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# Separation of sperm pairs and sperm–egg interaction in the opossum, *Didelphis virginiana*

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**Summary.** Oocytes, recovered from the oviduct or from ripe follicles, and spermatozoa flushed from the oviduct of opossums mated under laboratory conditions, were used to study the characteristics of marsupial gamete interaction and sperm incorporation. Opossum oocytes lose all granulosa cells before ovulation and are invested only by a thin zona pellucida when ovulated. The spermatozoa unpair in the oviduct but the motile population flushed at about the time of ovulation includes paired, separating and single spermatozoa. Separation of paired spermatozoa was associated with changes in the acrosome, the amorphous matrix being displaced by membrane-bound vacuoles. Fertilization is normally monospermic. Spermatozoa associated with the zona of unfertilized eggs by the flat acrosomal face of the head, whereas those trapped in mucoid eventually laid down around the zona were orientated at random. In penetrating the zona, the spermatozoon created a relatively large uneven hole that contrasts with the discrete penetration slit seen in the thicker zona of eutherian oocytes. Spermatozoa appeared to associate and fuse with the oolemma by the acrosomal face of the head. Ultrastructurally the sperm head, in oocytes fixed immediately after incorporation into the ooplasm, was devoid of surrounding membranes. This pattern of gamete interaction resembles the mode seen in non-mammalian vertebrates and invertebrates, and not that in eutherian mammals. Sperm penetration by tubal sperm samples occurred readily *in vitro* within 1 h of placing ripe follicular oocytes with the oviducal flushings containing spermatozoa. None of the oocytes penetrated *in vivo* or *in vitro* displayed extra perivitelline spermatozoa. This suggests that the oocyte is able to mount a block to polyspermy at the zona surface, although the mucoid deposited on the zona pellucida may be involved *in vivo*. The results indicate that the complex mode of sperm incorporation seen in eutherian mammals is unique to the infraclass and not shared by their closest relatives the marsupials.

## Introduction

Marsupials are the most closely related line to the eutherian mammals. The two diverged from a common ancestor probably in the early Cretaceous, more than 100 million years ago (Kielan-Jaworowska, Brown & Lillegraven, 1979), and in numerous aspects of their biology have developed remarkably parallel systems (see Tyndale-Biscoe, 1973). There are, however, well-known differences in the pattern of pregnancy and neonatal development, and structural differences between their gametes suggest that this may also be the case for the mode of

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fertilization. The pattern of gamete interaction in most invertebrates and non-mammalian vertebrates is in general a fusion of the inner acrosomal membrane covering the apex of the reacted sperm head and the oolemma, followed by incorporation into the ooplasm of the sperm nucleus entirely devoid of sperm head membranes or sequestered oolemma (Colwin & Colwin, 1967; Nicander & Sjoden, 1971; Okamura & Nishiyama, 1978). By contrast, in eutherian mammals, initial fusion occurs by way of a persistent region of plasmalemma overlying the equatorial segment of the reacted acrosome (Bedford, Moore & Franklin, 1979). The egg cortex then responds in a phagocytic manner engulfing the rostral portion of the sperm head encased by inner acrosomal membrane and a segment of oolemma is also internalized (Bedford, 1970, 1972; Yanagimachi & Noda, 1970, 1972). This complex of membranes persists around the decondensing sperm head nucleus within the ooplasm for some hours. Gamete interaction in the amphibian urodeles has been described as mammal-like, in light of the unusual complexity of sperm structure and the degree of –S–S– stabilization of nucleus, neck and tail (Picheral, 1977). However, this is unlikely, for although incorporation of the highly –S–S– stabilized tail undulating membrane does involve invagination of oolemma, there is no evidence of any such association of egg or sperm head membranes around the incorporated sperm nucleus (Picheral, 1977). Moreover, the urodele acrosome shows no specialization equivalent to the equatorial segment of the eutherian spermatozoon (Picheral, 1977, 1979).

Among other mammals, the pattern of sperm–egg interaction remains unknown. In monotremes, it is likely to resemble that described in the fowl (Okamura & Nishiyama, 1978). Monotreme spermatozoa have retained a sauropsid-, bird- and reptile-like character (Carrick & Hughes, 1978; Bedford & Rifkin, 1979) and the mature oocyte of monotremes has an extremely thin zona pellucida (Hughes, 1977). Consideration of gamete structure alone does not, however, allow one to predict with any confidence the pattern that will typify marsupial fertilization, not least because post-testicular sperm maturation seems equally as complex as that occurring in eutherians (Bedford, 1979). The marsupial ovum differs from that of eutherians in the rather flimsy character of the zona pellucida which is distinctly thinner than in eutherian ova of comparable size (Hill, 1910; Hartman, 1916; Bedford & Calvin, 1974; Renfree & Tyndale-Biscoe, 1978). There are a number of more distinct differences in the organization of the sperm head. Although the surface character of the marsupial spermatozoon differs regionally as in eutherians, significant –S–S– stabilization of head structures is not developed, and the acrosome is not differentiated to form a persistent equatorial segment (Bedford & Calvin, 1974; Temple-Smith & Bedford, 1976, 1980). In eutherian spermatozoa, the equatorial segment is stabilized by 'struts' that pass between the parallel inner and outer acrosomal membranes (Bedford *et al.*, 1979; Russell, Peterson & Freund, 1980). A striking feature confined to the spermatozoa of American marsupials is the phenomenon of sperm pairing which occurs during epididymal transit. The manner of pairing has been described at the ultrastructural level for two opossum species (Phillips, 1970; Olson, 1980; Temple-Smith & Bedford, 1980): it is established by way of a close association of the plasma membrane overlying the acrosomes of apposed spermatozoa, together with small peripheral junctions. What biological advantage this confers is unknown, nor is it clear whether both spermatozoa of a pair co-operate during fertilization, and if not where separation takes place.

The present study describes the early events of fertilization in the Virginia opossum, *Didelphis virginiana*.

## Materials and Methods

The source, housing and management of the animals used in this study, together with the techniques of gamete recovery and assessment are described in detail by Rodger & Bedford (1982).

