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# Testicular descent, sperm maturation and capacitation. Lessons from our most distant relatives, the monotremes

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The present review examines whether monotremes may help to resolve three questions relating to sperm production in mammals: why the testes descend into a scrotum in most mammals, why spermatozoa are infertile when they leave the testes and require a period of maturation in the specific milieu provided by the epididymides, and why ejaculated spermatozoa cannot immediately fertilise an ovum until they undergo capacitation within the female reproductive tract. Comparisons of monotremes with other mammals indicate that there is a need for considerable work on monotremes. It is hypothesised that testicular descent should be related to epididymal differentiation. Spermatozoa and ova from both groups share many of the proteins that are thought to be involved in gamete interaction, and although epididymal sperm maturation is significant it is probably less complex in monotremes than in other mammals. However, the monotreme epididymis is unique in forming spermatozoa into bundles of 100 with greatly enhanced motility compared with individual spermatozoa. Bundle formation involves a highly organised interaction with epididymal proteins, and the bundles persist during incubation in vitro, except in specialised medium, in which spermatozoa separate after 3 h incubation. It is suggested that this represents an early form of capacitation.

## **Keywords**

maturation, sperm, monotremes, relatives, distant, most, our, capacitation, descent, testicular, lessons

## **Disciplines**

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## Testicular descent, sperm maturation and capacitation. Lessons from our most distant relatives, the monotremes

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**Abstract.** The present review examines whether monotremes may help to resolve three questions relating to sperm production in mammals: why the testes descend into a scrotum in most mammals, why spermatozoa are infertile when they leave the testes and require a period of maturation in the specific milieu provided by the epididymides, and why ejaculated spermatozoa cannot immediately fertilise an ovum until they undergo capacitation within the female reproductive tract. Comparisons of monotremes with other mammals indicate that there is a need for considerable work on monotremes. It is hypothesised that testicular descent should be related to epididymal differentiation. Spermatozoa and ova from both groups share many of the proteins that are thought to be involved in gamete interaction, and although epididymal sperm maturation is significant it is probably less complex in monotremes than in other mammals. However, the monotreme epididymis is unique in forming spermatozoa into bundles of 100 with greatly enhanced motility compared with individual spermatozoa. Bundle formation involves a highly organised interaction with epididymal proteins, and the bundles persist during incubation *in vitro*, except in specialised medium, in which spermatozoa separate after 2–3 h incubation. It is suggested that this represents an early form of capacitation.

