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Protein profile of Dabry's sturgeon (*Acipenser dabryanus*) spermatozoa and relationship to sperm quality

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ABSTRACT

Knowledge of conditions affecting sperm quality is essential for efficient culture of fish for commercial purposes and conservation of species. Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry were used to characterize the proteomic profile of *Acipenser dabryanus* spermatozoa relative to motility and fertilization capacity. There were differential amounts of protein in 313 spots in spermatozoa of males classified to have relatively greater or lesser spermatozoa quality. The functions of 43 of 50 selected proteins were identified. The proteins in 14 spots were involved in metabolism, and of these, proteins in 11 spots were highly abundant in spermatozoa of males categorized to have spermatozoa of greater quality, including pyruvate kinase, enolase B, phosphoglycerate kinase, lactate dehydrogenase, cytosolic malate dehydrogenase, brain creatine kinase b, Ckmb protein, and nucleoside diphosphate kinase. The proteins involved in mechanics of flagellum movement were identified, including the dynein intermediate chain, radial spoke head 1 homolog; ropporin-1-like, Bardet-Biedl syndrome 5, ADP-ribosylation factor-like protein 3, tektin-4, gamma-actin, and tubulin cytoskeleton proteins to be differentially abundant in spermatozoa that were classified relatively greater or lesser quality. Heat shock proteins, copper/zinc superoxide dismutase and peroxiredoxins, which are involved in stress response were of differential abundance in spermatozoa from males with spermatozoa in the two different classification groups. Proteins were also detected that are involved in protein folding and binding, or hydrolase activity. The results are valuable for the prediction of sperm quality and for reproduction management in *A. dabryanus* and other threatened species.

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1. Introduction

Dabry's sturgeon *Acipenser dabryanus* (Acipenseriformes) are found only in the Yangtze River of China. It is listed in the first class of national protected animals in China, as critically endangered in the International Union for Conservation of Nature list, and in Appendix II of animal protection in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Zhuang et al., 1997; Kynard et al., 2003; Zhang et al., 2011). A sustainable population may be potentially restored via artificial reproduction and germplasm conservation.

Sperm quality has an important role in reproduction and in sperm cryopreservation, which is especially important for endangered species. In sturgeon, spermatozoa quality is usually characterized by the relative (i.e., greater or lesser) sperm motility, presence of an intact acrosome, membrane integrity, DNA integrity, reactive oxygen species (ROS) concentration, and fertilization capacity (Dzyuba et al., 2014; Shaliutina-Kolešová et al., 2015). Study of protein components that determine the specific properties of fish sperm associated with quality is limited. Proteomic studies of spermatozoa have been mostly conducted in mammals and humans and related to male fertility or disease (Martinez-Heredia et al., 2006; Baker et al., 2008; Peddinti et al., 2008; Wang et al., 2013). In sparids (*Sparus aurata, Lithognathus mormyrus*), Zilli et al. (2008) detected some proteins that were phosphorylated after spermatozoa motility activation include an A-kinase anchor protein (AKAP), an acetyl-coenzyme A synthetase, a protein phosphatase inhibitor, a phosphatase (myotubularin-related protein 1) and a kinase (DYRK3). Forne et al. (2009) found that F1 males in the farmed flatfish Senegalese sole (*Solea senegalensis*) reared in captivity often have less sperm production and fertilization capacity than wild-caught males. There was also detection of some proteins implicated in oxidoreductase activity, protein catabolism, formation of the zona pellucida receptor, cytoskeleton organization, and lipid binding and metabolism. These proteins were regulated in the F1 testes as germ cell development progressed and regulation of these proteins may be related to the poor reproductive performance of Senegalese sole F1 males. In carp (*Cyprinus carpio*), Li et al. (2010a, 2013) reported that cryopreservation caused changes in spermatozoa protein profiles, and subsequently may lead to a decrease in sperm velocity, motility, fertilization success, and ova hatching rate. Furthermore in this study, there was found to be phosphorylation and/or dephosphorylation modifications of sperm proteins that occur during cryopreservation that could stimulate a series of biochemical effects interfering with spermatozoa function and leading to a loss of motility and fertilization capacity. Research into the proteome of sturgeon sperm has been conducted in *Acipenser baerii, A. ruthenus, A. gueldenstaedtii, A. stellatus, Huso huso, Polyodon spathula,* and it was the first application of the proteomics for differential characterization and comparative studies of acipenseriform species at the molecular level (Li et al., 2010b).

The objective of the present study was to characterize the proteome of *A. dabryanus* spermatozoa to ascertain the relationship of these proteins with conventional sperm quality variables. This is the first report of the protein profile of *A. dabryanus* spermatozoa and the relationship of these proteins to sperm quality. This information could be of great value for optimization of reproduction and the development of cryopreservation techniques.

2. Materials and methods

2.1. Sperm and egg collection

Mature male and female *A. dabryanus* were reared at the Hatchery for Chinese Sturgeon at Jingzhou, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Spermiation was induced in ten males by intramuscular injection at ~5 μg/kg body weight (BW) luteinizing hormone-releasing hormone A2 (LRH-A2) and 0.5 mg/kg BW domperidone (DOM). After 12 h, sperm were obtained by gentle abdominal massage, taking special care to avoid blood, urine, or fecal contamination. Ovulation was induced in three females by intramuscular injection of 5 μg/kg BW LRH-A2 24 h before stripping, followed by a second injection of 9 μg/kg BW LRH-A2, 1 mg/kg DOM, and 1 mg/kg vitamin B1 12 h before stripping. Eggs were obtained by abdominal massage and stored in dry bowls. Sperm quality assessment, including motility and fertility, was conducted immediately.

