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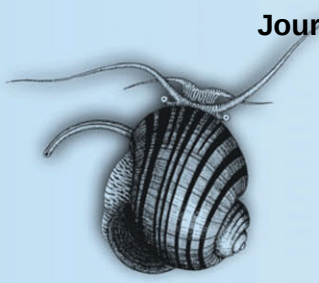

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Differential protein expression during sperm maturation and capacitation in an hermaphroditic bivalve, *Pecten maximus* (Linnaeus, 1758)

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ABSTRACT

In order to investigate the mechanisms of final maturation and capacitation of spermatozoa in *Pecten maximus*, we used a 2D proteomic approach coupled with MALDI-TOF/TOF mass spectrometry (MS) and bioinformatics search against the *Pecten* database, to set up a reference map of the proteome of spawned spermatozoa, and identified 133 proteins on the basis of the EST database. These proteins are mainly involved in energy production, ion and electron transport (44%), cell movement (22%) and developmental processes (10%). Comparison between proteomes of spermatozoa collected before and after transit through the genital ducts of *P. maximus* led to the identification of differentially expressed proteins. Most of them are associated with energy metabolism (aconitate hydratase, malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase), indicating important modifications of energy production during transit in gonoducts, potentially linked with acquisition of sperm motility. Three proteins involved in cell movement (Tektin-2, tubulin and microtubule-associated protein RP/EB family member 3) were down-regulated in spermatozoa stripped from the gonad. 40S ribosomal protein SA, involved in maturation of 40S ribosomal subunits, was also found to be down-regulated in spermatozoa obtained by induced spawning, suggesting reduction of the efficiency of RNA translation, a characteristic of late spermatozoon differentiation. These results confirm that maturation processes of *P. maximus* spermatozoa during transit through the gonoduct involve RNA translation, energy metabolism and structural proteins implicated in cell movement. Spermatozoa maturation processes clearly differ between *P. maximus* and gonochoric or alternately hermaphroditic bivalves, potentially in relation to reproductive strategies: the final maturation of the spermatozoon along the genital tract probably contributes to reduction of autofertilization in this simultaneously hermaphroditic species.

INTRODUCTION

In animal species, spermatozoa collected from the testis are morphologically complete, but generally immotile and unable to fertilize an oocyte. The initiation of motility relies on processes occurring during transit through the genital duct. During this transit, spermatozoa undergo discrete modifications controlled by the environment in the reproductive tract. For externally fertilizing species, transit through the male gonoduct and release into water lead to initiation of motility, allowing subsequent activation and chemotaxis of spermatozoa towards oocytes (Morisawa & Yoshida, 2005). The existence of capacitation processes leading to initiation of spermatozoan motility and fertility has been demonstrated in various bivalve species exhibiting external fertilization and factors that inhibit and stimulate sperm motility have also been investigated. In the Pacific oyster *Crassostrea gigas*, sperm capacitation takes place by simple dilution of spermatozoa in sea water. Consequently, for this species spermatozoa collected from the mature gonad of male specimens using a scarification procedure (known as 'stripping' in aquaculture) can easily be activated, facilitating controlled fertilization in oyster hatcheries. In pearl oysters, an alkaline treatment is required to activate testis-collected spermatozoa. Ohta *et al.* (2007) observed that spermatozoa of *Pinctada fucata martensii* obtained by dissecting the testis and then

diluted in sea water containing 2.0 mM NH_3 at pH 9.4 become motile after 30 s. Serotonin and potassium have also been found to induce initiation of sperm motility in various species of marine bivalves (*Ruditapes philippinarum*, *C. gigas* and *Patinopecten yessoensis*; Alavi *et al.*, 2014). Some authors have suggested that the artificial activation of spermatozoa by these various treatments may mimic the effects of substances that are delivered in the seminal fluid during the transit of gametes through the genital ducts (Demoy-Schneider *et al.*, 2012).

The king scallop *Pecten maximus* is a simultaneous hermaphroditic species, spawning sequentially male and female gametes through the same terminal genital duct (Barber & Blake, 2006). This situation is also observed in other simultaneously hermaphroditic pectinids (e.g. *Argopecten purpuratus* and *Pecten irradians*; Martinez *et al.*, 2007). For *P. maximus*, spermatozoa can be activated in sea water only if they have previously transited through the genital duct (Faure, Devauchelle & Girard, 1994) and spawning may be artificially induced by serotonin or heat treatment (Gibbons & Castagna, 1984). This observation suggests that, for pectinid species, transit through the genital duct is necessary for final maturation of spermatozoa and subsequent capacitation. However, knowledge of sperm maturation and capacitation process is limited in bivalve species.

Maturation of spermatozoa in the genital ducts has been investigated mainly in mammals, in order to understand the processes occurring during transit along the epididymal tubule. Here, maturation is controlled by extracellular factors from the epididymal environment. Mature spermatozoa then acquire the ability to fertilize a mature oocyte in the female reproductive tract. Proteins are key elements of these capacitation processes. It is now established that no nuclear transcription occurs in ejaculated spermatozoa and that translational activity is reduced, although some authors have demonstrated that some protein synthesis occurs during capacitation (Gur & Breitbart, 2008). In addition, post-translational protein modifications (including N-linked glycosylations, phosphorylations or ubiquitination) are involved in the process as well as membrane trafficking of proteins (reviewed by Amaral *et al.*, 2013). In mammals, the mechanisms of maturation and capacitation were largely investigated because of their contribution to spermatozoan fertility (Aitken *et al.*, 2007; Aitken & Nixon, 2013; Ashrafzadeh, Karsani & Nathan, 2013). Proteomic studies based on protein electrophoresis and excising the proteins out of the gels for mass spectrometry (MS) analysis is currently used to identify proteins from spermatozoa (reviewed by Oliva, De Mateo & Estanyol, 2009). The identified proteins that are related to sperm quality are classified as energy-related enzymes in mitochondrial and glycolytic pathways, structural proteins (mainly of the acrosome) and activating signal transducers (Martinez-Heredia *et al.*, 2008; Liao *et al.*, 2009; Siva *et al.*, 2010).

