# Acrosome formation during sperm transit through the epididymis in two marsupials, the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*)

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### ABSTRACT

In certain Australian marsupials including the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*), formation of the acrosome is not completed in the testis but during a complex differentiation process as spermatozoa pass through the epididymis. Using transmission and scanning electron microscopy this paper defined the process of acrosome formation in the epididymis, providing temporal and spatial information on the striking reorganisation of the acrosomal membranes and matrix and of the overlying sperm surface involved. On leaving the testis wallaby and possum spermatozoa had elongated 'scoop'-shaped acrosomes projecting from the dorsal surface of the head. During passage down the epididymis, this structure condensed into the compact button-like organelle found on ejaculated spermatozoa. This condensation was achieved by a complex process of infolding and fusion of the lateral projections of the 'scoop'. In the head of the epididymis the rims of the lateral scoop projections became shorter and thickened and folded inwards, to eventually meet midway along the longitudinal axis of the acrosome. As spermatozoa passed through the body of the epididymis the lateral projections fused together. Evidence of this fusion of the immature outer acrosomal membrane is the presence of vesicles within the acrosomal matrix which persist even in ejaculated spermatozoa. When spermatozoa have reached the tail of the epididymis the acrosome condenses into its mature form, as a small button-like structure contained within the depression on the anterior end of the nucleus. During the infolding process, the membranes associated with the immature acrosome are either engulfed into the acrosomal matrix (outer acrosomal membrane), or eliminated from the sperm head as tubular membrane elements (cytoplasmic membrane). Thus the surface and organelles of the testicular sperm head are transient structures in those marsupials with posttesticular acrosome formation and this must be taken into consideration in attempts to dissect the cell and molecular biology of fertilisation.

*Key words*: Sperm maturation; marsupials.

### INTRODUCTION

In eutherian mammals, spermatozoa leaving the testis are immotile and infertile. Fertilising capability is achieved by posttesticular maturation processes as spermatozoa pass through the epididymis. The epithelial cells of the epididymis, under the control of androgens, create the environment in which spermatozoa differentiate functionally to achieve full progressive motility, the ability to bind to the egg coat (zona pellucida) and to undergo fusion with the egg membrane. All are critical aspects of sperm function for successful fertilisation (reviewed in Cooper, 1986; Moore, 1990; Yanagimachi, 1994). There is a large body of evidence that epididymal maturation is also critical for marsupial spermatozoa to acquire fertilizing capability (Setchell, 1970; Harding et al. 1979; Jones et al. 1987; Rodger, 1991; Jones & Clulow, 1994; Temple-Smith, 1994; Mate & Rodger, 1996). However, the marsupial pattern of epididymal maturation involves far more structural change than is seen in eutherians.

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A major sperm structure formed in the marsupial epididymis is the midpiece fibre network. This set of helically wound fibres completely surrounds the posterior midpiece in the spermatozoa of all marsupials studied (Temple-Smith & Bedford, 1967; Harding et al. 1976, 1979, 1984; Lin et al. 1997). In the possum, the first ultrastructural evidence of this structure is found in spermatozoa from the distal caput epididymis and its formation is completed as spermatozoa pass through the proximal cauda epididymis (Temple-Smith & Bedford, 1976). In some groups of Australian marsupials there is an even more striking posttesticular structural differentiation even which results in the formation of the compact acrosome from the very extensive immature structure present at spermiation (Cummins, 1976; Temple-Smith & Bedford, 1976; Harding et al. 1983; Mate & Rodger, 1996; Setiadi et al. 1997). In American marsupials an equally dramatic posttesticular event is the pairing of spermatozoa at their acrosomal faces as they pass down the epididymis (Rodger, 1982; Temple-Smith, 1994).

The acrosome, an organelle that lies on the sperm head, plays vital roles in fertilisation. In most vertebrates the acrosome is fully formed in the testis and undergoes no morphological change in the excurrent ducts (e.g. fish, birds and monotremes; Jones & Lin, 1993), or only very minor modification (most eutherians and some marsupials; Harding et al. 1979; Bedford, 1991). However, in the phalangerid (large possums) and macropodid (wallabies and kangaroos) marsupial families, acrosome formation is not completed until spermatozoa have passed at least halfway through the epididymis (Temple-Smith, 1994). Such complex transformation of the acrosome is never seen in any other mammals (see review by Bedford, 1996). Our previous study on the tammar wallaby and the brushtail possum, the first to examine testicular sperm using scanning electron microscopy (SEM), found that when the mature spermatids are released into the lumen of the seminiferous tubule to become spermatozoa, their acrosomes are a 'scoop'-shaped sheet of folded tissue extending away from the dorsal surface of the head (Lin et al. 1997). This shape is completely different to the acrosome of ejaculated spermatozoa, which is a compact button lodged in a depression on the dorsal side of the nucleus (Harding et al. 1976; Cummins, 1976; Setiadi et al. 1997). Obviously, it is extremely difficult to unequivocally describe this extraordinary transformation of the marsupial acrosome by 2-dimensional transmission electron microscope (TEM) images alone. As a result, there is no general agreement on the pattern of the

acrosomal formation in the epididymis, even for the brushtail possum and tammar wallaby, the most studied species. For example, the acrosome structure in the head region of the epididymis, has been described as either a cup-like structure (Harding et al. 1976; Cummins, 1976; Setiadi et al. 1997) or like the fingers of a glove with many projections of membrane and matrix over its dorsal surface (Temple-Smith & Bedford, 1976). The present study thus set out to characterise in detail the formation of the tammar wallaby acrosome in the epididymis using both TEM and SEM to provide temporal and spatial information on this complex process of differentiation. Special attention was given to the reorganisation of the cell membrane over the sperm head and of the acrosomal membranes because of their critical role in fertilisation. A briefer description of the essentially similar morphological maturation of the brushtail possum acrosome during transit of the epididymis is also included.