Oocytes, small blocks of oviducal isthmus, and Graafian follicles were prepared for transmission electron microscopy. Three fixatives were tested: (1) 2·5% glutaraldehyde in 0·1 M-phosphate buffer, pH 7·2; (2) 2·4% glutaraldehyde and 0·013% picric acid in 0·175 M-cacodylate buffer pH 7·4; and (3) 2·5 or 5% glutaraldehyde with and without 1 mM-$CaCl_2$ in 0·16 or 0·18 M-collidine buffer, pH 7·2. Eggs were fixed for 1 h and tissues for 2 h at room temperature, post-fixed in 1% osmium tetroxide in the appropriate buffer for 1 and 2 h respectively, dehydrated in graded alcohols, then through propylene oxide to Epon 812. All fixatives gave satisfactory results but membranes, and in particular those of the sperm mitochondria, were best preserved when collidine buffer was used.

The ultrastructure of fertilization was studied in ova sectioned serially on a Sorvall MT2-B ultramicrotome with a diamond knife (25–40 grids per ovum, and 70–80 individual sections per grid). Sequential thin sections were stained in the conventional manner with lead-uranyl acetate and examined in a JEOL JEM100B electron microscope. By this method, it was possible to examine the whole oocyte systematically. The numbers so examined at each stage of fertilization are listed in Table 1.

**Table 1.** Numbers of ova examined in the electron microscope for each fertilization stage

| Stage | Numbers of ova | | |
|---|---|---|---|
| | Perforate zona | | |
| | 1 hole | 2 holes | Total |
| Unfertilized | | | |
| Not activated (metaphase) | 1* | 2* | 12 |
| Activated (anaphase) | 1* | | 1 |
| Fertilized | | | |
| Partial incorporation of spermatozoon | 2 | 2* | 4 |
| Polar body 2 formation | | | 5 |
| Pronuclei bound by membranes | | | 9 |

* The interacting spermatozoon was not present at the site of perforation of the zona.

Semi-thin 1 μm sections were cut from tissues embedded in Epon, with a glass knife and stained with a mixture of 1% methylene blue and 0·5% toluidine blue in 1% sodium borate. The stained sections were then examined in a light microscope. Oviducal sections were examined in this way to locate isthmic crypts, so that appropriate thin sections could be obtained for ultrastructural examination. The changing relationship between granulosa cells and the maturing preovulatory oocyte was evaluated from material examined at the light microscope level only.

Sperm penetration and incorporation were studied at the ultrastructural level mainly in oocytes fixed immediately after flushing from the oviduct. However, phase-contrast observation indicated that oviducal spermatozoa would readily penetrate and become incorporated by oocytes when incubated together in the flushing medium. In-vitro culture was then exploited to obtain further examples of the early phases of fertilization. The preparation of these samples involved either mixing naked oocytes from immediately preovulatory follicles with tubal flushings containing motile spermatozoa, or culture of freshly ovulated oocytes in tubal flushings. Culture was for 1–2 h at 37°C in water-saturated air.

## Results

### *Tubal spermatozoa*

A mixed population of spermatozoa was flushed from the oviducts. Some were immotile, others vibrated but were stationary, and others showed vigorous progressive motility. Of the motile spermatozoa, many were paired while some appeared to be in the process of separation, as judged by the independent gyrations of their still associated heads. When separate, these spermatozoa swam forward as vigorously motile single cells. Only single spermatozoa interacted with oocytes, and fertilization was monospermic. Spermatozoa apparently in the process of separation were also observed in fixed material in both the light and electron microscope in epithelial crypts in the lower isthmus of the oviduct. Separation began at the anterior end of the sperm heads reversing the apparent sequence of pairing (Krause & Cutts, 1979). In this intermediate phase, the most posterior connection persisted (Pl. 1, Figs 1–5), and in fresh material appeared to function as a hinge, the two acrosomal faces moving apart and together repeatedly. One of the pair often appeared to initiate the separation, its movements being the more forceful. Thus, both spermatozoa of a pair do not necessarily have equal fertilization potential. Ultrastructural examination of separating pairs revealed that the peripheral membrane junction disappears first (Pl. 2, Fig. 6), followed by a progressive lateral displacement of the

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PLATE 1

**Figs 1–5.** Light micrographs illustrating the sequence of separation of sperm pairs in the isthmic crypts. The relative position of the separating acrosomal faces is emphasized by two lines.

**Fig. 1.** A group of paired spermatozoa.

**Fig. 2.** Unpairing began as a small V-shaped cleft at the anterior border of the sperm pair.

**Figs 3 and 4.** This gap between the apposed acrosomes became progressively larger until only the posterior extremity remained connected.

**Fig. 5.** Two single, independent spermatozoa are shown, together with the orientation of the formerly apposed surfaces.

PLATE 2

**Fig. 6.** Unpairing is initiated by breakdown of the peripheral junction, and arrow heads indicate the point at which the sperm plasmalemmas were previously joined. The apposition of plasmalemma over the acrosomal surface (shafted arrow) persists. a = acrosomal matrix.

**Fig. 7.** The spermatozoa remain united over most of their acrosomal faces (shafted arrow), but there is a reduction in the density of the acrosomal matrix, and spaces (×) appear within it.