Comparative proteomics using 2D-PAGE electrophoresis coupled with MS should enable greater understanding of the processes of maturation and capacitation in the scallop and comparison of these processes with other molluscan species. In molluscs, some proteomic approaches have also been developed recently to identify the proteins involved in sperm maturation. In bivalves, proteins of *C. gigas* and *Mytilus galloprovincialis* spermatozoa have recently been analysed (Huang, Liu & Huan, 2015; Zhang *et al.*, 2015) and those involved in spermatozoon maturation were identified in *C. gigas* (Kingtong *et al.*, 2013). The recent development of a transcriptomic database for *P. maximus* has enabled us to establish a proteome map of the scallop spermatozoon. Additionally, proteomic-based comparison between spawned spermatozoa and spermatozoa collected from the gonad before spawning was used to identify proteins that are differentially expressed between both types of sperm. Their potential involvement in processes of maturation and capacitation of the spermatozoon is discussed below.

MATERIAL AND METHODS

Broodstock conditioning

Pecten maximus specimens were collected from the bay of Brest (Finistère, France) and conditioned in the Argenton Hatchery (Ifremer) in order to obtain broodstock, using the standard protocol described by Suquet *et al.* (2013).

Sperm collection

At the end of broodstock conditioning, animals ($n = 4$) were induced to spawn by injecting 100 µl of serotonin (10 mM) into the male part of the gonad. Animals were then placed in individual beakers containing 500 ml filtered sea water (FSW) until sperm emission. Spermatozoa were emitted after approximately 30 min. Spermatozoa were individually collected by centrifugation (700 g, 15 °C, 10 min). The pellets were frozen (−80 °C) until protein extraction was performed.

Stripped spermatozoa were individually collected from breeders ($n = 3$) after opening of the shells and dissection of the male part of the gonad. Male gonads were scarified and resuspended in 5 ml of FSW. They were then deposited onto a discontinuous Percoll gradient (80%, 60%, 40%) in order to isolate spermatozoa from other cell types present in the gonad (germline cells and somatic cells). Two main bands containing spermatozoa were obtained after 30 min centrifugation (600 g, 15 °C). The enrichment of spermatozoa in the lower band of the gradient was assessed by microscopic observation. The collected fraction was rinsed in 50 ml FSW for Percoll dilution and spermatozoa were collected by centrifugation (700 g, 15 °C, 10 min) and frozen (−80 °C) until protein extraction.

Histology

Transverse sections were taken through the male part of the gonad before stripping or after sperm emission in order to confirm gonadal maturity. The sections were fixed in Davidson fixative (10% v/v glycerol, 20% v/v 37% formaldehyde, 30% v/v 95% ethanol, 40% v/v FSW) for 48 h, then dehydrated and embedded in paraffin wax. Sections (5 µm) were then prepared and stained by the Prenant-Gabe trichrome method (Gabe, 1968).

Protein extraction and two-dimensional gel electrophoresis

Protein extractions for the stripped spermatozoa were performed in a lysis buffer containing 7 M urea, 2 M thiourea, 0.1 M DTT, 4% CHAPS, 5% ampholine (pH 3–10) and 1 mM protease-inhibitor aprotinin. The extract was sonicated on ice before being incubated at room temperature for 30 min and centrifuged at 12,000 g and 4 °C for 30 min. The supernatant was collected and precipitated in acetone (overnight at −20 °C). After centrifugation (15,000 g, 30 min, 4 °C) the pellet of proteins was rinsed in fresh acetone and dried in a speed vacuum. Protein extraction from both spawned and stripped spermatozoa followed the same process.

For protein separation using 2D gel, two sizes of immobilized pH gradient (IPG) strips, 7 cm and 18 cm, were used for two main purposes. The 7-cm strips were used for protein-profile comparison of stripped and spawned spermatozoa. The 18-cm strips were used to allow more of the protein to be loaded on the gel and were also used as a reference map of the spermatozoan proteome of *P. maximus*.

For isoelectric focusing (IEF) separation, proteins were resuspended in 200 µl of lysis buffer maintained at 4 °C, using pulse sonication. Concentration of protein in the sample was carried out by Bradford's method with bovine serum albumen as standard. For the first dimension separation, 250 µg (7-cm strip) or 750 µg

(18-cm strip) of protein was mixed with a rehydration solution containing 7 M urea, 0.1 M DTT, 4% CHAPS and 0.2% IPG buffer and then loaded on IEF 7-cm or 18-cm strips, pH 3–10 NL (BioRad). After overnight rehydration, the first dimension separation was achieved by the gradient-step method using the MultiPhore II electrophoresis system (GE Healthcare) with the following protocols: 1 min to reach 200 V, 1 h at 200 V, 2 h to reach 3,500 V then 2 h at 3,500 V for 7-cm strip; 1 h at 500 V, 1 h to reach 1,000 V, 3 h to reach 8,000 V, 2 h at 8,000 V for 18-cm strip. Focused strips were equilibrated in equilibration buffer (6 M urea, 50 mM Tris pH 6.8, 2% w/v SDS, 30% v/v glycerol) containing 1% w/v DTT for 15 min then in the same buffer containing 2.5% w/v iodoacetamide for another 15 min. The second dimension separation was performed by 12% SDS-PAGE with the following protocols: 500 V for 1 h for 18 cm gels and 100 V for 1 h for 7 cm gels in running buffer. IEF protein markers 3–10 and molecular weight (MW) marker were used to determine the isoelectric point (pI) and MW of each identified protein. Protein visualization was carried out using the colloidal Coomassie Brilliant Blue G-250 staining method.

Image analysis

After electrophoresis and staining, the gels were scanned with the ProEXPRESS 2D Proteomic Imaging System (Perkin Elmer, USA) and the images analysed using Progenesis Same Spot software (Nonlinear Dynamics, Newcastle-upon-tyne, UK). This program detected volume of protein spots, matched them across gels and expressed the results as average ± standard deviation. Then the amounts of same-protein spots were compared between experimental groups. To eliminate gel-to-gel variation, each spot volume was normalized as the percentage of the total volume of all spots for each gel. For differential analysis, three gels from the three stripped spermatozoa samples and three gels from the three spawned spermatozoa samples were used for comparison, with a cut-off of 1.5-fold volume difference with *t*-test *P*-value ≤ 0.05 as evidence of significant differences of protein expression. Differential proteins ($P \leq 0.05$) were further identified as described below.

