RESEARCH ARTICLE

Global cooling: Cold acclimation and the expression of soluble proteins in carp skeletal muscle

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The common carp (*Cyprinus carpio*) has a well-developed capacity to modify muscle properties in response to changes in temperature. Understanding the mechanisms underpinning this phenotypic response at the protein level may provide fundamental insights into the molecular basis of adaptive processes in skeletal muscle. In this study, common carp were subjected to a cooling regimen and soluble extracts of muscle homogenates were separated by 1-D SDS-PAGE and 2-DE. Proteins were identified using MALDI-TOF-MS and *de novo* peptide sequencing using LC-MS/MS. The 2-D gel was populated with numerous protein spots that were fragments of all three muscle isoforms (M1, M2 and M3) of carp creatine kinase (CK). The accumulation of the CK fragments was enhanced when the carp were cooled to 10°C. The protein changes observed in the skeletal muscle of carp subjected to cold acclimation were compared to changes described in a previous transcript analysis study. Genes encoding CK isoforms were downregulated and the genes encoding key proteins of the ubiquitin–proteasome pathway were upregulated. These findings are consistent with a specific cold-induced enhancement of proteolysis of CK.

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1 Introduction

Although muscle plasticity is well established, comparative approaches to investigate phenotypic responses in muscle typically rely upon nonphysiological adaptations such as denervation [1] or pathological processes [2] to produce a measurable effect at the molecular level. Such dramatic changes in phenotype can often prove incapacitating or fatal in vertebrates. However, many species of fish provide a natural model system to study the molecular changes resulting from the remodelling of muscle. The common carp (*Cyprinus carpio*) has a well-developed capacity to modify muscle properties in response to changes in temperature, which allows it to maintain physiological and mechanical homeostasis under severe thermal stress [3, 4]. Importantly, the carp model can be easily manipulated in the laboratory and does not require damaging interventions to change its muscle phenotype. The carp model also has the advantage of clearly defined anatomical segregation of fast-twitch (white) and slow-twitch (red) fibres, which provides easy access to samples of pure fibre-type.

During cold acclimation, extensive molecular and ultrastructural changes take place in the muscle of carp to compensate for the effect of reduced temperature. There is an increase in the number of mitochondria [5, 6] and elevated levels of fatty acid unsaturation in membrane phospholipids [7]. Microarray expression profiling has revealed upregula-

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Abbreviations: CK, creatine kinase; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HSP,** heat shock protein

tion of genes involved in mitochondrial production of ATP and the modification of lipid membranes in the muscle of cold-acclimated carp [8, 9]. However, to date, changes in the expression of muscle proteins in carp have only been examined in isolation. Conventional experimental approaches have typically studied the expression patterns of a single-type or class of protein rather than examining broad populations of muscle proteins. These studies have focused on the biochemical and immunochemical characterisation of proteins such as myosin heavy and light chains [10], the mitochondrial ATP synthase complex [11], sarcoplasmic reticulum Ca^{2+} ATPase and parvalbumin [12].

A perspective driven by comparative proteomics provides an integrated approach that encompasses a global view of protein expression in fish tissues under different environmental conditions. In this study, we have employed proteomic strategies to characterise the soluble protein complement of skeletal muscle of common carp. In order to define the protein response to cold acclimation, we have determined the changes in protein expression patterns in the skeletal muscle of carp subjected to a progressive cooling regimen. Understanding the mechanisms underpinning this phenotypic response at the protein level may provide fundamental insights into the molecular basis of adaptive processes in skeletal muscle.

2 Materials and methods

2.1 Experimental animals

Common carp weighing 500–800 g were maintained in the University of Liverpool aquarium. Throughout the experiment, the fish were fed *ad libitum*. Initially, they were held at 30°C for 2 months. Consistent with a previous study that tracked cold-induced gene expression changes in carp [9], for cooling, fish were subjected to a stepped regimen of 1°C/h and a maximum of 7°C/day to a temperature of 10°C over the course of 3 days. The temperature was then maintained at either 10 or 30°C for up to 42 days until the fish were sampled. Between three and six fish were sampled at 21 and 42 days. Fish were killed in accordance with UK Home Office Schedule One regulations. The cooling regimen was carried out under license granted by the UK Animal (Scientific Procedures) Act, 1986.

2.2 Sample preparation

Immediately after killing, axial white skeletal muscle from midway down the body of each fish (under the dorsal fin and above the lateral line) was swiftly dissected and weighed. The muscle samples (approximately 1 g) were then mechanically homogenised in 10 mL of ice-cold phosphate buffer (20 mM, pH 7.0) containing Complete Protease Inhibitor Cocktail (Roche, Lewes, UK). To minimise protein modification or degradation, all dissection and sample processing was performed on ice and completed within 5 min. The homogenates were centrifuged at 15 000 × *g* for 45 min at 4°C. The protein concentration of each supernatant fraction was determined using the Coomassie Plus Protein Assay (Pierce Biotechnology, Rockford, USA). The samples were then immediately subjected to IEF and gel electrophoresis. Any remaining sample was aliquoted and stored at −80°C until required.

2.3 1-D SDS-PAGE

The soluble proteins were initially separated by 1-D SDS-PAGE. Muscle samples (4–50 μg) were electrophoresed at a constant potential of 200 V through a 12.5% w/v polyacrylamide gel with a 4% w/v stacking gel. Samples were boiled at 100°C for 5 min in a reducing buffer (125 mM Tris-HCl; 140 mM SDS; 20% v/v glycerol; 200 mM DTT and 30 mM bromophenol blue), prior to loading. Gels were stained with colloidal CBB stain (BioRad, Hercules, USA).

2.4 2-DE

Soluble extracts of muscle homogenates were separated by 2-DE. The supernatant fraction from each muscle homogenisation was precipitated using acetone and the resulting pellet containing the proteins of interest was solubilised in 4% w/v CHAPS, 8 M urea, 20 mM DTT and 0.5% v/v carrier ampholytes. Samples (approximately 300 μg of protein) were incubated at room temperature for 1 h and were then applied to IPG strips (pH 3–10, 13 cm, GE Healthcare, Amersham, UK). In-gel rehydration of the strips at 150 V·h at 30 V and 300 V·h at 60 V was followed by IEF (500 V·h at 500 V, 1000 V·h at 1000 V and 48 000 V·h at 8000 V) using an Ettan IPGPhor unit (GE Healthcare). The focused strips were equilibrated in reducing buffer containing 50 mM Tris-HCl at pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 65 mM DTT and a trace of bromophenol blue. A second equilibration step was then performed using alkylating buffer with 135 mM iodoacetamide in place of the DTT. The second dimension separation was performed with 12.5% w/v acrylamide gels on a Hoefer SE600 system (GE Healthcare). Proteins were visualised with CBB stain (BioRad).

2.5 Gel image analysis

Gels were imaged by scanning densitometry using an Epson 160 pro flatbed scanner (266 dpi, 24-bit colour in transmission mode). The images were saved as uncompressed TIFF files. Replicate gels from at least three individual fish were used for each treatment group (10 or 30°C for 21 and 42 days, respectively). 1-D gel band volumes were analysed using Phoretix 1D Quantifier software (Non-Linear Dynamics, Newcastle, UK). Band volumes were normalised to the total protein on the gel after subtracting the background. The statistical significance of variation in abundance between the acclimation temperatures at 21 and 42 days was determined

using a Student's *t*-test. *p*-values of $\leq$0.05, modified further by the Bonferroni correction were considered statistically significant. The Bonferroni correction is a highly conservative safeguard against multiple tests of statistical significance on the same data. The correction for such multiple significance testing is simply to multiply the *p*-value by the number of tests carried out. If the corrected value is still $\leq$0.05, the data are considered significant [13]. 2-D gels were compared using Phoretix 2D Evolution software (Non-Linear Dynamics). The expression levels of key proteins were determined by calculating the spot volume corresponding to each protein as a fraction of the total integrated spot density on the gel.

2.6 In-gel trypsin digestion

Gel plugs containing protein from areas of interest were excised and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep™ digestion robot (Waters, Manchester, UK). Briefly, gel plugs were excised and placed in distilled deionised water (50 μL). The water was then removed and the gel piece was treated with destain solution (10 μL of ACN/100 mM ammonium bicarbonate 1:1 v/v). The protocol included a second cycle of reduction and alkylation. The gel plug was dehydrated in ACN and then rehydrated with 25 μL of trypsin (3 ng/μL) in buffer. After digestion for 5 h at 37°C, tryptic peptides were extracted from the gel matrix by addition of 30 μL of 2% v/v ACN containing 1% v/v formic acid. Peptide samples were mixed 1:1 with a saturated solution of CHCA in ethanol/ACN/0.4% v/v TFA/water 1:1:1:1 v/v/v/v and 2 μL of the resultant mixture were spotted onto a MALDI target.

