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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 in the testis are morphologically complete but generally immotile and unable to fertilize an oocyte. The initiation of motility relies on processes occurring during transit in genital duct. During this transit, spermatozoa undergo discrete modifications controlled by the environment in the reproductive tract. For external fertilizer, transit through male gonoduct and release in water led 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 spermatozoa motility and fertility has been demonstrated in various bivalve species exhibiting external fertilization and factors that inhibit and stimulate sperm motility have been investigated: for the Pacific oyster *Crassostrea gigas*, sperm capacitation takes place by simple dilution of spermatozoa in sea water. Subsequently, for this species, spermatozoa collected in the mature gonad of male specimens using scarification procedure (or stripping) may be easily activated, facilitating the realisation of controlled fertilizations in hatcheries. In the 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 testis and diluted in sea water containing 2.0 mM NH_3 at pH 9.4 become motile after only 30 s. Moreover serotonin and potassium were also found to induce initiation of sperm motility in various species of marine bivalve molluscs (*Ruditapes philippinarum*, *C. gigas* and *Patinopecten yessoensis*) (Alavi *et al*., 2014). Some authors suggested that the artificial activation of spermatozoa by those various treatments may mime the effect of substances that would be delivered in seminal fluid during transit of gametes in genital ducts (Demoy-Schneider *et al.,* 2012).

The king scallop *Pecten maximus* is a simultaneous hermaphrodite species, spawning sequentially male and female gametes through the same terminal genital duct (Barber & Blake, 2006). This situation is also observed in other simultaneous hermaphrodite pectinids (e.g., *Argopecten purpuratus* and *Pecten irradians*) (Martinez et al., 2007), both sperm and oocytes are emitted from the gonad via a common gonoduct. For *P. maximus*, spermatozoa may be activated in sea water only if they have previously transited through genital ducts (Faure, Devauchelle & Girard, 1994) and spawning may be artificially induced by serotonin or heat treatment. This observation suggests that for pectinid species, transit through 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 through genital ducts has been mainly investigated in mammals in order to understand the processes occurring during transit along the epididymal tubule. The 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 but some authors

demonstrated that some protein synthesis occurs during capacitation (Gur & Breitbart, 2008). Moreover, 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 spermatozoa fertility (Aitken & Nixon, 2013; Aitken *et al*., 2007; Ashrafzadeh, Karsani & Nathan, 2013). Proteomic studies based on protein electrophoresis and excising the proteins out of the gels for MS analysis is currently used to identify proteins from spermatozoa (reviewed by Oliva, De Mateo & Estanyol, 2009). The identified proteins related to sperm quality are categorized in energy related enzymes in mitochondrial and glycolytic pathways, structural proteins mainly of acrosome and activating signal transducers (Siva *et al*., 2010; Martinez-Heredia *et al*., 2008; Liao *et al.*, 2009).

Comparative proteomics using 2D-Page electrophoresis coupled with mass spectrometry should be of great interest to understand the processes of maturation and capacitation in the scallop and to compare these processes with other molluscan species. In invertebrates, some proteomic approaches have also been developed recently to identify the proteins involved in sperm maturation. In bivalve species, proteins of *C. gigas* and *Mytilus galloprovincialis* spermatozoa have been recently analyzed (Huang, Liu & Huan, 2015; Zhang *et al.*, 2015) and the ones involved in spermatozoon maturation were identified in the oysters *C. gigas* (Kingtong *et al*., 2013). The recent development of a transcriptomic database for *P. maximus* enabled us to establish the proteome map of the scallop spermatozoa. Additionally, the proteomic-based comparison between spawned spermatozoa and spermatozoa collected in the gonad before spawning will be used to identify proteins differentially expressed between both types of sperm. Their potential involvement in processes of maturation/capacitation of the spermatozoon is discussed hereafter.

Material and method

Broodstock conditioning

The *P. maximus* collected from the bay of Brest (Finistère, France) were conditioned in the Argenton Hatchery (Ifremer) in order to obtain breeders, using the standard protocol described in 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) in the male part of the gonad. Animals were then placed in individual beakers containing 500mL 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 animals and dissection of the male part of the gonad. Male gonads were scarified and resuspended in 5mL 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 (germ line cells and somatic cells). Two main bands containing spermatozoa were obtained after 30 min centrifugation (600 g, 15°C). The enrichment in spermatozoa of 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 performed 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 formaldehyde (37%), 30% v/v ethanol (95%), 40% v/v FSW) for 48 hours then dehydrated and embedded in paraffin wax. Five micrometer sections 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), 1mM protease inhibitor aprotinin. The extract was sonicated on ice before being incubated at room temperature for 30 minutes and centrifuged at 12000 g, 4°C for 30 minutes. The supernatant was collected and acetone precipitated (overnight at -20°C). After centrifugation (15000 g, 30 min, 4°C) the pellet of proteins was rinsed in fresh acetone and dried by speed vacuum. The protein extraction from spawn spermatozoa used the same condition as the stripped spermatozoa.

For protein separation using 2-D gel, 2 sizes of strips 7 cm and 18 cm were used for 2 main purposes. The 7 cm strips were used for protein profile comparison of stripped and spawned spermatozoa. The 18 cm strips were used to improve amount of protein loading on gel and were also used as reference map of spermatozoa proteome of *P. maximus*.