**Additional keywords:** epididymis, fertilisation.

### Introduction

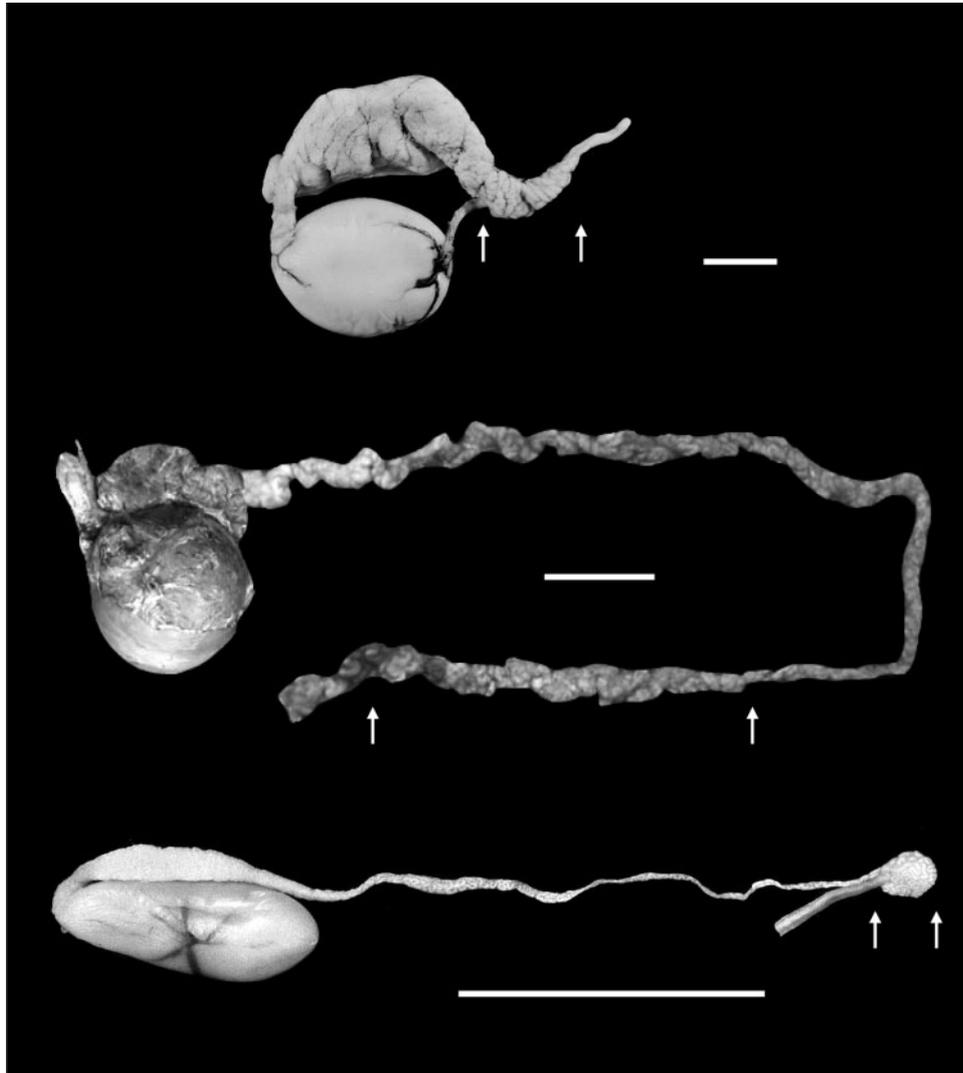
It is generally recognised that there are three unresolved questions relating to sperm production in mammals: why the testes descend into a scrotum in most mammals, why spermatozoa are infertile when they leave the testes and require a period of maturation in the specific milieu provided by the epididymides, and why ejaculated spermatozoa cannot immediately fertilise an ovum until they undergo capacitation within the female reproductive tract. Whilst these characteristics are considered to be unique to mammals it is well established that the testes of the monotremes and some other mammals remain near the kidneys as in the sauropsids. Also, the paradigms about post-testicular sperm development and capacitation are based on studies of marsupial and eutherian mammals, but have not been tested in monotremes. Consequently, considering the key phylogenetic position of the monotremes, it is suggested that a better understanding of sperm production in this Order may provide some insight into the need for testicular descent, sperm maturation and capacitation in mammals. Further, the monotremes are of interest as they exhibit a unique form of sperm cooperation involving the formation, in the epididymis, of bundles of ~100 individual spermatozoa. The present review examines sperm production in the two extant Australian monotremes that we are studying,

the platypus (*Ornithorhynchus anatinus*) and the short-beaked echidna (*Tachyglossus aculeatus*).

### Testicular and epididymal descent

Testicular descent is an important process in humans, as men with cryptorchid testes are infertile, and even when the condition is surgically corrected the risk of testicular cancer is much greater than in the normal population (Morrison 1976; Hughes and Acerini 2008). Consequently, a knowledge of the processes involved in and the origins and biological significance of testicular descent may be most informative for understanding the condition. In this respect, it is established that, in mice and humans, INSL3 (insulin-like factor 3) acts through the receptor LGR8 (RXFP2, GREAT) to mediate testicular descent via its effect on growth of the gubernaculum's primordia and caudal genitoringuinal ligament, and mutation of the genes for either of these proteins is associated with failure of testicular descent in developing males (Nef and Parada 1999; Zimmermann *et al.* 1999; Bogatcheva *et al.* 2003; Kamat *et al.* 2004; Wilhelm and Koopman 2006; Feng *et al.* 2009; Ferlin *et al.* 2009).

The position of the testes is highly variable in mammals; from not descended, to descended but ascrotal and descended and scrotal (Carrick and Setchell 1977). However, the relationship



**Fig. 1.** Testis and epididymis of three testicond mammals showing varying degrees of development and descent of the sperm storage region (shown with arrows) of the epididymis. From top to bottom the tracts are from the platypus (scale line = 10 mm), African elephant (scale line = 100 mm) and elephant shrew (scale line = 10 mm).