Protein identification

Differentially expressed proteins ($P \leq 0.05$, see above) were manually picked from the gel, destained in a solution of 25 mM NH_4HCO_3 in 50% acetonitrile and digested with trypsin (37 °C for 15 h) before mass analysis. MS experiments were carried out on an AB Sciex 5,800 proteomics analyser equipped with TOF TOF ion optics and an OptiBeam™ on-axis irradiation laser running at a repetition rate of 1,000 Hz. The system was calibrated immediately before analysis with a mixture of Angiotensin I, Angiotensin II, Neurotensin, ACTH clip (1–17) and ACTH clip (18–39), and mass precision was greater than 50 ppm. After tryptic digestion, the dry sample was resuspended in 10 µl of 0.1% TFA. A 1 µl volume of this peptide solution was mixed with 10 µl volumes of solutions of 5 mg/ml CHCA matrix prepared in a diluant solution of 50% ACN with 0.1% TFA. The mixture was spotted on a stainless steel Opti-TOF™ 384 target; the droplet was allowed to evaporate before the target was introduced into the mass spectrometer. All acquisitions were taken in automatic mode. A laser intensity of 4,300 was typically employed for ionizing. MS spectra were acquired in positive reflector mode by summarizing 1,000 single spectra (5 × 200) in the mass range 700–4,000 Da. MS/MS spectra were acquired in positive MS/MS reflector mode by summarizing a maximum of 2,500 single spectra (10 × 250) with a laser intensity of 4,500. For the tandem MS experiments the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as setting. The fragmentation pattern was used to determine the sequence of the peptide. MS/MS data files were used to query the *Pecten* EST database using the Mascot algorithm (Mascot server v. 2.2.04; http://www.matrixscience.com). The *P. maximus* database, containing a total of 60,180 annotated contigs, was obtained by assembling Roche 454 and Illumina sequencing reads (EU project REPROSEED). The variable modifications allowed were as follows: K-acetylation, methionine oxidation and dioxidation. 'Trypsin' was selected as enzyme, and three miscleavages were also allowed. Mass accuracy was set to 100 ppm and 0.6 Da for MS and MS/MS mode, respectively. All identifications were then annotated in the Universal Protein Resource Knowledgebase (http://www.uniprot.org/). The calculated peptide mass and pI of the translated EST were evaluated on the EXPASY website (http://web.expasy.org/compute_pi/).

RESULTS

Efficiency of broodstock conditioning

Microscopic observation of histological slides of male gonads of stripped and spawned animals confirmed the reproductive maturity of the breeders, the gonadal tubules containing mainly spermatozoa. Other types of germ cells (spermatids, spermatocytes) were nevertheless observed around the gonadal tubules (Supplementary material, Fig. S1). However, the gonoduct sections of serotonin-induced animals (spawned) and unspawned animals differed in appearance, spawned animals having spermatozoa in the gonoducts while the others had empty gonoducts.

Reference map of stripped spermatozoa

The reference map established with 750 µg of total protein extracted from stripped spermatozoa of *Pecten maximus* using 18-cm IPG strips gave a high-resolution protein map (Fig. 1). One hundred and sixty-two spots were submitted to MS/MS analysis and 133 proteins (82%) were identified on the basis of the EST database from *P. maximus* (Table 1). The proteins were putatively identified according to gene ontology as shown in Figure 2. Most of those identified (44%) were known to be connected with energy production, ion or electron transport, while others were involved in cell movement, developmental processes, transcription/biogenesis, proteasome, oxidoreductase processes and apoptotic processes, or were unclassified proteins.

Proteins differentially expressed between stripped and spawned spermatozoa

To understand the differences between fertile sperm obtained by serotonin-stimulated spawning and unfertile stripped sperm (collected from the gonad by scarification), a comparative proteomic approach was used. The proteome profiles of stripped and spawned spermatozoa (Fig. 3) looked very similar. Comparing these profiles produced a list of proteins that were up-and down-regulated in stripped spermatozoa. The identification of these proteins (name and putative functions) is provided in Table 2.

DISCUSSION

Among the 133 proteins found in spermatozoa of *Pecten maximus*, the most abundant are involved in energy production and ion or electron transport; these can be broken down further into three major classes according to their metabolic function. The first is involved in glycolysis or the tricarboxylic acid cycle. Examples of proteins belonging to this class are aconitate hydratase (spots B4-B5), fructose-bisphosphate aldolase (spot B38), citrate synthase