For proteome analysis, 1 ml sperm from each male was individually centrifuged at 1000 × *g* for 10 min immediately after collection at 4 °C. After centrifugation, the supernatant was discarded and the pellets were stored at -80 °C until analysis.

2.2. Sperm quality analysis

Spermatozoa were activated with distilled water, and values for motility variables were determined using microscopy (×10, Olympus CX-22). Percent of motile spermatozoa immediately after activation and duration of motility were recorded by three independent observers. The period of duration for rapid motility was measured from activation to the time point at which spermatozoon movement slowed sufficiently to enable distinguishing approximate shape and trajectory of movement of most spermatozoa. The mean of three repetitions per male was calculated.

For fertilization assessment, eggs from three females were combined. Sperm from each male was used to fertilize 100 to 120 eggs (~0.4 g). The spermatozoon:egg ratio was 10^5:1 with spermatozoa density calculated by hematocytometer. Fertilization rate was measured at Stage 17, the small yolk plug period, as the morphological characteristics of Stage 17 are easily distinguished. The fertilization studies were performed in triplicate.

An ANOVA was used to compare mean % motile spermatozoa, motility duration, and fertility among males. Differences were considered significant at $P < 0.05$. Spermatozoa of males categorized to have spermatozoa of relatively greater quality (Males 1, 2 and 7) and lesser quality (Males 5, 6 and 8) were grouped based on the motility and fertility data.

2.3. Protein extraction

Spermatozoa from individuals in each group were pooled for protein extraction. Total protein was extracted using the cold acetone method. Ethylenediaminetetraaceticacid (EDTA, 2 mM), phenylmethanesulphonyl fluoride (PMSF, 1 mM), and dithiothreitol (DTT, 10 mM) were added, and the samples were homogenized to disrupt the cells. The samples were centrifuged at 25,000 × *g* for 20 min at 4 °C, and the pellets were discarded. Dithiothreitol (10 mM) at five-fold the volume of cold acetone was added to the samples, followed by 12 h incubation at −20 °C and centrifugation at 25,000 × *g* at 4 °C for 20 min. The supernatant was discarded. To reduce acidity, the pellet was washed with 10 mM DTT in 1.5 mL cold acetone, centrifuged at 25,000 × *g* at 4 °C for 20 min, and the acetone wash was repeated. The precipitate was dried in a vacuum concentrator for 5 min, and the dried pellet was stored at −80 °C.

2.4. 2-DE analysis

The dried protein pellets were homogenized in 200 μL lysis buffer (2 M thiourea, 7 M urea, 2% DTT, 20 mM Tris base, 4% CHAPS, 1% protease inhibitor cocktail, 20 μL/mL Bio-Lytes 3/10, 0.5 μL benzonase). The solution was centrifuged at 12,000 × *g* for 20 min at 4 °C, the supernatant was collected, and its protein content was quantified using a 2-DE Quant Kit (GE Healthcare, Piscataway, NJ, USA). Immobilized pH gradient (IPG) strips (pH 4–7, 24 cm, GE Healthcare) for the first dimension were used with running conditions of 20 °C, Step 1: 300 V for 0.5 h, Step 2: 700 V for 0.5 h, Step 3: 1500 V for 1.5 h, Step 4: 9900 V for 3 h, Step 5: 9900 V for 6.5 h, Step 6: 600 V for 20 h, Step 7: 8000 V constant for a total of 56 000 Vh. After completion of the isoelectric focusing program, the strips were equilibrated by 15 min in an IPG equilibration buffer comprising 6 M urea, 2% SDS, 30% glycerol, 0.375 M Tris (pH 8.8), 20 mg/mL DTT, and a trace of bromophenol blue and 15 min alkylation. Subsequently, 12.5% SDS-PAGE 2-DE was conducted. Electrophoresis was conducted at 20 mA per gel for 40 min and then at 30 mA per gel until the dye front reached the bottom of the gel. The spots containing proteins were revealed via either silver staining or Coomassie Brilliant Blue G-250 staining. Triplicate 2-DE gels were used to assess spermatozoa of males categorized to have spermatozoa with relatively greater (Males 1, 2 and 7) and lesser quality (Males 5, 6 and 8). The gels were analyzed for spot intensity using Image Master 2D Platinum software (GE Healthcare) according to the manufacturer's protocol. The criterion for differences in abundance of protein was a difference ≥ than two-fold for spermatozoa of males that were categorized to have spermatozoa of relatively greater or lesser quality.

2.5. Protein identification

There were fifty spots in the gels with significant abundances of protein that were selected and excised from the 2-DE gels. Gel spots containing proteins were washed and digested with sequencing-grade trypsin (Promega, Madison, WI, USA). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and TOF/TOF tandem MS were performed on a MALDI-TOF-TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) set in reflector mode. Peptide mass fingerprints coupled with peptide fragmentation patterns were used to identify the protein in the International Protein Index (IPI) (http://www.ebi.ac.uk/IPI/IPIhelp.html) database (v3.67) using the MASCOT search engine (http://www.matrixscience.com). The functions and specific processes of these proteins were obtained by searching Gene Ontology (www.geneontology.org).