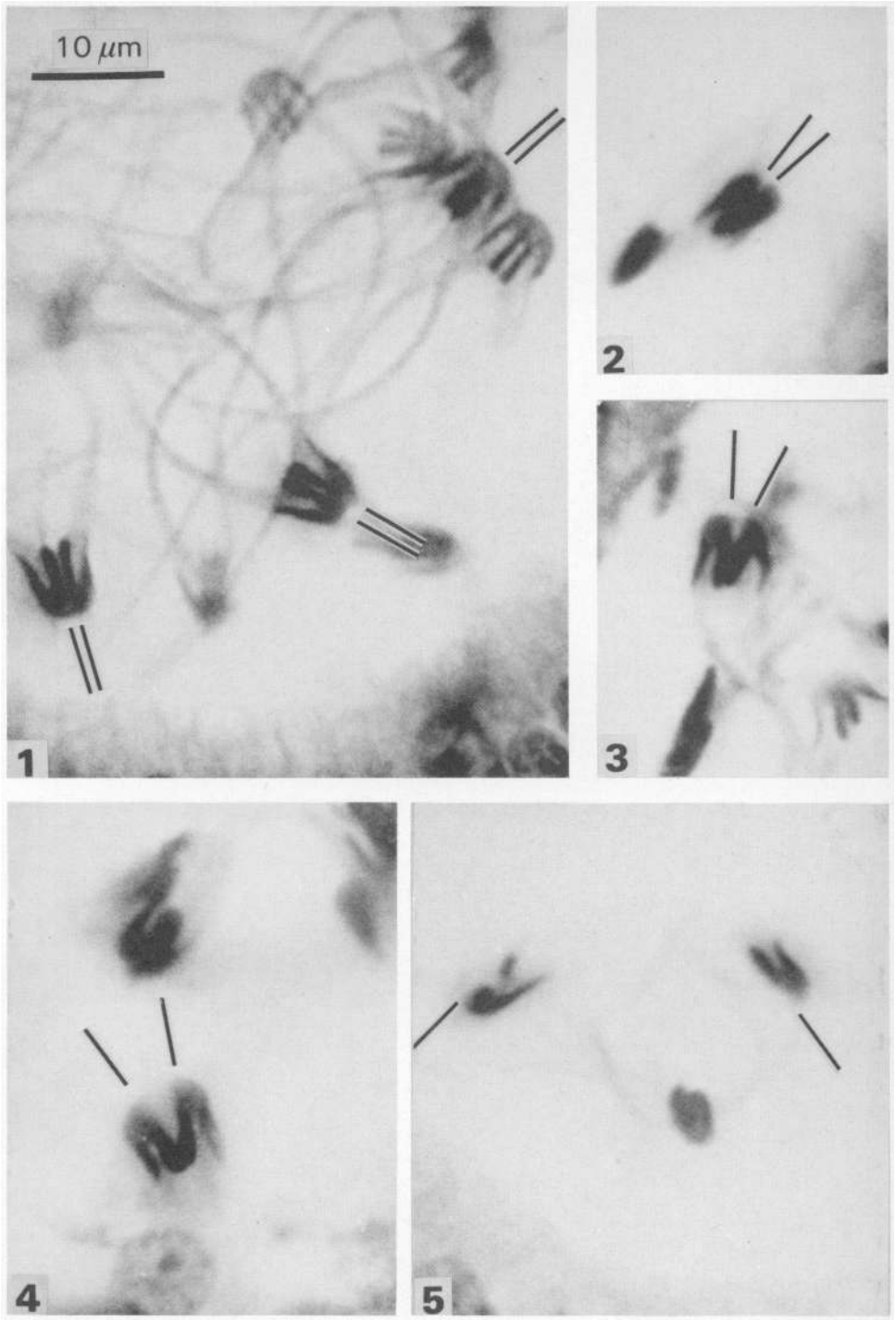
**Fig. 8.** Change in the acrosomal contents progressed until the acrosome was filled with membrane-bound vesicles and an apparently reduced matrix. Separation is advanced in the sperm pair at left and complete in the right pair, which are no longer in contact. Throughout this process, the plasmalemma overlying the acrosome (shafted arrows where united, arrow heads when free), and the outer acrosomal membrane appeared to remain intact.

PLATE 3

**Fig. 9.** A semi-thin section of a mature follicular oocyte obtained close to ovulation. Most of the zona pellucida is naked. The small remaining mass of granulosa cells attached to the zona will be lost before ovulation.

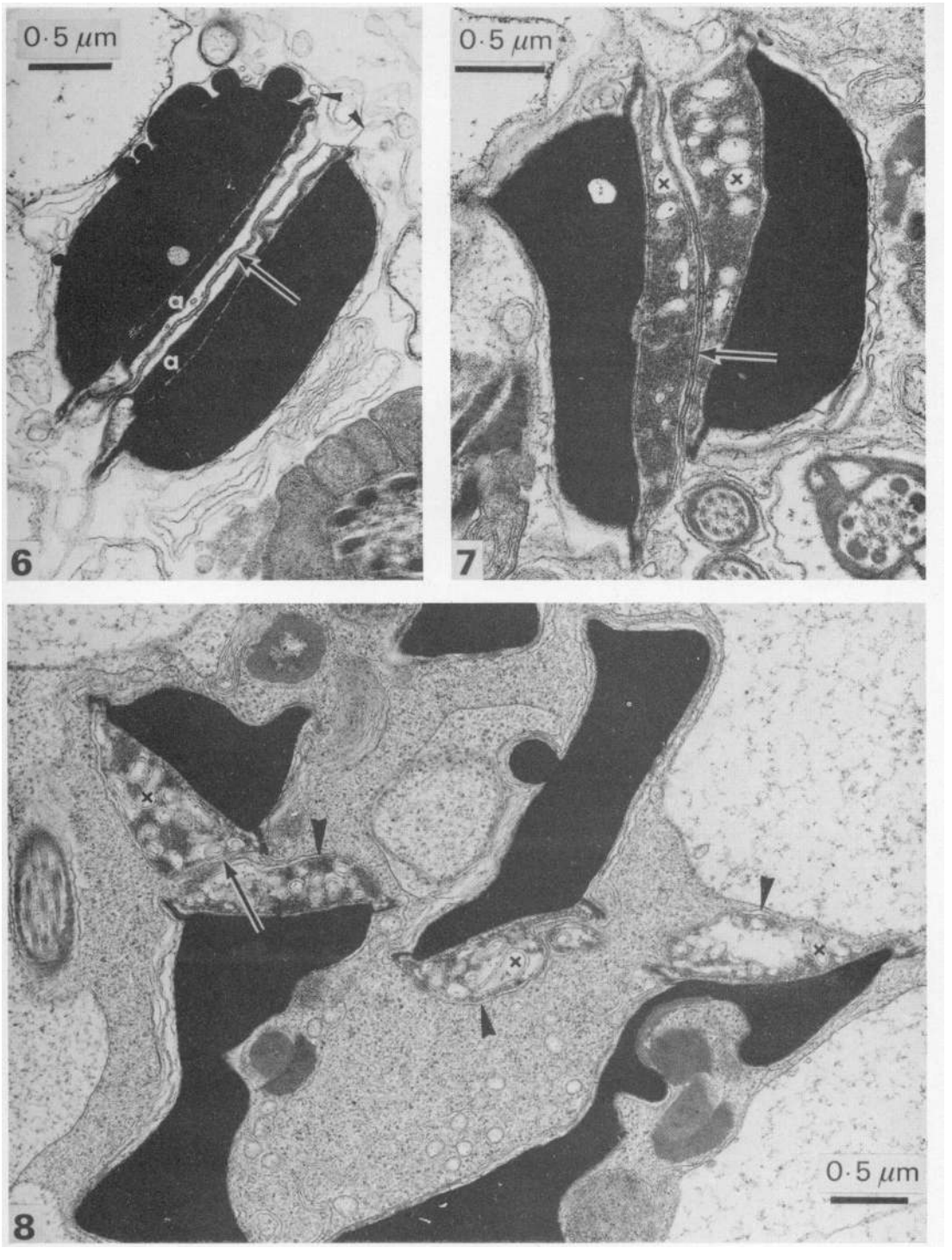
**Fig. 10.** Electron micrograph of an opossum spermatozoon within the egg cytoplasm (fertilization *in vitro*). Incorporation of this spermatozoon was incomplete in that most of the principal piece of the sperm tail protruded from the surface of the ovum. Unlike eutherian mammals at this early stage of fertilization, there is no shroud of incorporated membrane around the decondensing sperm head.