For IEF separation, proteins were resuspended in 200 µL of lysis buffer maintained at 4°C using pulse sonication. Protein concentration of the sample was carried out by Bradford's method with BSA (Bovine Serum Albumin) as standard. For the first dimension 250 µg (7 cm) or 750 µg (18 cm) 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 was achieved by gradient step method using 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 3500 V then 2 h at 3500 V for 7 cm strip; 1 h at 500 V, 1 h to reach 1000 V, 3 h to reach 8000 V, 2h at 8000 V for 18cm 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 was performed by 12% SDS-PAGE with the following protocols: 500 V for 1 hour for 18 cm gels and 100 V for 1 hour for 7 cm gels in running buffer. IEF protein markers 3-10 and molecular weight marker were used to determine the

pI and MW of each identified protein. Protein visualization was carried out by 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 were analyzed using Progenesis Same Spot software (Nonlinear Dynamics, Newcastle-upon-tyne, UK). This program detected volume of protein spots, matched them across gels and expressed as average ± standard deviation. Then the amounts of same protein spot 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, 3 gels from 3 stripped spermatozoa samples and 3 gels from spawned spermatozoa samples were used for comparison with a cut-off of 1.5-fold volume difference with t-test p-value ≤ 0.05 to evidence significant differences of protein expression. Differential proteins (P≤0.05) were further identified as described in the next section.

Protein identification

Differential proteins (P≤0.05) were manually picked up from gel, destained in a solution of 25 mM NH_4HCO_3 in 50% acetonitrile and digested with trypsin (37°C for 15h) before mass analysis. MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeam™ on-axis irradiation laser running at a 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of Angiotensin I, Angiotensin II, Neurotensin, ACTH clip (1-17), 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 4300 was typically employed for ionizing. MS spectra were acquired in positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range 700 to 4000Da. MS/MS spectra were acquired in positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 4500. For the tandem MS experiments the acceleration voltage applied was 1kV and air was used as the collision gas. Gas pressure medium was selected as settings. 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 v2.2.04; 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 100ppm 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 p*I* 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. Indeed, gonadal tubules appeared to contain mainly spermatozoa. Other types of germ cells (spermatids, spermatocytes) were nevertheless observed around the gonadal tubules (shown in Supplementary material, Fig. S1). However, the gonoduct sections of serotonin-induced animals (spawned) and unspawned animals differ in their aspect: spawned animals exhibit gonoduct containing spermatozoa while the others have empty gonoducts.

Reference map of *P. maximus* stripped spermatozoa

A reference map was established with 750µg of total protein extracted from stripped spermatozoa of *P. maximus* using 18cm IPG strips allowing high resolution of protein map (Fig. 1). One hundred sixty two spots were submitted to MS/MS analysis and 133 proteins (82%) were identified on the basis of EST database from *P. maximus* (Table 1). The proteins were putatively identified according to gene ontology as shown in Figure 2. Most of them (44%) were annotated as performing energy production, ion or electron transport whereas other proteins were classified as involved in cell movement, development process, transcription/biogenesis, proteasome, oxidoreductase process, apoptotic process and as unclassified proteins.

Differentially expressed proteins between stripped and spawned spermatozoa of *P. maximus*

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

Discussion

Among the 133 proteins found in scallop spermatozoa, the most abundant are involved in energy production, ion or electron transport which can be broken down into three major classes according to their metabolic function. The first class proteins, is involved in glycolysis or tricarboxylic acid cycle. Examples of proteins belonging to this class are aconitate hydratase (spots B4-B5), fructose-bisphosphate aldolase (spot B38), citrate synthase (spots B30-B31), succinate dehydrogenase

(spots A12-A13-A14), glyceraldehyde-3-phosphate dehydrogenase (spot D2). The second class of proteins involved in energy production is the lipid metabolism proteins which 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 isomerise (spot D24) belong to this class. The third class is 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), cytochrome b-c1 complex (spots A24-A33). The high number of proteins (45 protein spots) involved in energy production/ion or electron transport found in spermatozoa of *P. maximus* seems to correlate with the high energy requirement for sperm motility. Indeed sperm movement duration is generally long for bivalve species, as compared to what is observed in vertebrates, reaching 10 hours in *P. maximus* (Suquet *et al*., 2013). Some authors suggest that scallop sperm could partially restore the energy content catabolised during movement time (Faure *et al*., 1993).

Subcellular fractioning should be of interest to identify less abundant proteins in the spermatozoa of *P. maximus*. Indeed, 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 would be particularly interesting to identify the proteins involved in oocyte recognition.

The number of up regulated proteins in spawned spermatozoa is quite important considering the short process of the transfer through the germinal tract 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. Moreover, 2-D gel electrophoresis allowed the separation of different modified forms of protein such as phosphorylations or glycosylations (Seo & Lee, 2004).

The proteome profiles of stripped and spawned spermatozoa led to identification of differentially expressed proteins. Most down-regulated proteins in the stripped spermatozoa, such as Tektin-2, tubulin and MAPRE3 are associated with energy metabolism and cell movement. Tektin-2, the protein involved in cell movement, has been found to be required for normal flagellum structure and function in mouse (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). The flagellum bending and shortening found in Tektin-2 null sperm is due to the dynein inner arm disruption (Shimasaki *et al*., 2010). In immotile human spermatozoa, lower levels of tektin-2 are also related with decreasing sperm motility and fertilization rates (Bhilawadikar *et al*., 2013). Interestingly, Tektin-2 was also found to be up-regulated in mature spermatozoa of *C. 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). Post-translational modifications (PTMs) of this protein generate functional diversity of microtubules. Recently, acetyled 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, microtubule-associated protein RP/EB family

member 3 (MAPRE3) was also found to be down-regulated. This protein binds to the end of microtubules and promotes microtubule growth, a property which may play a role in cell migration. The decreasing levels of tektin-2, tubulin and MAPRE3 proteins in stripped scallop spermatozoa indicate a limitation of sperm motility which correlates with Faure's observation that the motility of scarified spermatozoa directly from gonad was lower than that of the spawned ones (Faure *et al*., 1993).

