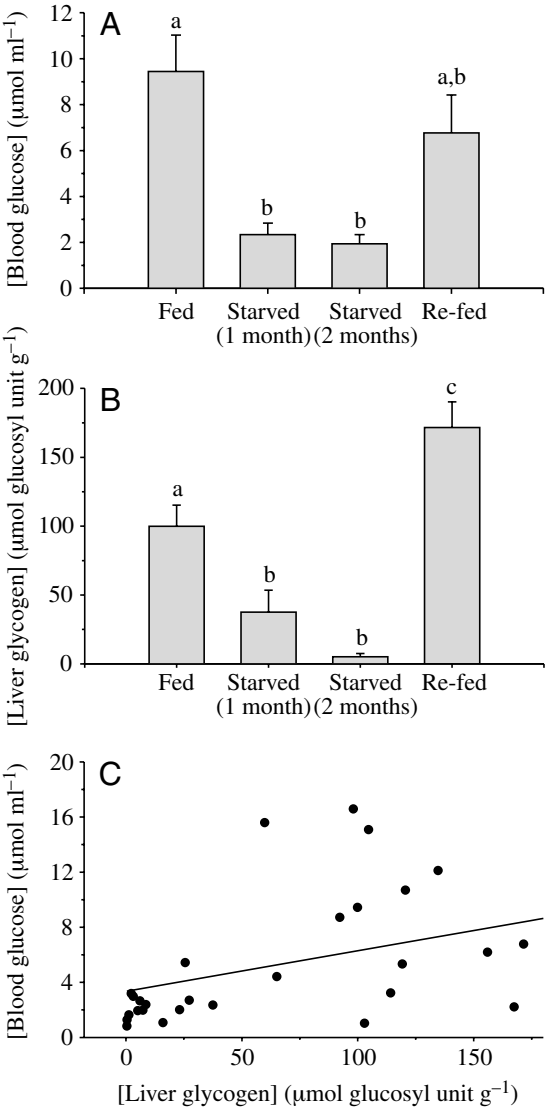


Fig. 6. The effects of fasting/re-feeding on blood glucose and liver glycogen levels of Atlantic cod. (A) Blood glucose; (B) liver glycogen; (C) blood glucose *versus* liver glycogen from individual fish (y=0.29x+3.4; R^2=0.20, P=0.017); fish were fed (N=10 for glucose; N=12 for glycogen) or deprived of food for 1 month (N=6) or 2 months (N=6). Starved fish were then re-fed (N=6) for 3 weeks. Columns with different letters are significantly different (P<0.05).

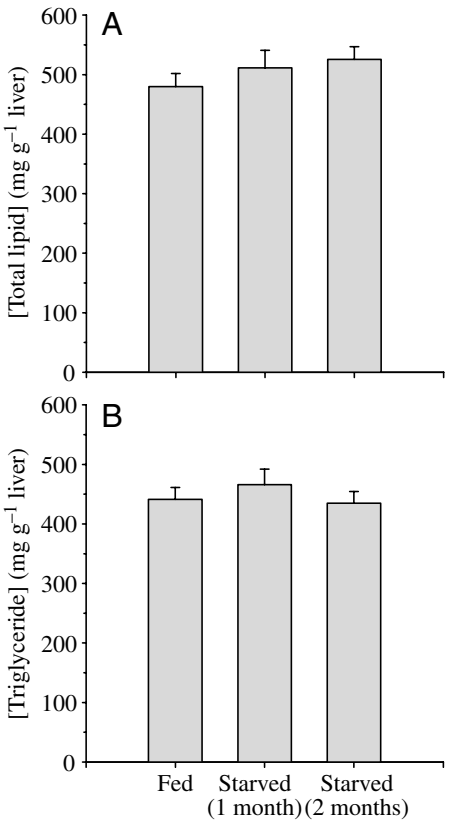


Fig. 7. The effects of fasting/re-feeding on total lipid and triglyceride levels in liver of Atlantic cod. (A) Total lipid; (B) triglyceride. Fish were fed (N=12) or deprived of food for 1 month (N=6) or 2 months (N=6).

reported by Black and Love (Black and Love, 1986). We note here for the first time a correlation between glycogen content in liver and blood glucose, suggesting that the former sets the glucose level available to other tissues.

Expression of GLUT4 in heart decreased during starvation, increased with re-feeding and correlated with blood glucose. The most plausible explanation for this is that high levels of blood glucose are associated with elevated levels of insulin and this in turn results in activation of GLUT4 transcription. The scenario depicted for heart of Atlantic cod matches that in red muscle of brown trout, in which starvation is associated with decreases in blood insulin and glucose in association with decreases in red muscle GLUT4 (Capilla et al., 2002).