between the position of the testes and the role of the epididymides has not been satisfactorily resolved. The finding that mammals with undescended testes have relatively larger testes (which produce sperm at a greater rate per animal) than scrotal mammals (Kenagy and Trombulak 1986; Freeman 1990; Werdelin and Nilsson 1999) indicates that there is less need for the former to develop a specialised region to store extragonadal sperm. Nevertheless, it is unresolved whether the functions of the epididymis were determined early in mammalian evolution and then subsequently adapted to different positions of the testes or whether other factors are involved. Glover (1973) and Bedford (1977, 1978, 1979) proposed that the epididymis was the prime mover in the evolution of testicular descent because in some testicond mammals the distal, sperm storage region of the epididymis develops near the urethra, rather than adjacent to the testis, and close to the surface of the body where it may be kept lower than

deep body temperature (Fig. 1). However, it is not obvious why some species, such as the elephant (Jones and Brosnan 1981) and dugong (Marsh *et al.* 1984), retain an intermediate position of descent of the sperm storage region of the epididymis. Further, in an assessment of the reasons for testicular descent and its relationship with the phylogeny of Mammalia, Werdelin and Nilsson (1999) hypothesised that evolution has proceeded from the scrotal to ascrotal condition, but never the reverse. Clearly, both testicular and epididymal descent should be considered together and it is suggested that the monotremes may provide important clues to understanding the biological significance and evolution of the processes as, if the above interpretation is correct, the monotremes may be the only extant, primitive testicond mammals. It has been established that the specificity of INSL3 for LGR8 is absent in monotremes and evolved in therian mammals by a single point mutation (Park *et al.* 2008). Significantly,

in the monotremes that we have studied, the abdominal cavity is so short that the distal ‘sperm storage’ region of the epididymis is adjacent to the testis and empties into the urethra through a very short duct (Fig. 1) so that there is no scope for the cauda epididymidis to descend.

### Sperm maturation in the epididymis

This section examines sperm structure in relation to fertilisation and the role of the epididymis. Although it is well established that the mammalian epididymis has two essential functions, sperm maturation and storage (Bedford 1979), as well as transporting spermatozoa from the testis, there is little understanding of the biological significance of the organ. It has been noted that daily sperm production is less than the number of spermatozoa in a normal ejaculate (Jones 1999), but this only explains the role of the epididymis in ensuring that mammals can ejaculate many more spermatozoa than are needed to achieve fertilisation. It has also been suggested that the unique functions of the mammalian epididymis are adaptations to enhance a male’s chances of parenthood in competitive mating systems, that is, in sperm competition (Jones 1998; Jones *et al.* 2007). Nevertheless, it is unlikely that sperm competition is very important in humans (Smith 1984a; Jones *et al.* 2007) and, as many male infertility problems have been solved by procedures based on *in vitro* fertilisation (IVF) of the egg, there has been little recent interest in epididymal function. However, it is timely to reassess epididymal functions as the use of procedures based on IVF will increase the proportion of infertile men in a population (and so increase the cost of public health) and there is an increasing number of reports of birth defects in babies conceived through assisted reproduction (Nair 2008). Further, as sperm competition is a significant factor in achieving paternity in many other species (Smith 1984b; Birkhead 1995; Gomendio *et al.* 1998) and there is increasing evidence that evolution is male-driven (Crow 1997; Ellegren and Fridolfsson 1997; Hurst and Ellegren 1998; Li *et al.* 2002), it is timely to reassess how epididymal function has evolved from monotremes to higher mammals.

### Sperm structure and fertilisation proteins

Monotremes have sauropsid-like spermatozoa that have some mammalian characteristics. Their shape is vermiform like the sauropsids (Carrick and Hughes 1982) and quite unlike the shapes of spermatozoa from eutherians and marsupials. Like eutherian spermatozoa, the acrosome extends laterally over the head rather than being limited to the rostral surface as in the sauropsids (Okamura and Nishiyama 1976; Gunawardana and Scott 1977; Lin and Jones 1993). Also, as in the other mammals, a cytoplasmic droplet is retained in the neck region during spermiation and persists during transport through most of the epididymis (Lin and Jones 1993). Unlike in the higher mammals, the middle piece has only rudimentary coarse fibres that don’t extend into the principal piece, and the fibrous sheath is arranged spirally as in many sauropsids (Nicander 1970; Lin and Jones 1993) and unlike the circumferential arrangement of the higher mammals (Fawcett 1975). Bedford showed that the nuclei and tails of monotreme (and lower vertebrate) spermatozoa are less rigid than those of other mammals and that this is because disulfide

Table 1. Percentage amino acid identity of ZP-associated genes identified in the platypus genome with their orthologues in other vertebrate species

Zona pellucida proteins	Ensemble gene ID – platypus ( <i>Ornithorhynchus anatinus</i> )	Human ( <i>Homo sapiens</i> )	Mouse ( <i>Mus musculus</i> )	Short-tailed opossum ( <i>Monodelphis domestica</i> )	Chicken ( <i>Gallus gallus</i> )	Anole lizard ( <i>Anolis carolinensis</i> )	Zebra fish ( <i>Danio rerio</i> )
ZPA/ZP2	ENSOANG000000002223	51	46	56	44	31	–
ZPB1/ZP1	ENSOANG000000015211	53	54	58	47	51	34
ZPB2/ZP4	ENSOANG000000006364	38	–	–	44	–	25
ZPC/ZP3	ENSOANG000000022309	52	51	53	57	49	38
ZPAX	ENSOANG000000002187	–	–	–	51	48	29