Other differentially expressed proteins are linked to mitochondrial energy metabolism and could be correlated to the energy and ATP content of spermatozoa in *P. maximus*. Aconitate hydratase, an enzyme of the tricarboxylic acid cycle that catalyzes the isomerization of citrate to isocitrate, was down-regulated in scallop stripped spermatozoa, whereas this protein was up-regulated in *C. gigas* stripped spermatozoa, together with a success in fertilization (Kingtong *et al*., 2013). This main difference between both molluscan species may be related to the different fertility levels observed between stripped oyster spermatozoa and stripped *Pecten* spermatozoa. Moreover, both species exhibited strong differences in ATP management associated to sperm movement, as described by Suquet *et al*. (2013), possibly related with aconitate hydratase expression level.

One of the up-regulated proteins in stripped scallop spermatozoa is Glyceraldehyde-3-phosphate dehydrogenase (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 the 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 also known to initiate apoptotic cell death (Hara *et al.,* 2005). The GAPDH expression level of apoptotic cells is three time higher than that of non-apoptotic cells (Dastoor & Dreyer, 2001). Malate dehydrogenase (MADH), an enzyme that catalyzes the last step of the Krebs cycle by oxidizing malate to oxaloacetate , is another up-regulated protein found in *P. maximus* stripped spermatozoa. This enzyme is located in the sperm mitochondria of the midpiece in spermatozoa of various vertebrate species (Kohsaka *et al*., 1992; Auger *et al*., 2010). This respiratory enzyme, mitochondrial malate dehydrogenase, (MDH2) was found to be significantly overexpressed in human spermatozoa under oxidative stress (Sharma *et al*., 2013). Thus, up-regulation of the GAPDH and MADH enzymes implies that some oxidative stress or cell apoptosis found in stripped scallop spermatozoa may reflect a stress of the cell in response to the stripping procedure and could also be involved in infertilization.

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. This protein may also function as a laminin receptor after post-translational modification and was already identified in another scallop *Chlamys Farreri* (Sun *et al.*, 2014). As mature spermatozoa were supposed to be translationally inactive (Dacheux & Dacheux, 2014), the diminishing level of this protein between stripped and spawn spermatozoa suggests the possible reduction of translation capacities, a characteristic of late spermatozoon differentiation. Moreover, this observation

indicates that in *P. Maximus*, this late maturation step takes place during the transit of gametes through the genital duct.

In conclusion, proteomes of stripped and spawned spermatozoa of *P. maximus* differ by expression of proteins mainly involved in motility and energy supply for the spermatozoon. Moreover, some proteins implied in stress reactions are more expressed in stripped spermatozoa. The results confirmed 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 that respect the maturation pattern of the spermatozoa appeared different according to the bivalve species considered: 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 observation leads to draw a parallel with the different reproductive strategies of both species: considering the simultaneous hermaphrodism of the scallop, the final maturation step of spermatozoa occurring in the genital duct may contribute to reducing autofertilisation.

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Figure S1: Histological sections of male gonad of breeders. A: characteristic aspect of the section of the male gonad containing germ cells from spermatogonia to spermatozoa, with a large majority of spermatozoa. B: gonadal duct full of spermatozoa of animals after serotonin injection and spawning Cc: empty gonadal duct of a non-induced scallop. sz: spermatozoa, sd: spermatid, sc: spermatocyte, sg: spermatogonia, gd: gonadal duct

Figure 1: Referent Map of *P. maximus* stripped spermatozoa. Details of protein identification are shown in table 1. Mr: molecular weight marker, reference molecular weight are indicated on the left of gel.

Figure 2: Repartition of the identified proteins on the basis of putative function according to gene ontology.

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

Table 1: List of proteins identified in the referent map of stripped *P. maximus* spermatozoa.

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

Table 1. List of proteins identified in the referent map of Stripped *P. maximus* spermatozoa

