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New proteins identified in epididymal fluid from the platypus (*Ornithorhynchus anatinus*)

Abstract

The platypus epididymal proteome is being studied because epididymal proteins are essential for male fertility in mammals and it is considered that knowledge of the epididymal proteome in an early mammal would be informative in assessing the convergence and divergence of proteins that are important in the function of the mammalian epididymis. Few of the epididymal proteins that have been identified in eutherian mammals were found in platypus caudal epididymal fluid, and the major epididymal proteins in the platypus (PXN-FBPL, SPARC and E-OR20) have never been identified in the epididymis of any other mammal.

Keywords

platypus, anatinus, fluid, epididymal, ornithorhynchus, proteins, identified

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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New proteins identified in epididymal fluid from the platypus (*Ornithorhynchus anatinus*)

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Abstract. The platypus epididymal proteome is being studied because epididymal proteins are essential for male fertility in mammals and it is considered that knowledge of the epididymal proteome in an early mammal would be informative in assessing the convergence and divergence of proteins that are important in the function of the mammalian epididymis. Few of the epididymal proteins that have been identified in eutherian mammals were found in platypus caudal epididymal fluid, and the major epididymal proteins in the platypus (PXN-FBPL, SPARC and E-OR20) have never been identified in the epididymis of any other mammal.

Additional keywords: epididymis, monotreme, proteome, sperm maturation.

Introduction

In all mammalian species, the production of fertile spermatozoa is a result of active spermatogenesis in the testis and several sperm surface modifications during the transit of the gametes along the epididymis. The presence of a specific sperm environment produced by the epididymis during the post-gonadal differentiation stages is believed to play an essential role in preparing spermatozoa to fertilise ova. This specific luminal environment is controlled by the Sertoli cells in the testis and the epididymal epithelium, and is protected from plasma proteins by the presence of blood-luminal barriers in the testis and epididymis.

Studies on the composition of epididymal proteins in several different domestic mammals (see recent review, Dacheux *et al.* 2009) have shown significant differences between species with regard to the sequential changes in the luminal proteome along the length of the epididymal duct and the surface proteins that are present on spermatozoa. Each species appears to have developed its own strategy for sperm maturation and preservation, but evolutionary trends are not obvious in the species that have been studied. It was therefore considered that an understanding of the epididymal proteome of early mammals might provide an insight into which epididymal proteins have been conserved or have diverged during mammalian evolution, and for this reason the platypus (*Ornithorhynchus anatinus*), an extant egg-laying (monotreme) mammal, has been studied. During the active breeding period of the platypus, spermatozoa are produced in abdominal testes, develop the capacity for motility in the

proximal epididymides (initial segments) and are stored in the distal epididymides in bundles of ~100 individual spermatozoa (Djakiew and Jones 1981, 1983).

Materials and methods

Animals

Three mature male platypus (*Ornithorhynchus anatinus*) were captured by netting the Upper Barnard River, New South Wales, Australia, according to procedures accepted by the University of Newcastle's Animal Ethics Committee. The captured animals were sacrificed using an intraperitoneal injection of pentobarbitone sodium (Nembutal; Boehringer Ingelheim, Sydney, NSW, Australia) at a dose of 60 mg kg⁻¹ bodyweight. Epididymides and testes were removed from sacrificed animals near the capture site. The luminal contents of the caudal epididymal region (Fig. 1) were obtained by perfusing the duct with PBS buffer (137 mM NaCl, 15 mM KCl, 17 mM Na₂HPO₄, 1.5 mM KH₂PO₄) as previously described (Dacheux 1980). Spermatozoa were separated from the fluids by centrifugation (2000g for 10 min at 10°C), and the supernatant was centrifuged at 4°C at 15 000g for 15 min and stored at -80°C until analysis.