2.7 MALDI-TOF-MS

Peptide analysis was performed in positive ion mode on a M@LDI R mass spectrometer (Waters). The instrument was calibrated for each batch of analysis using a standard mix containing *des*-Arg bradykinin (M_r 903.47), neurotensin (M_r 1671.92), adrenocorticotrophic hormone (M_r 2464.20) and insulin-β chain (M_r 3493.65). All standards were purchased from Sigma (Poole, UK). Samples were mixed 1:1 with a saturated solution of CHCA in 50% v/v ACN containing 0.1% v/v TFA. Monoisotopic peptide masses were collected in the range of *m/z* 800–4000. Proteins were identified from their peptide mass fingerprints by manual searching using a locally implemented MASCOT server version 1.9 (www.matrixscience.com) against the MSDB and Swiss-Prot databases. Initial search parameters allowed a mass tolerance of ± 250 ppm, a variable trypsin missed cleavage, carbamidomethyl modification of cysteine residues and variable oxidation of methionine. The taxonomic search space was restricted to *Chordata*.

2.8 LC-MS/MS

De novo sequencing of peptides was performed using a Q-TOF Micro tandem mass spectrometer (Waters) equipped with a nanospray ESI source and coupled to an Ultimate nano-HPLC (Dionex, Camberley, UK) system. All analyses were performed in positive ion mode. The product ion spectrum from human [Glu^1]-fibropeptide B (M_r 1570.62, Sigma) was used as the mass calibrant. The samples were initially desalted and concentrated on a C18 peptide trap. The peptides were then separated on a C18 PepMap column (150 mm × 75 μm) nanocolumn (Dionex) at a flow rate of 200 nL/min using an ACN/water gradient; 0% ACN for 3 min, followed by 0–50% ACN over 30 min, 50–100% ACN for 2 min, 100% ACN for 5 min and 0% ACN for 3 min. MS/MS spectra were acquired in the data-dependent mode. Doubly or triply charged peptide ions were identified from a survey scan (*m/z* 400–2000), following which individual precursor ions were automatically selected for fragmentation. The collision energy was determined automatically and argon was used as the collision gas at a pressure of 15 psi. The resulting product ion data were processed by MaxENT 3 software and the peptide sequence determined using PepSeq within MassLynx software. Peptide sequences were searched using National Center for Biotechnology Information (NCBI) BLAST (www.ncbi.mlm.gov).

2.9 Western blotting

The expression levels of ubiquitinated proteins were evaluated by Western blotting [14]. Soluble extracts of muscle homogenates were separated by 2-DE. Pooled samples of three individual fish, acclimated at each temperature for 21 days were solubilised in 4% w/v CHAPS, 8 M urea, 20 mM DTT and 5% v/v carrier ampholytes. The samples (approximately 90 μg of protein for each) were incubated at room temperature for 1 h and were then applied to 7 cm IPG strips (pH 3–10, BioRad). In-gel rehydration of the strips at 150 V·h at 50 V and 200 V·h at 100 V was followed by IEF (1000 V·h at 1000 V and 35 000 V·h at 8000 V) using a PROTEAN^R IEF Cell (BioRad). The focused strips were equilibrated as described in Section 2.4, and then loaded onto 12% w/v acrylamide minigels and the proteins separated using a Mini-PROTEAN^R 3 Electrophoresis Cell (BioRad). The separated proteins were transferred to a 0.2 μm pore NC membrane (Schleicher and Schuell, Dassel, Germany). After protein transfer, the blot was boiled in distilled deionised water for 30 min and then blocked with 1% w/v fat free milk in 0.1% v/v Tween–TBS for 4 h at room temperature. The blot was then probed with a 1:1000 dilution of rabbit (polyclonal) anti(human) (poly)ubiquitin antibody (Sigma) for 2 h at room temperature. After six washes with Tween–TBS, the blot was incubated for 2 h with a 1:2000 dilution of goat antirabbit HRP-conjugated secondary antibody (Sigma). The blot was washed six times with Tween–TBS, followed by an additional two washes with PBS, developed with the Pico

ECL kit (Pierce Biotechnology). The images were recorded by exposing the blots simultaneously for 3 min to Hyperfilm (GE Healthcare). Control gels blotted with the secondary antibody demonstrated that there was no nonspecific binding.

2.10 Transcript analysis

The mRNA abundance for selected genes was extracted from a large-scale dataset that describes the gene expression changes associated with cold exposure of carp [9] (ArrayExpress Accession number E-MAXD-1). To make the studies directly comparable, the carp used in the present proteomic study were maintained under identical laboratory conditions in the same facility, and subjected to an identical cooling regimen as that used for the study of transcript expression. For the gene expression study, fish were obtained from a commercial supplier, held for 2 months at 30 ± 0.5°C in large 2000 L aquaria and fed twice daily on trout pellets. For cooling exposure, fish were transferred to 1000 L tanks and subjected to a stepped cooling regime of 1°C/h to a maximum of 7°C/day, to 10°C over 3 days and then maintained at 10°C for 22 days. Five replicate fish were killed at 1, 2, 3, 5, 8, 12 and 22 days after the onset of cooling, and skeletal white muscle was rapidly dissected out, frozen on dry ice and transferred to −80°C. Control 30°C acclimated animals were subjected to an identical handling regime and sampled at three time points during the 22 days experiment. For each time-point, total RNA was isolated from skeletal white muscle samples and pooled across the five replicate fish. Then fluorescently labelled cDNA was synthesised in a reverse transcription reaction using aminoallyl adducts coupled to Cy3 and Cy5 fluors. Changes in mRNA abundance associated with cooling were determined by competitive hybridisation of the labelled cDNA to carp microarrays consisting of 13440 cDNAs using a common reference design with a dye-swap [9].

3 Results and discussion

3.1 Identification of common carp skeletal muscle proteins

A major focus of this work was to define the proteome of carp skeletal muscle. The soluble muscle proteins were initially separated by 1-D SDS-PAGE (Fig. 1). The 1-D SDS-PAGE analysis revealed a large dynamic range in protein expression, with the distributions and intensities of protein bands similar to those described in the skeletal muscle of other fish species [15–17]. Individual protein bands were excised from the gel, subjected to in-gel digestion with trypsin and the resultant peptides were analysed by MALDI-TOF-MS and LC-MS/MS (Table 1). Many of the proteins identified were enzymes involved in glycolysis and the most abundant protein in the gel was creatine kinase (CK), comprising approximately 25% of total stained intensity.

Identification of proteins by PMF depends on database searches of peptide masses, but fish genomes are underrepresented in sequence databases. In the nonredundant Swiss-Prot database approximately 2500 sequences correspond to ray-finned fish of which 89 are common carp sequences. Therefore, to accurately determine the identity of fish muscle proteins we have combined cross-species matching with *de novo* sequencing of peptides by LC-MS/MS. As the full genome sequence of common carp is not

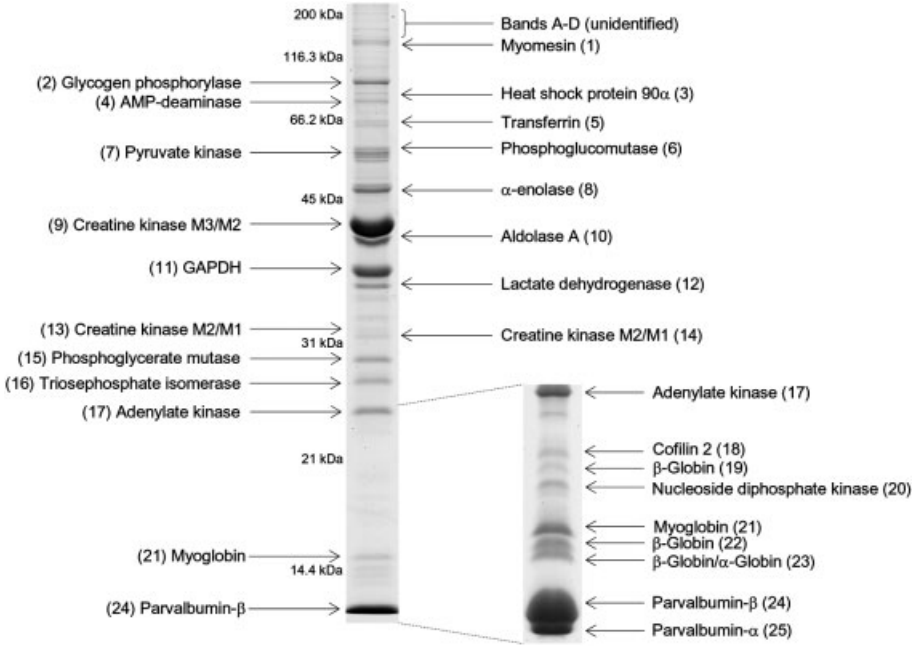

Figure 1. 1-D SDS-PAGE of soluble proteins in carp skeletal muscle. Muscle proteins (40 μg protein) were separated by 1-D SDS-PAGE and visualised by staining with colloidal CBB. An additional muscle sample (250 μg protein) was resolved on a separate gel to increase identifications of proteins of low molecular weight (inset). The proteins were identified by PMF and *de novo* peptide sequencing by LC-MS/MS (Table 1). Bands A–D were not identified.