**Table 2. Percentage amino acid identity of sperm-associated genes identified in the platypus genome with their orthologues in other vertebrate species**  
NP, not present

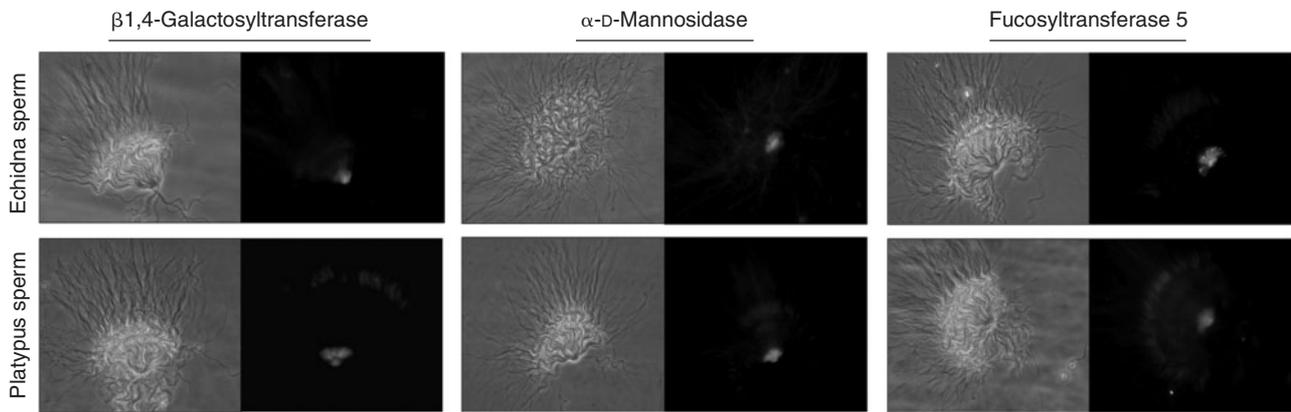
	Ensemble gene ID – platypus ( <i>Ornithorhynchus anatinus</i> )	Human ( <i>Homo sapiens</i> )	Mouse ( <i>Mus musculus</i> )	Short-tailed opossum ( <i>Monodelphis domestica</i> )	Chicken ( <i>Gallus gallus</i> )	Anole lizard ( <i>Anolis carolinensis</i> )	Zebra fish ( <i>Danio rerio</i> )
<b>Proteins associated with ZP binding</b>							
ZP binding protein (ZBPB; Sp38)	ENSOANG000000000287	68	71	66	62	64	—
ZP binding protein 2 (ZBPB2)	ENSOANG000000005691	60	58	54	50	60	—
ZP binding protein 3 receptor (Zp3r; Sp56)	ENSOANG00000014004	45	39	43	20	—	—
Sperm autoantigenic protein 17 (SPA17; Sp17)	ENSOANG000000003468	67	65	63	—	35	—
Milk fat globule-EGF factor 8 protein (MFG8; Sed1)	ENSOANG000000006383	46	56	59	60	59	54
$\beta$ 1,4-galactosyl transferase (B4GALT1)	ENSOANG000000002834	74	73	80	81	80	73
Fucosyltransferase 5 (FUT5)	ENSOANG000000005427	53	—	51	53	53	—
$\alpha$ -D-mannosidase (MAN2B2)	ENSOANG000000003982	54	51	56	53	—	—
Acidic seminal fluid protein (ASFP)	NP	—	—	—	—	—	—
<b>Proteins associated with oolemma binding</b>							
A disintegrin and metalloproteinase domain 3 (ADAM3; cyritestin)	ENSOANG000000020160	54	48	64	38	45	—
A disintegrin and metalloproteinase domain 1a (ADAM1; fertilin $\alpha$ )	ENSOANG000000009507	—	39	46	34	—	—
Izumo sperm-egg fusion 1 (IZUMO1)	ENSOANG000000010023	31	29	20	—	18	—

bonds do not form during epididymal maturation as in eutherians and marsupials (Bedford and Calvin 1974a, 1974b; Bedford and Rifkin 1979). It is now known that the lack of disulfide bonds in the nucleus is due to a lack of cysteine residues in the protamine-1 protein used to package the DNA (Retief *et al.* 1993).