2.6. Western blot analysis

Of the proteins in differential abundance in spermatozoa from males with the two sperm quality categorizations, creatine kinase (CKMT1A), L-lactate dehydrogenase B-A chain (LDHB), and malate dehydrogenase (MDH2) were selected for western blot analysis to validate abundances in spermatozoa of males with the two sperm quality categorizations, and the specificity of the three antibodies (anti-CKMT1A, anti-LDHB and anti-MDH2) was confirmed. Western blot samples were prepared in ice-cold RIPA Lysis Buffer containing 1 mM PMSF. The protein concentration was determined using the BCA assay kit (Beyotime, Shanghai, China). Equal quantities of total protein from each male were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Milipore). After blocking with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with each primary antibodies, respectively, overnight at 4 °C [anti-CKMT1A 1:400 (Atagenix); ATApla8239, rabbit anti-LDHB 1:400 (Atagenix); ATApla5470, rabbit; anti-MDH2 1:400 (Atagenix); ATApla1906, rabbit; GAPDH 1:5000 (Proteintech) 10494-1-AP, rabbit]. After washing with TBST three times, the membrane was incubated with the corresponding secondary antibody conjugated to Horseradish Peroxidase (1:5000, Atagenix) for 1 h at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Western blot analysis was triplicated, and Student's *t*-test was used to compare abundances of three proteins abundances in spermatozoa of males with the two sperm quality categorizations.

3. Results

3.1. Sperm quality analysis

There were differences among individuals for values of spermatozoon quality. There were relatively greater proportions of motile spermatozoa for Males 1, 2, 7, and 9 while Males 5, 6, and 8 had relatively lesser spermatozoa motility with Males 3, 4, and 10 having spermatozoa motility that was categorized as being at a medium between that of the other two groups of males. The values for duration, of rapid spermatozoa motility were greater for Males 1, 2, 7, therefore, spermatozoa from these males were categorized as

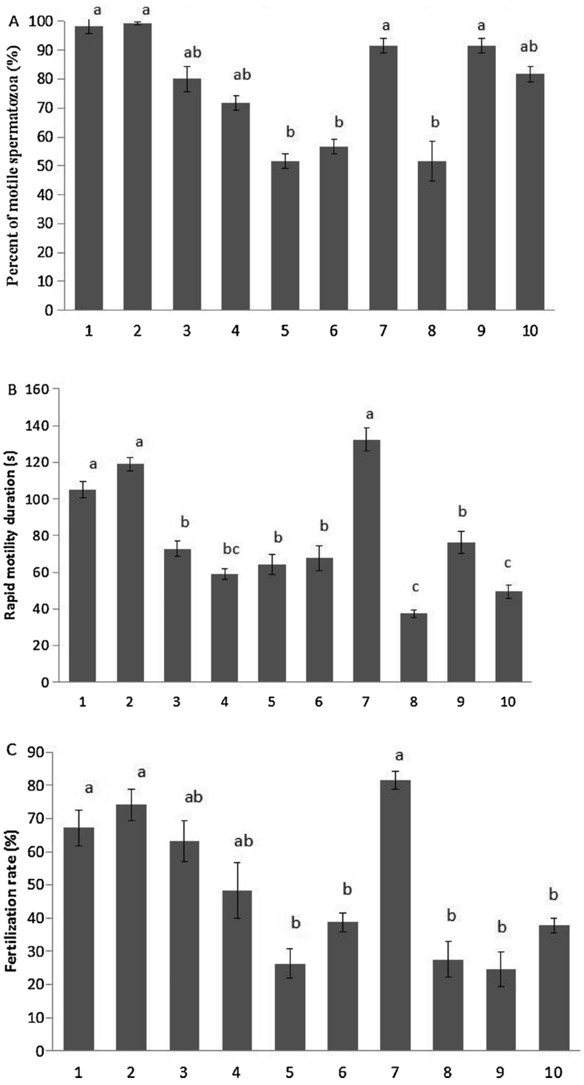

Fig. 1. Values for sperm quality variables of individual *Acipenser dabryanus.* (A) percent motile spermatozoa, (B) duration of period of rapid motility, (C) fertilization rate; Values are presented as the mean ± SD; Values with different alphabetical superscripts are different (ANOVA – Tukey test at $P < 0.05$).

being of relatively greater quality and those from the other males being categorized as being of relatively lesser quality. The fertilization rate for Males 1, 2, and 7 was relatively greater than for Males 5, 6, 8, 9, 10 which were categorized as having relatively lesser fertilization rates Furthermore, Males 3 and 4 were categorized as having a medium fertilization rate. When values for all sperm quality variables were considered, Males 1, 2, and 7 were considered to have spermatozoa with relatively greater quality, and Males 5, 6 and 8 were categorized as having spermatozoa with relatively lesser quality (Fig. 1).

3.2. Proteomics analysis of spermatozoa

The spermatozoon proteins of males categorized to have spermatozoa with relatively greater and lesser quality were analyzed by 2D polyacrylamide gel electrophoresis map (Fig. 2). On average, more than 3000 spots containing proteins were detected in each gel. There were a total of 168 proteins that were in lesser abundance (Spots A1-168, Fig. 2A) in spermatozoa of males that were categorized to have lesser quality spermatozoa compared with the abundance of these proteins in males categorized to have spermatozoa of greater quality. Furthermore, there were 145 proteins (spots B1-145, Fig. 2B; greater than 2-fold difference) in relatively greater abundance in spermatozoa of males that were categorized to be of lesser as compared with those categorized to be of greater quality. Fifty spots with proteins of greatest abundance were subjected to peptide analysis using MALDI-TOF-TOF MS, and 43 spots were identified (Table 1). Of the identified proteins, 14 were involved in metabolism, 11 were characterized as cell cytoskeleton proteins, five were active in stress response, and the other 13 were categorized as having folding or binding functions.