## METHODS AND MATERIALS

### Animals

Five adult male tammar wallabies (*Macropus eugenii*) were obtained from Kangaroo Island, South Australia, and maintained in the breeding yard of the Marsupial Cooperative Research Centre at the University of Newcastle, New South Wales, Australia. These animals had been previously used as semen donors and were known to produce ejaculates containing large numbers of highly motile spermatozoa. Three adult brushtail possums (*Trichosurus vulpecula*) were trapped in the Canterbury region of New Zealand and housed for up to 3 wk in the Animal House of Landcare Research at Lincoln prior to collection of tissues. The possums used were part of a study of spermatogenesis and all had apparently normal sperm production. The use of protected animals and animal experimentation were approved by the appropriate Australian state authorities and by the Animal Care and Ethics Committees of the University of Newcastle and Landcare Research respectively.

### Dissection of tissues

The animals were killed with an overdose of sodium pentobarbitone (30 mg/kg, intravenously) via a lateral tail vein (wallaby) or intracardiac (possum) after sedation with $CO_2/O_2$ (Jolly, 1993). The testis and epididymis were freed from the scrotum and prepared for electron microscopic examination. Each epididy-
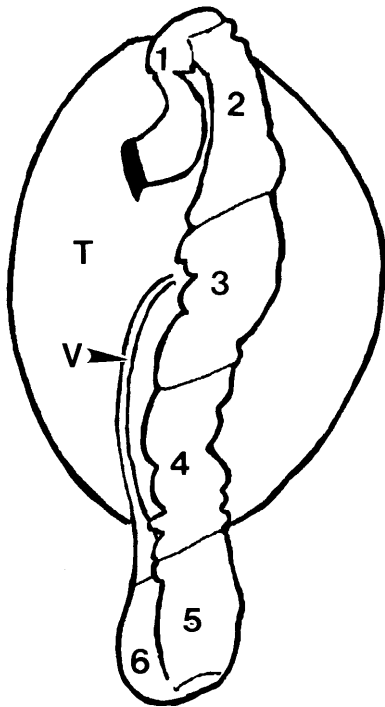
Fig. 1. Diagram of the epididymis of the tammar wallaby showing the dissected epididymal segments for this study: 1, proximal head; 2, distal head; 3, proximal body; 4, distal body; 5, proximal tail; 6, distal tail. T, testis; V, vas deferens.

mis was dissected and divided into segments of (1) the proximal head, (2) the distal head, (3) the proximal body, (4) the distal body, (5) the proximal tail and (6) the distal tail (see Fig. 1). These segments corresponded to the 22 regions of the tammar wallaby epididymis described by Jones et al. (1984) as (1) regions 1–4, (2) regions 5–8, (3) regions 9–12, (4) regions 13–18, (5) regions 19–20 and (6) regions 21–22. The regionalisation of the epididymis of the brushtail possum was similar to that for the tammar wallaby.

*Preparation for transmission electron microscopy*

The dissected tissues were fixed in 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 4 h at room temperature or overnight at 4 °C. The tissues were postfixed in 1% osmium tetroxide for 1 h. After dehydration through serial concentrations of acetone, the tissues were embedded in Spurr's resin (Agar Scientific, Essex, UK). Sections (70–100 nm) were cut on an Ultracut E ultramicrotome (Reichert-Jung, Austria) with a diamond knife (Diatome, Bienne, Switzerland), and
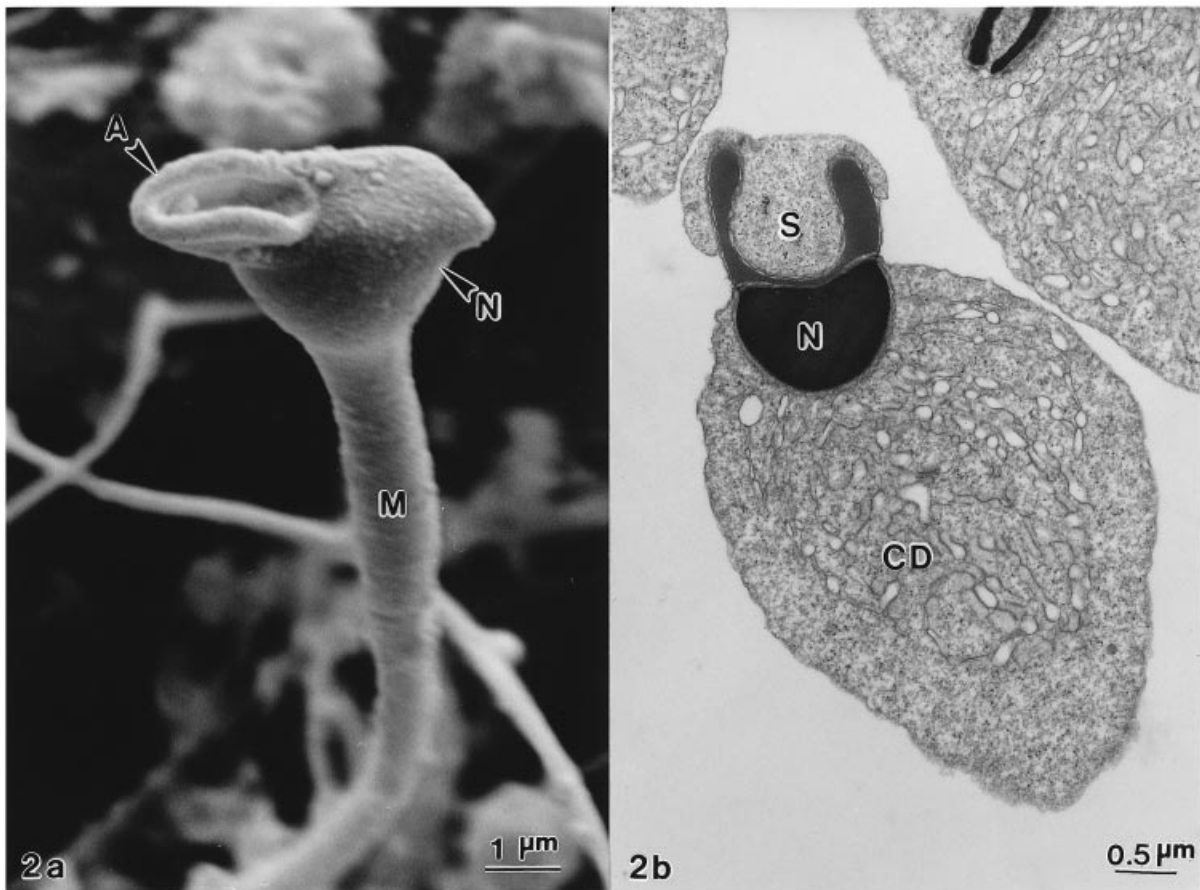