Gel electrophoresis and protein quantification

SDS-PAGE separation was carried out for all samples in 6–16% linear polyacrylamide gels (14 × 16 cm) according to Laemmli's method (Laemmli 1970). Isoelectric focusing (IEF) was performed using the O'Farrell technique, modified as previously

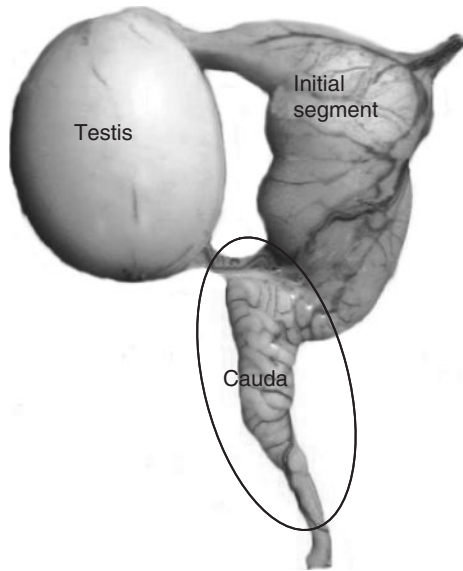


Fig. 1. Testis and epididymis of the platypus. The epididymal fluid was sampled from the cauda epididymidis (circled) by retrograde perfusion of the duct from the distal end.

described (Blangarin *et al.* 1984; Syntin *et al.* 1996). For protein IEF the pH gradient was obtained by mixing 1% ampholytes (pH 3–10) and 1% servalytes (pH 2–11). Isoelectric focusing was run in two steps, first at 20 mA, 0.1 W/tube, 700 V for a total of 10 000 V h⁻¹, followed by a second run at 20 mA, 0.1 W/tube, 3000 V for a total of 2000 V h⁻¹. Two-dimensional separation was performed on a 6–16% acrylamide gel (16 × 16 cm, 1.5 mm thick) at 40 mA. After two-dimensional gel electrophoresis, proteins were stained with Coomassie blue.

Protein quantification was obtained from three replicates of samples separated by 2D gels and stained with Coomassie blue. Densitometric values were obtained by transmission acquisition with an ImageScanner (GE Healthcare, Orsay, France), whilst characteristics (pI and MM) and quantification of the spots were obtained using Samespot software (NonlinearDynamics, Newcastle Upon Tyne, UK). The major protein spots observed on 2D gels were identified by mass spectrometry. Coomassie blue-stained spots were excised and cut into small blocks that were rinsed with water and acetonitrile, then reduced with dithiothreitol, alkylated with iodoacetamide and incubated overnight at 37°C in 25 mM NH₄HCO₃ with 12.5 ng μL⁻¹ trypsin (Sequencing Grade; Roche, Paris, France) (Shevchenko *et al.* 2007). Tryptic peptides were analysed by nano-LC-MS/MS with an Ettan MDLC system coupled to a linear ion trap LTQ mass spectrometer (Thermo Electron, Waltham, MA, USA). The peptide and fragment masses obtained were matched automatically to proteins in a non-redundant database (nr NCBI) using MASCOT software (Matrix Science Ltd, London, UK). Enzyme specificity was set for trypsin with two missed cleavages using carbamidomethylcysteine, methionine oxidation and cys propionamide as variable modifications. The tolerance of the ions was set at 1.4 Da for parent and 1 Da for fragment ion matches. All hits with a *P* value <0.05 were manually verified

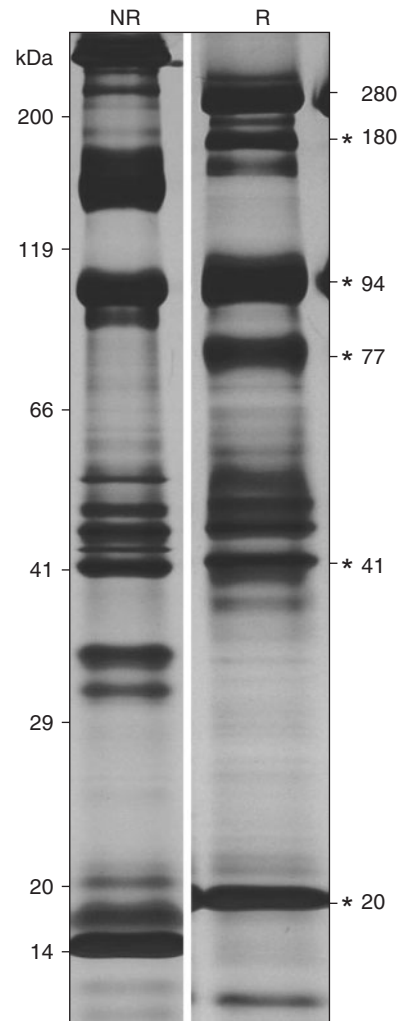


Fig. 2. One-dimensional gel electrophoresis separation of the caudal epididymal proteins under reducing (R) and non-reducing (NR) conditions. Silver-stained. Asterisks indicate the major bands.

and proteins detected by one peptide were considered to be positively identified with five consecutive fragment ions.