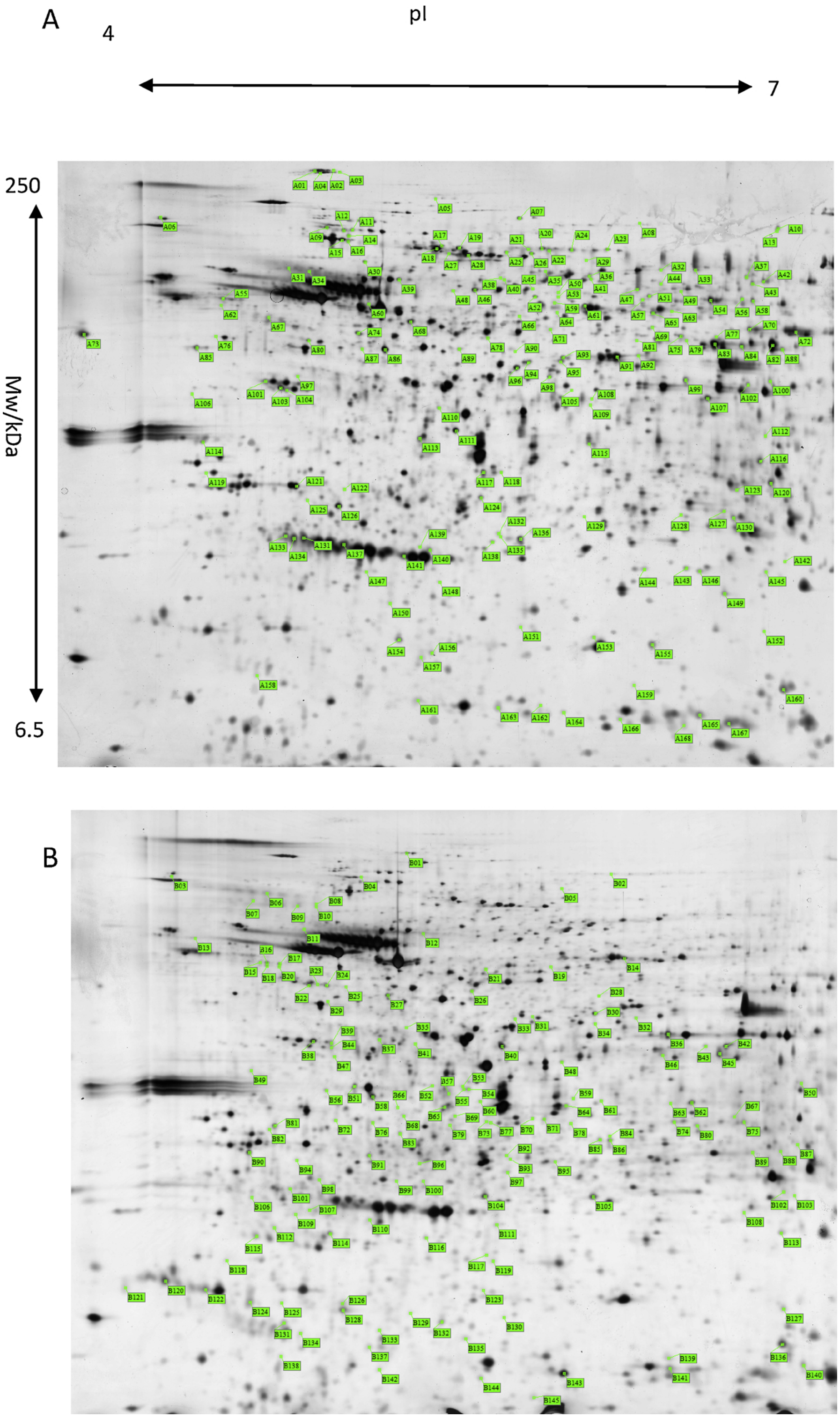

Fig. 2. Proteins in differential abundance from *A. dabryanus* males with spermatozoa categorized to have a relatively greater and lesser spermatozoa quality; Protein abundances determined with two-dimensional polyacrylamide gel electrophoresis; (A) Group with relatively greater (Males 1, 2, and 7) and (B) relatively lesser (Males 5, 6, and 8) spermatozoa quality.

Table 1
Proteins in differential abundances as identified by 2DE and MALDI-TOF-MS/MS in *A. dabryanus* males categorized to have spermatozoa of relatively greater or lesser quality.