Table 1. Identification of proteins from soluble fraction of carp skeletal muscle in 1-D SDS-PAGE

ID no.	Protein identification	Accession no.	M_r (kDa)	p*I*	PMF			Species
					MOWSE-score[b]	No. of peptides-matched	% Cover-age	
1	Myomesin 1[a] (theoretical protein)	XP_699443[c]	190.0	7.27				*D. rerio*
2	Glycogen phosphorylase	Q6PUS4	97.4	6.11	150 (58)	19	22	*D. rerio*
3	HSP 90-α	Q90474	83.5	4.95	87 (68)	16	20	*D. rerio*
4	AMP deaminase	Q6P3G5	82.9	6.35	159 (58)	22	27	*D. rerio*
5	Transferrin (variant A)[a]	Q8UVE7	73.5	5.75				*C. carpio*
6	Phosphoglucomutase	Q7SXW7	61.1	5.74	129 (68)	18	37	*D. rerio*
7	Pyruvate kinase	Q8QGU8	58.0	7.96	91 (58)	11	20	*Fugu rubripes*
8	α-Enolase	Q6TH14	47.4	6.25	100 (68)	10	29	*D. rerio*
9	CK M3	Q9YI14	42.9	6.25	172 (57)	17	53	*C. carpio*
	CK M2	Q9YI15	42.9	6.22	128 (57)	15	45	*C. carpio*
10	Aldolase A	Q8JH72	39.7	8.27	109 (68)	11	29	*D. rerio*
11	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Q8AWX8	36.1	7.74	107 (68)	7	30	*Gadus morhua*
12	Lactate dehydrogenase	Q9W7K5	36.1	7.31	142 (68)	17	38	*C. carpio*
13	CK M2 (fragment)	Q9YI15	42.9	6.22	110 (58)	14	32	*C. carpio*
	CK M1 (fragment)	Q9YI16	42.7	6.32	77 (58)	11	29	*C. carpio*
14	CK M2 (fragment)	Q9YI15	42.9	6.22	109 (58)	15	33	*C. carpio*
	CK M1 (fragment)	Q9YI16	42.7	6.32	90 (58)	13	31	*C. carpio*
15	Phosphoglycerate mutase	Q7T3G4	28.8	8.83	122 (68)	14	54	*D. rerio*
16	Triosephosphate isomerase	Q7T315	26.8	6.90	202 (68)	14	49	*D. rerio*
17	Adenylate kinase	P12115	21.3	6.89	308 (68)	19	76	*C. carpio*
18	Cofilin 2	Q6NZW3	18.6	7.62	104 (68)	10	57	*D. rerio*
19	β-Globin	P70073	16.3	8.39	85 (68)	8	59	*C. carpio*
20	Nucleoside diphosphate kinase	Q9PTF5	17.3	7.70	91 (68)	8	42	*D. rerio*
21	Myoglobin	P02204	15.6	8.05	199 (68)	16	90	*C. carpio*
22	β-Globin	P70073	16.3	8.39	122 (68)	11	67	*C. carpio*
23	β-Globin	P70073	16.3	8.39	89 (68)	8	50	*C. carpio*
	α-Globin	P02016	15.3	8.78	74 (68)	5	29	*C. carpio*
24	Parvalbumin-β	P02618	11.4	4.67	176 (68)	11	62	*C. carpio*
25	Parvalbumin-α	P09227	11.3	4.43	93 (68)	7	47	*C. carpio*

The skeletal muscle proteins were identified by PMF and *de novo* peptide sequencing using LC-MS/MS. Proteins were identified by PMF on the basis of their MOWSE score using the MSDB and Swiss-Prot databases. Each identification number (ID no.) refers to proteins identified in Fig. 1.

a) Proteins identified by *de novo* peptide sequencing (myomesin 1 (ID No. 1) VNGYYIDIK, VGYYIESS; transferrin (ID No. 5) TDFSINDLK; YSGDEGALQCLK; TPDCTLYNFFSK; TPGCTLYNFFSK; LLFSDT*TK).

b) MOWSE baseline significance score in brackets.

c) Gene sequence.

available, the carp muscle proteins were identified by matching to other fish species, in particular zebrafish (*Danio rerio*), which has been sequenced and extensively annotated. This approach is facilitated by the close taxonomic relationship of carp to zebrafish (both are cyprinids). Whilst the availability of further sequence data for carp will enhance the identification of proteins in this type of study, our results demonstrate that it is possible to confidently identify fish muscle proteins through cross-species matching to a taxonomic near neighbour.

2-DE was used to enhance separation of the soluble muscle proteins (Fig. 2) and revealed a relatively simple pattern. The gels were dominated by a few proteins between 20 and 66 kDa with p*I* 6–9. Significant clusters of low molecular weight protein spots were resolved across the entire p*I* range of the gels. A total of 18 individual proteins were identified from the 2-D gel on the basis of their peptide mass fingerprints (Table 2). The majority of protein identifications matched those determined from 1-D SDS-PAGE and were glycolytic enzymes. Additional protein spots were matched to apolipoprotein A1, tropomyosin and fibroblast growth factor. In many cases, the same protein was identified in multiple gel spots of similar molecular mass (Fig. 2, Table 2). These spots differed only in charge, forming trains, and may represent a PTM such as phosphorylation or proteolysis.

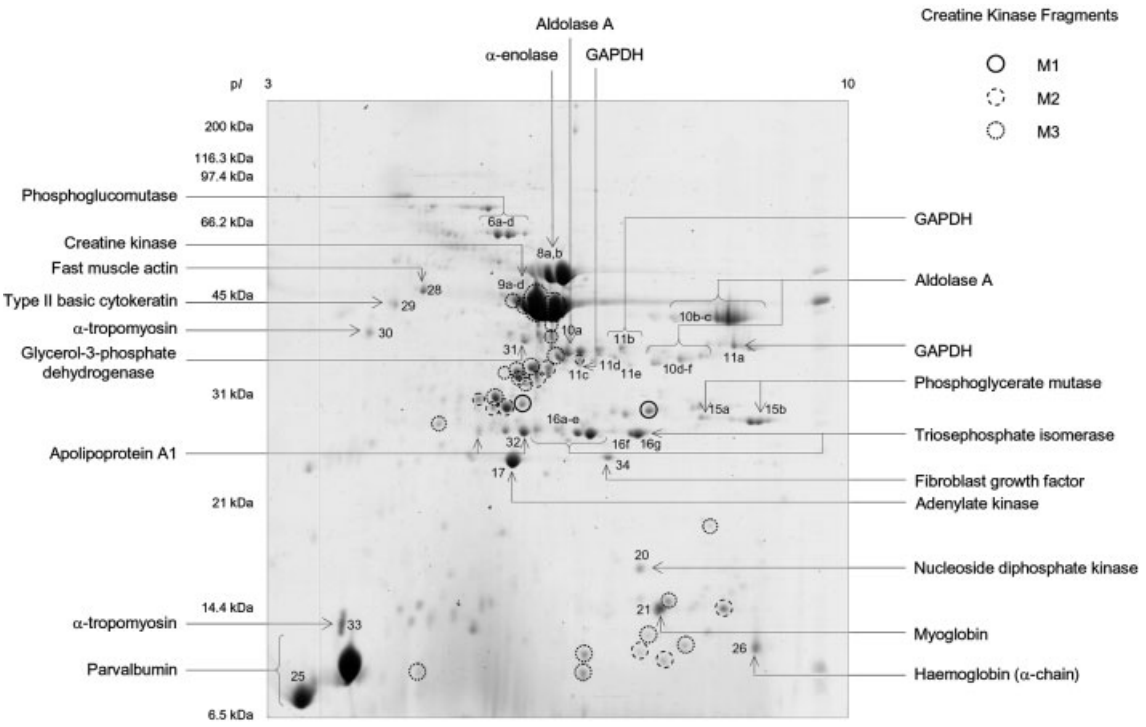

Figure 2. 2-DE of soluble protein complement from carp skeletal muscle. Proteins were separated in the first dimension on pH 3–10 IPG strips followed by SDS-PAGE on 12.5% w/v gels. Gels were stained with colloidal CBB. Proteins were identified by PMF, as listed on the gel. Subscripted letters refer to clearly resolved protein spots that yielded an identical identification to another spot. Protein identifications from the 2-DE analyses are detailed in Table 2. Spots circled by unbroken or dashed lines indicate fragments of CK.