Fig. 2. Scanning (*a*) and transmission (*b*) electron micrographs showing the testicular sperm of the tammar wallaby. Note the immature acrosome (A) is in a 'scoop' shape and holding a patch of Sertoli cell cytoplasm (S). The acrosome (A) is lodged in the anterior end of dorsal surface of the nucleus (N) which is perpendicular to the midpiece (M) of the sperm. CD, cytoplasmic droplet.

stained with 1 % uranyl acetate in 30 % (v/v) ethanol (Watson, 1958) for 5–10 min, followed by lead citrate (Reynolds, 1963) for 10–20 min. Transmission electron micrographs (TEM) were taken with a JEOL-100CX electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

### Preparation for scanning electronmicroscopy

The tissues for SEM were prepared by using the same fixative procedures as for TEM, except that the tissues were fixed overnight at 4 °C. They were then treated with 1 % osmium tetroxide for 4 h. After dehydration and critical point drying, the tissues were coated with gold and examined in a JSM 840 scanning electron microscope (JEOL, Tokyo, Japan) operated at 15 kV.

### RESULTS

### Tammar wallaby testis

Testicular spermatozoa in the lumen of the seminiferous tubules and rete testis had an elongated posterior facing 'scoop'-shaped acrosome formed of a sheet of folded tissue with its base sitting on the anterior half of the dorsal surface of the nucleus. The sides of the scoop protruded up from the nucleus anteriorly and laterally but not posteriorly (Fig. 2a). At this stage the acrosome scoop contained a remnant of Sertoli cell cytoplasm which in SEM appeared as a small circular body (Fig. 2a). In TEM the remnant of Sertoli cell cytoplasm was closely attached to the sperm cytoplasmic membrane filling the 'scoop' and projecting over the lateral projections (Fig. 2b). The spermatozoa were roughly T-shaped with the head at a right angle to the tail (Fig. 2a).

### Proximal head of the tammar wallaby epididymis

There was no dramatic change in the morphology of the acrosome when spermatozoa migrated into the proximal head region of the epididymis. The acrosome was scoop-shaped sitting on the roughly T-shaped sperm (Fig. 3a). However, the rim of the scoop projection was notably thickened compared with that
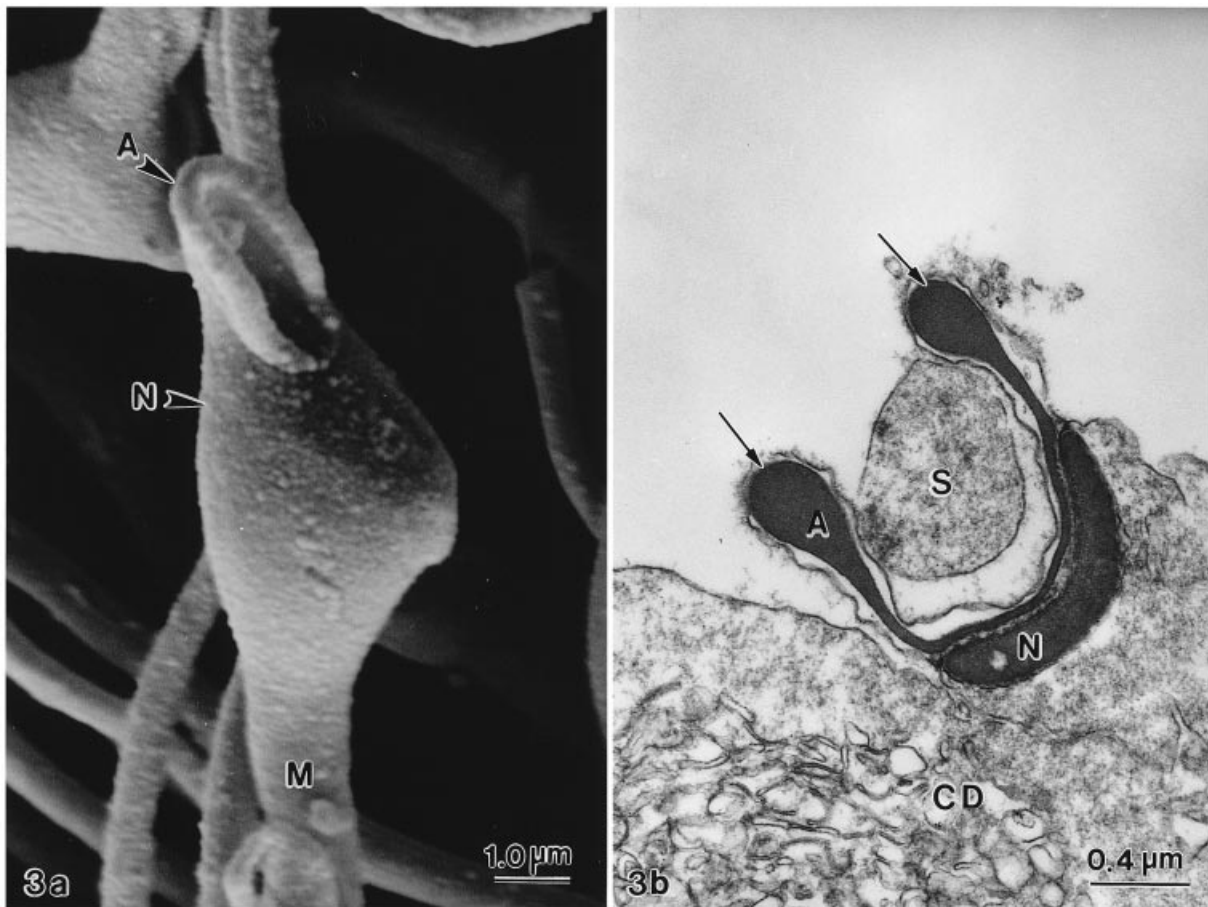