Expression of GLUT4 in white muscle was the mirror image of that in heart. Food deprivation resulted in an increase in mRNA levels, which returned to control values with re-feeding. GLUT4 expression in white muscle did not correlate with blood glucose. Differences between red muscle and white muscle, with respect to alterations in GLUT4 levels, have been previously noted in trout although the significance of it may have gone unrecognized (Capilla et al., 2002). GLUT4 mRNA levels decreased in red muscle of starved brown trout but in white muscle, although the there was no significant difference in expression level, the average value increased by about 25%. In rainbow trout injected with porcine insulin, there was an increase in GLUT4 mRNA in red muscle but no change in white muscle. It appears that the GLUT4 responsiveness to insulin and/or high blood glucose levels noted in heart of Atlantic cod and red muscle of trout does not carry over to white muscle. Indeed starvation is associated with increases in GLUT4 mRNA. An explanation to account for this is that during starvation there is proteolysis in white muscle of Atlantic cod (Black and Love, 1986) that probably includes GLUT4. The data presented here are for GLUT 4 mRNA and

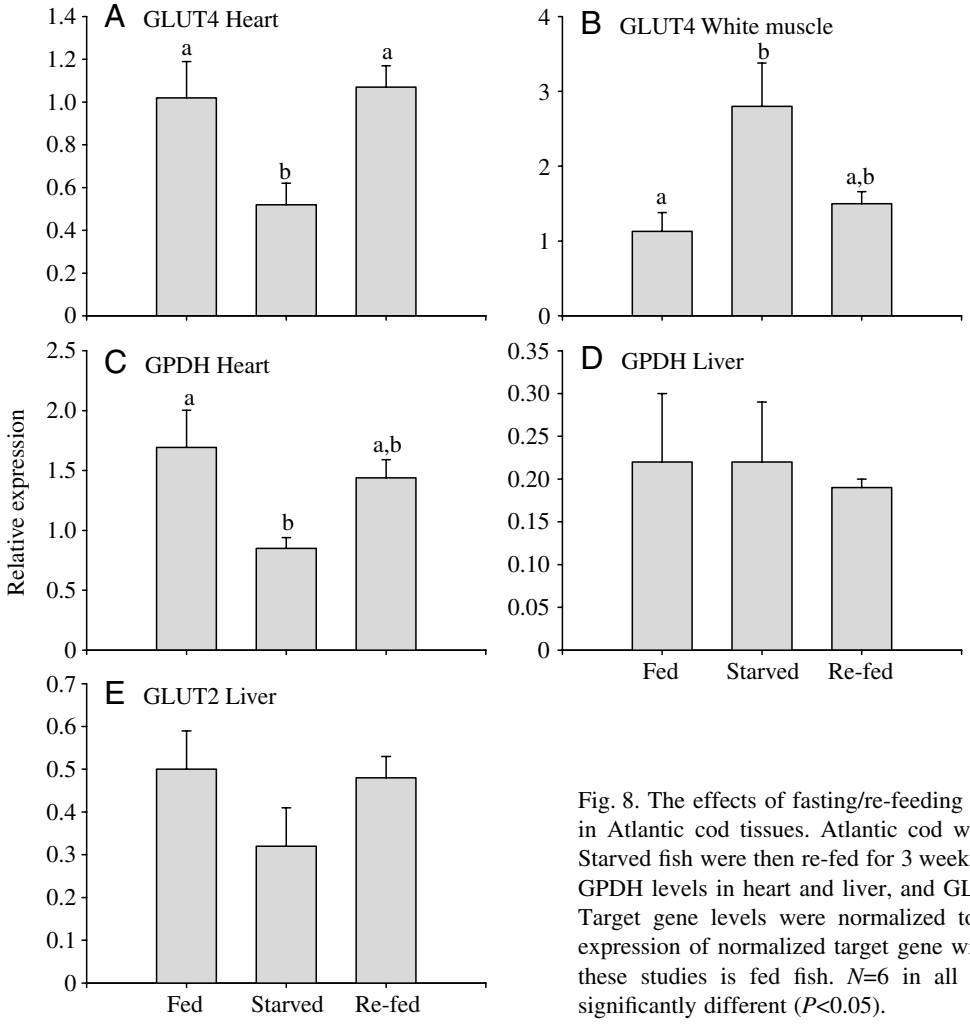


Fig. 8. The effects of fasting/re-feeding on GLUT4, GLUT2 and GPDH expression in Atlantic cod tissues. Atlantic cod were fed or deprived of food for 2 months. Starved fish were then re-fed for 3 weeks. GLUT4 levels in heart and white muscle, GPDH levels in heart and liver, and GLUT2 in liver were analyzed by qRT–PCR. Target gene levels were normalized to 18S rRNA. Units represent the relative expression of normalized target gene with respect to a calibrator sample, which in these studies is fed fish. N=6 in all cases. Columns with different letters are significantly different (P<0.05).

not transporter protein. In order to maintain glucose transport there may need to be an increase in synthesis of this protein and this is reflected in increased GLUT4 expression. A response similar to that reported here for Atlantic cod has been observed in rats, in which a 3-day fast led to a two- to threefold increase in GLUT4 transcription and GLUT4 mRNA in white muscle but no change in red muscle (Neufer et al., 1993).