The most unusual finding was the large number of protein spots across a broad range of M_r and p*I* that corresponded to the muscle isoforms of CK. Complex patterns of CK isoforms exist in the skeletal muscle of fish [16–18] and three isoforms of CK have previously been identified in carp skeletal muscle [19]. These are referred to as M1, M2 and M3 and have predicted masses of approximately 43 kDa and a p*I* range of 6.22–6.32. This would locate the protein in the 2-D gel around spots 9a–9d (Fig. 2). From the MALDI-TOF mass spectra obtained from these four spots, we identified the M2 and M3 CK isoforms. However, additional CK spots covering a mass range of 10–30 kDa were also identified across the charge dimension, a finding consistent with the skeletal muscle proteome of zebrafish [20].

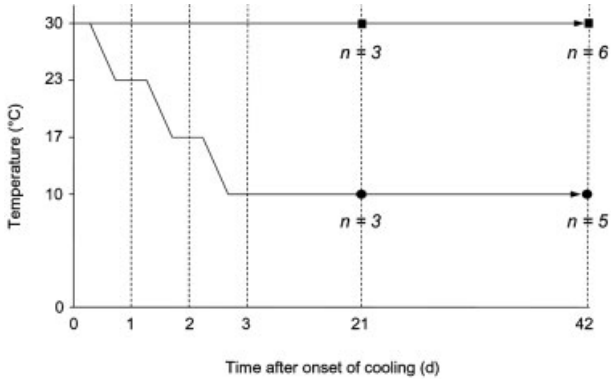

Figure 3. Schematic diagram showing the cooling time-course and sampling regimen. Warm-acclimated control carp (30°C) were sampled at 21 and 42 days and compared with fish cooled down to 10°C over the same time period.

3.2 Effects of thermal acclimation on skeletal muscle protein profile

In order to characterise the changes in the soluble protein complement of skeletal muscle induced by cold acclimation, we investigated the protein patterns of carp cooled to 10°C and compared them with fish maintained at a control temperature of 30°C. Fish were sampled at 21 and 42 days after the onset of cooling (Fig. 3). At each time-point, the soluble protein complement from the muscle of between three and six fish was analysed using 1-D SDS-PAGE. Scanning densitometry of the gels was used to determine the relative concentrations of specific proteins. The pattern of protein expression in carp skeletal muscle underwent subtle but statistically significant changes following thermal acclimation. After cooling for 21 days at 10°C, 1-D SDS-PAGE analysis failed to reveal any significant changes in the expression of soluble proteins from carp skeletal muscle (Fig. 4a). At 42 days acclimation, four protein bands showed significant

Table 2. Identification of proteins from soluble fraction of carp skeletal muscle in 2-DE

ID no.	Protein identification	Accession no.	M_r (kDa)	p*I*	PMF			Species
					MOWSE score[a)]	No. of peptides-matched	% Cover-age	
6a	Phosphoglucomutase	Q7SXW7	61.1	5.74	119 (57)	11	25	*D. rerio*
6b	Phosphoglucomutase	Q7SXW7	61.1	5.74	104 (57)	16	36	*D. rerio*
6c	Phosphoglucomutase	Q7SXW7	61.1	5.74	86 (57)	13	28	*D. rerio*
6d	Phosphoglucomutase	Q7SXW7	61.1	5.74	83 (57)	10	33	*D. rerio*
8a	β-Enolase	Q568G3	47.8	6.20	83 (67)	8	25	*D. rerio*
8b	β-Enolase	Q568G3	47.8	6.20	111 (69)	11	36	*D. rerio*
9a	CK M3	Q9YI14	42.9	6.25	128 (57)	16	38	*C. carpio*
9b	CK M3	Q9YI14	42.9	6.25	157 (57)	16	43	*C. carpio*
9c	CK M3	Q9YI14	42.9	6.25	184 (57)	17	54	*C. carpio*
9d	CK M2	Q9YI15	42.9	6.22	123 (57)	11	36	*C. carpio*
10a	Aldolase A	Q8JH72	39.7	8.27	75 (67)	8	22	*D. rerio*
10b	Aldolase A	Q8JH72	39.7	8.27	70 (67)	14	36	*D. rerio*
10c	Aldolase A	Q8JH72	39.7	8.27	145 (67)	14	54	*D. rerio*
10d	Aldolase A	Q8JH72	39.7	8.27	79 (67)	8	26	*D. rerio*
10e	Aldolase A	Q8JH72	39.7	8.27	74 (67)	7	19	*D. rerio*
10f	Aldolase A	Q8JH72	39.7	8.27	83 (67)	10	26	*D. rerio*
11a	GAPDH	Q8AWX8	36.1	7.74	70 (67)	5	21	*G. morhua*
11b	GAPDH	Q8AWX8	36.1	7.74	76 (67)	7	23	*G. morhua*
11c	GAPDH	Q8AWX8	36.1	7.74	71 (67)	5	21	*G. morhua*
11d	GAPDH	Q8AWX8	36.1	7.74	82 (67)	9	32	*G. morhua*
11e	GAPDH	Q90ZF1	35.9	8.63	81 (67)	7	29	*Oncorhychus mykiss*
15a	Phosphoglycerate mutase 2	Q7T3G4	28.8	8.83	64 (57)	8	41	*D. rerio*
15b	Phosphoglycerate mutase 2	Q7T3G4	28.8	8.83	83 (57)	7	35	*D. rerio*
16a	Triosephosphate isomerase B	Q90XG0	26.8	6.45	82 (67)	6	18	*D. rerio*
16b	Triosephosphate isomerase B	Q90XG0	26.8	6.45	76 (67)	7	26	*D. rerio*
16c	Triosephosphate isomerase B	Q90XG0	26.8	6.45	145 (67)	13	51	*D. rerio*
16d	Triosephosphate isomerase 1b	Q7T315	26.8	6.90	158 (67)	13	47	*D. rerio*
16e	Triosephosphate isomerase 1b	Q7T315	26.8	6.90	168 (67)	13	50	*D. rerio*
16f	Triosephosphate isomerase 1b	Q7T315	26.8	6.90	98 (67)	9	31	*D. rerio*
16g	Triosephosphate isomerase B	Q90XG0	26.8	6.45	114 (67)	12	43	*D. rerio*
17	Adenylate kinase	P12115	21.3	6.89	154 (58)	12	55	*C. carpio*
20	Nucleoside diphosphate kinase	Q9PTF5	17.3	7.77	116 (57)	8	44	*Danio rerio*
21	Myoglobin	P02204	15.6	8.05	120 (67)	7	54	*C. carpio*
25	Parvalbumin	Q918V0	11.5	4.46	63 (57)	5	50	*D. rerio*
26	Hemoglobin α-chain	P02016	15.3	8.78	106 (67)	6	50	*C. carpio*
28	Fast muscle actin	Q90Z02	42.0	5.16	89 (67)	10	29	*Scyliorhinus retifer*
29	Type II basic cytokeratin	Q9PV92	54.2	5.42	99 (67)	11	17	*D. rerio*
30	α-Tropomyosin	P13104	32.7	4.70	144 (57)	15	42	*D. rerio*
31	Glycerol-3-phosphate dehydrogenase	Q7T1E0	38.2	6.38	74 (67)	6	21	*D. rerio*
32	Apolipoprotein A1 (fragment)	Q98SI3	20.8	8.63	110 (57)	13	46	*C. carpio*
33	α-Tropomyosin (fragment)	P13104	32.7	4.70	80 (57)	7	25	*D. rerio*
34	Fibroblast growth factor	Q9DFC9	21.2	10.05	85 (57)	8	32	*D. rerio*

The skeletal muscle proteins were identified by PMF. The ID number refers to proteins identified in Fig. 2. Proteins were identified on the basis of their MOWSE score using the MSDB and Swiss-Prot databases.

a) MOWSE baseline significance score in brackets.

increases in intensity (at *p*-value of $\leq$0.05 after Bonferroni correction [13]) in the 10°C gel, compared to the 30°C gel (Fig. 4b).