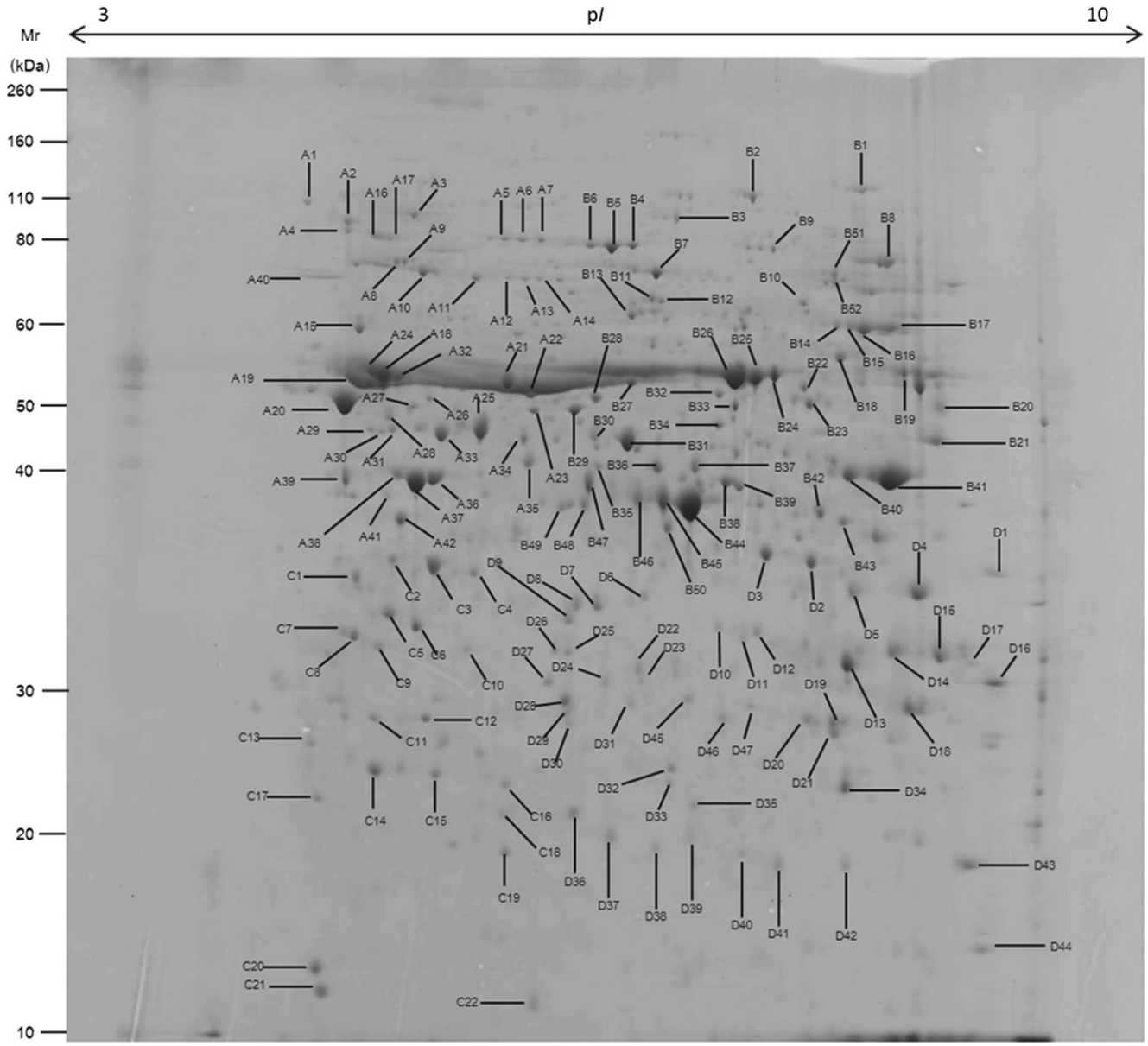

Figure 1. Reference map of stripped spermatozoa of *Pecten maximus*. Details of protein identification are shown in Table 1. Reference molecular weights (Mr) are indicated on the left of the gel.

Table 1. List of proteins identified in the reference map of stripped spermatozoa of *Pecten maximus*.

Spot	Swiss prot accession number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
A1	Q95YJ5	Thioredoxin domain-containing protein 3 homologue	4.56	73,038.5	4.37	107,857	1	67.48
A2	Q9D2H2	Adenylate kinase 7	4.9	70,675.4	4.68	92,589	3	164.59
A3	Q7ZU99	Transitional endoplasmic reticulum ATPase	5.14	89,423.8	5.19	99,018	6	287.11
A4	Q18688	Heat shock protein 90	4.96	80,283.2	4.68	89,911	4	300.52
A5	Q16959	Dynein intermediate chain 2	5.7	79,138.4	5.88	79,919	2	170.43
A6	Q16959	Dynein intermediate chain 2	5.7	79,138.4	6.02	79,919	2	224.45
A7	Q16959	Dynein intermediate chain 2	5.7	79,138.4	6.16	79,757	3	76.89
A8	Q0MQG2	NADH-ubiquinone oxidoreductase 75 kDa subunit	5.55	76,988.5	5.05	74,575	5	354.30
A9	Q0MQG2	NADH-ubiquinone oxidoreductase 75 kDa subunit	5.55	76,988.5	5.12	74,575	7	598.37
A10	Q06248	Heat shock 70 kDa protein IV	5.55	69,749.8	5.26	71,822	16	1114.22
A11	P29845	Heat shock 70 kDa protein cognate 5	6.02	74,066.1	5.73	71,498	3	90.78

Continued

Table 1. *Continued*

Spot	Swiss prot accession number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
A12	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67,845.5	5.91	71,012	2	107.88
A13	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67,845.5	6.04	70,526	3	141.51
A14	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67,845.5	6.15	70,364	6	242.75
A15	O02649	60 kDa heat shock protein	5.07	54,604.6	4.77	59,322	9	866.41
A16	Q16960	Dynein intermediate chain 3	4.99	68,224.5	4.88	82,411	4	216.85
A17	Q16960	Dynein intermediate chain 3	4.99	68,224.5	4.93	82,411	3	165.64
A18	P18288	Tubulin alpha chain, testis-specific	4.98	49,993.6	4.98	53,432	8	683.81
A19	P11833	Tubulin beta chain	4.73	50,051.2	4.76	52,542	13	847.28
A20	P10719	ATP synthase subunit beta, mitochondrial	4.95	51,710.1	4.65	48,696	17	1,556.49
A21	P11833	Tubulin beta chain	4.73	50,051.2	5.91	52,034	9	557.50
A22	O02654	2-phosphoglycerate dehydratase	5.78	47,426.2	6.14	51,695	3	153.15
A23	Q26648	Tektin-B1	5.34	46,147.6	6.11	49,022	8	578.82
A24	P18288	Tubulin alpha chain, testis-specific	4.98	49,993.6	4.95	53,136	9	694.38
A25	P98080	Cytochrome b-c1 complex subunit 1, mitochondrial	6.07	51,735.9	5.7	45,000	9	541.17
A26	P11833	Tubulin beta chain	4.73	50,051.2	5.31	50,593	9	473.39
A28	Q90512	Dihydrolipoyllysine-residue succinyltransferase	5.25	39,848.8	5	48,261	3	195.32
A30	P12716	Actin, cytoplasmic	5.3	41,614.5	4.94	45,761	2	82.61
A31	P91929	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex	5.18	40,414	5.02	45,978	4	197.37
A32	P18288	Tubulin alpha chain, testis-specific	4.98	49,993.6	5.05	53,220	8	624.11
A33	P32551	Cytochrome b-c1 complex subunit 2, mitochondrial	8.83	46,751.2	5.39	44,674	4	244.05
A34	Q0MQG3	Probable NADH dehydrogenase	6.26	49,235.7	6.03	44,348	7	372.71
A35	Q6P5L8	Hydroxysteroid dehydrogenase-like protein 2	7.08	44,420.9	6.07	40,543	1	113.51
A36	P12716	Actin, cytoplasmic	5.3	41,614.5	5.33	39,422	7	452.02
A37	P18603	Actin, clone 403	5.3	41,603.5	5.19	38,965	11	932.36
A38	P53445	Fructose-bisphosphate aldolase, muscle type	8.84	39,239	5.06	39,665	4	305.25
A39	Q9Z2I9	Succinyl-CoA ligase [ADP-forming] subunit	5.33	44,422.5	4.66	39,422	3	154.53
A40	A1L0Z6	Radial spoke head protein 6 homologue A	4.26	58,497	4.56	70,769	5	290.50
A41	P53590	Succinyl-CoA ligase [GDP-forming] subunit	5.1	42,572.9	4.98	38,782	2	82.88
A42	Q9VWH4	Probable isocitrate dehydrogenase [NAD] subunit alpha	6.96	40,844	5.08	37,595	6	410.86
B2	Q5RCB8	2-oxoglutarate dehydrogenase	6.07	11,1326.9	7.8	111,381	2	79.82
B3	P09812	Glycogen phosphorylase	6.9	97,142.24	7.21	95,268	2	135.84
B4	Q9ER34	Aconitate hydratase	7.15	82,461.86	6.87	78,219	4	166.392
B5	Q9ER34	Aconitate hydratase	7.15	82,461.86	6.7	77,328	9	454.132
B7	Q9D9T8	EF-hand domain-containing protein 1 (Myoclonin-1)	5.71	75,141.64	7.05	71,174	8	388.0152
B8	Q8BMS1	Trifunctional enzyme subunit alpha	9.06	78,765.22	8.8	73,927	11	478.8753
B9	Q32TF8	EF-hand domain-containing family member C2	5.81	85,457.36	7.95	77,814	6	213.062
B12	Q921G7	Electron transfer flavoprotein-ubiquinone oxidoreductase	7.34	68,090.93	7.09	65,344	2	65.71331
B13	Q6AXQ8	Meiosis-specific nuclear structural protein 1	7.09	61,239.82	6.86	61,781	4	117.2953
B14	Q2YDI7	Tektin-5	7.95	56,503.87	8.45	59,746	1	31.9
B16	Q2YDI7	Tektin-5	7.95	56,503.87	8.65	58,898	4	153.73
B17	Q2YDI7	Tektin-5	7.95	56,503.87	8.84	59,237	1	43.98
B19	P41383	Tubulin alpha-2/alpha-4 chain	4.94	50,210.72	8.96	53,602	8	480.15
B20	Q9YIC0	Elongation factor 1-alpha (EF-1-alpha)	9.22	50,443.26	9.23	49,457	5	232.64
B21	P34255	Uncharacterized protein B0303.3	9.15	47,874.47	9.21	43,913	6	281.5199
B22	P35381	ATP synthase subunit alpha, mitochondrial (Protein bellwether)	8.22	54,553.87	8.18	52,034	6	367.3533
B23	Q91YT0	NADH dehydrogenase [ubiquinone] flavoprotein 1	7.92	48,626.48	8.24	49,891	5	200.8133
B24	P82264	Glutamate dehydrogenase, mitochondrial (GDH) (EC 1.4.1.3)	7.34	55,394.09	7.97	53,136	11	620.0233
B25	P41383	Tubulin alpha-2/alpha-4 chain	4.94	50,210.72	7.82	53,051	9	715.16
B26	Q5R546	ATP synthase subunit alpha, mitochondrial	9.16	59,780.66	7.65	52,203	14	1112.652
B27	P11833	Tubulin beta chain (Beta-tubulin)	4.73	50,051.16	6.86	53,051	3	166.47
B28	O02654	Enolase	5.78	47,426.22	6.59	50,763	5	252.3866
B29	Q922G7	Tektin-2 (Tektin-t) (Testicular tektin)	5.98	50,311.09	6.42	48,913	5	318.6766
B30	Q4S5X1	Citrate synthase, mitochondrial (EC 2.3.3.1)	6.69	48,727.71	6.57	45,000	4	151.03