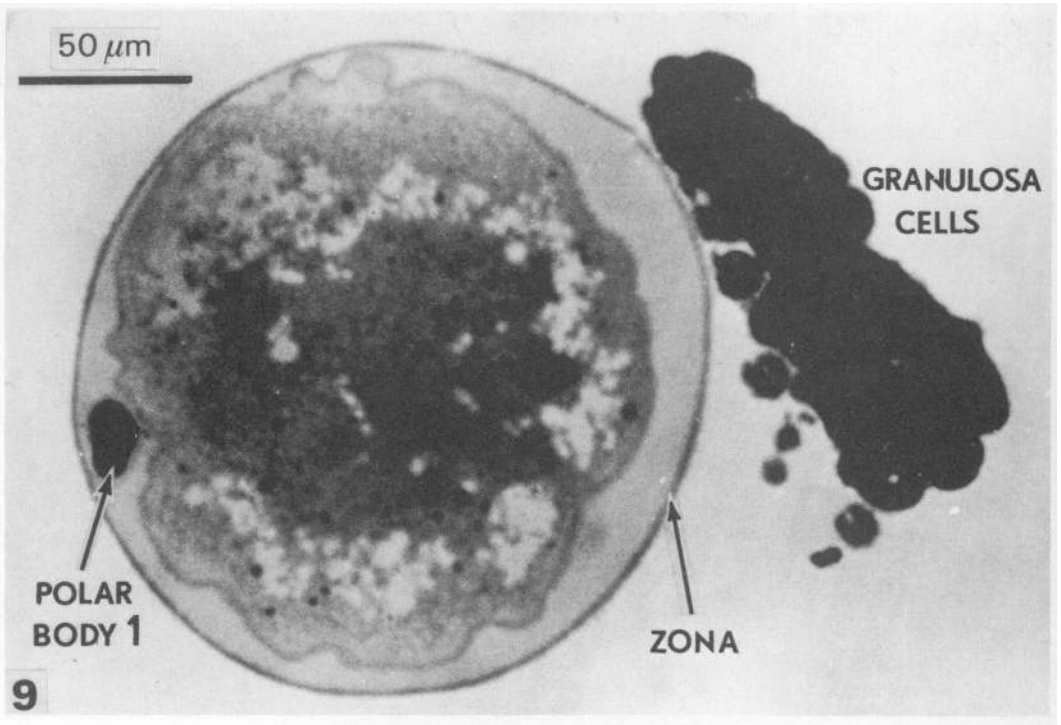
PLATE 1

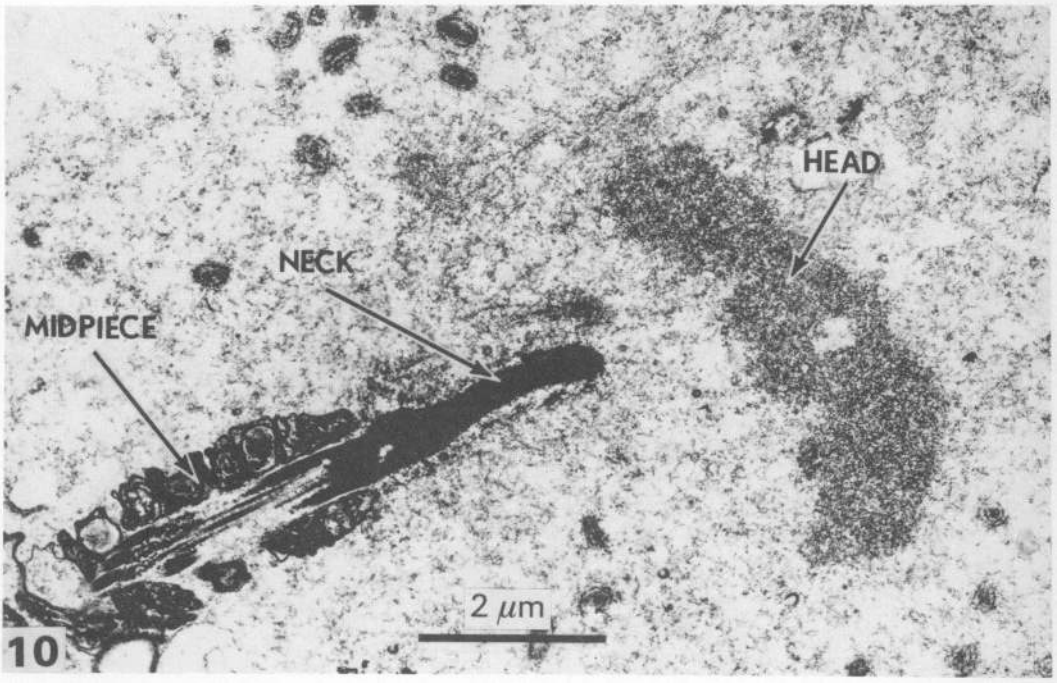



(*Facing p.* 174)