Two of these protein bands (Bands A and C) could not be to be identified. The other proteins were identified as heat shock protein (HSP) 90-α and AMP-deaminase. HSPs are

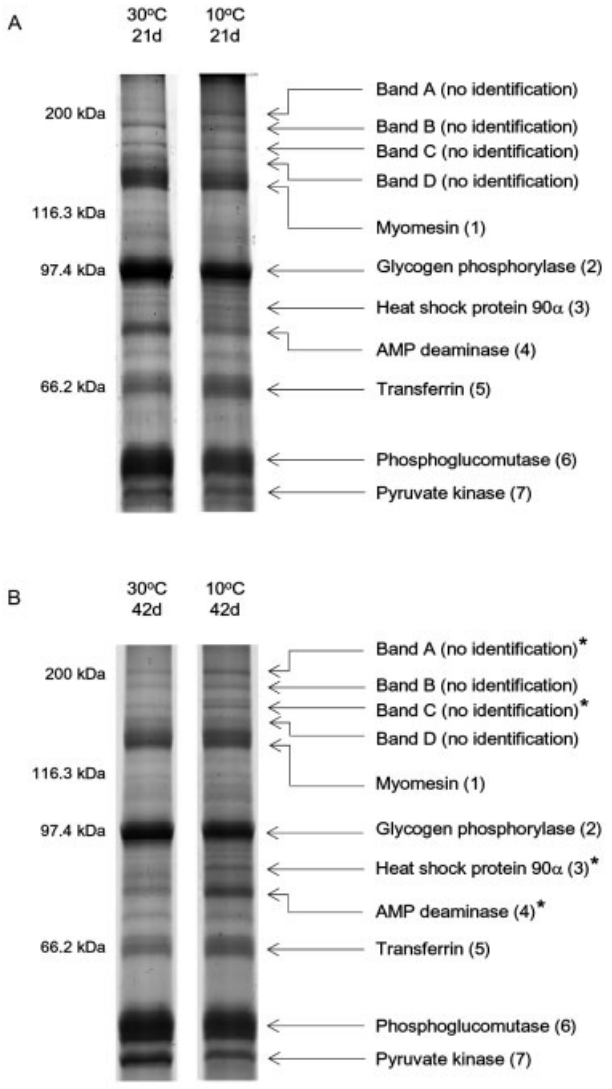

Figure 4. 1-D SDS-PAGE comparison of soluble muscle protein expression in carp cooled to 10°C for 21 and 42 days. Carp were cooled down to 10°C and maintained at that temperature for 21 days (panel A) and 42 days (panel B), respectively. Proteins were separated by 1-D SDS-PAGE and the protein expression profiles compared to fish maintained for the same time period at 30°C. Representative gels from individual fish for each time and temperature are shown. Statistically significant changes in the expression of soluble proteins (at p-value of $\leq$0.05 after Bonferroni correction) are indicated by an asterisk.

molecular chaperones that assist in the folding and translocation of newly synthesised proteins. They have also been implicated in coping with stress-induced denaturation of other proteins. Therefore, it might be expected to observe changes in this protein during thermal acclimation. AMP deaminase catalyses the deamination of AMP to inosine monophosphate. This enzyme has a stabilising function in the regulation of purine metabolism during intense energy demand, particularly in muscle.

Using 2-DE, the general pattern of muscle protein expression was similar in 30°C and cold-acclimated (10°C) fish. Qualitative inspection of the 2-D gels revealed that in the cooled fish, there was a notable increase in intensities of a number of spots that had previously been identified as CK fragments (Fig. 5a). These CK fragments were consistently seen in each of the replicate sets and were more apparent at 21 than at 42 days of acclimation. New CK fragment spots in the 10°C gels were not apparent in the 30°C gels. The expression levels of intact CK and CK fragments key proteins were also assessed quantitatively by determining the spot volume corresponding to each protein as a fraction of the total integrated spot density on the gel (Fig. 5b). The intact CK showed a decrease in its level of expression on cooling at both 21 and 42 days. In contrast, there was an increased accumulation of CK fragments in the skeletal muscle of fish cooled to 10°C.

3.3 CK isoform mapping

The 2-DE of soluble proteins from carp muscle revealed a complex pattern of M1, M2 and M3 CK isoforms and fragments. This effect was particularly manifest in fish at 10°C. Whilst some of the spots corresponded to the predicted M_r of the intact protein (42 kDa), the majority of these spots migrated at M_r between 10 and 30 kDa. This behaviour was specific to CK; no other fragments of abundant, well characterised, soluble muscle protein were identified.

Tryptic peptides derived from each of the fragment spots were analysed by MALDI-TOF-MS and LC-MS/MS and then mapped to the amino acid sequence of the intact proteins to determine the origin of the low molecular weight CK fragments (Fig. 6a). The peptide coverage maps for each CK fragment could be attributed to a specific isoform and showed clear evidence for scission of the protein into N- or C-terminal fragments. For example, spot 'vii' relates to a full length M3 isoform with peptides covering the entire primary sequence; whereas spot 'xxix' corresponds to a C-terminal fragment of M3 where peptides are only observed from sequence covering the tryptic fragments, T39-T49. No peptides N-terminal to T39 were observed, even though many of these peptides were readily determined by MS. CK isoforms with C-terminal truncations were also identified. Spot 'xiv' contains only peptides covering the sequence T1-T29. These truncations of CK are apparent from the M_r and pI of the fragments on the 2-D gel and mass spectrometric analysis.

Comparisons with theoretical charge distribution of the M1, M2 and M3 isoforms of CK suggest that the cleavage site occurs in the region of Phe_{278} of the intact protein (Fig 6b). However, although the evidence for the site of separation of the N-terminal and C-terminal domains was clear, this does not reveal the primary proteolytic event, which separated the two regions. In addition, nontryptic fragments have been observed, for example T35, which may arise from further exoproteolytic processing.

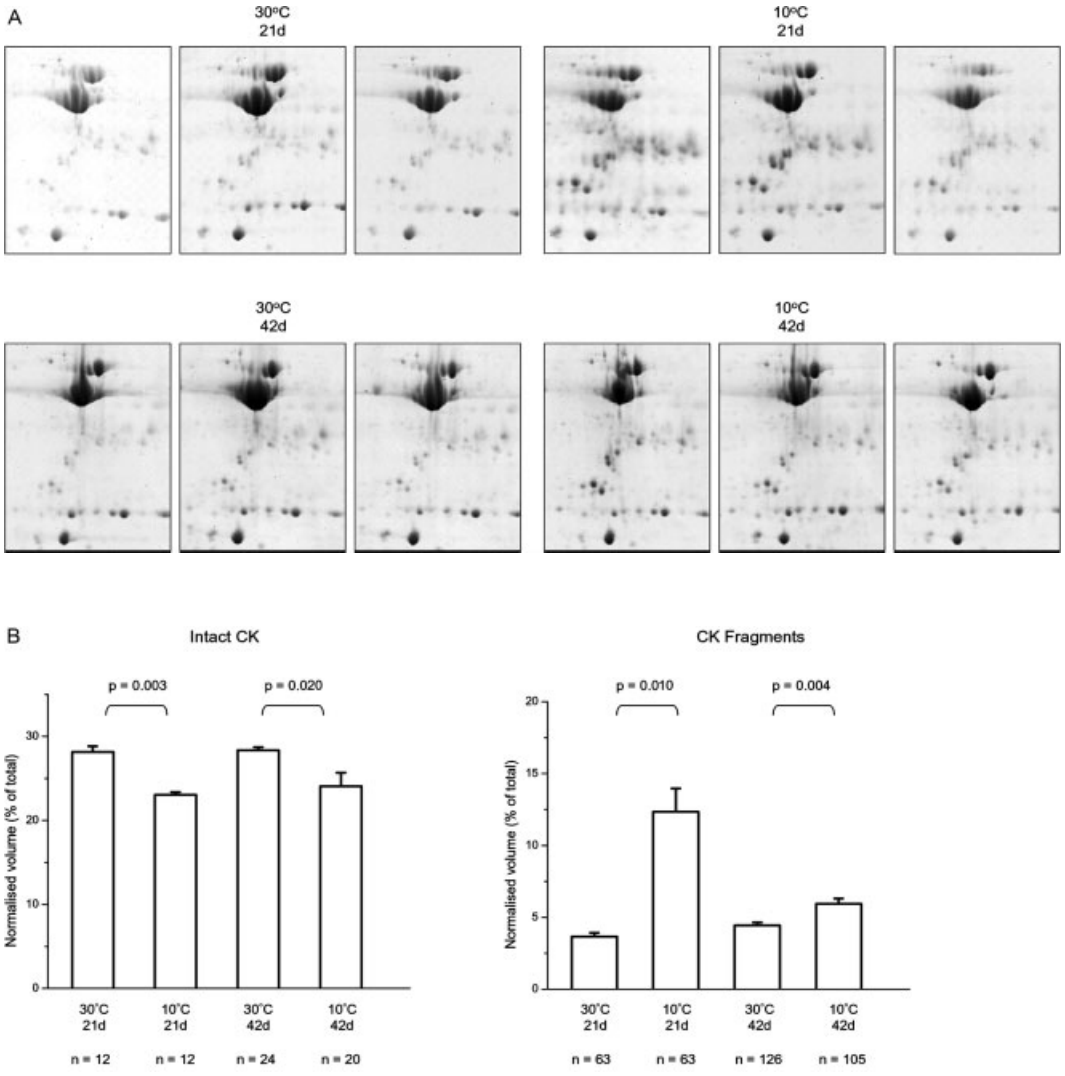

Figure 5. 2-DE comparison of soluble muscle proteins from acclimated carp cooled down to 10°C for 21 and 42 days. Carp were cooled down to 10°C and maintained at that temperature for 21 and 42 days. Proteins were separated by 2-DE and the protein expression profiles compared to fish maintained for the same time period at 30°C. In Fig. 5a, representative gels from three separate fish for each time and temperature are shown. Fig. 5b shows the expression levels of the total intact CK and CK fragments for the fish muscle samples at each time and temperature (mean ± SEM). The number of observations are indicated.