Continued

Table 1. *Continued*

Spot	Swiss prot accession number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
B31	Q4S5X1	Citrate synthase, mitochondrial (EC 2.3.3.1)	6.69	48,727.71	6.82	43,043	9	486.1933
B32	Q9JLI7	Sperm-associated antigen 6 (Axoneme central apparatus protein)	6.33	55,269.25	7.54	51,186	3	203.75
B33	P35623	Serine hydroxymethyltransferase, cytosolic (SHMT)	8.03	52,893.44	7.66	49,674	15	741.81
B35	Q2KIW6	26 S protease regulatory subunit 10B	6.74	44,073.85	6.6	40,217	4	162.06
B36	Q3SZB4	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial (MCAD) (EC 1.3.8.7)	7.02	43,586.83	7.05	39,970	5	262.36
B37	Q3T0R7	3-ketoacyl-CoA thiolase, mitochondrial	8.06	42,131.21	7.4	40,652	3	133.99
B38	Q9GP32	Fructose-bisphosphate aldolase (EC 4.1.2.13)	8.03	39,727.38	7.59	39,209	6	289.26
B39	Q9QXL8	Nucleoside diphosphate kinase 7 (NDK 7) (NDP kinase 7) (EC 2.7.4.6) (nm23-M7)	6.72	44,433.9	7.7	39,087	2	162.79
B40	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39,101.69	8.53	39,543	13	855.9066
B41	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39,101.69	8.83	39,117	13	861.3966
B42	O77784	Isocitrate dehydrogenase [NAD] subunit beta	8.2	38,959.96	8.31	37,900	5	200.7099
B43	Q80Y75	DnaJ homologue subfamily B member 13 (Testis and spermatogenesis cell-related protein 6)	7.02	36,154.51	8.49	37,504	4	176.37
B44	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39,101.69	7.29	37,778	10	752.9266
B45	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39,101.69	7.11	38,356	7	456.5133
B46	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39,101.69	6.92	38,539	4	283.4233
B47	P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial (LCAD) (EC 1.3.8.8)	6.5	44,627	6.53	39,117	6	240.9866
B48	Q27928	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic (GPD-C) (GPDH-C) (EC 1.1.1.8)	6.52	39,448	6.49	38,295	5	335.5387
B49	P11833	Tubulin beta chain (Beta-tubulin)	4.73	50,051.16	6.32	38,143	6	214.8933
B50	P41564	Isocitrate dehydrogenase [NAD] subunit gamma	8.66	38,767.48	7.14	37,291	4	174.9733
B51	Q9JID6	Long-chain-fatty-acid—CoA ligase 1	7.48	77,697.99	8.42	71,336	3	158.66
B52	Q4R755	Glycerol-3-phosphate dehydrogenase	6.42	76,309.31	8.43	69,879	2	98.98
C3	Q5NVR2	Malate dehydrogenase	8.54	33,046.5	5.34	35,464	11	599.36
C4	P11833	Tubulin beta chain	4.73	50,051.2	5.65	35,282	6	203.02
C5	P11833	Tubulin beta chain	4.73	50,051.2	5	33,425	2	126.31
C6	Q5TYW6	Radial spoke head protein 9 homologue	5.48	30,924.2	5.2	32,846	9	447.16
C8	Q6PC29	14-3-3 protein gamma-1	4.86	28,234.5	4.72	32,481	4	164.24
C9	Q6VTH5	Radial spoke head 1 homologue	5.09	24,566.4	4.92	32,024	4	152.11
C11	Q25117	ATP synthase subunit beta, mitochondrial	4.91	54,020.4	4.88	28,117	6	451.11
C12	Q0MQG7	NADH dehydrogenase [ubiquinone] iron–sulfur protein 3	5.48	26,414.9	5.28	28,068	10	597.90
C13	P11833	Tubulin beta chain	4.73	50,051.2	4.39	26,357	3	210.42
C14	Q66IC9	Ropporin-1-like protein	5.61	24,170.8	4.88	24,352	6	363.22
C15	Q0MQI9	NADH dehydrogenase [ubiquinone]	5.71	23,760.3	5.36	24,108	3	121.55
C17	Q25117	ATP synthase subunit beta	4.91	54,020.4	4.45	22,543	3	169.60
C19	Q5FB30	Superoxide dismutase [Mn], mitochondrial	6.82	22,241.2	5.88	19,028	1	39.19
C20	P55954	Cytochrome c oxidase subunit 5 A	5.12	17,115.4	4.5	14,044	5	324.45
C21	Q15370	Transcription elongation factor B polypeptide 2	4.73	13,132.8	4.47	12,633	3	348.05
D1	A2RRW4	Protein FAM166B	9.55	33,272.8	9.69	35,205	5	227.46
D2	P10096	Glyceraldehyde-3-phosphate dehydrogenase	8.52	35,736.9	8.24	35,647	8	511.58
D10	P76536	Probable deferrochelatase/peroxidase YfeX	5.34	33,052.3	7.53	32,846	3	145.43
D12	P76536	Probable deferrochelatase/peroxidase YfeX	5.34	33,052.3	7.82	32,633	2	73.13
D13	P82013	Voltage-dependent anion-selective channel	8.62	30,066.6	8.52	31,050	12	1025.69
D15	P05631	ATP synthase subunit gamma	9.16	30,255.7	9.23	31,476	7	487.40
D16	A3KQA5	Outer dense fibre protein 3-B	9.78	27,895.6	9.65	30,350	8	441.14
D17	Q6SP97	Enkurin	9.58	29,527.4	9.48	31,187	3	133.18
D18	P13619	ATP synthase subunit b, mitochondrial	9.14	24,668.7	9	28,655	9	592.97
D19	O70250	Phosphoglycerate mutase 2	8.65	28,827.1	8.41	27,824	7	352.48
D20	Q9PTW9	Proteasome subunit alpha type-7	8.96	28,084.2	8.21	27,922	3	207.65