When no match was obtained, *de novo* sequencing was performed on tryptic peptide extracts. Manual MS and MS/MS were performed on a nanoESI Q-TOF UltimaGlobal mass spectrometer (Waters, Manchester, UK). Data acquisition and analyses were performed using MassLynx v4.0 software (Waters, Milford, MA, USA). *De novo* sequences were compared with the nr database with a similarity search program (BLAST NCBI; Altschul *et al.* 1990). The theoretical molecular weights (MWs) shown in Table 1 are from the database according to the amino acid sequence of the protein, and the observed MWs were calculated from 2D gels with MW markers using 'SameSpot' software.

Results

The 1D gel electrophoresis separation of platypus caudal epididymal fluid revealed several major bands at 280, 94, 77, 41

Table 1. Identification of proteins from platypus caudal epididymal fluid

| Spot number ^A | Gene name | Protein name | Gene ID | % sequence | Mascot score ^B | Theoretical molecular mass (kDa) | Observed molecular mass (kDa) ^C | % Total proteins ^D |
|--|------------|--|--------------------|------------|---------------------------|----------------------------------|--|-------------------------------|
| <u>1</u> , 27 | ? | E-OR20 (new lipocalin) | ENSOANT00000015815 | 26 | dNS ^E | 16.09 | 21.91 | 19.90 |
| <u>2</u> , 3 | SPARC | SPARC precursor | gi 149412550 | 16 | dNS | 34.15 | 38.09 | 7.67 |
| 9 | VTDB | Vitamin D-binding protein precursor | XP_001511847 | 4 | dNS | 65.37 | 46.63 | 0.6 |
| <u>14</u> , 25 | CEGP1 | Guanine nucleotide binding protein | gi 149409503 | 7 | 249 | 103.54 | 89.65 | 0.02 |
| <u>4</u> , 12, <u>14</u> , <u>15</u> –28, 34, 37, 38 | PXN-FBPL | PXN-FBPL, partial | gi 149580809 | 28 | 3073 | 176.45 | 75.69 | 26.00 |
| <u>17</u> , 27 | IgE | IgE immunoglobulin | gi 17223803 | 11 | 211 | 63.32 | 58.94 | 0.07 |
| <u>17</u> , 27 | LCP1 | Lymphocyte cytosolic protein 1 | gi 149635926 | 10 | 275 | 75.32 | 58.94 | 0.07 |
| <u>19</u> | EFEMP1 | EGF-containing fibulin-like extracellular matrix protein 1 | gi 149640868 | 3 | 62 | 55.17 | 38.75 | 0.05 |
| <u>22</u> , 23 | TUB | β tubulin | gi 30088884 | 40 | 1325 | 50.29 | 48.79 | 0.13 |
| <u>22</u> , <u>23</u> , 47 | DPEP2 | Dipeptidase 2, partial | gi 149523934 | 13 | 210 | 36.69 | 43.89 | 0.14 |
| 22 | GK | Glycerol kinase | gi 149642522 | 6 | 148 | 65.22 | 48.79 | 0.13 |
| 22 | TCP1 | T-complex protein 1 | gi 149634817 | 5 | 119 | 40.77 | 48.79 | 0.13 |
| 23 | NUCB2 | Hypothetical nucleobindin 2 | gi 149634496 | 12 | 397 | 54.72 | 43.89 | 0.01 |
| 23 | TXNDC4 | hCG28655, thioredoxin domain-containing 4 | gi 149567274 | 4 | 107 | 46.95 | 43.89 | 0.01 |
| 24 | CTSB | Putative cathepsin B variant 2 | gi 149436731 | 23 | 176 | 23.05 | 39.07 | 0.3 |
| 25, 26 | FGL1 | Fibrinogen-like protein 1 precursor | gi 149441799 | 24 | 268 | 20.34 | 34.15 | 0.06 |
| 26, <u>35</u> , 37, 43–46 | N6-PLA2 | N6b basic phospholipase A2, partial | gi 149491846 | 44 | 478 | 17.58 | 24.64 | 1.3 |
| <u>27</u> , 41 | ALB | Serum albumin | gi 149466340 | 49 | 1365 | 46.37 | 22.29 | 1 |
| <u>30</u> , 34 | Oncoprot 1 | Oncoprotein induced transcript 1, partial | gi 149587479 | 5 | 62 | 19.62 | 24.71 | 1 |
| 31 | PRDX1 | Peroxisome proliferator-activated gene | gi 149632365 | 19 | 230 | 22.18 | 24.90 | 0.08 |
| 41 | REG4 | Regenerating islet-derived family, member 4 | gi 149478572 | 14 | 113 | 30.03 | 20.02 | 0.3 |
| 41 | PSP-1 | Spermadhesin PSP-1 | gi 108346 | 44 | 118 | 11.98 | 20.02 | 0.3 |
| 44 | S100A11 | S100 calcium-binding protein A11 | gi 149432668 | 36 | 166 | 12.18 | 12.88 | 0.5 |
| 46, <u>47</u> , 48 | SOD3 | Superoxide dismutase 3, extracellular | gi 149412311 | 8 | 224 | 44.12 | 32.46 | 0.6 |
| 48 | CA2 | Carbonic anhydrase II | gi 149637125 | 9 | 189 | 47.06 | 28.77 | 0.07 |
| 48 | ZNHIT4 | Thyroid hormone receptor interactor 4 | gi 149411660 | 17 | 101 | 29.78 | 28.77 | 0.07 |