PLATE 2





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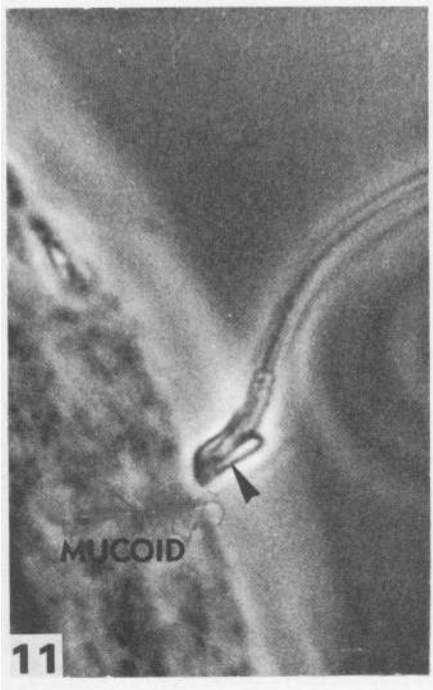


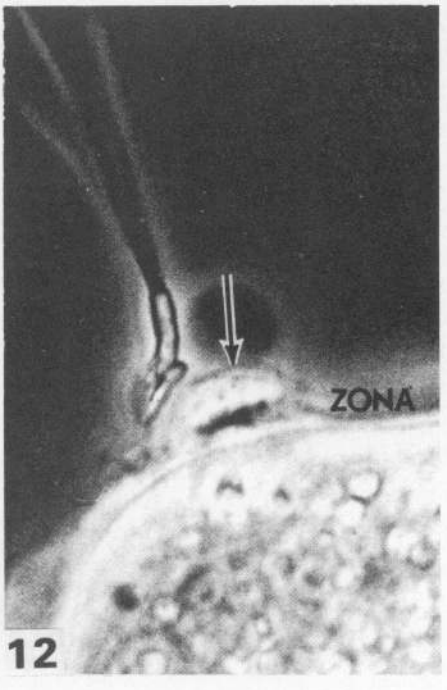


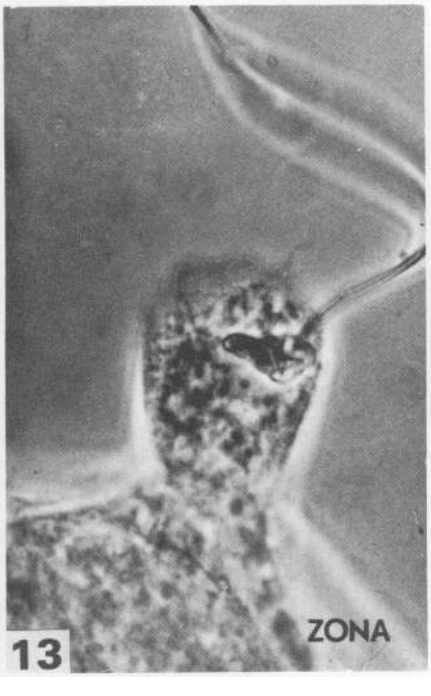