The molecular events involved in the union of spermatozoon and egg during fertilisation in mammals have not been definitively established. In the ovum, the zona pellucida (ZP)-associated proteins are thought to mediate the binding of spermatozoa and subsequently initiate the acrosome reaction. The ZP proteins of humans are ZPA (ZP2), ZPB1 (ZP1), ZPB2 (ZP4/ZPB) and ZPC (ZP3) (Lefièvre *et al.* 2004; Wassarman *et al.* 2004) and the platypus genome has single orthologues that display a high level of similarity (between 54 and 39% identity at the amino acid level) for the genes of all these proteins (Warren *et al.* 2008; Table 1). The platypus genes also share a high level of similarity with orthologous genes in the chicken (between 47 and 44% identity at the amino acid level). Further, the platypus genome encodes at least one ZP-associated gene (*ZPAX*) that has only been found in birds, amphibians and fish and is thought to be lost from the eutherian lineage (Spargo and Hope 2003; Smith *et al.* 2005; Warren *et al.* 2008). For practical purposes we have successfully applied this knowledge of homologies between the platypus and chicken genes to develop an assay to test binding of monotreme spermatozoa to preparations of the perivitelline membrane of the chicken egg (which is analogous to the ZP of mammals). It is anticipated that this assay will be valuable for assessing the post-testicular development of monotreme spermatozoa.

Although ZP genes are present in the platypus genome, caution is required in directly extrapolating the presence of protein(s) from genomic comparisons. In this respect, recent analysis has shown that the major sperm-binding proteins that are expressed on the inner perivitelline membrane of the chicken egg are homologues of human ZPB1 and ZPC, and there is also a unique ZPD protein (Okumura *et al.* 2004), which is absent in the platypus and eutherian mammals. However, although gene orthologues of the ZPA and ZB2 families are predicted products of the fowl genome (Smith *et al.* 2005), significant amounts of the ZPA or ZB2 homologues were not detected by 2D-SDS-PAGE in the fowl egg envelope (Okumura *et al.* 2004).

The sperm-specific ligands responsible for binding to the ZP in mammals have not been established, although several candidates have been suggested (Primakoff and Myles 2002; Nixon *et al.* 2007). Orthologues of many of the eutherian candidates are present in the platypus genome (Table 2) and most of them share a high level of identity at the amino acid level. For example, several proteins that bind to the ZP and oolemma of mammals are homologous to predicted platypus proteins (Table 2). The conservation of these genes provides evidence for their functional importance in spermatozoa and is indicative of the conservation of the molecular processes that mediate fertilisation. Our immunocytochemical localisation of some of these proteins, using commercially available antibodies, are shown in Fig. 2. These preliminary studies indicate that antibodies to fucosyltransferase 5,  $\alpha$ -D-mannosidase 2 and  $\beta$ 1,4-galactosyltransferase all cross-react with monotreme spermatozoa and the proteins are all predominately present on the sperm



**Fig. 2.** Immunolocalisation of proteins associated with ZP binding in monotreme spermatozoa. Echidna (top panels) and platypus (bottom panels) spermatozoa, present in sperm bundles, were diluted from the cauda epididymidis and fixed with methanol. Antibodies against  $\beta$ 1,4-galactosyltransferase,  $\alpha$ -D-mannosidase and fucosyltransferase 5 indicated that homologues of these proteins are present in monotreme spermatozoa and localised predominantly to the sperm head.

head, a localisation consistent with a role in binding to the ovum. We intend to refine the localisation of these proteins and establish their pattern of expression.

In contrast to the above, some of the sperm proteins that can bind to the ZP and oolemma in eutherian models do not appear to be present in the platypus genome. For example, acidic seminal fluid protein (ASFP) is absent. Other proteins associated with sperm function also appear to be absent in the platypus genome including acrosomal vesicle protein 1 (Acrv1; SP10), sperm-associated cation channel 3 (Catsper3), preproacrosin precursor (Acr) and proacrosin-binding protein (Acrbp; sp32). Interestingly, male mice with gene knockouts for Catsper3 are infertile due to problems associated with sperm motility and hyperactivation (stimulation of motility characterised by high-amplitude, asymmetrical beating pattern of the flagellum) of spermatozoa (Ren *et al.* 2001). An orthologous Catsper3 gene is present in the opossum genome (57% identity at the amino acid level to the mouse homologue) indicating that the essential role of Catsper3, and other proteins not present in monotreme spermatozoa, may be a relatively recent evolutionary development. The apparent absence of testis-specific proteases, Acr and Acrbp, which are thought to play a key role in digesting and degrading the ZP of eutherians during fertilisation, may explain the much thinner ZP of monotremes compared with that of eutherians (Bedford 1991), the thicker ZP in the eutherians providing the evolutionary pressure for the emergence of these proteins in spermatozoa.