^A Spot numbers correspond to the spots in Fig. 3.

^B The values of the Mascot score given in the table correspond to those obtained for the protein identification (from the spot for which the number is underlined).

^C Molecular mass corresponding to the spot number, or underlined spot number.

^D Percentage of the total protein calculated from the 2D gel separation (Fig. 3) and illustrated also in Fig. 4.

^E dNS: Protein identification obtained after *de novo* sequencing.

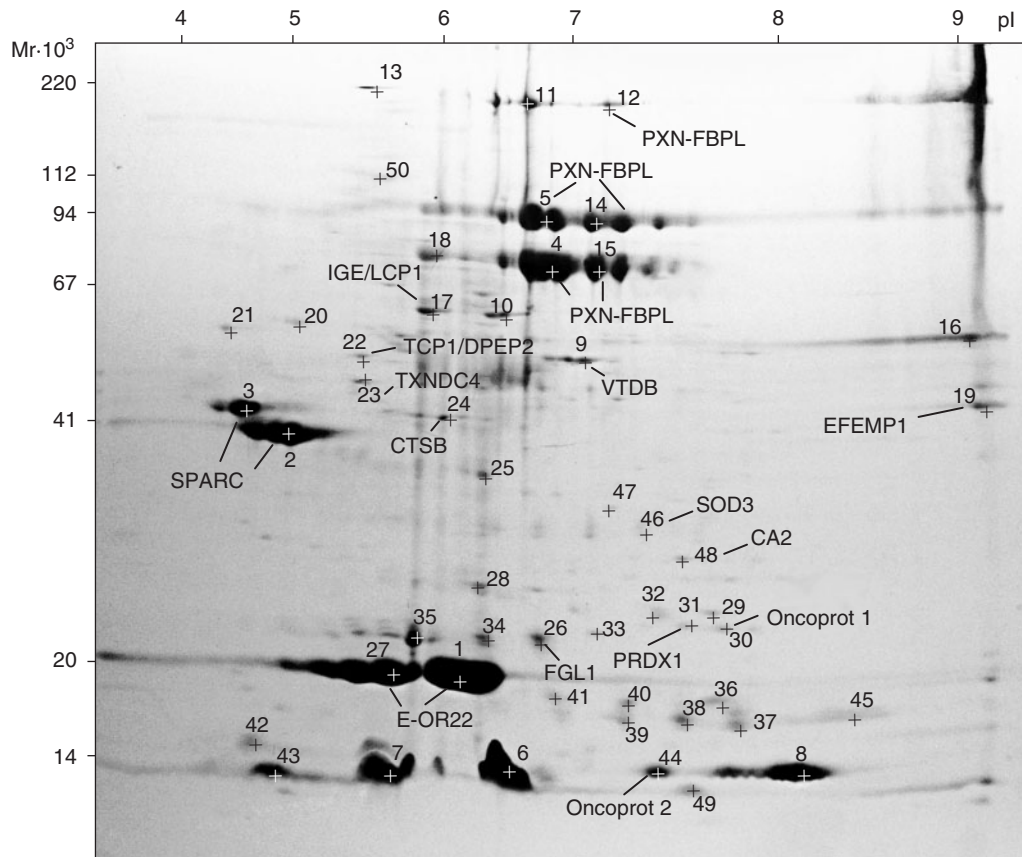


Fig. 3. Two-dimensional gel electrophoresis of the caudal epididymal proteins stained by Coomassie blue. Identification of the proteins in the numbered spots was carried out by LC-MS/MS analysis (shown in Table 1).