Fig. 3. Scanning (a) and transmission (b) electron micrographs showing the tammar wallaby sperm in the proximal head of the epididymis. In a the nucleus of the sperm (N) is still perpendicular to the midpiece (M) and the acrosome (A) remains in the scoop shape. Note the rims of the lateral scoop projections (arrows in b) of the acrosome (A) are thicker than those of the testicular sperm (see Fig. 2). A patch of Sertoli cell cytoplasm (S in b) is still restrained by the lateral projections of the acrosome (a). CD, cytoplasmic droplet.
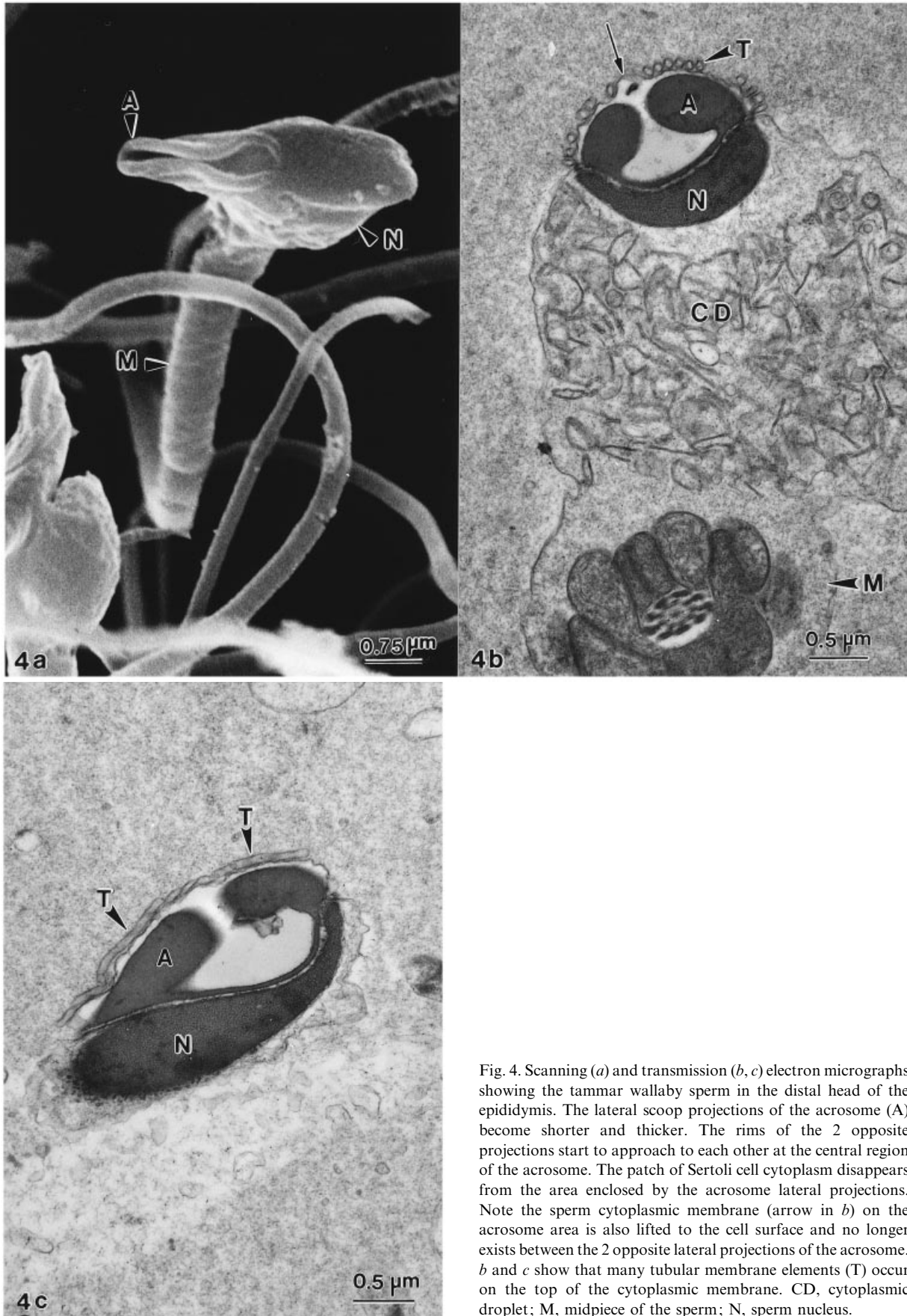
Fig. 4. Scanning (*a*) and transmission (*b*, *c*) electron micrographs showing the tammar wallaby sperm in the distal head of the epididymis. The lateral scoop projections of the acrosome (A) become shorter and thicker. The rims of the 2 opposite projections start to approach to each other at the central region of the acrosome. The patch of Sertoli cell cytoplasm disappears from the area enclosed by the