Carp muscle CK exhibits over 80% sequence identity to the equivalent enzyme in the bovine, for which 3-D structures are available. We confirmed that homology modelling yielded a structure of similar overall fold (results not shown) and mapped the site of scission of the N- and C-terminal domains onto the rabbit muscle CK dimeric structure (Fig. 6c). The site of hydrolysis was buried within the protein structure, and it is unlikely that proteases would have access to the protein without some local unfolding. This is consistent with the invocation of a specific mechanism to selectively remove CK from the muscle, as part of the adaptation to cooling. It is possible that the fragments remain in muscle because the enhanced commitment to degradation (possibly mediated by ubiquitin conjugation) is not matched by an equivalently enhanced completion of degradation, such that intermediates accumulate. Consistent with this idea is the observation that the CK fragments are more prominent at the early time of cooling.

In fish, endogenous proteases such as calpains and lysosomal cathepsins play a role in altering the protein profile of skeletal muscle *postmortem* [21]. We minimised *postmortem* degradation of skeletal muscle proteins by rapidly processing all tissues at a low temperature in phosphate buffer containing protease inhibitors. The entire process from netting to homogenisation of the muscle samples took less than 5 min during which time the tissue was maintained close to 0°C. The muscle samples were also analysed by 2-DE, immediately following homogenisation. Bosworth *et al.* [20] observed

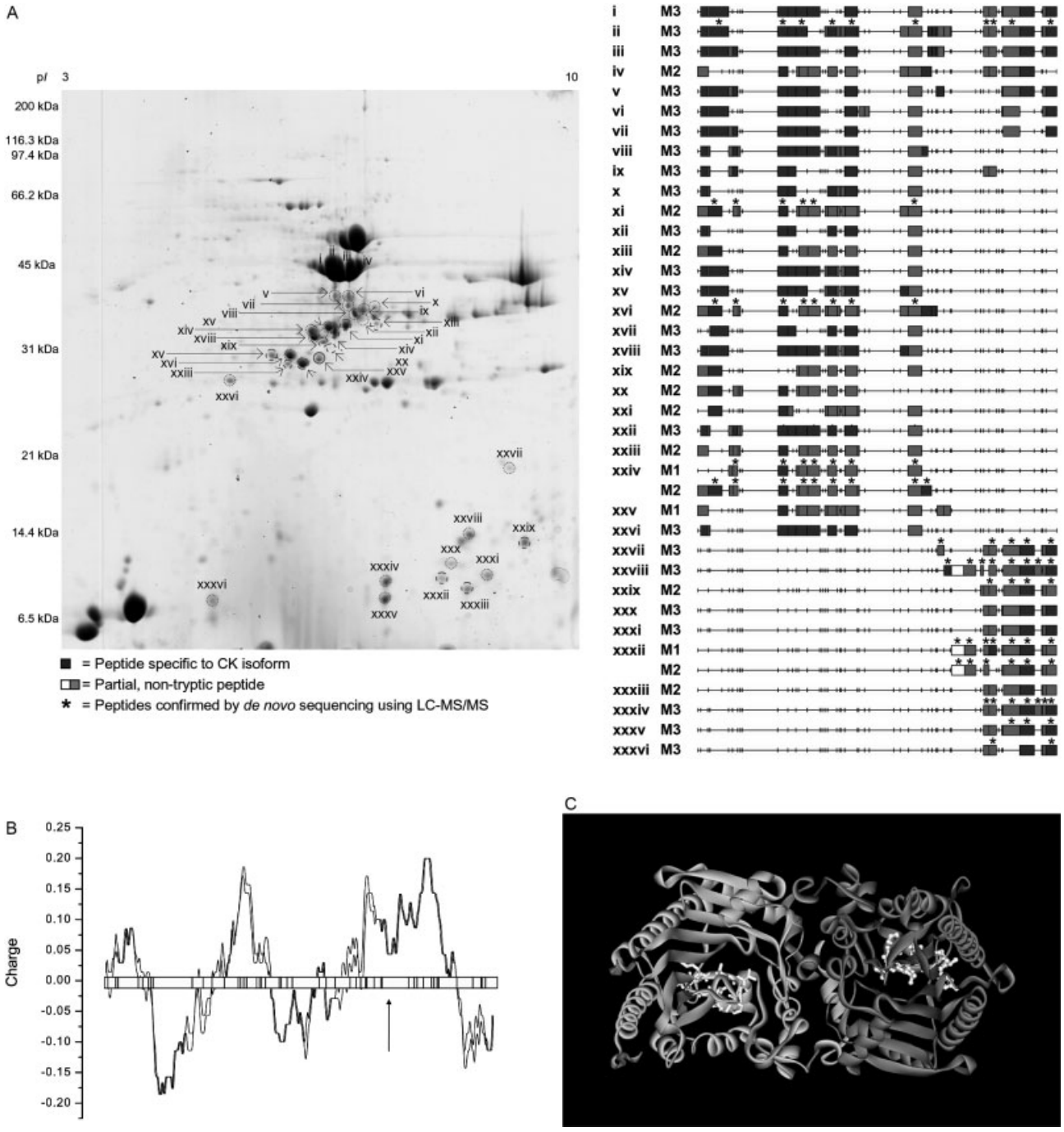

Figure 6. Peptide coverage maps for the isoforms of CK isoforms and fragments. In Fig. 6a, the tryptic peptides of the M1, M2 and M3 isoforms of CK were identified by PMF and mapped to *in silico* tryptic digests of the proteins. Filled boxes indicate a positive match between an observed peptide ion and a theoretical tryptic peptide. Unfilled boxes indicate partial nontryptic peptides. * = peptides identified by *de novo* peptide sequencing using LC-MS/MS. The theoretical charge distribution of each CK isoform is shown in Fig. 6b. The truncations of CK observed experimentally agree with the theoretical M_r and p*I* of the fragments. Data were smoothed over a charge window of 35 residues. An *in silico* tryptic map of the M3 isoform of CK is also shown to indicate charge distribution along the length of the intact protein. An arrow indicates the approximate site of scission of CK in carp skeletal muscle. In Fig. 6c, the dimer of rabbit muscle CK (1U6R.PDB), which shows over 80% sequence identity to carp muscle CK (M3 isoform), is displayed to highlight the approximate region of cleavage into the N-terminal and C-terminal domains. The sequence corresponding to the site of proteolysis is highlighted as a ball and stick structure on each subunit, and the two chains of the dimer are shaded with a different colour grey to emphasise the monomer–monomer interface.

CK fragments despite freezing zebrafish in liquid nitrogen to prevent *postmortem* protein degradation of skeletal muscle. Since we did not identify significant degradation products from other skeletal muscle proteins in common carp, we are confident that these fragments are the products of proteolysis *in vivo*.

CK catalyses the reversible phosphoryl transfer from phosphocreatine to ADP and is found in high concentrations in tissues that have high-energy demands. Multiple isoforms of CK are thought to constitute an intricate transport and energy buffering system, which connects high-energy phosphate production to sites of energy consumption [22, 23]. As such CK is involved intimately in the maintenance of cellular energy homeostasis. The specific activity of different CK isoforms is temperature dependent [17], therefore the fact that thermal acclimation of fish results in changes in the expression of this key muscle protein should cause no surprise. More surprising perhaps was the accumulation of such partially degraded products in the cell. Most intracellular protein degradation is considered to be 'all or nothing', and to be deconstructed at least conceptually into two phases: a commitment step for degradation, and a completion/proteolysis step. The rate of the second process is generally held to be substantially higher than the commitment step, which ensures that the cell does not accumulate partial degradation products that might possess inappropriate or ectopic activity.

This study has focused on defining changes in protein expression of soluble proteins in skeletal muscle of carp undergoing thermal acclimation. Our results indicate that CK was significantly altered as result of cooling. It is possible that other proteins may be proteolysed in a similar manner but our data show no evidence of this occurrence and we were unable to detect fragments of other soluble muscle proteins.