#### *Epididymal sperm maturation*

Spermatozoa take 8 to 17 days to pass through the epididymal ducts of marsupial (Setchell and Carrick 1973) and eutherian (Amann *et al.* 1976) mammals and it is well established that the epididymis plays an essential role in sperm maturation and storage (Jones 1999). The maturation processes that are unique to mammals involve distal migration of the cytoplasmic droplet along the middle piece, condensation of the acrosome in some species, development of the capacity for motility and modification of the plasmalemma, which enables binding to the ovum and possibly other functions. In monotremes the cytoplasmic droplet

migrates along the middle piece during epididymal transit as in other mammals (Djakiew and Jones 1983). However, no other change in structure has been identified after spermatozoa leave the testis of monotremes (Bedford and Rifkin 1979; Carrick and Hughes 1982; Lin and Jones 2000).

Testicular spermatozoa from marsupial and eutherian mammals show no motility, or only weak vibrations of the tail with no progressive motility (Voglmayr *et al.* 1966; Setchell 1970; Tuck *et al.* 1970); this form of motility and the capacity to undergo hyperactivation develop during epididymal transit (Orgebin-Crist 1967; Jones *et al.* 1984; Clulow *et al.* 1992; Pérez-Sánchez *et al.* 1996). Testicular spermatozoa from the echidna also do not display progressive motility, and although we found that soluble forms of cAMP or phosphodiesterase inhibitors can stimulate activity, the flagellum beat remains weak and motility is non-progressive (K. Krivanek, B. Nixon and R. C. Jones, unpubl. data). Echidna spermatozoa become motile during epididymal transit (Djakiew and Jones 1983), but it is unresolved whether there is any change in the form of motility, or whether exposure to the epididymis is necessary for them to undergo hyperactivation. Consequently, further study is required to resolve the role of the monotreme epididymis in developing sperm motility.

The acquisition of proteins and remodelling of the plasma membrane (Aitken *et al.* 2007) during epididymal sperm maturation in higher mammals is important for the development of sperm motility (Mohri and Yanagimachi 1980; White and Voglmayr 1986; Ishijima and Witman 1987; Clulow *et al.* 1992) as well as the ability to bind to the ovum. This process involves the sequential interaction of spermatozoa with the milieu of the epididymal ducts, which is important since the spermatozoa have little transcriptional capacity, and are unlikely to generate new proteins. However, there is little evidence for plasma-membrane remodelling during sperm transit through the epididymis of monotremes, although there is evidence for the acquisition of proteins associated with sperm bundle formation (see below). Consequently, it is possible that for the two most important aspects of post-testicular development of eutherians and marsupials (sperm maturation in the epididymis and

capacitation), the process is no more involved in monotremes than in the domestic fowl, in which testicular spermatozoa can achieve a high conception rate if inseminated into the oviduct (Munro 1938; Howarth 1970, 1983) and epididymal transit only involves an increase in the proportion of motile spermatozoa.

### Division of labour along the epididymis

The epididymis of marsupial and eutherian mammals (including the testiconds) is structurally differentiated into at least six segments (although the differentiation is less distinct in humans) indicating a considerable division of labour along the duct, the proximal regions being mainly involved in sperm maturation and the distal region in sperm storage (Nicander 1957, 1958; Nicander and Glover 1973; Jones and Brosnan 1981; Jones *et al.* 1984). The segmentation has been confirmed in studies of gene expression (Douglass *et al.* 1991; Winer *et al.* 1993; Cornwall and Hann 1995; Turner *et al.* 2006) and protein secretion (Dacheux *et al.* 2003, 2006; Gatti *et al.* 2004).

The epididymis of monotremes is longer than that of the sauropsids and as long, relative to testis or body size, as in other mammals (Djakiew and Jones 1982a; Chaturapanich and Jones 1991). However, the monotreme epididymis is unique among the mammals in being differentiated into only two distinctly different segments, including the characteristic 'initial segment', which is unique to mammals (Jones 1998). The initial segment has a tall actively secretory epithelium with long, coarse stereocilia, the presence of narrow (mitochondria-rich) cells and few spermatozoa in the lumen (Djakiew and Jones 1981, 1982b). The structure and function of the initial segment epithelium is dependent on a luminal connection with the testis (Jones *et al.* 1992). Also, compared with the rest of the duct, it has very high activity of 5 $\alpha$ -reductase, which converts testosterone to the more potent androgen, dihydrotestosterone. The initial segment plays an essential role in post-testicular sperm maturation in marsupial and eutherian mammals as indicated by modifications to sperm structure and function (Jones *et al.* 1987). Also, when the segment does not develop, as in targeted mutations of the c-ros tyrosine kinase or apolipoprotein E receptor-2 receptors, animals are sterile even though spermatogenesis is unaffected (Sonnenberg-Riethmacher *et al.* 1996; Wagenfeld *et al.* 2002; Andersen *et al.* 2003; Cooper 2007).