Spot no.	Reproduction_Unigene_BMK	Protein name	MM	PI	MS	SC %	Matches	Sequence	IS	FC	Function
	Metabolism										
A61	75696|g.139545	pyruvate kinase, muscle isoform 1 [*Xenopus (Silurana) tropicalis*]	58543	6.59	166	29	11	R.EAEAAIFHR.Q	36	1/∞	pyruvate metabolic process
A72	57714|g.75625	enolase B [*Acipenser baerii*]	48239	7.26	377	42	15	R.AAVPSGASTGIYEALELR.D	142	0.45	pyruvate metabolic process
A82	69611|g.113862	phosphoglycerate kinase [*Acipenser baerii*]	51065	8.32	420	41	13	K.LGDVYINDAFGTAHR.A	106	0.39	glucose catabolic process; pyruvate metabolic process
A83	69611|g.113862	phosphoglycerate kinase [*Acipenser baerii*]	51065	8.32	397	43	14	K.LGDVYINDAFGTAHR.A	102	0.49	glucose catabolic process; pyruvate metabolic process
A160	81168|g.172453	RecName: Full = L-lactate dehydrogenase B-A chain; Short = LDH-B-A	36629	6.51	88	11	4	K.SADTLWGIQK.D	59	1/∞	pyruvate metabolic process; carbohydrate metabolic process
A107	68095|g.108173	cytosolic malate dehydrogenase [*Acipenser brevirostrum*]	36472	6.25	359	38%	13	K.IVDGLAINDFSR.G	106	0.33	carbohydrate metabolic process; dicarboxylic acid metabolic process; citrate metabolic process
B91	77085|g.146371	PREDICTED: glycogen phosphorylase, muscle form-like, partial [*Anolis carolinensis*]	13630	9.61	145	23%	3	K.LITAIGDIVNNDPVVGDR.L	119	∞	Carbohydrate metabolism
A91	59440|g.80152	brain creatine kinase b [*Danio rerio*]	43109	5.69	358	41	14	R.GTGGVDTAAVGGVFDISNA-DR.L	78	0.43	Amino acid metabolism
A93	59440|m.80152	brain creatine kinase b [*Danio rerio*]	43109	5.69	153	31	14	K.TFLVWVNEEDHLR.V	50	0.27	Amino acid metabolism
A117	27642|g.27940	Ckmb protein [*Danio rerio*]	19503	7.92	28	7	1	K.TFLVWVNEEDHLR.V	28	0.25	Amino acid metabolism
B45	70460|g.117146	PREDICTED: glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic-like [*Takifugu rubripes*]	38697	6.28	376	56	18	K.DADILVFVIPHQFIGR.L	94	7.5	Lipid metabolism
B62	70460|g.117146	PREDICTED: glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic-like [*Takifugu rubripes*]	38697	6.28	325	47	16	K.DADILVFVIPHQFIGR.L	82	2.92	Lipid metabolism
A28	56162|g.71712	NME/NM23 family member 9 [*Xenopus laevis*]/ nucleoside diphosphate kinase	12053	5	208	51	6	K.LQEAGFILVTEEQR.H	114	0.35	cellular energy production
A155	57523|g.75132	Nme2 protein [*Danio rerio*]/nucleoside diphosphate kinase 2	17316	6.75	420	54	10	R.TFIAIKPDGVQR.G	95	0.15	cellular energy production
	Cytoskeleton										
A68	70591|g.117632	dynein intermediate chain [*Cynops pyrrhogaster*]	37600	5.01	133	31	8	K.DVNPQEMEQTIR.Y	65	0.44	microtubule-based process
A103	45264|g.50828	radial spoke head 1 homolog [*Xenopus laevis*]	31951	4.84	290	46	12	K.YEGGWVDDQR.F	76	0.35	mechanical movement of the flagellum; cellular developmental process
A134	60823|g.84147	PREDICTED: ropporin-1-like [*Anolis carolinensis*]	24021	5.3	235	23	3	R.ALTYLEEEVVGTEGLVR.V	117	0.22	mechanical movement of the flagellum
A86	72847|g.126751	Bardet-Biedl syndrome 5 [*Xenopus (Silurana) tropicalis*]	41834	5.36	222	30	10	R.FEFIFTNVVPGSPR.L	62	0.42	mechanical movement of the flagellum
A143	39947|g.43104	PREDICTED: ADP-ribosylation factor-like protein 3 [*Xenopus (Silurana) tropicalis*]	20674	6.75	197	52	7	K.QDLLTAAPASEITEGLNLHV-IR.D	52	0.21	nucleoside-triphosphatase activity; tubulin binding
B14	52979|g.64621	tektin-4 [*Xenopus laevis*]	17164	5.23	179	44	6	K.DSVEVELLNELDLIR.S	84	2.67	mechanical movement of the flagellum
B27	76474|g.143255	RecName: Full = Actin, cytoplasmic 2; AltName: Full = Gamma-actin; N-terminally processed	42080	5.3	551	47	14	K.SYELPDGQVITIGNER.F	130	2.42	structural molecule activity; transcription factor binding; kinase binding; cytoskeletal protein binding
B30	74221|g.132871	PREDICTED: tubulin alpha-1 A chain isoform 1 [*Danio rerio*]	42691	5.78	245	19	6	R.FDGALNVDLTEFQTNLVPY-PR.I	94	2.28	mechanical movement of the flagellum
B40	74221|g.132871	PREDICTED: tubulin alpha-1 A chain isoform 1 [*Danio rerio*]	42691	5.78	207	15	4	R.AVFVDLEPTVIDEVR.T	69	2.32	mechanical movement of the flagellum
B58	74941|g.136132	Zgc:55461 [*Danio rerio*]/tubulin, beta 2B [*Danio rerio*]	50241	4.79	423	27	14	K.GHYTEGAELVDSVLDVVR.K	123	2.64	mechanical movement of the flagellum

(*continued on next page*)

Table 1 (*continued*)