Spot	Accession Number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
A1	Q95YJ5	Thioredoxin domain-containing protein 3 homolog	4.56	73038.5	4.37	107857	1	67.48
A2	Q9D2H2	Adenylate kinase 7	4.9	70675.4	4.68	92589	3	164.59
A3	Q7ZU99	Transitional endoplasmic reticulum ATPase	5.14	89423.8	5.19	99018	6	287.11
A4	Q18688	Heat shock protein 90	4.96	80283.2	4.68	89911	4	300.52
A5	Q16959	Dynein intermediate chain 2	5.7	79138.4	5.88	79919	2	170.43
A6	Q16959	Dynein intermediate chain 2	5.7	79138.4	6.02	79919	2	224.45
A7	Q16959	Dynein intermediate chain 2	5.7	79138.4	6.16	79757	3	76.89
A8	Q0MQG2	NADH-ubiquinone oxidoreductase 75 kDa subunit	5.55	76988.5	5.05	74575	5	354.30
A9	Q0MQG2	NADH-ubiquinone oxidoreductase 75 kDa subunit	5.55	76988.5	5.12	74575	7	598.37
A10	Q06248	Heat shock 70 kDa protein IV	5.55	69749.8	5.26	71822	16	1114.22
A11	P29845	Heat shock 70 kDa protein cognate 5	6.02	74066.1	5.73	71498	3	90.78
A12	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67845.5	5.91	71012	2	107.88
A13	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67845.5	6.04	70526	3	141.51
A14	Q7ZVF3	Succinate dehydrogenase [ubiquinone] flavoprotein	5.74	67845.5	6.15	70364	6	242.75
A15	O02649	60 kDa heat shock protein	5.07	54604.6	4.77	59322	9	866.41
A16	Q16960	Dynein intermediate chain 3	4.99	68224.5	4.88	82411	4	216.85
A17	Q16960	Dynein intermediate chain 3	4.99	68224.5	4.93	82411	3	165.64
A18	P18288	Tubulin alpha chain, testis-specific	4.98	49993.6	4.98	53432	8	683.81
A19	P11833	Tubulin beta chain	4.73	50051.2	4.76	52542	13	847.28
A20	P10719	ATP synthase subunit beta, mitochondrial	4.95	51710.1	4.65	48696	17	1556.49
A21	P11833	Tubulin beta chain	4.73	50051.2	5.91	52034	9	557.50
A22	O02654	2-phosphoglycerate dehydratase	5.78	47426.2	6.14	51695	3	153.15
A23	Q26648	Tektin-B1	5.34	46147.6	6.11	49022	8	578.82
A24	P18288	Tubulin alpha chain, testis-specific	4.98	49993.6	4.95	53136	9	694.38
A25	P98080	Cytochrome b-c1 complex subunit 1, mitochondrial	6.07	51735.9	5.7	45000	9	541.17
A26	P11833	Tubulin beta chain	4.73	50051.2	5.31	50593	9	473.39
A28	Q90512	Dihydrolipoyllysine-residue succinyltransferase	5.25	39848.8	5	48261	3	195.32
A30	P12716	Actin, cytoplasmic	5.3	41614.5	4.94	45761	2	82.61
A31	P91929	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex	5.18	40414	5.02	45978	4	197.37
A32	P18288	Tubulin alpha chain, testis-specific	4.98	49993.6	5.05	53220	8	624.11
A33	P32551	Cytochrome b-c1 complex subunit 2, mitochondrial	8.83	46751.2	5.39	44674	4	244.05
A34	Q0MQG3	Probable NADH dehydrogenase	6.26	49235.7	6.03	44348	7	372.71
A35	Q6P5L8	Hydroxysteroid dehydrogenase-like protein 2	7.08	44420.9	6.07	40543	1	113.51
A36	P12716	Actin, cytoplasmic	5.3	41614.5	5.33	39422	7	452.02
A37	P18603	Actin, clone 403	5.3	41603.5	5.19	38965	11	932.36
A38	P53445	Fructose-bisphosphate aldolase, muscle type	8.84	39239	5.06	39665	4	305.25
A39	Q9Z2I9	Succinyl-CoA ligase [ADP-forming] subunit	5.33	44422.5	4.66	39422	3	154.53
A40	A1L0Z6	Radial spoke head protein 6 homolog A	4.26	58497	4.56	70769	5	290.50
A41	P53590	Succinyl-CoA ligase [GDP-forming] subunit	5.1	42572.9	4.98	38782	2	82.88
A42	Q9VWH4	Probable isocitrate dehydrogenase [NAD] subunit alpha	6.96	40844	5.08	37595	6	410.86
B2	Q5RCB8	2-oxoglutarate dehydrogenase	6.07	111326.9	7.8	111381	2	79.82
B3	P09812	Glycogen phosphorylase	6.9	97142.24	7.21	95268	2	135.84
B4	Q9ER34	Aconitate hydratase	7.15	82461.86	6.87	78219	4	166.392
B5	Q9ER34	Aconitate hydratase	7.15	82461.86	6.7	77328	9	454.132
B7	Q9D9T8	EF-hand domain-containing protein 1 (Myoclonin-1)	5.71	75141.64	7.05	71174	8	388.0152
B8	Q8BMS1	Trifunctional enzyme subunit alpha	9.06	78765.22	8.8	73927	11	478.8753