3.4 Ubiquitin-conjugated proteins

Ubiquitin is a small (8.5 kDa), highly conserved protein that has been implicated in a range of signalling roles. Ubiquitin conjugation to proteins is a PTM that dictates the function and/or fate of that protein. Addition of a single ubiquitin molecule to a protein (monoubiquitination) is a signal for endocytosis [24], membrane trafficking [25] and DNA repair [26]. In contrast, addition of individual ubiquitin molecules at multiple sites on a protein (multiubiquitination) directs that protein to endocytosis or lysosomal degradation. Ubiquitin can also polymerise to form chains of ubiquitin molecules, linked through the ε-amino groups of one of several lysyl residues. The position of this linkage has implications for the fate of the protein to which the polyubiquitin chain binds. For example, ubiquitin chains linked through Lys_{63} will affect signalling pathways and endocytosis [24]. Lys_{48}-ubiquitin on the other hand is implicit in the targeting of proteins for proteasomal degradation [27].

Increased protein degradation is often mediated by upregulation of the ubiquitin–proteasome pathway [28]. Therefore, we performed Western blots (five replicates) for ubiquitinated proteins in the skeletal muscle from carp maintained at 30 and 10°C for 21 days (Fig. 7). These experiments revealed a number of spots that reacted to the antibody, all of which could be readily mapped to the M2 and M3 isoforms of CK and their fragments. An intense spot of low molecular weight was observed, which was attributed to ubiquitin. Interestingly, the data showed an apparent increase in the level of ubiquitinated CK fragments in the muscle of carp cooled down to 10°C at 21 days. This observation indicates that the cooling of carp may affect the degradation of CK in skeletal muscle by the ubiquitin–proteasome pathway. An increase in CK proteasomal degradation could result in the increase in protein fragmentation observed. Increased proteolysis has been implicated in atrophy of the muscle [29]. It is possible that the downregulation of metabolic enzymes such as CK may diminish energy levels in the cell leading to hampered ATP-dependent initiation of protein translation.

3.5 Transcript analysis

In order to explore the mechanisms underpinning this response, we examined relevant genes from a large dataset to determine whether mRNA levels for key proteins changed in the skeletal muscle of carp during cooling regimen [9]. This analysis revealed a decrease in the expression of M2 and M3 CK genes and increased expression of the HSP 90-β gene (Fig. 8). The relative mRNA expression of AMP deaminase increased (1.31-fold) in 10°C cooled fish. In addition, a number of key proteins involved in the ubiquitin–proteasome pathway; namely the muscle-specific ring finger protein 28 (*TRIM63*) and the F box protein 32 (*FBXO32*) had changed levels of expression (Fig. 9). These enzymes are ubiquitin ligases that promote both the ubiquitination and degradation of muscle myofibrillar contractile proteins [30], while *TRIM63* is also responsible for the ubiquitination of proteins involved in energy production, including CK [31].

Cooling down from 30 to 23°C did not affect *TRIM63* and *FBXO32* transcript levels, though transitions to 17°C and then to 10°C induced substantial, but transient, increases in transcript levels. This suggests that the response was induced only following more extreme cooling and that the response was graded according to the magnitude of the cooling imposed. Similarly, the transcript of the M2 and M3 isoforms of CK only changed significantly on cooling down to 10°C in 22 days at which point its transcript levels were eight-fold depressed. The expression of *TRIM63* mRNA was greatest after long-term acclimation (22 days) at 10°C, a pattern that is consistent with the finding that the degree of CK fragmentation is increased following 21 days of cold acclimation. Furthermore, cold acclimation was associated with a

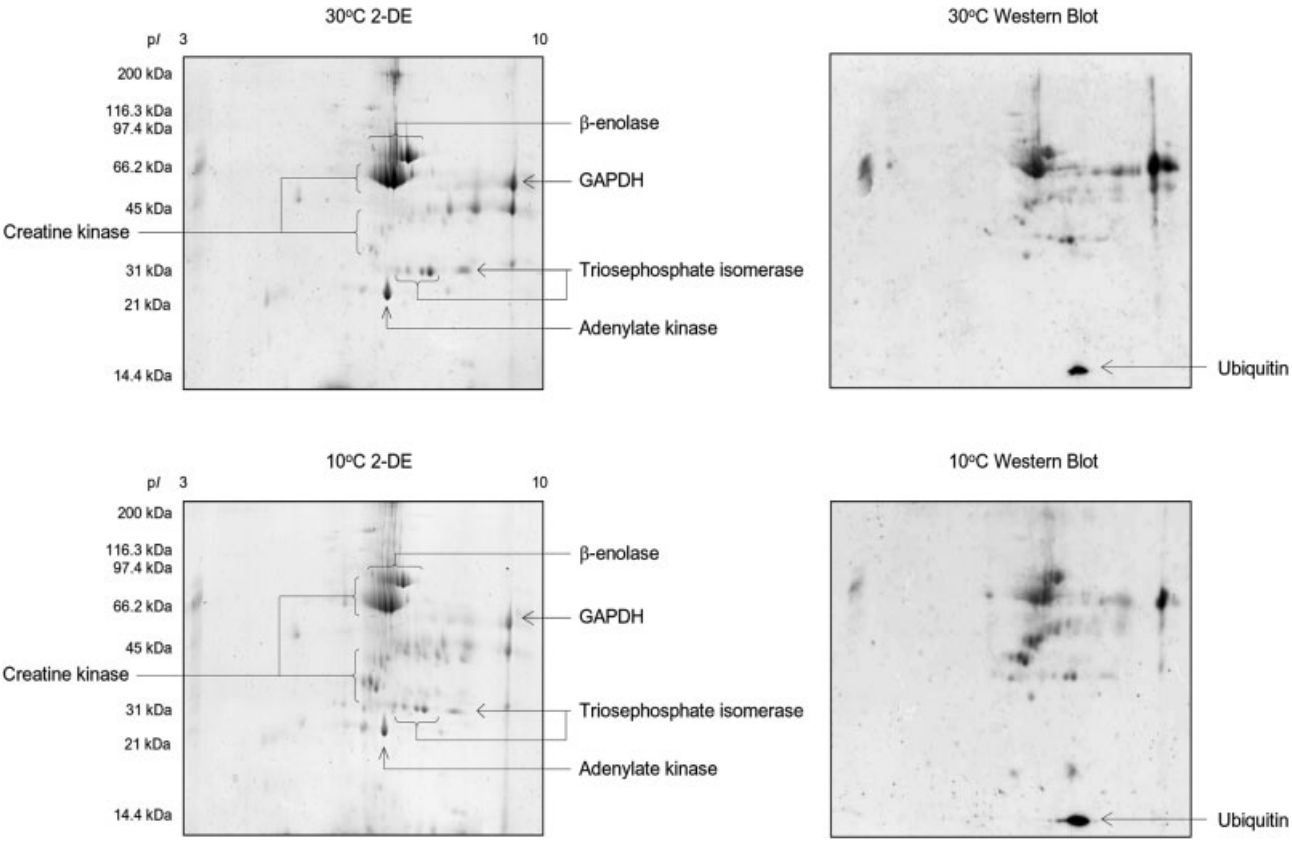

Figure 7. Western blot analysis of ubiquitinated proteins in muscle extracts from carp acclimated to 30 and 10°C for 21 days. 2-DE was performed on soluble muscle protein extract (approximately 90 μg of protein) and the separated proteins were transferred to an NC membrane prior to probing with a rabbit antihuman ubiquitin antibody. The images were recorded simultaneously. Spots of high intensity indicate a large degree of ubiquitination. Each sample was a combination of protein extracts from three individual fish.

wholesale induction of transcripts of other genes involved in ubiquitin-dependent protein catabolism and proteasomal function including proteasome subunits as well as ubiquitin itself [9].

These results lend further support to the hypothesis that remodelling and metabolic reprogramming of muscle tissue are components of its response to cooling [32]. The transcript data are consistent with an intention to reduce the abundance of CK, M2 and M3 isoforms. The rate at which this reduction in transcript can be effected is of course controlled in turn by the rate of turnover of the protein – a protein that has a low rate of turnover (=degradation) cannot respond rapidly to a change in protein synthesis. The substantial activation of the degradative machinery implies a strong signal for protein removal, and the increased CK fragments and their ubiquitination is consistent with this model.

Why then should the muscle be populated by partially degraded fragments of CK? Proteasome-mediated proteolysis is thought to be processive, and once a protein is committed to degradation, it cannot leave the substrate:26S complex until degradation is complete. However, if the degradative machinery is to function effectively, ATP must be supplied in adequate amounts. If ATP was limiting, then the 26S proteasome might slip into a 'stalled' conformation, such that the residual substrate might dissociate, or a non-covalent substrate:26S might persist sufficiently that it would be dissociated by the buffers used for 2-DE. In either instance the outcome would be the same; a gel populated with partially degraded fragments. A rather more challenging view of the accumulation of CK fragments is that they are generated to disrupt normal CK function, whether through interference in dimerisation, or by modulation of interactions between CK and other proteins in the sarcoplasm or myofibril [33]. At present, this remains conjecture, but serves to illustrate the complexity of such changes and their interpretation.