Continued

Table 1. *Continued*

Spot	Swiss prot accession number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
D21	P12716	Actin, cytoplasmic precursor	5.3	41,848.8	8.41	27,139	7	445.04
D24	Q62651	Delta(3,5)-Delta(2,4)-dienoyl-CoA	6.26	32,474.2	6.66	30,381	3	204.68
D25	Q9BKU4	Mitochondrial prohibitin complex protein	6.94	29,988.4	6.37	31,750	10	580.93
D26	Q9BKU4	Mitochondrial prohibitin complex protein	6.94	29,988.4	6.27	31,842	7	426.64
D27	P18053	Proteasome subunit alpha type-4	6.75	29,411.7	6.22	30,441	4	110.87
D28	Q68FU3	Electron transfer flavoprotein subunit	7.81	27,556.2	6.35	29,242	7	378.98
D29	P60901	Proteasome subunit alpha type-6	6.34	27,399.5	6.36	28,509	4	221.43
D31	Q58DM8	Enoyl-CoA hydratase, mitochondrial	6.86	28,095.4	6.85	29,095	6	321.24
D32	P56597	Nucleoside diphosphate kinase homologue 5	5.89	24,236.3	7.17	24,499	6	477.52
D33	Q3T108	Proteasome subunit beta type-4	5.45	24,361.8	7.15	23,472	3	192.39
D34	Q69BJ8	Cytochrome b-c1 complex subunit 11	6.98	21,655.7	8.51	23,081	4	202.02
D35	Q90384	Peroxiredoxin	7.63	22,339.6	7.34	21,858	4	259.07
D36	Q5R546	ATP synthase subunit alpha	8.28	55,239.4	6.41	21,320	4	239.94
D38	Q3MHN0	Proteasome subunit beta type-6 precursor	4.9	25,542.1	7.02	19,279	6	305.73
D39	Q2KID4	Dynein light chain 1, axonemal	5.63	21,535.9	7.38	19,812	4	207.38
D40	Q2TBP0	Proteasome subunit beta type-7	5.84	25,305	7.72	18,997	3	117.73
D41	A8Y5T1	Uncharacterized protein C1orf194 homologue	6.75	18,800.7	7.99	18,401	6	376.72
D42	Q1HPL8	NADH dehydrogenase [ubiquinone] 1 beta	5.94	18,999.3	8.51	18,887	5	307.93
D43	P13621	ATP synthase subunit O, mitochondrial	9.83	20,929.8	9.56	18,652	5	248.82
D44	O01369	Cytochrome b-c1 complex subunit 7	6.84	15,331.5	9.55	14,545	9	531.78
D45	Q9CQ24	F-box only protein 36	8.29	22,075.4	7.3	29,438	2	63.91
D47	Q32L77	Uncharacterized protein C9orf135 homologue	6.66	26,623	7.78	28,998	3	155.18

MW, molecular weight (Da); pI, isoelectric point.

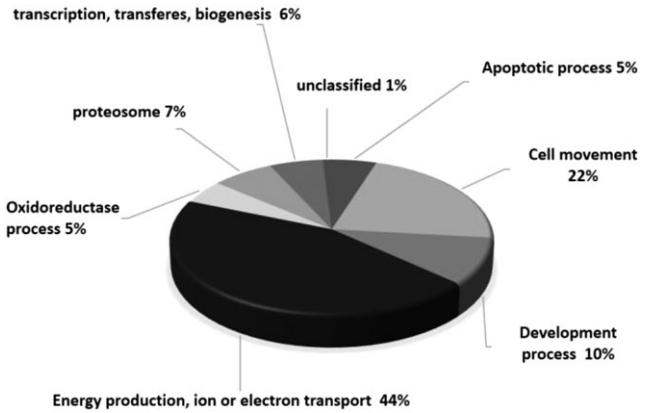

Figure 2. Breakdown of the identified proteins in *Pecten maximus* spermatozoa on the basis of putative function according to gene ontology.