Spot	Accession Number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
B9	Q32TF8	EF-hand domain-containing family member C2	5.81	85457.36	7.95	77814	6	213.062
B12	Q921G7	Electron transfer flavoprotein-ubiquinone oxidoreductase	7.34	68090.93	7.09	65344	2	65.71331
B13	Q6AXQ8	Meiosis-specific nuclear structural protein 1	7.09	61239.82	6.86	61781	4	117.2953
B14	Q2YDI7	Tektin-5	7.95	56503.87	8.45	59746	1	31.9
B16	Q2YDI7	Tektin-5	7.95	56503.87	8.65	58898	4	153.73
B17	Q2YDI7	Tektin-5	7.95	56503.87	8.84	59237	1	43.98
B19	P41383	Tubulin alpha-2/alpha-4 chain	4.94	50210.72	8.96	53602	8	480.15
B20	Q9YIC0	Elongation factor 1-alpha (EF-1-alpha)	9.22	50443.26	9.23	49457	5	232.64
B21	P34255	Uncharacterized protein B0303.3	9.15	47874.47	9.21	43913	6	281.5199
B22	P35381	ATP synthase subunit alpha, mitochondrial (Protein bellwether)	8.22	54553.87	8.18	52034	6	367.3533
B23	Q91YT0	NADH dehydrogenase [ubiquinone] flavoprotein 1	7.92	48626.48	8.24	49891	5	200.8133
B24	P82264	Glutamate dehydrogenase, mitochondrial (GDH) (EC 1.4.1.3)	7.34	55394.09	7.97	53136	11	620.0233
B25	P41383	Tubulin alpha-2/alpha-4 chain	4.94	50210.72	7.82	53051	9	715.16
B26	Q5R546	ATP synthase subunit alpha, mitochondrial	9.16	59780.66	7.65	52203	14	1112.652
B27	P11833	Tubulin beta chain (Beta-tubulin)	4.73	50051.16	6.86	53051	3	166.47
B28	O02654	Enolase	5.78	47426.22	6.59	50763	5	252.3866
B29	Q922G7	Tektin-2 (Tektin-t) (Testicular tektin)	5.98	50311.09	6.42	48913	5	318.6766
B30	Q4S5X1	Citrate synthase, mitochondrial (EC 2.3.3.1)	6.69	48727.71	6.57	45000	4	151.03
B31	Q4S5X1	Citrate synthase, mitochondrial (EC 2.3.3.1)	6.69	48727.71	6.82	43043	9	486.1933
B32	Q9JLI7	Sperm-associated antigen 6 (Axoneme central apparatus protein)	6.33	55269.25	7.54	51186	3	203.75
B33	P35623	Serine hydroxymethyltransferase, cytosolic (SHMT)	8.03	52893.44	7.66	49674	15	741.81
B35	Q2KIW6	26S protease regulatory subunit 10B	6.74	44073.85	6.6	40217	4	162.06
B36	Q3SZB4	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial (MCAD) (EC 1.3.8.7)	7.02	43586.83	7.05	39970	5	262.36
B37	Q3T0R7	3-ketoacyl-CoA thiolase, mitochondrial	8.06	42131.21	7.4	40652	3	133.99
B38	Q9GP32	Fructose-bisphosphate aldolase (EC 4.1.2.13)	8.03	39727.38	7.59	39209	6	289.26
B39	Q9QXL8	Nucleoside diphosphate kinase 7 (NDK 7) (NDP kinase 7) (EC 2.7.4.6) (nm23-M7)	6.72	44433.9	7.7	39087	2	162.79
B40	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39101.69	8.53	39543	13	855.9066
B41	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39101.69	8.83	39117	13	861.3966
B42	O77784	Isocitrate dehydrogenase [NAD] subunit beta	8.2	38959.96	8.31	37900	5	200.7099
B43	Q80Y75	DnaJ homolog subfamily B member 13 (Testis and spermatogenesis cell-related protein 6)	7.02	36154.51	8.49	37504	4	176.37
B44	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39101.69	7.29	37778	10	752.9266
B45	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39101.69	7.11	38356	7	456.5133
B46	O15990	Arginine kinase (AK) (EC 2.7.3.3)	7.12	39101.69	6.92	38539	4	283.4233
B47	P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial (LCAD) (EC 1.3.8.8)	6.5	44627	6.53	39117	6	240.9866
B48	Q27928	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic (GPD-C) (GPDH-C) (EC 1.1.1.8)	6.52	39448	6.49	38295	5	335.5387
B49	P11833	Tubulin beta chain (Beta-tubulin)	4.73	50051.16	6.32	38143	6	214.8933
B50	P41564	Isocitrate dehydrogenase [NAD] subunit gamma	8.66	38767.48	7.14	37291	4	174.9733
B51	Q9JID6	Long-chain-fatty-acid--CoA ligase 1	7.48	77697.99	8.42	71336	3	158.66
B52	Q4R755	Glycerol-3-phosphate dehydrogenase	6.42	76309.31	8.43	69879	2	98.98
C3	Q5NVR2	Malate dehydrogenase	8.54	33046.5	5.34	35464	11	599.36
C4	P11833	Tubulin beta chain	4.73	50051.2	5.65	35282	6	203.02
C5	P11833	Tubulin beta chain	4.73	50051.2	5	33425	2	126.31
C6	Q5TYW6	Radial spoke head protein 9 homolog	5.48	30924.2	5.2	32846	9	447.16
C8	Q6PC29	14-3-3 protein gamma-1	4.86	28234.5	4.72	32481	4	164.24
C9	Q6VTH5	Radial spoke head 1 homolog	5.09	24566.4	4.92	32024	4	152.11
C11	Q25117	ATP synthase subunit beta, mitochondrial	4.91	54020.4	4.88	28117	6	451.11