acrosome lateral projections. Note the sperm cytoplasmic membrane (arrow in *b*) on the acrosome area is also lifted to the cell surface and no longer exists between the 2 opposite lateral projections of the acrosome. *b* and *c* show that many tubular membrane elements (T) occur on the top of the cytoplasmic membrane. CD, cytoplasmic droplet; M, midpiece of the sperm; N, sperm nucleus.
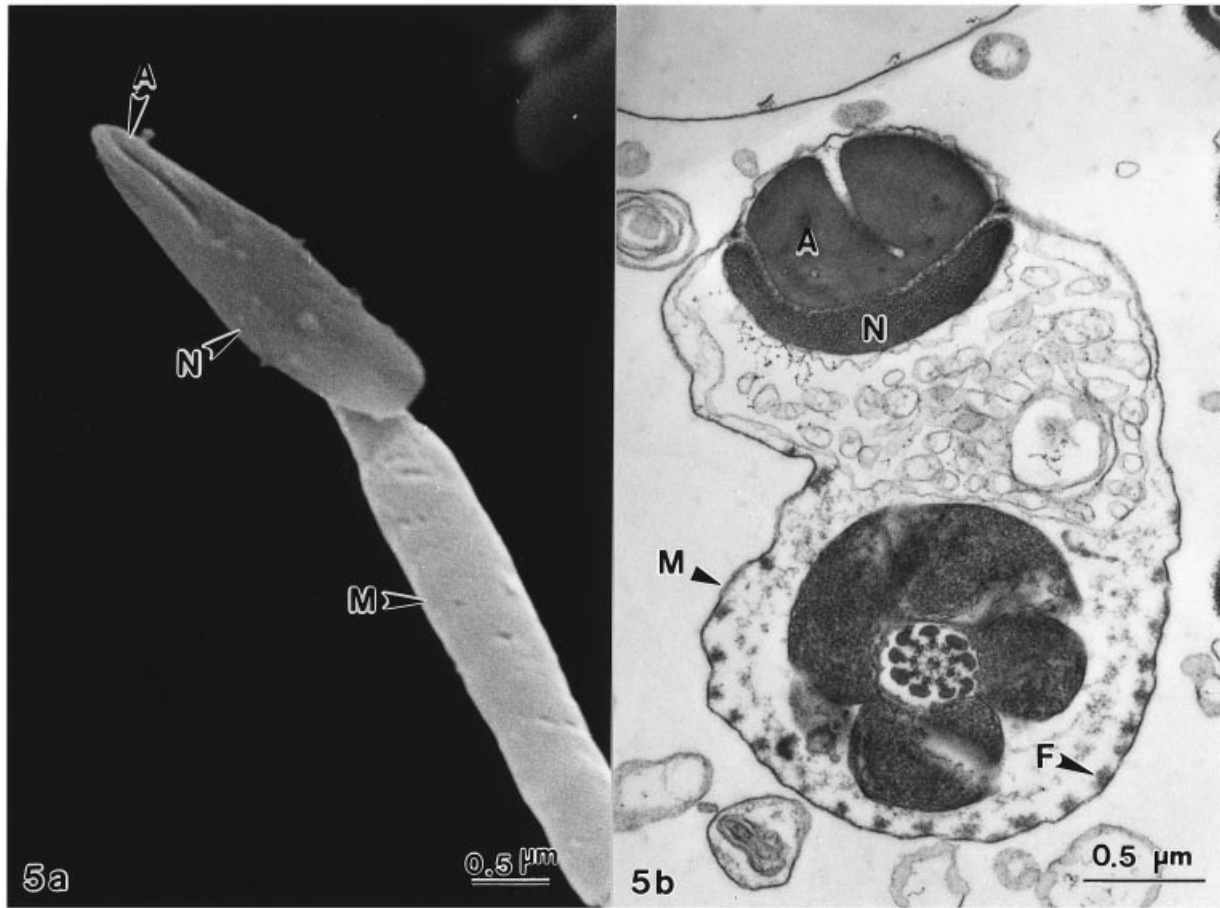
Fig. 5. Scanning and transmission electron micrographs showing the tammar wallaby sperm in the proximal (*a*) and distal body (*b*) of the epididymis. The sperm nucleus (N) is oriented to be nearly parallel to the midpiece (M) of the sperm in the proximal epididymis body (*a*). In *b* the acrosome (A) is further condensed and its lateral projections start to fuse along the longitudinal axis of the acrosome when sperm are in the distal epididymis body. (*b*) Note the midpiece fibre network (F) is produced when the sperm transit through the epididymis body.