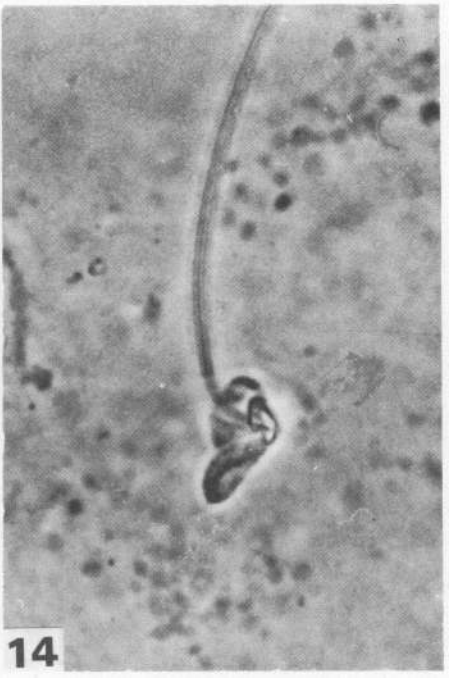

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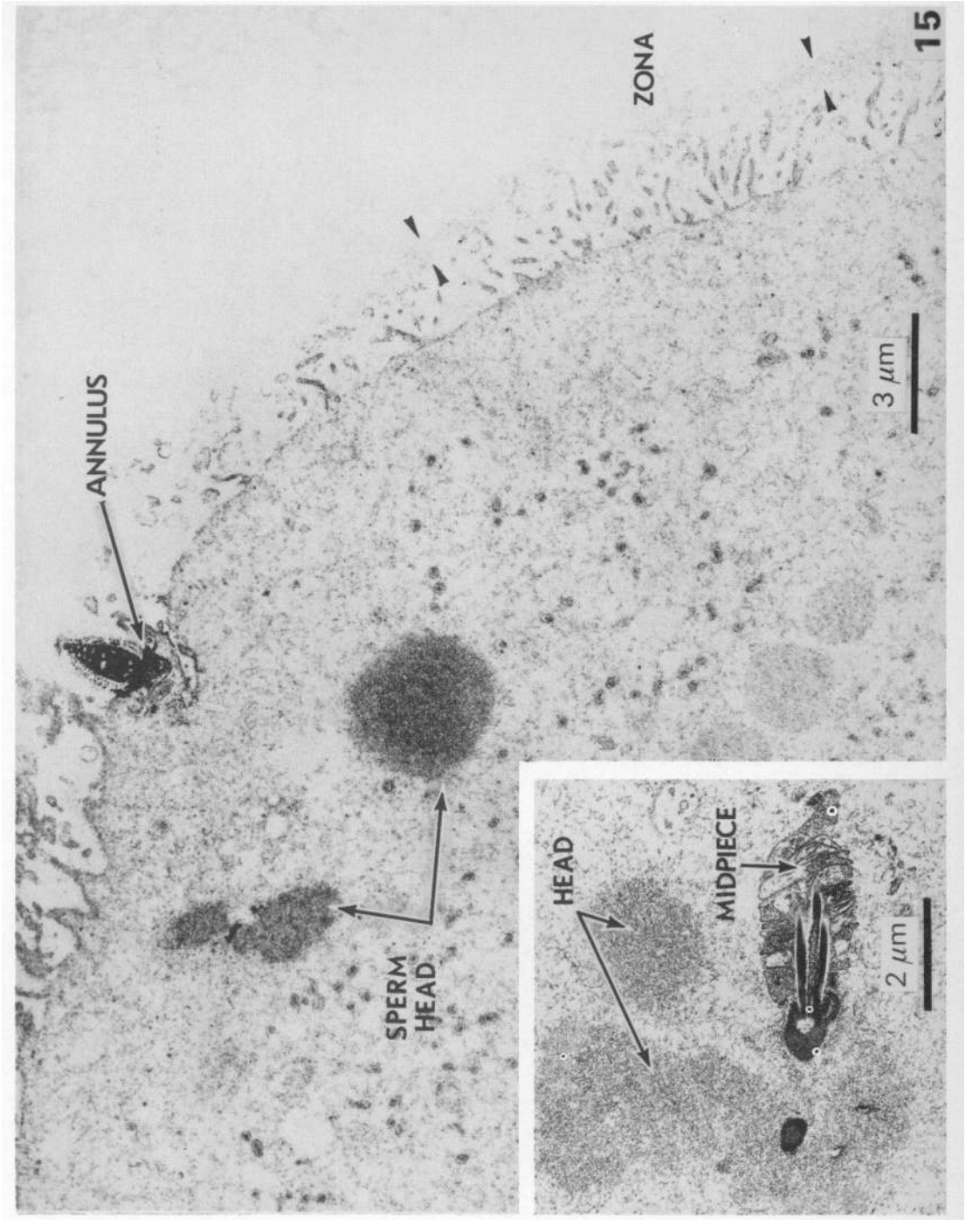

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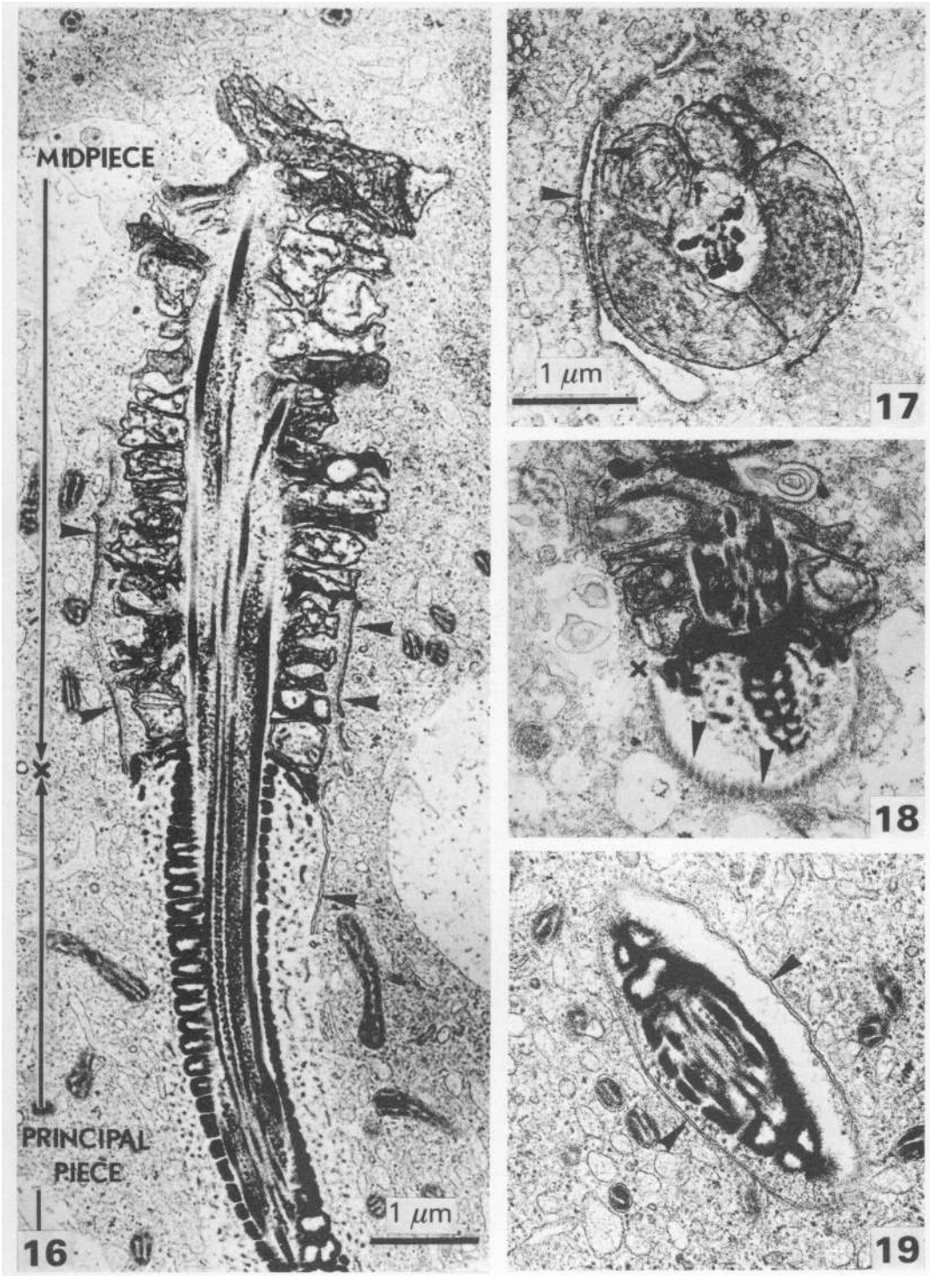