Spot no.	Reproduction_Unigene_BMK	Protein name	MM	PI	MS	SC %	Matches	Sequence	IS	FC	Function
B120	74941|g.136132	Zgc:55461 [*Danio rerio*]/tubulin, beta 2B [*Danio rerio*]	50241	4.79	172	14	9	R.ISEQFTAMFR.R	70	2.23	mechanical movement of the flagellum
	Response to stress										
A19	64954|g.97200	heat shock 70 kDa protein-like isoform X1 [*Lepisosteus oculatus*]	26013	5.17	371	35	6	K.VSNAVITVPAYFNDSQR.Q	91	0.21	response to stress
A80	64955|g.97204	heat shock protein 70 [*Gobiocypris rarus*]	30011	7.94	221	22	7	K.LLQDFFHGR.E	59	0.22	response to stress
A165	57711|g.75610	copper/zinc superoxide dismutase [*Anguilla japonica*]	16149	5.5	321	28	4	K.GGNDESLVTGNAGGR.L	122	1/∞	antioxidant activity; cation binding; catalytic activity
B104	49758|g.58341	peroxiredoxin-1-like [*Lepisosteus oculatus*]	15006	6.14	197	42	5	R.QITVNDLPVGR.S	56	18.5	protein binding; peroxidase activity/RNA binding
B105	58364|g.77249	Thioredoxin-dependent peroxide reductase, mitochondrial precursor [*Salmo salar*]	28931	7.25	238	23	6	R.DYGVLLEGPGIALR.G	115	∞	peroxidase activity; cysteine-type endopeptidase inhibitor activity; kinase binding
	Other functions										
A12	72402|g.125006	Protein disulfide isomerase associated 4 [*Danio rerio*]	73020	4.94	238	20	12	K.YSMEPEEFDSDVLR.E	73	0.42	protein folding
A46	67897|g.107468	protein disulfide-isomerase A3 precursor [*Danio rerio*]	63841	5.54	337	32	16	K.ALEQFLQDYFDGNLKR.Y	91	0.41	protein folding
A39	62085|g.87833	T-complex protein 1 subunit theta [*Oncorhynchus mykiss*]	57456	5.05	152	22	11	-.YYSGLEEVVHR.N	31	0.18	protein binding; sperm-egg recognition
A126	64935|g.97160	T-complex protein 1 subunit theta [*Oncorhynchus mykiss*]	60041	5.33	34	1	1	K.FAEAFEAIPR.A	34	0.44	protein binding; sperm-egg recognition
A73	69755|g.114380	reticulocalbin 2, EF-hand calcium binding domain [*Xenopus (Silurana) tropicalis*]	38168	4.34	430	48	12	R.DPGAENDAEWTIVER.E	114	0.17	calcium binding
A121	63509|g.92502	ubiquitin carboxyl-terminal hydrolase isozyme L1 [*Danio rerio*]	25421	4.94	83	20	3	R.ATHDEVAAEGQCR.V	57	1/∞	endopeptidase activity; exopeptidase activity; adrenergic receptor binding; small conjugating protein binding
A167	44067|g.48988	cystatin B (stefin B) [*Gallus gallus*]	11457	6.28	204	62	5	K.VHVGADEYLHLR.V	105	1/∞	RNA binding; endopeptidase inhibitor activity; enzyme binding
B01	78173|g.152100	transitional endoplasmic reticulum ATPase [*Danio rerio*]	89942	5.14	247	23	22	K.MDELQLFR.G	39	3.1	peptidase activator activity; ubiquitin binding; protease binding; phosphatase binding; nucleoside-triphosphatase activity; RNA binding
B16	62980|g.90794	UV excision repair protein RAD23 homolog A [*Salmo salar*]	41397	4.75	100	19	6	R.AVEYLLTGIPVER.E	48	3.05	Replication and repair; Folding, sorting and degradation
B17	62980|g.90794	UV excision repair protein RAD24 homolog A [*Salmo salar*]	41397	4.75	70	9	4	R.AVEYLLTGIPVER.E	39	∞	Replication and repair; Folding, sorting and degradation
B22	62721|g.89919	PREDICTED: NSFL1 cofactor p47-like isoform 1 [*Danio rerio*]	44105	5.12	253	32	15	R.NYSDPGNAQFLESIR.R	66	∞	Folding, sorting and degradation
B03	28643|g.29147	uncharacterized protein	17434	4.78	65	11	2	K.EYQVVVFQR.V	43	∞	unknown
B06	28643|g.29147	uncharacterized protein	17434	4.78	69	5%	1	K.EYQVVVFQR.V	63	∞	unknown

Spot numbers refer to those in Fig. 1.
'∞' means infinity.
MM - Molecular mass (Mr).
MS - Mascot score.
SC - Sequence coverage.
IS - Ion score.
FC - Fold change.