of the testicular spermatozoa (Fig. 3*b*, cf Fig. 2*b*). The remnant of Sertoli cell cytoplasm looked similar in SEM (Fig. 3*a*) but appeared reduced in volume into a circular droplet contained within the scoop projections in TEM (Fig. 3*b*). In addition, the Sertoli cell remnant and cytoplasmic membrane lining the scoop were no longer in close contact.

### Distal head of the tammar wallaby epididymis

The rims of the acrosome lateral projections were markedly thickened and shortened (Fig. 4*b*). The lateral projections were inward folding and approached each other to meet in the midline of the acrosome and midway along its length (Fig. 4*a*). As the acrosomal projections condensed, the acrosomal surface area was substantially reduced and the cytoplasmic membrane which previously was closely applied to the acrosomal membrane and lined the inner surface of the scoop was lifted to above the projections (Fig. 4*b*). This lifting of the cytoplasmic

membrane was associated with the formation of tubular membrane elements across the surface of the acrosome area (Fig. 4*c*). At this stage the sperm head remained perpendicular to the midpiece but the Sertoli cell remnant was no longer evident.

### Proximal and distal body of the tammar wallaby epididymis

Within the proximal body of the epididymis, the sperm head had rotated to a position so that its long axis was nearly parallel to the sperm midpiece and the base of the acrosome was restricted to its final site, a depression on the anterior third of the dorsal surface of the nucleus (Fig. 5*a*). Shortening and thickening of the acrosome lateral projections were advanced and the space between the 2 lateral projections was reduced to a narrow slit along the length of the acrosome (Fig. 5*a*,*b*). At this stage tubular membrane elements overlying cytoplasmic membrane above the acrosome were no longer present, and the first evidence of the
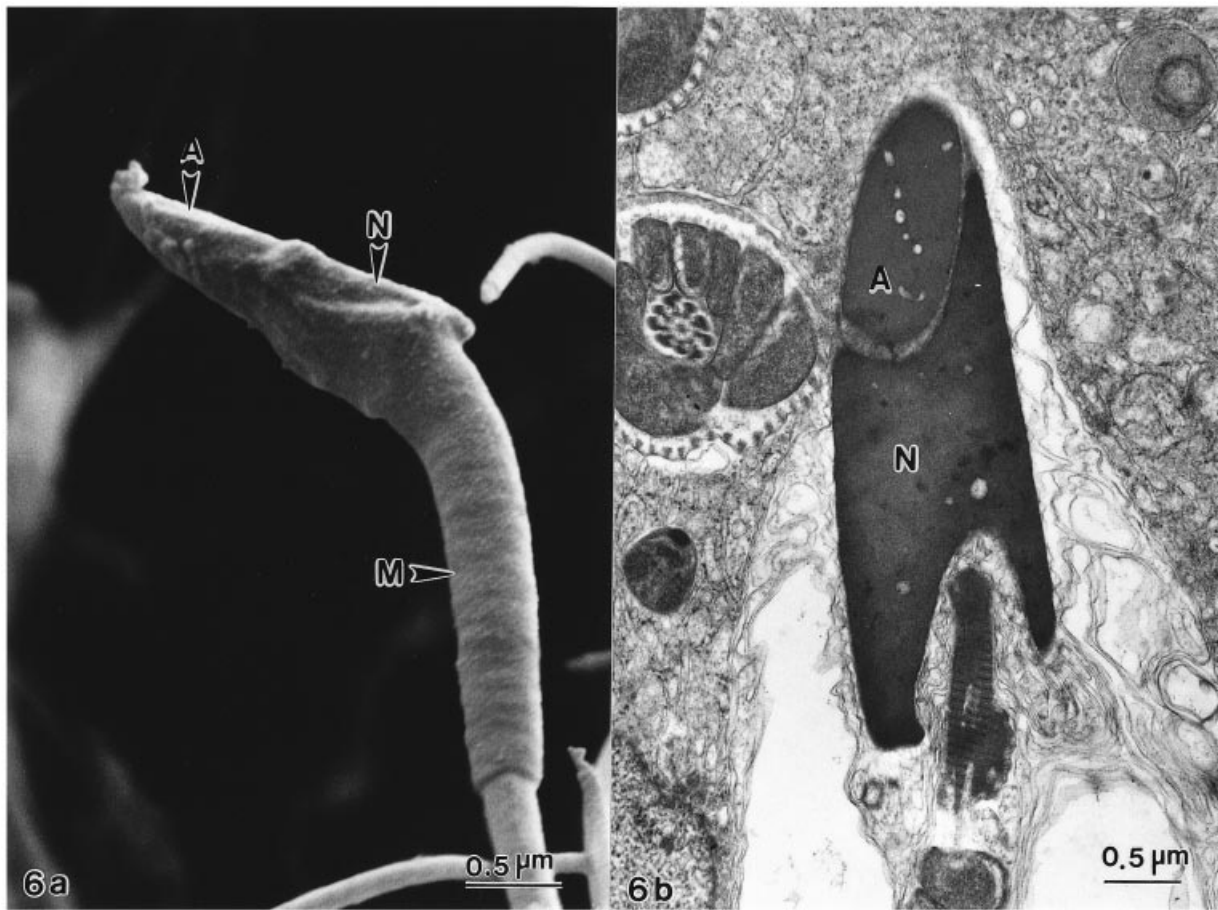
Fig. 6. Scanning and transmission electron micrographs showing the tammar wallaby sperm in the proximal (*b*) and distal tail (*a*) of the epididymis. The multiple-point fusion of the lateral projections can still be seen in the acrosome matrix (see *b*) of the sperm in the distal tail of the epididymis. The acrosome (A) condenses to its final compact button shape, lodged into the depression on the distal third of the dorsal surface of the nucleus (N). M, sperm midpiece.

midpiece fibre network was seen (Fig. 5*b*). When spermatozoa passed through the distal body of the epididymis the lateral projections of the acrosome fused together to produce a compact unified structure.

### Proximal and distal tail of the wallaby epididymis

SEM indicated that the acrosome had condensed into its mature form as a small compact button-like structure as found in ejaculated spermatozoa (Fig. 6*a*). However, the fusion process continued in the acrosome interior evidenced by a line of fusion vacuoles within the matrix (Fig. 6*b*).

### Brushtail possum

SEM and TEM demonstrated that acrosome formation in the epididymis of the brushtail possum is very similar to that described above for the tammar wallaby. On leaving the testis the acrosome of the possum was also a scoop-shaped sheet of folding

tissue on the dorsal side of the sperm head (Fig. 7*a*) As the possum spermatozoa pass down the epididymis, the acrosome condenses into a compact button-like structure (Fig. 7*b*). condensation of the acrosome occurred by a complex process of infolding and fusion of the scoop projections, which was essentially the same as occurred in the tammar wallaby (Fig. 8*a–c*).