and 20 kDa (Fig. 2). Using 2D PAGE, at least 400 spots could be detected after Coomassie blue staining (Fig. 3). However, the protein present in the 280-kDa band (Fig. 2) did not appear on the 2D gel, due to the limited resolution of the 2D-gel method (Fig. 3).

Protein identification by mass spectrometry (MS) was performed on the 50 most intensely stained spots, and 27 proteins were identified from these spots (Table 1). Three of them represented ~54% of the total protein present in the 2D gel of the caudal epididymal fluid. The most abundant of these proteins (26% of the total protein, Fig. 4) was a multimeric protein identified at three separate molecular masses (180, 94 and 77 kDa, the last two representing 10 and 15% of the total protein, respectively) and several isoforms (with pI from 6 to 8) for each mass (Fig. 3). Based on the sequences obtained by MS, this multimeric protein was predicted to be similar to PXN-FBPL (XP_001518889.1; Table 1), deduced to be the gene annotated: 'GeneID: 100089494' in the NCBI database from the platypus genome. This protein has a predicted mass of 176 kDa. In our analysis, the 77-kDa protein was present only after reduction (Fig. 2); thus it is probable that native PXN-FBPL was composed of at least two subunits of 94 and 77 kDa. The amino acid sequence of PXN-FBPL is characterised by several conserved domains that belong to the FA58C, LamG and the PTX

super-families. PXN-FBPL has seven conserved domains, including an eel-fucolectin tachylectin-4 pentraxin-1 domain (FTP) and two pentraxin (PTX) domains. This protein is involved in laminin, carbohydrate and Ca^{2+} -dependent ligand binding and is probably characterised by pentameric discoid assembly, as with the plasma protein pentraxin. Among other vertebrates, similar proteins are found in *Xenopus laevis* (gb|AAI70505.1) and *oncorhynchus mykiss* (NP_001118028.1).

The second-most abundant protein found in the platypus caudal epididymal fluid was a 20-kDa protein with a pI of 4.5–6.5 (Spots 1 and 27). This protein represented 20% of the total protein in the caudal epididymal fluid (Fig. 4). After *de novo* sequencing, this protein was identified as a novel member of the lipocalin family (protein ID: ENSOANP00000015812), which we have named E-OR20 (Epididymal ORnithorhynchus protein 20 kDa). This new lipocalin shows similarity with the conserved motifs of the lipocalin family (Flower 1996). It is probably glycosylated since a difference of 4 kDa was observed between the calculated and the observed mass (16 v. 20 kDa). Homologous lipocalins have also been identified in the epididymides of other mammals, making up 15–20% of the secreted epididymal proteins from several species, such as the rat, ram, bull, stallion and lizard (*Lacerta vivipara*) (Morel *et al.* 1993; Fouchécourt *et al.* 1999; Ong *et al.* 2000).