The initial segment of the echidna epididymis comprises more than 95% of the total length of the duct (Djakiew and Jones 1982a) and there is no variation in the ultrastructure of its epithelium or the composition of the proteins along the segment (Djakiew and Jones 1982b, 1983). The terminal segment partly compensates for its short length by its mucosa-forming folds, or villi, a unique feature of the epididymis of monotremes, elephants and dugongs (Jones and Brosnan 1981; Marsh *et al.* 1984), which ensures that the relatively wide lumen is faced by a considerable area of epithelium. However, as in the elephant (Jones *et al.* 1974) the terminal segment of the echidna holds only a small proportion of extragonadal spermatozoa (25 and 33% respectively compared with 75% in the rat). Further, whereas it has been determined that scrotal marsupials and eutherians (White 1932; Paüfler and Foote 1968; Chaturapanich *et al.* 1992b) can maintain motile spermatozoa for 6 weeks when

isolated between ligatures in the cauda epididymis, there have been no comparable studies in monotremes.

Hundreds of proteins are present in epididymal luminal fluid from eutherian mammals (see companion paper in this volume by Dacheux *et al.* 2009). The duct mucosa secretes and reabsorbs proteins and some of the secreted proteins are then modified or may be reabsorbed downstream, and some proteins originate from spermatozoa. There is considerable evidence that the epididymal proteins are involved in sperm maturation including modifying the composition of the sperm membrane so that it can bind to an ovum and undergo capacitation. 1-D gels of micropuncture samples of luminal fluid collected along the epididymis of the echidna (Djakiew and Jones 1983) show a similar pattern of luminal fluid proteins collected from eutherians (Dacheux *et al.* 2003) and marsupials (Chaturapanich *et al.* 1992a). However, whereas clusterin is the most abundant epididymal protein in the eutheria that have been studied, including humans (Dacheux *et al.* 2003, 2006; Gatti *et al.* 2004), the most abundant secreted protein in the platypus epididymis is lipocalin (see companion paper by Dacheux *et al.* 2009); its homologues most closely resemble proteins in the reptilian epididymis (Morel *et al.* 2000). Of particular interest are the proteins that are secreted by the epididymis and interact with spermatozoa to modify their function. A number have been identified in eutherian mammals, including SPAM1 (formerly PH20; Chen *et al.* 2006), SED1 (Ensslin and Shur 2003),  $\alpha$ -D-mannosidase (Jin *et al.* 1999), CRISP1 (Eberspaecher *et al.* 1995), CD52 (Kirchhoff *et al.* 1993), ASF (Thomas *et al.* 1984), HE1 (Kirchhoff *et al.* 1996) and p26H (Coutu *et al.* 1996). However, whilst knockout mice for SPAM1 (Baba *et al.* 2002) and SED1 (Ensslin and Shur 2003) have reduced fertility compared with wild-type, none of the knockouts is infertile. Whilst there have been no studies of epididymal proteins that interact with monotreme spermatozoa, there is evidence that epididymal proteins are involved in the formation of sperm bundles (see below).

### Sperm bundles

A unique characteristic of monotremes is that their spermatozoa form into bundles of ~100 individuals as they transit through the isthmus joining the initial and terminal segments of the epididymis (Djakiew and Jones 1981, 1983). This bundle formation is quite different from other forms of mammalian sperm cooperation: the pairing of marsupial sperm (Biggers and Creed 1962; Biggers and Delamater 1965), the rouleaux formation of guinea-pig spermatozoa (Tung *et al.* 1980) or the sperm 'trains' formed in the European wood mouse (Moore *et al.* 2002). In monotremes, the process of formation and ultrastructure of the bundles suggests that proteins are involved in an organised, sequential process (Djakiew and Jones 1981). Formation of sperm bundles appears to be mediated by specific protein(s) secreted by the epididymis. The spermatozoa initially group into a sphere with the rostral ends of their heads facing the centre, before re-orientating such that they form a V-shaped bundle. The significance of these bundles is highlighted by the fact that they greatly enhance sperm motility, and therefore can be interpreted as an adaptation presumably arising in response to sperm competition pressures between males (Jones *et al.* 2004, 2007).