(spots B30-B31), succinate dehydrogenase (spots A12-A13-A14) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot D2). The second class of proteins involved in energy production are the lipid-metabolism proteins that function in fatty acid beta-oxidation. Proteins such as acyl-CoA dehydrogenase (spots B36-B47), enoyl-CoA hydratase (spot D31), delta(3,5)-delta(2,4)-dienoyl-CoA isomerize (spot D24) belong to this class. The third class consists of ion- or electron-transport proteins such as ATP synthase (spots A20-B22-B26-C11-C17-D15-D18-D36-D43), cytochrome *c* oxidase (spot C20), NADH dehydrogenase (spots A31-A33-A34-B23-C15-D42) and the cytochrome *b-c1* complex (spots A24-A33). The high number of proteins (45 spots) involved in energy production and ion or electron transport found in the spermatozoa of *P. maximus* seems to agree with the high energy requirement for sperm motility. The duration of sperm movement is generally long for bivalve species, as compared with vertebrates, reaching 10 h in *P. maximus* (Suquet *et al.*, 2013). These authors have suggested that scallop sperm could partially replenish the energy content catabolized during this prolonged movement.

Subcellular fractioning should be of interest to identify less abundant proteins in the spermatozoa of *P. maximus*. This strategy is especially appropriate for compartmentalized cells such as sperm, where distinct compartments have clear and specific cellular roles (Byrne *et al.*, 2012). For example, isolation of sperm membrane proteins could lead to identification of the proteins involved in oocyte recognition.

The number of up-regulated proteins in spawned spermatozoa is large, considering the short process of transfer through the gonadal duct and the low translation level in spermatozoa. However, it should be noticed that up- and down-regulation of proteins may reflect the effects of post-translational modifications (PTMs). Two-dimensional gel electrophoresis allows the separation of different modified forms of protein such as are produced by phosphorylation or glycosylation (Seo & Lee, 2004).

The proteome profiles of stripped and spawned spermatozoa allowed identification of differentially expressed proteins. Most down-regulated proteins in the stripped spermatozoa, such as tektin-2, tubulin and microtubule-associated protein RP/EB family member 3 (MAPRE3), are associated with energy metabolism and cell movement. Tektin-2, the protein involved in cell movement, is required for normal structure and function of the flagellum in mouse sperm (Shimasaki *et al.*, 2010). Tektins are conserved components of the flagellar proteins and are co-assembled with tubulins to form flagellar axonomes and centrioles (Amos, 2008). In sperm lacking tektin-2 the flagellum is bent and shortened, owing to the disruption of the dynein inner

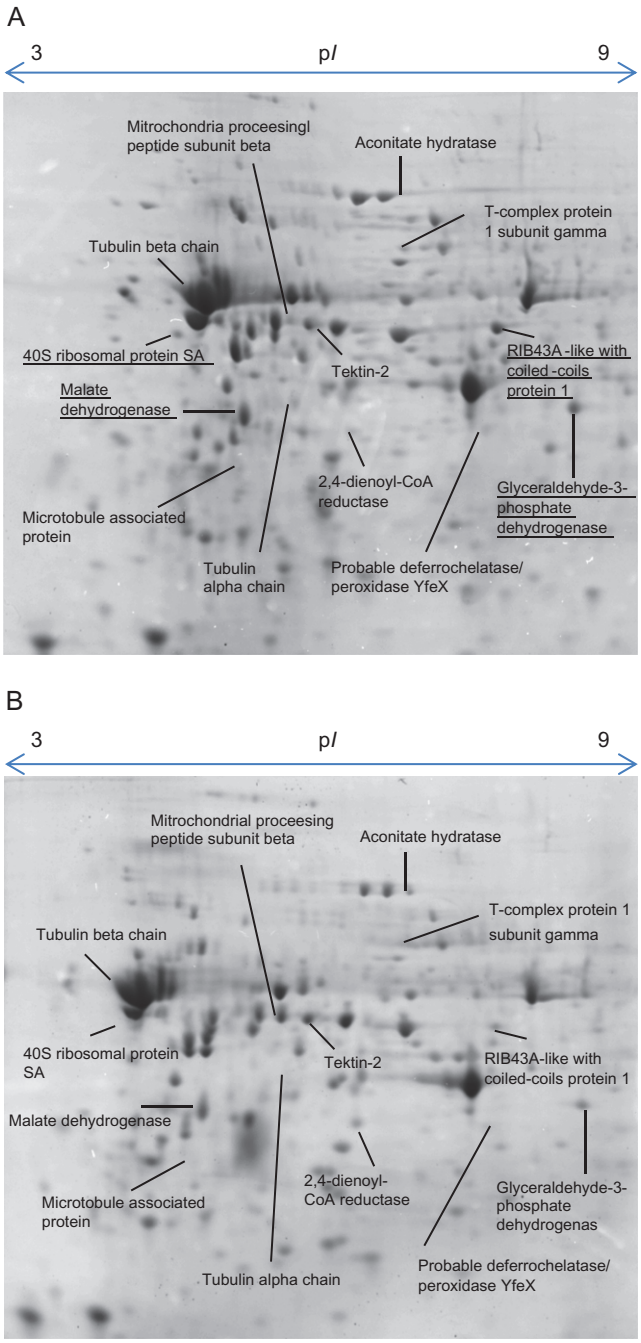

Figure 3. Representative 2D gels of proteome from stripped (**A**) and spawned (**B**) spermatozoa of *Pecten maximus*. Proteins were extracted and separated (first dimension by IEF on IPG strip pH 3–10 NL, second dimension on 10% SDS-PAGE 7 cm gels). Up-regulated proteins (underlined) and down-regulated proteins (not underlined) in stripped spermatozoa are indicated on the gels. Details of protein identification are given in Table 2.

arm (Shimasaki *et al.*, 2010). In human spermatozoa, low levels of tektin-2 are also related to decreasing sperm motility and fertilization rates (Bhilawadikar *et al.*, 2013). Interestingly, tektin-2 was also found to be up-regulated in mature spermatozoa of *Crassostrea gigas* (Kingtong *et al.*, 2013). One of three cytoskeletal proteins present in the head of mammalian spermatozoa is tubulin, the main component of microtubles (Dvoráková *et al.*, 2005). PTMs of this protein generate a functional diversity of microtubules. Recently, acetylated α-tubulin was found to decrease in asthenozoosperms as compared with normal human spermatozoa, which suggests that α-tubulin acetylation may be one of the determinants of sperm motility (Bhagwat *et al.*, 2014). In stripped spermatozoa, the MAPRE3 was also found to be down-regulated. This protein binds to the end of microtubules and promotes microtubule growth, a property that may play a role in cell migration. The decreased levels of tektin-2, tubulin and MAPRE3 proteins in stripped scallop spermatozoa indicate a limitation of sperm motility, which correlates with the observation by Faure *et al.* (1993) that the motility of spermatozoa removed directly from gonad was lower than that of the spawned ones.