Spot	Accession Number	Protein name	Theoretical		Experimental		Peptide matched	Score
			pI	MW	pI	MW		
C12	Q0MQG7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	5.48	26414.9	5.28	28068	10	597.90
C13	P11833	Tubulin beta chain	4.73	50051.2	4.39	26357	3	210.42
C14	Q66IC9	Ropporin-1-like protein	5.61	24170.8	4.88	24352	6	363.22
C15	Q0MQI9	NADH dehydrogenase [ubiquinone]	5.71	23760.3	5.36	24108	3	121.55
C17	Q25117	ATP synthase subunit beta	4.91	54020.4	4.45	22543	3	169.60
C19	Q5FB30	Superoxide dismutase [Mn], mitochondrial	6.82	22241.2	5.88	19028	1	39.19
C20	P55954	Cytochrome c oxidase subunit 5A	5.12	17115.4	4.5	14044	5	324.45
C21	Q15370	Transcription elongation factor B polypeptide 2	4.73	13132.8	4.47	12633	3	348.05
D1	A2RRW4	Protein FAM166B	9.55	33272.8	9.69	35205	5	227.46
D2	P10096	Glyceraldehyde-3-phosphate dehydrogenase	8.52	35736.9	8.24	35647	8	511.58
D10	P76536	Probable deferrochelatase/peroxidase YfeX	5.34	33052.3	7.53	32846	3	145.43
D12	P76536	Probable deferrochelatase/peroxidase YfeX	5.34	33052.3	7.82	32633	2	73.13
D13	P82013	Voltage-dependent anion-selective channel	8.62	30066.6	8.52	31050	12	1025.69
D15	P05631	ATP synthase subunit gamma	9.16	30255.7	9.23	31476	7	487.40
D16	A3KQA5	Outer dense fiber protein 3-B	9.78	27895.6	9.65	30350	8	441.14
D17	Q6SP97	Enkurin	9.58	29527.4	9.48	31187	3	133.18
D18	P13619	ATP synthase subunit b, mitochondrial	9.14	24668.7	9	28655	9	592.97
D19	O70250	Phosphoglycerate mutase 2	8.65	28827.1	8.41	27824	7	352.48
D20	Q9PTW9	Proteasome subunit alpha type-7	8.96	28084.2	8.21	27922	3	207.65
D21	P12716	Actin, cytoplasmic precursor	5.3	41848.8	8.41	27139	7	445.04
D24	Q62651	Delta(3,5)-Delta(2,4)-dienoyl-CoA	6.26	32474.2	6.66	30381	3	204.68
D25	Q9BKU4	Mitochondrial prohibitin complex protein	6.94	29988.4	6.37	31750	10	580.93
D26	Q9BKU4	Mitochondrial prohibitin complex protein	6.94	29988.4	6.27	31842	7	426.64
D27	P18053	Proteasome subunit alpha type-4	6.75	29411.7	6.22	30441	4	110.87
D28	Q68FU3	Electron transfer flavoprotein subunit	7.81	27556.2	6.35	29242	7	378.98
D29	P60901	Proteasome subunit alpha type-6	6.34	27399.5	6.36	28509	4	221.43
D31	Q58DM8	Enoyl-CoA hydratase, mitochondrial	6.86	28095.4	6.85	29095	6	321.24
D32	P56597	Nucleoside diphosphate kinase homolog 5	5.89	24236.3	7.17	24499	6	477.52
D33	Q3T108	Proteasome subunit beta type-4	5.45	24361.8	7.15	23472	3	192.39
D34	Q69BJ8	Cytochrome b-c1 complex subunit 11	6.98	21655.7	8.51	23081	4	202.02
D35	Q90384	Peroxiredoxin	7.63	22339.6	7.34	21858	4	259.07
D36	Q5R546	ATP synthase subunit alpha	8.28	55239.4	6.41	21320	4	239.94
D38	Q3MHN0	Proteasome subunit beta type-6 precursor	4.9	25542.1	7.02	19279	6	305.73
D39	Q2KID4	Dynein light chain 1, axonemal	5.63	21535.9	7.38	19812	4	207.38
D40	Q2TBP0	Proteasome subunit beta type-7	5.84	25305	7.72	18997	3	117.73
D41	A8Y5T1	Uncharacterized protein C1orf194 homolog	6.75	18800.7	7.99	18401	6	376.72
D42	Q1HPL8	NADH dehydrogenase [ubiquinone] 1 beta	5.94	18999.3	8.51	18887	5	307.93
D43	P13621	ATP synthase subunit O, mitochondrial	9.83	20929.8	9.56	18652	5	248.82
D44	O01369	Cytochrome b-c1 complex subunit 7	6.84	15331.5	9.55	14545	9	531.78
D45	Q9CQ24	F-box only protein 36	8.29	22075.4	7.3	29438	2	63.91
D47	Q32L77	Uncharacterized protein C9orf135 homolog	6.66	26623	7.78	28998	3	155.18

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

Swiss Prot accession number	Protein name	Function	pI/MW(kDa) theoretical	pI/MW(kDa) 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	Catalyzes 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 heme 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