For instance, we have found that when epididymal and ejaculated spermatozoa from the echidna are diluted and incubated *in vitro*, the sperm bundles move forward three times faster ( $140\ \mu\text{m s}^{-1}$ ) than individual spermatozoa and much faster than spermatozoa with a similar structure, such as from Japanese quail ( $50\ \mu\text{m s}^{-1}$ ), faster than human spermatozoa  $40\text{--}50\ \mu\text{m s}^{-1}$  (Davis and Katz 1992) and as fast as bull and ram spermatozoa (Farrell *et al.* 1998; Arman *et al.* 2006).

Our studies of the synthesis and secretion of proteins along the monotreme epididymis (see companion paper in this issue by Dacheux *et al.* 2009) showed that at least two new proteins are secreted into the region of the duct where spermatozoa form into bundles. In the echidna these proteins have molecular masses of  $\sim 60\ 000\ \text{Da}$  and  $76\ 000\ \text{Da}$  (Jones *et al.* 2007). They were not detected in small-format gels used in our early micropuncture studies (Djakiew and Jones 1983), but were subsequently detected in large-format gels of luminal fluids. They were shown to be secreted, and they appear to be involved in the formation of sperm bundles. The heads of spermatozoa within the bundles appear to be held in place by proteinaceous material that is electron-dense. We are conducting further analyses of these proteins in order to identify them and determine their role. We hypothesise that, after assisting the delivery of spermatozoa to the site of fertilisation, the sperm bundle protein(s) must be either shed or inactivated to enable spermatozoa to dissociate and bind to the oocyte.

### Capacitation

The term capacitation was coined following *in vivo* studies investigating the fertilisation of freshly ovulated eggs in rats and rabbits (Austin 1951, 1952; Chang 1951). In these studies, a final time-dependent maturational step was recognised as an essential part of a spermatozoon achieving the ability to fertilise an ovum. Although recognised for more than 50 years, capacitation remains an ill-defined process, in which a series of morphological and biochemical changes render mammalian spermatozoa fully functional. Previous work has led to the dogma that, although differences exist between mammalian species, there are obligatory components of capacitation that occur across all species. However, the requirement for capacitation has not been tested in monotremes and therefore not definitively established for all mammals.

The culmination of the processes that take place during capacitation is the ability of the spermatozoa to participate in the recognition events that occur on the surface of the zona pellucida. Although capacitation normally takes place in the female reproductive tract, for most species this maturational process can now be accomplished *in vitro* using well defined media (Yanagimachi 1970; Miyamoto and Chang 1973). Several correlates of capacitation have been identified in a variety of higher mammals, including plasma-membrane remodelling and cholesterol efflux (Davis *et al.* 1979; Davis 1981), an increase in sperm metabolic rate (Murdoch and White 1967; Hicks *et al.* 1972; Rogers 1979), the activation of a cAMP-mediated signalling pathway leading to tyrosine phosphorylation (Visconti and Tezon 1989), the expression of hyperactivated motility (Yanagimachi 1970; Fraser 1977) and the ability to undergo the acrosome reaction on the surface of the zona pellucida (Bedford 1970; Ward and Storey 1984).

However, besides the latter, there is no certainty which of these, if any, is obligate for capacitation and none of these processes have been reported to occur in monotreme spermatozoa incubated *in vitro*.

In preliminary studies, we incubated monotreme spermatozoa in a medium that supports capacitation in other species and found no evidence for a cAMP-mediated signalling pathway leading to protein tyrosine phosphorylation (unpubl. data). However, the sperm bundles, which initially remain intact *in vitro* upon dilution from the epididymal fluid or ejaculates of the echidna, begin to dissociate after  $\sim 2\text{--}3\ \text{h}$  in medium. It is considered unlikely that spermatozoa within bundles could possibly fertilise an ovum since most of the heads of the spermatozoa are embedded in proteinaceous material that mediates and maintains bundle formation, thus making sperm membrane proteins inaccessible for ZP binding. Dispersion from the bundles would facilitate unmasking of ZP ligands on the sperm head and, therefore, fertilisation. Consequently, it is interpreted that sperm bundle dispersion represents an early form of capacitation that is consistent with the definition in higher mammals of being a time-dependent process. However, it remains to be resolved how complex the dissociation is, and whether it is associated with specific changes to the spermatozoon with a level of complexity similar to that of higher mammals.

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