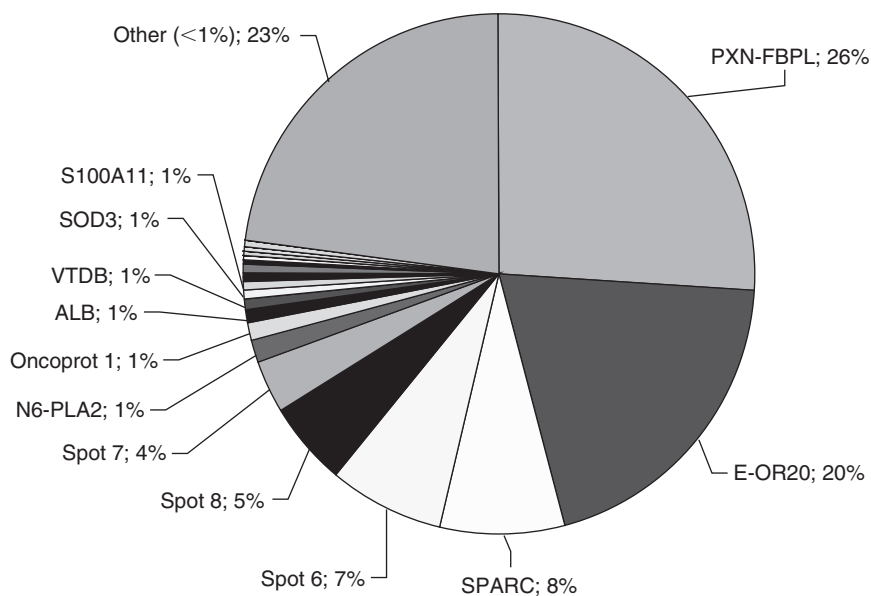


Fig. 4. Schematic distribution of the total proteins obtained from platypus epididymal fluid. Protein quantification was determined from 2D gel electrophoresis of the epididymal proteins stained by Coomassie blue.

The third major protein present in the platypus epididymal fluid was a 38–41-kDa protein with a pI of 4.5–5 (Fig. 3, Spots 2 and 3), representing 8% (Fig. 4) of the total proteins. This protein appeared as two separate spots of 41 and 38 kDa. The protein identified from these two spots is predicted to be a precursor of SPARC (also known as secreted protein, acidic and rich in cysteine or osteonectin). In the platypus genome, the SPARC gene has two putative transcripts (ENSOANT00000008117, ENSOANT00000008118) each encoding one protein, a 302-residue protein (ENSOANP00000008115) and a 297-residue protein (ENSOANP00000008116), respectively. These two proteins only differ from one another by 19 amino acids and may have accounted for the two distinct isoforms identified by 2D PAGE. However, the peptides identified from each spot by MS did not discriminate between these two variants. SPARC consists of three modular domains: a low-affinity, high-capacity, Ca^{2+} -binding N-terminal domain, a central region with a follistatin-like domain and a C-terminal domain that contains a high-affinity Ca^{2+} -binding EF-hand motif. The C-terminal region of the protein is involved in extracellular matrix binding and cell-surface interaction (Hohenester *et al.* 1997).

Among the other major proteins observed, Spot 6 (14.1 kDa, pI 6.5), Spot 7 (14.4 kDa, pI 5.6) and Spot 8 (16.7 kDa, pI 8.18) together represented 16% of the total protein in the cauda epididymal fluid. These proteins were not successfully identified by MS although several amino acid sequences were obtained by *de novo* sequencing for Spots 6 and 8. Of the other proteins that could be identified (Table 1), eleven were associated with protein binding and six were enzymes.

Discussion

Only a few of the epididymal proteins that have been identified in eutherian mammals were found in the platypus. Most

of the major proteins identified in the cauda epididymidis of eutherian mammals are enzymes (such as glycosidases, including mannosidase, hexosaminidase, galactosidase), peroxidases (GPX5) or binding proteins (PGDS, clusterin, lactoferrin and HE1/NCP2) (Kirchhoff *et al.* 1996; Okamura *et al.* 1997; Fouchécourt *et al.* 2002). These epididymal proteins have not been identified in the platypus, and the major epididymal proteins in the platypus (PXN-FBPL, SPARC and E-OR20) have never been identified in the epididymis of any other mammal.

The role of the major platypus epididymal proteins and possible interactions with spermatozoa during their transit through the epididymis have not been established. However, PXN-FBPL could have an immunological role, since fucoselectins and pentraxins have been described as immune-recognition molecules in both invertebrates and vertebrates (horseshoe crab, Saito *et al.* 1997; Japanese eel, Honda *et al.* 2000) and pentraxins are also involved in immune surveillance (Garlanda *et al.* 2002). Other minor proteins, such as peroxiredoxin, superoxide dismutase and thioredoxin, are probably involved in sperm protection against oxidative stress during their storage in the epididymis.

In conclusion, among all the mammals studied to date, the protein composition of the epididymal luminal fluid in the platypus appears to be unique, and several new proteins were identified in the present study. The origin of the epididymal luminal proteins and their role in sperm maturation should be studied, in particular their role in the formation of sperm bundles, a strategy of sperm co-operation that is unique to monotremes among the mammals.

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