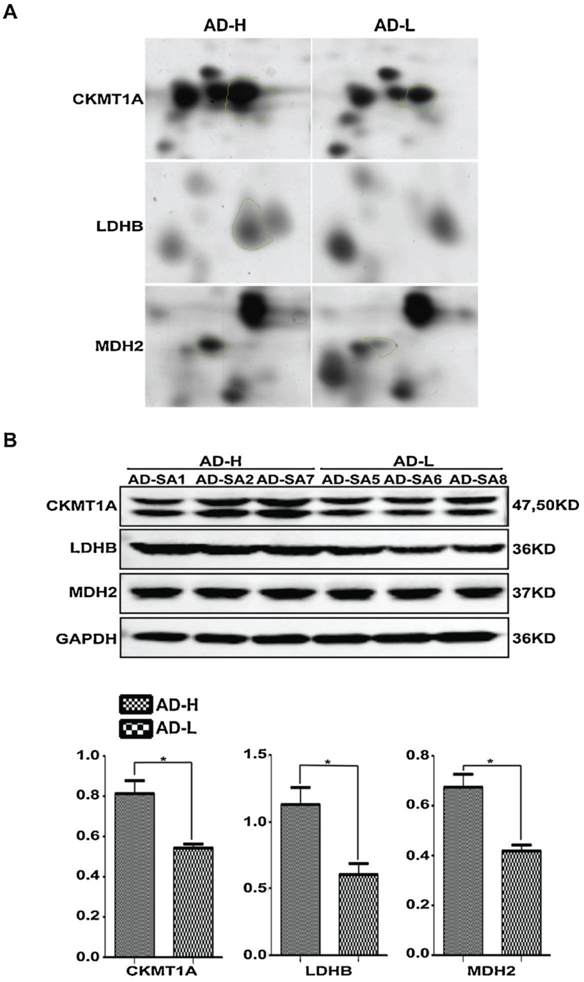

Fig. 3. Verification of proteins in differential abundance from *A. dabryanus* spermatozoa of males with relatively greater (Males 1, 2, and 7) and lesser (Males 5, 6, and 8) spermatozoa quality. Verifications were conducted using the western blot procedure; (A) Magnified images of spots containing *A. dabryanus* proteins CKMT1A, LDHB, and MDH2 from 2-DE gels; (B) Western blot analysis of CKMT1A, LDHB, and MDH2 proteins in spermatozoa from *A. dabryanus* males categorized to have relatively greater (AD-H, Males AD1, AD2, and AD7) and lesser (AD-L, Males AD5, AD6, and AD8) quality spermatozoa and quantification of abundance of protein; $^*P < 0.05$; GAPHD was used as the loading control.

3.3. Verification by western blot

Of the candidates, CKMT1A, LDHB, and MDH2 were selected for western blot analysis to validate the abundance of protein (Fig. 3). Differential abundances of the selected proteins was consistent with the 2-DE results (Fig. 3A and B). The abundances of CKMT1A, LDHB, and MDH2 were greater ($P < 0.05$) in spermatozoa categorized to be of relatively greater as compared with those categorized to have spermatozoa of relatively lesser quality.

4. Discussion

Studies have reported fish sperm quality to be influenced by a variety of factors, including species, breed, age, nutrition, season, general health condition, endocrine system function, amount of sexual stimulation, stress factors, and testicular size (Hajiahmadian et al., 2016; İnanan and Yılmaz, 2018; Valcarce and Robles, 2018). Proteomic analyses are emerging as techniques for characterizing specific protein profiles, particularly for studying the protein composition of spermatozoa and seminal plasma with respect to the regulation of motility, capacitation, acrosome reaction, and fertilization, in addition to establishing biomarkers for male infertility (Pilch and Mann, 2006; Yamakawa et al., 2007; Liao et al., 2009; Batruch et al., 2011; Thacker et al., 2011; Milardi et al., 2013; Sharma et al., 2013).

In the present study, 14 spots containing proteins involved in metabolism were in differential abundances in spermatozoa of males with the two sperm quality categorizations. Of these, 11 proteins were in greater relative abundance in the spermatozoa of males categorized as having spermatozoa of greater quality, including pyruvate kinase, enolase B, phosphoglycerate kinase, lactate dehydrogenase, cytosolic malate dehydrogenase, brain creatine kinase b, Ckmb protein, and nucleoside diphosphate kinase (Table 1). The metabolism rate in male gametes is greater than in many cell types, with there being an abundance of enzymes involved in metabolic pathways including glycolysis, the tricarboxylic acid cycle, fatty acid oxidation, and the respiratory chain (Hafez and Hafez, 2002). Pyruvate kinase, enolase, phosphoglycerate kinase, and lactate dehydrogenase are major enzymes in the glycolytic pathway that when activated result in an energy transfer that is required for spermatozoon motility and fertilization and these enzymes have been considered to be potentially important biomarkers of sperm quality in boars (Feiden et al., 2007), bulls (Fernández and Córdoba, 2017), mice (Burgos et al., 1995), and humans (Jiang et al., 2015), as well as in fish including carp and sturgeon (Lahnsteiner et al., 1996; Piros et al., 2002; Li et al., 2010a,b; Aramli, 2015; Dzyuba et al., 2016). Malate dehydrogenase is a key enzyme in the malate/aspartate shuttle across the mitochondrial membrane, and in the tricarboxylic acid cycle within the mitochondrial matrix (Minarik et al., 2002). The creatine kinase shuttle is a source of extra-mitochondrial ATP and responsible for transfer of energy from mitochondria to the cytosol (Kaldis et al., 1996; Wallimann et al., 2011). Nucleotide diphosphate kinase, which catalyzes phosphoryl exchanges between nucleoside di- and tri-phosphates, may have functions in the phosphotransfer network involved in spermiogenesis and flagellar movement (Munier et al., 2003). These proteins that are in differential abundance could be evaluated to study processes of spermatogenesis in vivo and sperm quality in vitro, potentially serving as biomarkers of male infertility (McCarrey et al., 1992; Yoshioka et al., 2007; Danshina et al., 2010). In the present study, CKMT1A, LDHB, and MDH2 were detected by western blot, consistent with the proteomic results; hence these proteins could be used as potential biomarkers of sperm quality in *A. dabryanus*.