PLATE 7

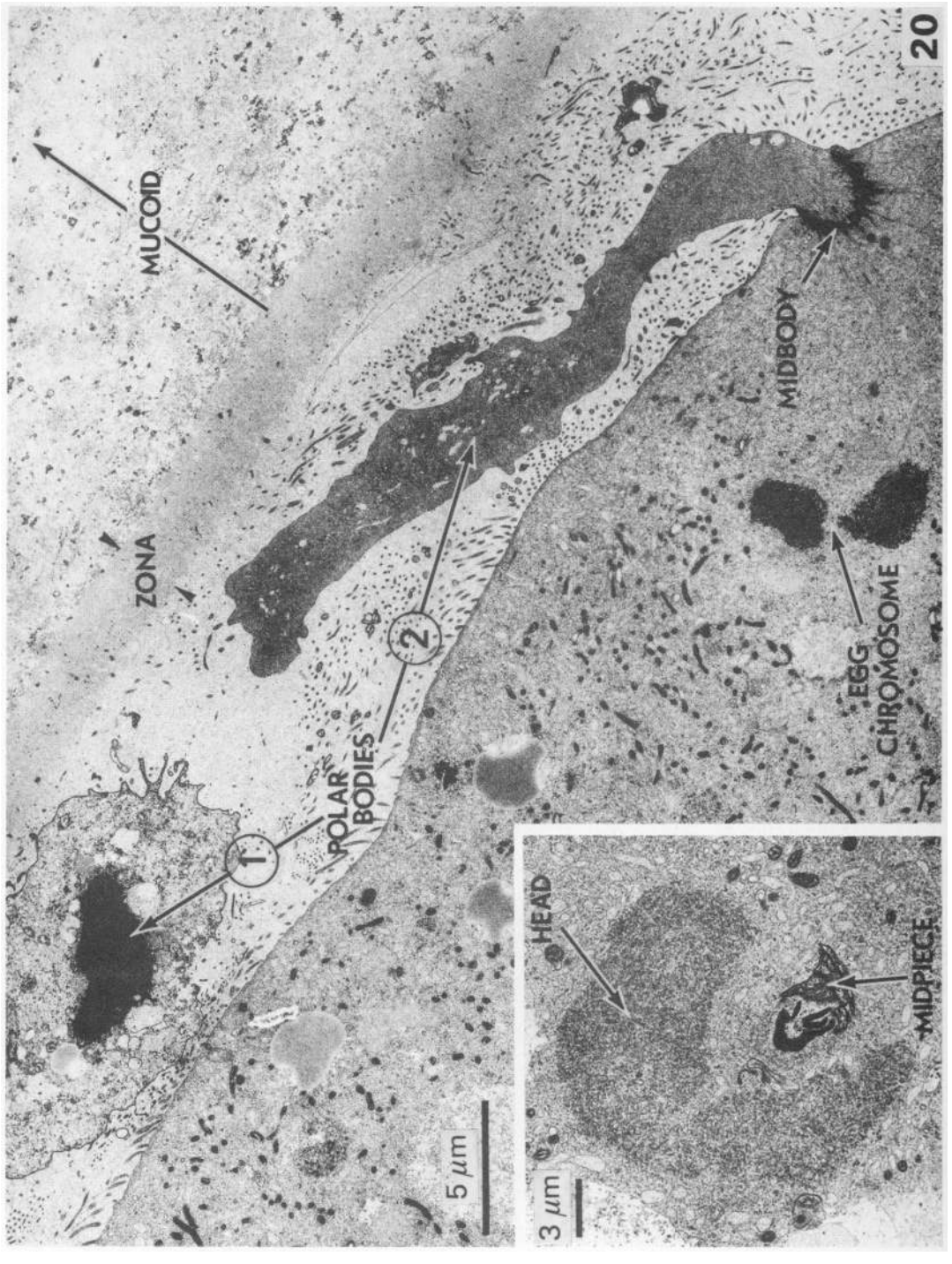
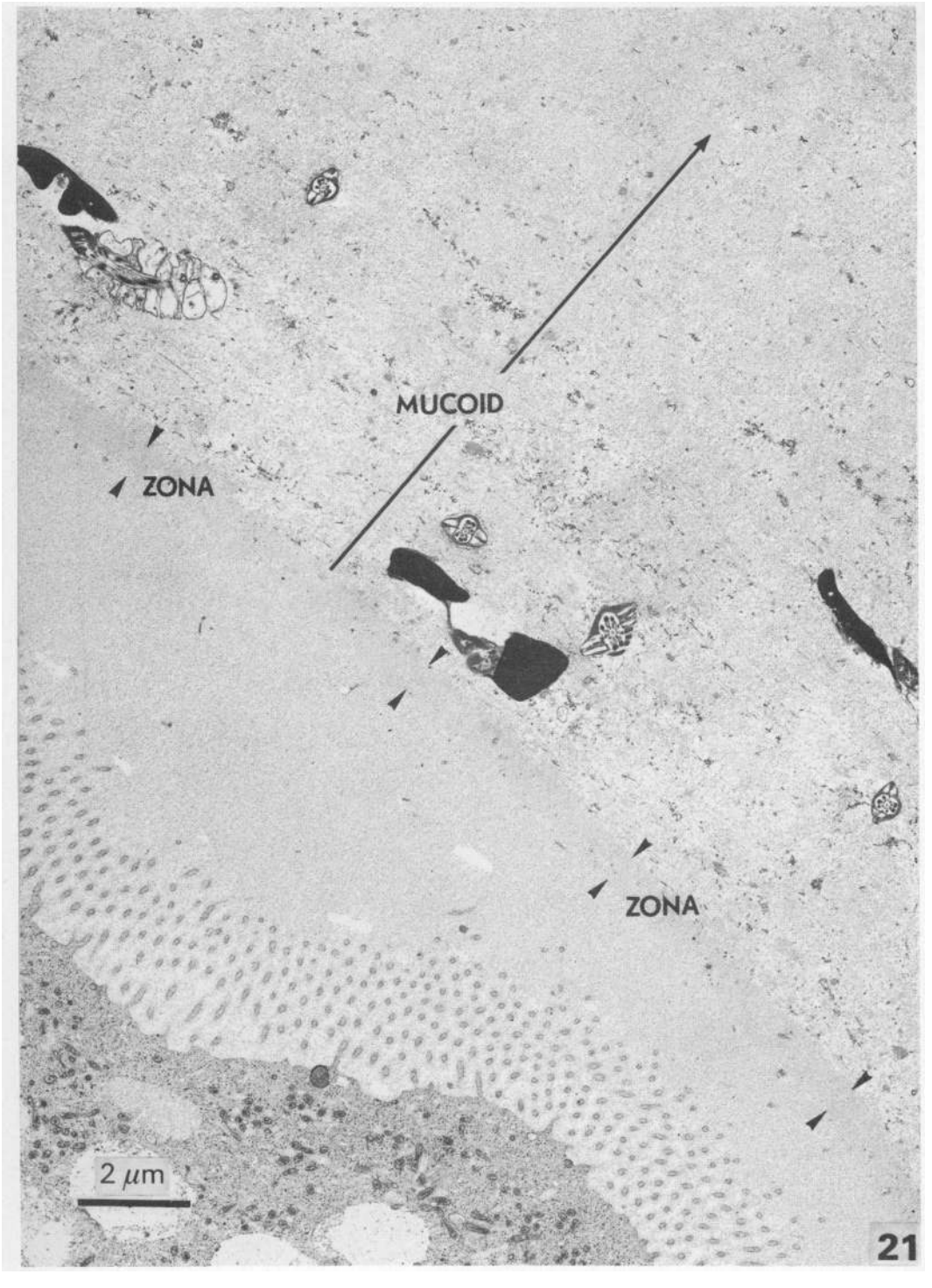