GLUT2 mRNA levels correlated with blood glucose. In mammals, GLUT2 serves to facilitate glucose transport either into or out of liver cells, dependent on dietary and hormonal status. One scenario to account for the data is that as starvation proceeds, liver glycogen is depleted and delivery of glucose from liver to blood is diminished therefore reducing the need to maintain high levels of GLUT2 protein. In the only other study that we are aware of, regarding GLUT2 in fish, the transporter was highly expressed in liver of rainbow trout and the level was not influenced by 4 days of food deprivation (Panserat et al., 2001).

Glycerol-3-phosphate dehydrogenase

GPDH from Atlantic cod encodes a deduced amino acid sequence that is very similar to that found in a number of other fish species. The enzyme GPDH plays a pivotal role in the synthesis of glycerol 3-phosphate required for triglyceride/phospholipid synthesis and in the conversion of glycerol 3-phosphate to dihydroxyacetone in the breakdown of these components. GPDH expression was apparent at fertilization, increased by about threefold by halfway to hatching, and remained elevated throughout the remainder of the developmental period. In juvenile fish, GPDH was detected in all tissues sampled with the highest levels, typically occurring in those tissues with high lipid turnover, such as brain, intestine and liver. The starvation challenge was not substantive enough to induce changes in liver triglyceride levels. In this context it is not surprising that GPDH mRNA levels did not change in liver. GPDH expression in heart decreased with starvation and recovered during re-feeding. The simplest explanation for this is a decrease in phospholipid/triglyceride synthesis in heart during starvation associated with decreased synthesis of glycerol 3-phosphate.

Conclusion

In summary, GLUT4, GLUT2 and GPDH cDNAs from Atlantic cod were cloned and sequenced. In accordance with

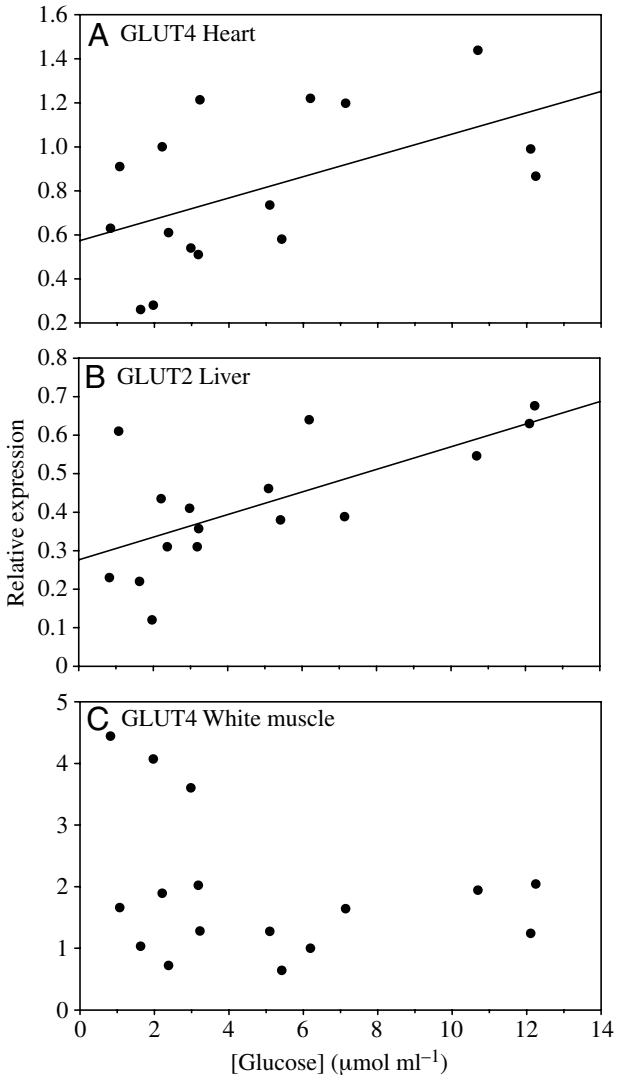


Fig. 9. The relationship between GLUT expression and blood glucose level in Atlantic cod. (A) Heart GLUT4 *versus* blood glucose (y=0.048x+0.57; R^2=0.28, P=0.035); (B) liver GLUT2 *versus* blood glucose (y=0.29x+0.28; R^2=0.46, P=0.0037); (C) white muscle GLUT4 *versus* blood glucose. Values are for individual fish from fasting/re-feeding experiments.

the mammalian model GLUT4 is expressed primarily in heart and muscle, whereas GLUT2 is expressed at the highest levels in liver. GPDH is expressed in all tissues assessed, with highest levels in tissues that have high rates of lipid turnover. Starvation was associated with decreases in blood glucose, liver glycogen and heart GLUT4 and GPDH. These parameters recovered with re-feeding. By contrast, white muscle GLUT4 increased with starvation and returned to pre-starved levels with re-feeding. The physiological significance of this is yet to be resolved. GLUT2 expression in liver correlated with blood glucose levels, probably reflecting glycogen depletion in liver during starvation with reduced movement of glucose from liver to blood.

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