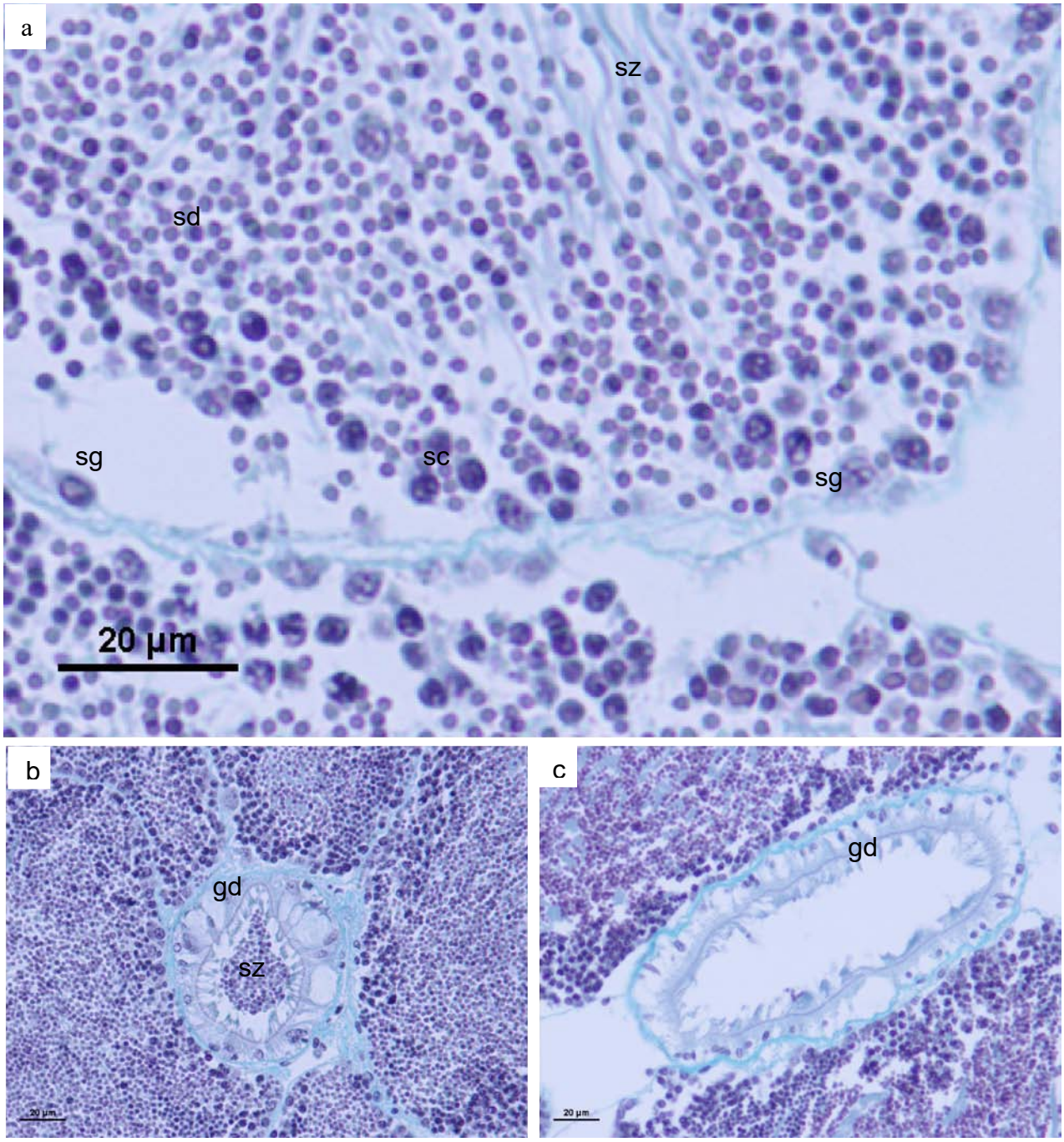

Figure 1S: Histological sections of male gonad of breeders. a: characteristic aspect of the section of the male gonad containing germ cells from spermatogonia to spermatozoa, with a large majority of spermatozoa. b: gonadal duct full of spermatozoa of animals after serotonin injection and spawning c: empty gonadal duct of a non-induced scallop. sz: spermatozoa, sd: spermatid, sc: spermatocyte, sg: spermatogonia, gd: gonadal duct

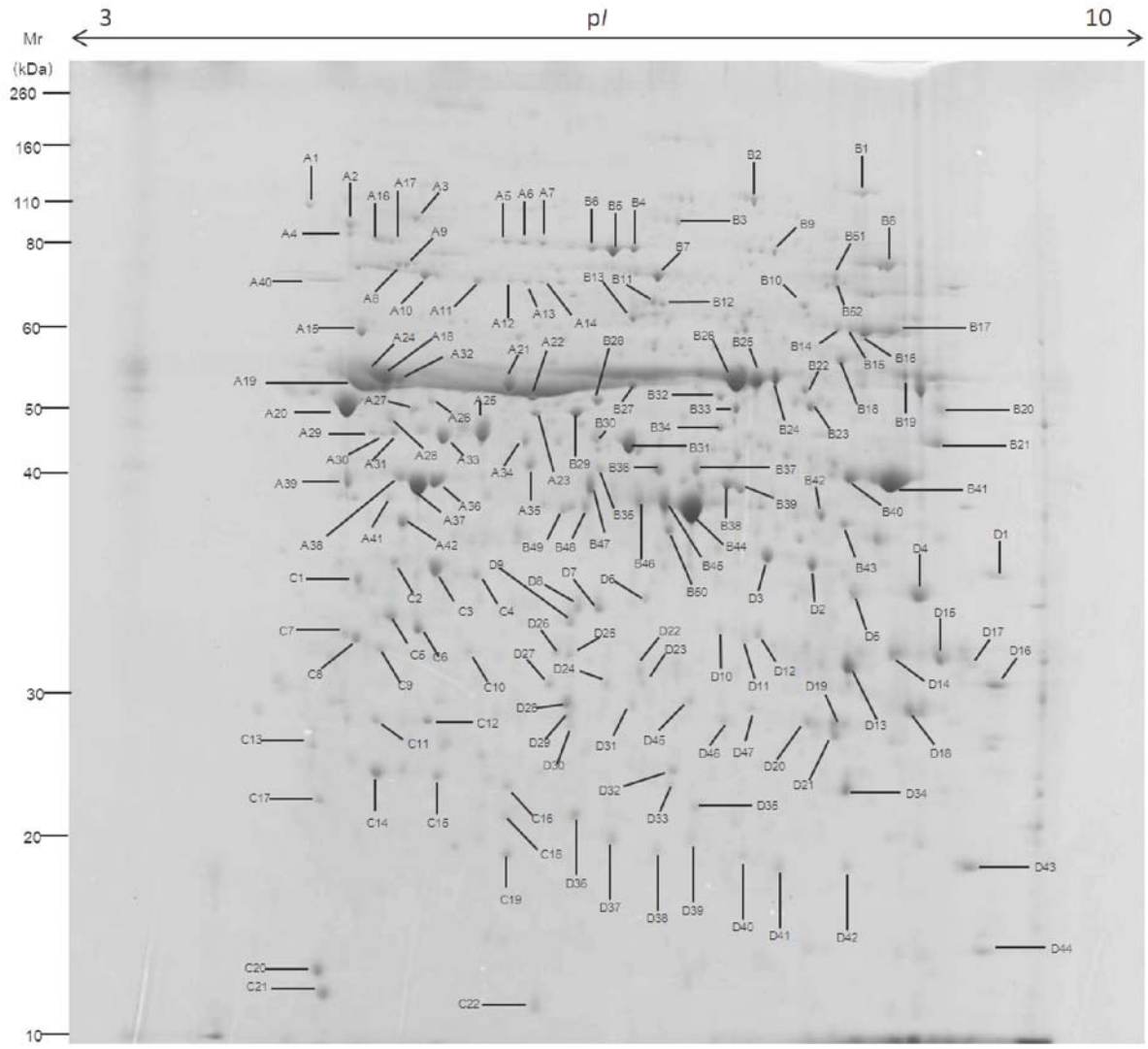

Figure 1: Referent Map of *Pecten maximus* stripped spermatozoa. Details of protein identification are shown in table 1. Mr: molecular marker, reference molecular weight are indicated on the left of gel.