### DISCUSSION

Using both TEM and SEM, the present study has been able to visualise the 3-dimensional process of acrosome formation which occurs as spermatozoa pass through the epididymis in the tammar wallaby and brushtail possum. It has been known for some time that on leaving the testis the acrosomes of both species are in an immature form but the exact nature of this structure and the manner by which it was transformed into the compact button-like mature acrosome seen in ejaculated spermatozoa was not
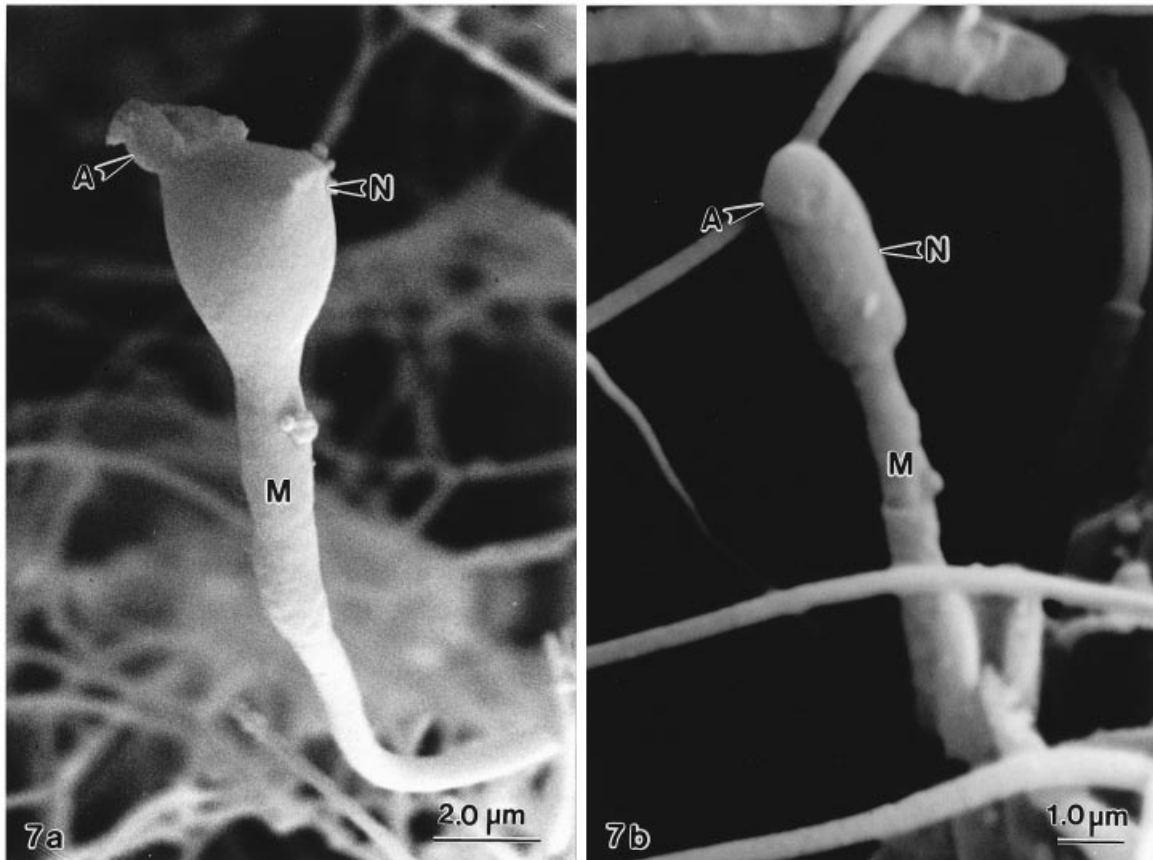
Fig. 7. Scanning electron micrographs of the brushtail possum sperm. (*a*) The acrosome (A) with a scoop shape is located on the anterior dorsal surface of the nucleus (N). (*b*) Possum sperm from the distal tail of the epididymis. Note the acrosome (A) is in a compact button-like shape. M, the sperm midpiece; N, the sperm nucleus.

clear. In our early study of the tammar wallaby (Lin et al. 1997) we reported, based on SEM, that the immature acrosome was a backward facing scoop-shaped structure. The present study confirmed this finding and showed that the immature possum acrosome is also a rear-facing scoop. Earlier studies had interpreted TEM images of the immature possum acrosome as a cup-like structure (Harding et al. 1976) or as resembling fingers of a glove (Temple-Smith & Bedford, 1976). Given these different views of the 3-dimensional structure of the immature acrosome, it has been difficult to interpret the large number of TEM images of the process of maturation produced since the original reports for the possum (Cummins, 1976; Harding et al. 1976, Temple-Smith & Bedford, 1976). Our recent studies in the tammar wallaby (Setiadi et al. 1997; Lin et al. 1997), although providing substantial further TEM data on the process of acrosome formation, still lacked the clear 3-dimensional view revealed by the present SEM observations. In addition, we have confirmed that an essentially similar process also occurs in the possum.

Posttesticular acrosome formation in both species, and presumably other marsupials where the phenom-