There may be many reasons as to why significant changes in the expression of other soluble muscle proteins were not observed. Conventional comparative proteomic approaches do not address the intricate dynamics of the proteome but simply provide a 'snap-shot' of the proteome at a given time under defined conditions. As such, it provides little insight into the mechanism creating differences between biological states. Changes in the amount of a specific protein

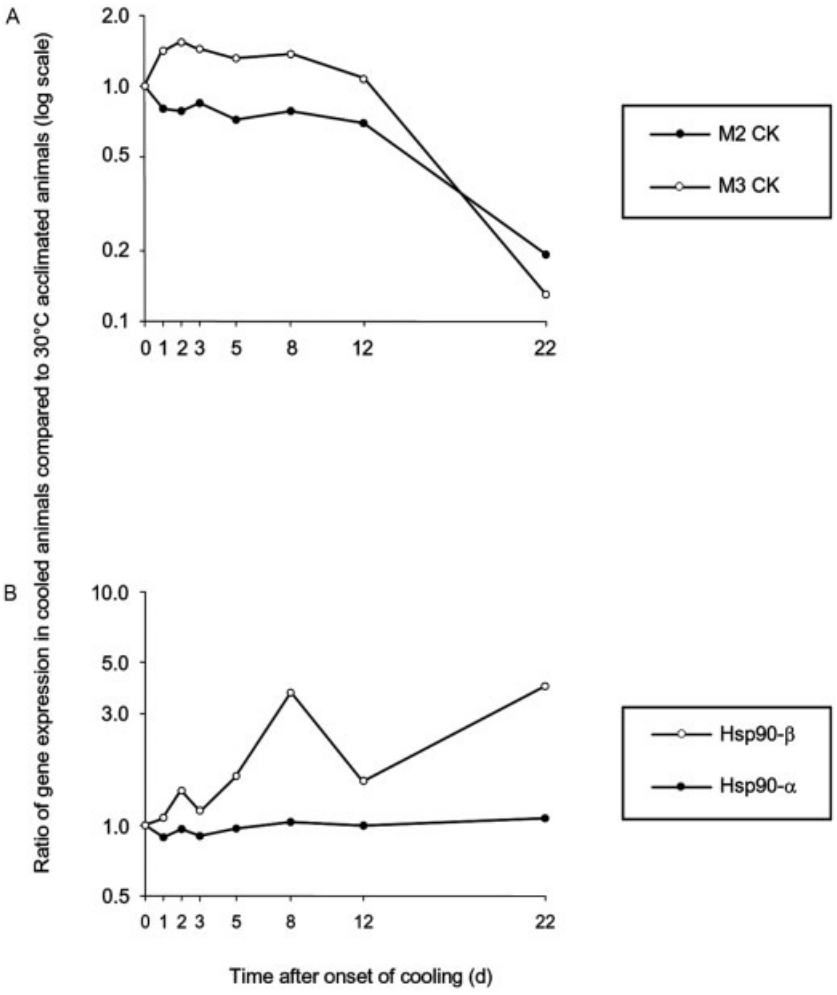

Figure 8. Cold-induced mRNA expression changes of CK and HSP-90 genes. The mRNA abundance for selected genes was extracted from a large-scale dataset that describes the gene expression changes associated with cold exposure of carp [9]. Carp acclimated to 30°C were exposed to reduced temperatures for increasing lengths of time, as indicated. The mRNA expression of both the M2 and M3 CK isoforms (panel A) was repressed in long-term cold-acclimated carp (22 days cooled). HSP 90-β was upregulated after 22 days of cold acclimation, whilst HSP 90-α showed no change in expression (Panel B).

reflect the net result of the opposing processes of protein synthesis and protein degradation [34]. However, even when the size of a protein pool remains constant, individual protein molecules are constantly being replaced. In this case, however, the rate of replacement (synthesis) is equal to the rate of removal (degradation). Therefore, monitoring only relative protein concentrations between two states may mask physiologically meaningful differences in the rate of synthesis and/or the rate of degradation of a given protein.

4 Concluding remarks

We have characterised the soluble protein complement of carp skeletal muscle and explored the changes in protein expression that accompany the transition to a cold-adapted phenotype. Chronic cold treatment of carp produces subtle changes in the pattern of soluble proteins, most notably in the appearance of multiple breakdown fragments of CK isoforms in cold-treated muscle. These findings were evident through the use of 2-DE and would almost certainly not have been picked up by a global LC-MS/MS approach. The CK fragments are ubiquitinated and likely to be undergoing proteolytic fragmentation. These protein responses can be linked to changes at the level of transcription, with two CK isoforms showing downregulated transcript expression; two ubiquitin ligases, ubiquitin and a large number of proteasomal subunits being upregulated. The data are consistent with a cold-induced enhancement of proteolysis and with a remodelling of cellular CK expression either through changes in relative isoform expression or in overall CK abundance. In future studies we propose to investigate other cellular fractions from muscle such as myofibrillar and membrane fractions, a proteome simplification strategy that has the advantage of retaining functional associations but reducing the complexity of the analyte.

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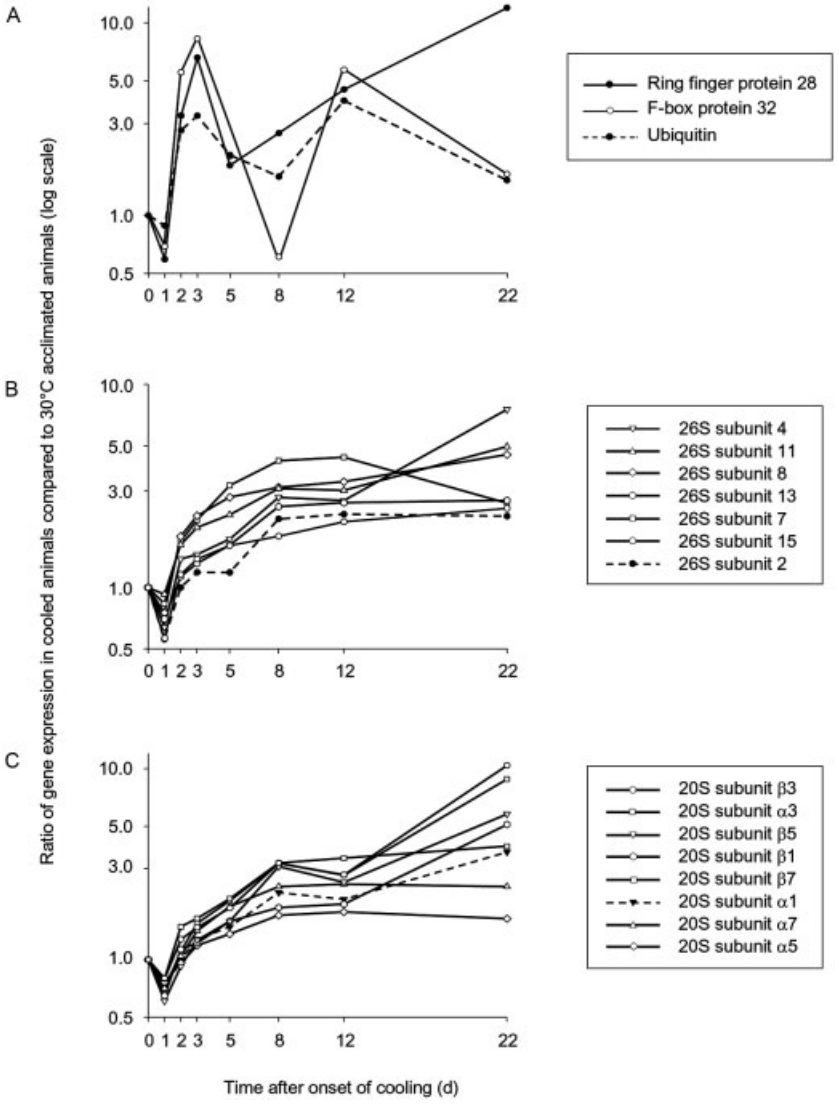

Figure 9. Cold-induced mRNA expression changes of genes in the ubiquitin–proteasome pathway. The mRNA abundance for selected genes was extracted from a large-scale dataset that describes the gene expression changes associated with cold exposure of carp [9]. Carp acclimated to 30°C were exposed to reduced temperatures for increasing lengths of time, as indicated. Changes in expression of ubiquitin and ubiquitin ligase genes are shown in panel A. Panel B and panel C show changes in gene expression of components of 26S and 20S proteasome, respectively. The onset of cooling induces a strong induction of ubiquitin transcript together with the increased expression of two pivotal ubiquitin ligases that mediate muscle atrophy. Cooling is also associated with the coregulated induction of most of the subunits of the proteasome apparatus.

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