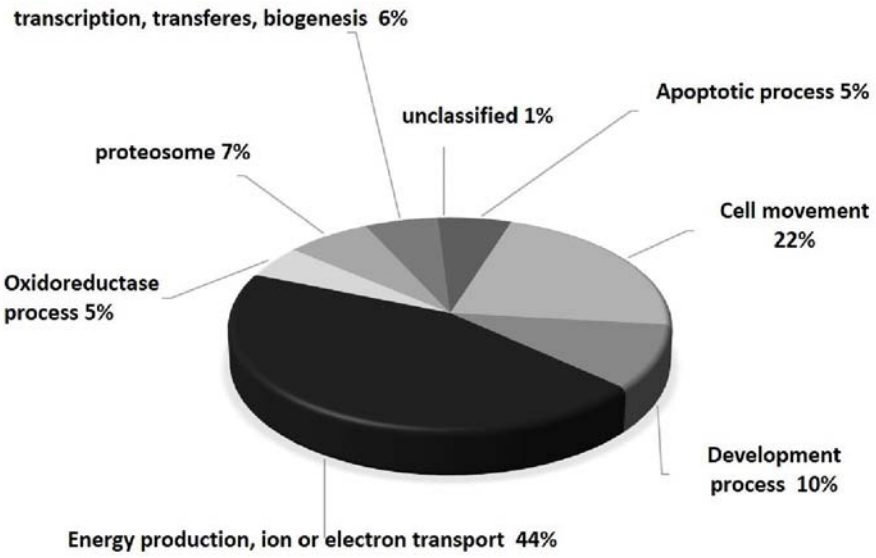

Figure 2: Distribution of the identified proteins on the basis of putative function according to gene ontology.

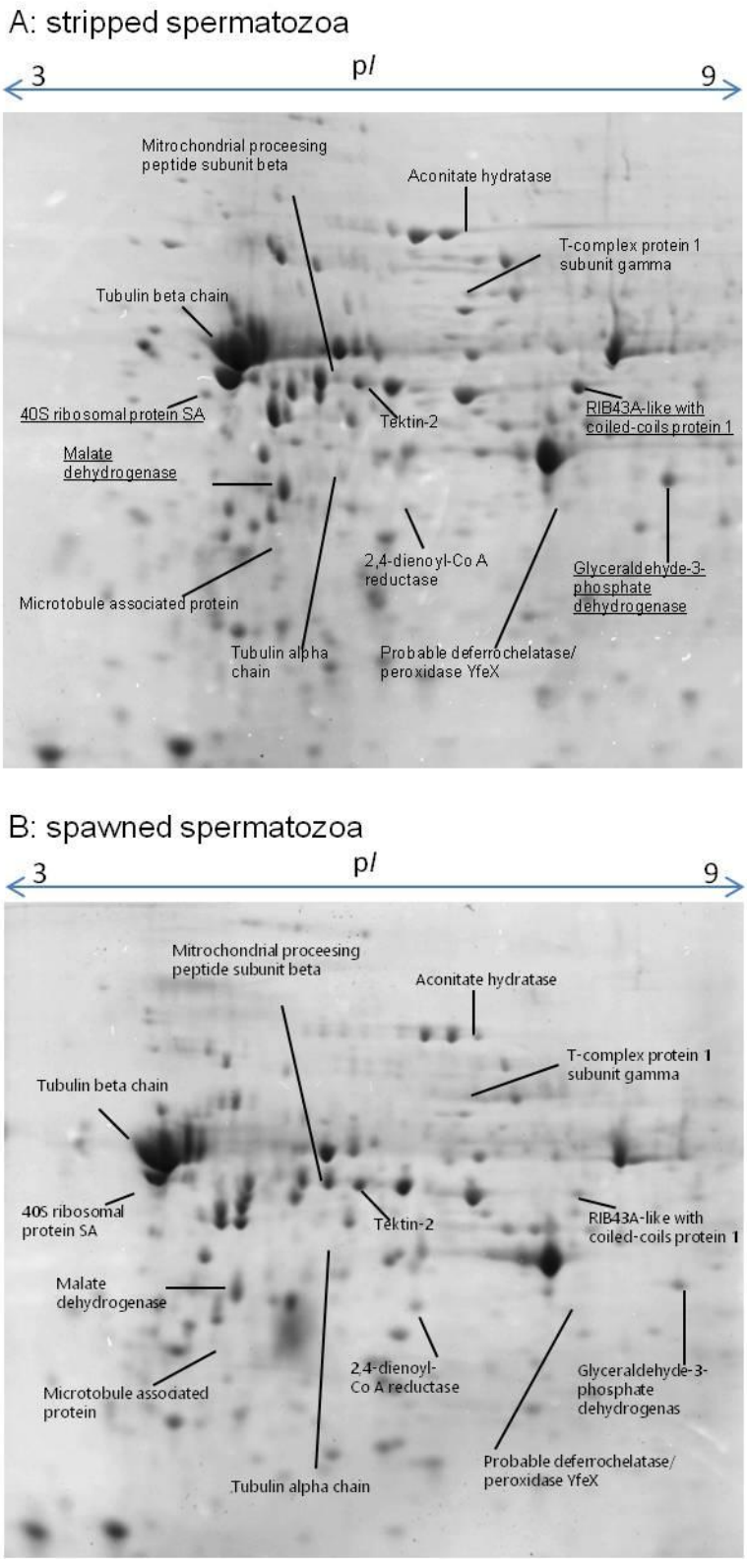

Figure 3: Representative 2-DE gels of proteome from stripped (A) and spawned (B) spermatozoa of *Pecten maximus.* Proteins were extracted and separated (first dimension by IEF on IPG strip pH 3-10NL, second dimension on 10% SDS-PAGE 7cm gels). Up-regulated proteins (underlined) and down-regulated proteins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