In the present study, there was identification of the dynein intermediate chain, radial spoke head 1 homolog; ropporin-1-like; Bardet-Biedl syndrome 5; ADP-ribosylation factor-like protein 3; tektin-4; gamma-actin; and tubulin cytoskeleton proteins involved in spermatozoon flagellum assembly, flagellum motility, and spermatozoon motility. These proteins were in differential abundances in spermatozoa of males with the two sperm quality categorizations. Dynein intermediate chain, radial spoke head 1 homolog, ropporin-1-like, Bardet-Biedl syndrome 5, and ADP-ribosylation factor-like protein 3 were in a lesser relative abundance in spermatozoa of males that were classified to be of lesser compared with those with spermatozoa of a relatively greater quality. The dynein intermediate chain, radial spoke head protein 9, ropporin, and Bardet-Biedl syndrome 5 are localized in the flagellum, and mutations adversely affect spermatozoon motility and fertility (Fujita et al., 2000; Eddy et al., 2003; Smith and Yang, 2004; Wemmer and Marshall, 2004; Li et al., 2004; Fiedler et al., 2008; Fatima, 2011).

Tektin-4, gamma-actin, and tubulin were in greater abundance in the spermatozoa of males categorized to have relatively lesser compared with those with spermatozoa of greater quality. Roy et al. (2007) observed that tektin-4-null spermatozoa had a marked reduction in forward velocity and uncoordinated waveform propagation along the flagellum. Results of studies have confirmed that the relatively abundance of tubulin is related to sperm motility: Carbon ion irradiation induces a reduction of tubulin in mouse sperm, resulting in lesser motility of these cells (Hong et al., 2014). Results of human studies indicate the abundance of beta-tubulin is less in asthenozoospermic than normospermic individuals (Peknicova et al., 2007; Shen et al., 2013). In the present study, it is unclear why there are greater abundances of these proteins in the gametes from males with lesser quality spermatozoa.

Antioxidants in the male reproductive tract provide a primary defense against oxidative stress caused by ROS, which compromise sperm function and male fertility. Heat shock proteins (HSP) are a group of proteins that provide thermo-tolerance in cells and protect cells against apoptosis during injury and oxidative stress (Beere and Green, 2001). Heat shock protein 70 (HSP70) has a protective role in hyperthermia as well as other stress conditions (Santoro, 2000) by preserving the balance between synthesis and degradation of cell proteins (Shi et al., 1998). The concentrations of HSP70 have been reported to affect sperm quality in boars (Huang et al., 2000). Knockout HSP70 mice had structural abnormalities in spermatocytes as well as interrupted development and increased apoptosis of primary spermatocytes (Christians et al., 2003).

Copper/zinc superoxide dismutase is an important antioxidant in spermatozoa. Garratt et al. (2013) reported that copper/zinc superoxide dismutase deficiency impaired spermatozoon motility and in vivo fertility in mice. In the present study with *A. dabryanus*, both HSP70 and copper/zinc superoxide dismutase were less in the spermatozoa of males categorized to have spermatozoa with relatively lesser quality, which may be associated with the lesser motility of these cells and the resulting lesser fertilization capacity.

Peroxiredoxins, also known as thioredoxin peroxidases and thiol-specific antioxidants, are antioxidant enzymes with wide species distribution in spermatozoa (Ozkosem et al., 2016) that reduce hydrogen peroxide, peroxynitrite, and alkyl hydroperoxides. Thioredoxins and peroxiredoxins are the prevalent redox proteins in a wide variety of tissues and cells (Arner and Holmgren, 2000; Wood et al., 2003). Gong et al. (2012) reported that spermatozoa of infertile men with lesser concentrations of, and inactive, peroxiredoxins to have an increased lipid peroxidation compared with normal spermatozoa. In the present study, these proteins were in relatively greater abundance in gametes of males categorized to have spermatozoa of lesser quality. The greater abundances of peroxiredoxins and thioredoxins in the spermatozoa of *A. dabryanus* males that were classified to have lesser quality spermatozoa must be confirmed and the biological significance investigated.

In the present study, there were also proteins in the relevant gel spots that were involved in protein folding and binding, or hydrolase activity, including disulfide isomerase-associated 4 and A3 precursor and ubiquitin carboxyl-terminal hydrolase isozyme L1. Protein-disulfide isomerase-associated 3 was previously described in boar epididymal spermatozoa (Akama et al., 2010) and is also associated with fertility in humans with relative abundance of this protein in spermatozoa being related to obesity-associated asthenozoospermia (Liu et al., 2015). Ubiquitin carboxyl-terminal hydrolase isozyme L1 is reported to be present in marked abundances in both the testis and epididymis and may have an important role in regulation of spermatogenesis (Martin et al., 1995; Fraile et al., 1996; Kon et al., 1999; Kwon et al., 2003). Kwon et al. (2005) suggested that this protein is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis. The lesser abundance of protein disulfide isomerase-associated 4 and A3 precursor and ubiquitin carboxyl-terminal hydrolases isozyme L1 in the present study appeared to affect fertility in *A. dabryanus*.

The results from the comparative proteomic analysis that was conducted with *A. dabryanus* spermatozoa in the present study indicate that sperm quality (i.e., motility and fertility) is associated with the abundance of certain proteins. These proteins were mainly involved in processes related to energy metabolism, mechanics of flagellum movement, and stress response. The results contribute to basic information of the proteins involved in sturgeon sperm biology and provide insights as to the role of these proteins in spermatozoon function, which may aid in development of effective reproductive technologies for the conservation and management of *A. dabryanus*.

Conflict of interest

We declare that all authors agree with the content of the submitted manuscript and that there are no conflicts of interest.

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