enon occurs (Harding et al. 1979; Temple-Smith 1994), involves a complex folding and fusing of the anterior and lateral projections of the scoop-shaped acrosome which commenced as spermatozoa entered the distal head of the epididymis. This folding process was completed by the time spermatozoa reached the proximal tail region of the epididymis, confirming earlier observation (possum: Harding et al. 1979; Temple-Smith, 1994; wallaby: Setiadi et al. 1997). In the tammar wallaby remnants of the fusion of the infolded acrosomal membrane persisted within the acrosomal matrix as a variable number of small vesicles in many spermatozoa in the distal tail of the epididymis. Such vesicles or vacuoles have been reported in ejaculated possum sperm (Sistina et al. 1993). During the infolding process the cystoplasmic membrane which covered the lateral projections of the immature acrosome and the underlying outer acrosomal membrane were completely remodelled. The cytoplasmic membrane appeared to be eliminated from the spermatozoa as tubules of budded off membrane, which is in agreement with earlier interpretations (Harding et al. 1976; Lin et al. 1997). This study and our earlier study (Setiadi et al. 1997) clearly
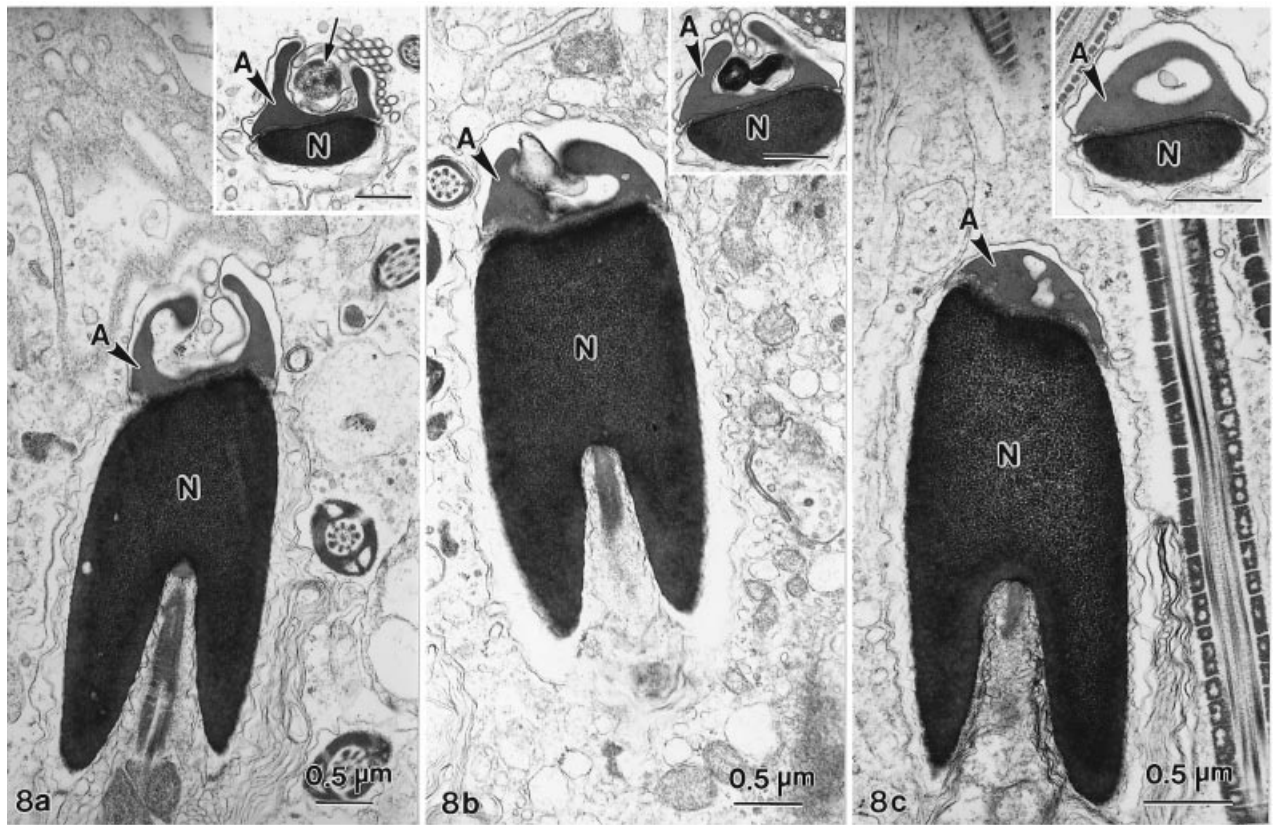
Fig. 8. Transmission electron micrographs of brushtail possum sperm in the epididymis body, showing the fusion of acrosome lateral projections. (*a*) The acrosome (A) before the fusion and its lateral projections are engulfed a droplet of Sertoli cell cytoplasm (arrow in the insert) when the sperm is in the proximal epididymis body. (*b*) Two acrosome lateral projections (A) infolding and approaching each other (see insert) during passage of the sperm through the proximal epididymis body. (*c*) Acrosome (A, also in the insert) after its lateral projections have fused together when the sperm is in the distal epididymis body. The multiple-point fusion of the lateral projections can be seen in the acrosome matrix. Bars in inserts, 0.5 μm. N, Nucleus.

indicate that the fate of the outer acrosomal membrane which lines the immature acrosome surface within the scoop is also complex but quite different to the cytoplasmic membrane. Acrosomal membrane was never observed to be shed during the maturation processes but appeared to reduce in surface area with the contracting matrix to which it closely adhered. The midline fusion of the acrosome was achieved by multiple-point fusion of the immature outer acrosomal membrane and evidence of this fusion event persisted as vesicles within the acrosomal matrix.

Thus the cytoplasmic membrane over the acrosomal surface of the mature sperm head, the most likely site of sperm zona pellucida binding and the site at which the acrosome reaction is probably initiated (reviewed in Rodger, 1991; Mate & Rodger 1996), is likely to be a very different, or highly modified, structure from that present at spermiation. Similarly the outer acrosomal membrane which is involved in fusion with the cytoplasmic membrane at the acrosome reaction is probably also a different, or highly modified structure, to that presented at spermiation. Therefore in those marsupial species where the acrosome is formed after

spermiation, attempts to understand the role of these structures in fertilisation must focus on the molecular character of the spermatozoa which have completed acrosome formation, that is spermatozoa from the vas deferens or ejaculate, and not on testicular sperm. This contrasts with the situation in eutherian mammals where it has been the practice to seek critical sperm antigens and receptors in testicular sperm to eliminate the complication of later interactions between the sperm surface and products of the epididymis which may mask functional integral membrane proteins.

This study found that the remnant of Sertoli cell cytoplasm held in the scoop shape of the acrosome of testicular spermatozoa (Lin et al. 1997) remains within the immature acrosome scoop when spermatozoa pass through the proximal head of the epididymis. In SEM this remnant appeared as a small droplet sitting within the scoop. However, TEM cross sections revealed that the Sertoli cell cytoplasmic remnant fully filled the space of the acrosome scoop and was firmly attached to the inner surface of the lateral projections of the immature scoop. This implied that the remnant may

play a role in supporting the delicate acrosomal projections which make up the sides of the scoop. When the acrosomal lateral projections condensed and started to fuse together in the distal head of the epididymis, the Sertoli cell cytoplasmic droplet lost contact with the scoop projections and appeared to be eliminated from the shrinking scoop. It is also possible that the Sertoli cell cytoplasmic remnant contributes materials or regulates the process of acrosome maturation although presumably the major factor in the process of posttesticular sperm maturation is the epididymal environment. Although the morphological changes described here are unique marsupial phenomena it suggests that these species are excellent models for studies of the fundamentals of mammalian sperm maturation and its regulation by the epididymal environment since such overt and readily visible morphological indicators occur. In eutherian mammals the equivalent maturation events can only be monitored indirectly by complex studies of function such as IVF. In contrast, our work would suggest that simple light microscopy on fresh or fixed sperm samples would in many experimental situations be a definitive indicator of sperm maturation or its disruption in the wallaby or possum.

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