Other differentially expressed proteins are linked to mitochondrial energy metabolism and could be connected with the energy demand and ATP content of spermatozoa in *P. maximus*. Aconitate hydratase, an enzyme of the tricarboxylic acid cycle that catalyses the isomerization of citrate to isocitrate, was down-regulated in stripped spermatozoa in our study. In contrast, this protein was found to be up-regulated in stripped spermatozoa of *C. gigas*, corresponding to their success in fertilization (Kingtong *et al.*, 2013). Stripped *Pecten* spermatozoa, on the other hand, have low levels of fertility. Moreover, the two species exhibit differences in ATP management associated with sperm movement, as described by Suquet *et al.* (2013), possibly related to the expression level of aconitate hydratase.

One of the up-regulated proteins in stripped spermatozoa of *P. maximus* is GAPDH, the enzyme that catalyses the conversion of glyceraldehyde-3-phosphate to D-glycerate-1,3-bisphosphate in the sixth step of the glycolysis pathway. Sharma *et al.* (2013) found that GAPDH was overexpressed in human spermatozoa under oxidative stress, as did Wang *et al.* (2009) who also found GAPDH overexpressed in asthenozoospermia seminal samples. Additionally, S-nitrosylated GAPDH is known to initiate apoptotic cell death (Hara *et al.*, 2005). The GAPDH expression level of apoptotic cells is three times higher than that of nonapoptotic cells (Dastoor & Dreyer, 2001). Malate dehydrogenase (MADH), an enzyme that catalyses the last step of the Krebs cycle by oxidizing malate to oxaloacetate, is another protein that is up-regulated in stripped spermatozoa of *P. maximus*. This enzyme is located in the mitochondria of the midpiece in spermatozoa of various vertebrate species (Kohsaka *et al.*, 1992; Auger *et al.*, 2010) and was found to be significantly overexpressed in human spermatozoa under oxidative stress (Sharma *et al.*, 2013). Thus, the up-regulation of GAPDH and MADH enzymes found in stripped scallop spermatozoa implies some oxidative stress or cell apoptosis, perhaps reflecting stress of the cells in response to the stripping procedure, and could also be involved in fertilization.

The 40S ribosomal protein SA is also up-regulated in stripped spermatozoa. This protein is required for the assembly and/or stability of the 40S ribosomal subunit. It may also function as a laminin receptor after PTM and has already been identified in another scallop, *Chlamys farreri* (Sun *et al.*, 2014). As mature spermatozoa are supposed to be translationally inactive (Dacheux & Dacheux, 2014), the diminished level of this protein in spawned compared with stripped spermatozoa suggests the possible reduction of translation capacities, a characteristic of late spermatozoan differentiation. This observation also indicates that, in *P. maximus*, this late maturation step takes place during the transit of gametes through the genital duct.

In conclusion, the proteomes of stripped and spawned spermatozoa of *P. maximus* differ by expression of proteins mainly involved in motility and energy supply for the spermatozoon. Furthermore, some proteins implicated in stress reactions are more highly expressed in stripped spermatozoa. The results confirm that, during the transit of spermatozoa through the genital tract, a late maturation step occurs, allowing spermatozoa to acquire an energetic pathway and structural proteins crucial for the final capacitation step. In this respect, the maturation pattern of the spermatozoa appears to differ according to the bivalve species considered,

Table 2. Identification of proteins differentially expressed between stripped and spawned spermatozoa of *Pecten maximus*.

Swiss prot accession number	Protein name	Function	pI/MW theoretical	pI/MW experimental	Average fold change	Unique peptide matched	Score
Down-regulation in stripped spermatozoa							
Q9UIF3	Tektin-2	Assembly or attachment of the inner dynein arm to microtubules in sperm flagella and tracheal cilia	5.39/49.67	5.72/50.78	1.4	15	827.13
P11833	Tubulin beta chain	GTP binding	4.73/50.05	4.83/54.31	1.4	19	1069.74
P18288	Tubulin alpha chain, testis-specific	GTP binding	4.98/49.99	5.61/38.54	1.7	2	88.35
O75439	Mitochondrial-processing peptidase subunit beta	Cleaves presequences from mitochondrial protein precursors	5.76/49.49	5.59/51.31	2.7	12	540.48
Q3T0K2	T-complex protein 1 subunit gamma	Molecular chaperone	6.38/60.59	6.55/69.88	2.0	3	115.24
Q16698	2,4-dienoyl-CoA reductase	Auxiliary enzyme of beta-oxidation	8.79/32.15	6.07/35.31	1.7	4	194.96
Q9ER34	Aconitate hydratase	Catalyses the isomerization of citrate to isocitrate	7.15/82.46	6.87/78.22	2.3	8	335.04
Q5XIT1	Microtubule-associated protein RP/EB family member 3	Promotes microtubule growth	5.33/31.97	5.10/31.02	1.5	2	98.89
P76536	Probable deferrochelatase/peroxidase YfeX	Involved in the recovery of exogenous haeme iron	5.34/33.05	7.4/33.33	4.1	6	333.66
Up-regulation in stripped spermatozoa							
Q5NVR2	Malate dehydrogenase	Tricarboxylic acid cycle	8.54/33.05	5.18/36.71	1.4	13	951.43
A3RLT6	40S ribosomal protein SA	Required for the assembly and/or stability of the 40S ribosomal subunit	5.24/33.39	4.59/49.59	2.7	2	81.75
Q5R2J2	Glyceraldehyde-3-phosphate dehydrogenase	Has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities	8.70/35.84	8.03/37.70	1.5	5	303.37
Q0VC09	RIB43A-like with coiled-coils protein 1	No function information	5.56/44.25	7.35/50.12	1.8	7	285.27

MW, molecular weight (kDa); pI, isoelectric point.

because in the oyster *C. gigas*, late differentiation of spermatozoa occurs in the gonad and seems to involve a different energy production mode (Shimasaki *et al.*, 2010). This difference may relate to the different reproductive strategies of the species: the scallop is a simultaneous hermaphrodite and the occurrence of the final maturation step of spermatozoa in the genital duct may contribute to reducing autofertilization.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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