PLATE 8



apposed acrosomal faces (Pl. 2, Figs 6–8). This separation was accompanied consistently by the appearance of spaces in the acrosomal matrix which in single spermatozoa were vacuole-like and membrane-bound. Little of the acrosomal matrix was visible at the end of this process and the vesicles appeared to be empty (Pl. 2, Fig. 8). The origin of intra-acrosomal membrane appearing at this stage (Pl. 2, Figs 7 and 8) is obscure. The plasmalemma overlying the acrosome or the acrosomal membranes did not seem to be involved, and the change did not resemble the eutherian acrosome reaction (Barros, Bedford, Franklin & Austin, 1967; Bedford & Cooper, 1978). At the completion of the process of separation, the formerly compact acrosome appeared as a swollen bag containing membrane vesicles. Membrane and particulate material were often

PLATE 4

Phase-contrast micrographs illustrating the pattern of opossum fertilization.

**Fig. 11.** The head of a spermatozoon typically associated at random with mucoid laid down on the zona surface after fertilization. Note the free acrosomal face (arrow).

**Fig. 12.** The fertilizing spermatozoon binds to and penetrates the zona by its acrosomal face, then attaches by its acrosomal face to the oolemma.

**Fig. 13.** Decondensation of the sperm head begins immediately it is incorporated by the ovum. In Figs 12 and 13, the spermatozoon is interacting with a small section of oolemma and ooplasm of the unfixed egg forced by coverslip pressure through the site of zona penetration.

**Fig. 14.** The decondensing head and tail of a completely incorporated spermatozoon within an oocyte fixed in acetic alcohol and stained with lacmoid.

PLATE 5

**Fig. 15.** A spermatozoon at a very early stage of incorporation during in-vitro fertilization, only the head and midpiece being within the ooplasm. The zona pellucida (defined by arrow heads) is absent at the point of sperm entry, and for some distance from this point it appears disrupted (cf. zona, Pl. 7, Pl. 8). The insert is a detail of the fertilizing spermatozoon from another section of the same ovum. In both views, there is no evidence that membrane has been incorporated with the decondensing sperm head.

PLATE 6

Fertilization stages *in vivo*.

**Fig. 16.** Although membrane was not incorporated with the sperm head (cf. Pl. 3, Fig. 10; Pl. 5, Fig. 15) or anterior midpiece, membrane was often associated with the posterior midpiece and principal piece.

**Figs 17 and 18.** The membrane (arrow heads) surrounding the midpiece and annulus (×) had the scalloping and submembranous material that characterizes the plasmalemma overlying the posterior two thirds of the midpiece.

**Fig. 19.** More distant from the annulus the principal piece remains invested by its plasmalemma, and probably oolemma (arrow heads).

PLATE 7

**Fig. 20.** Formation of the second polar body (2) begins during sperm head decondensation (insert). There is already a substantial coat of mucoid surrounding the fertilized ovum. Cortical granules are absent, and there are no supplementary perivitelline spermatozoa. In this particular egg, fertilized *in vivo*, there were very few spermatozoa trapped in the inner layers of the mucoid, and none in the region illustrated here (cf. Pl. 8).

PLATE 8

**Fig. 21.** A pronucleate ovum covered by a thick coat of mucoid (only about a quarter of the total thickness is shown here). The innermost region of the mucoid contains numbers of trapped spermatozoa, but none is in contact with the zona surface. The thin character of the opossum zona is clearly illustrated. Fertilization *in vivo*.

found in the crypt lumina associated with unpairing spermatozoa (Pl. 2, Fig. 8), but other cells (e.g. phagocytes) were never seen, nor in general were the spermatozoa closely applied to the epithelium of the crypts (Rodger & Bedford, 1982; Pl. 2, Fig. 3).

*Oocytes and sperm–egg contact*

The opossum ovum is quite naked at the time of fertilization, and all tubal ova lacked any form of follicular cell investment. Maturing follicles fixed about 5 h before ovulation contained oocytes that still had a few cells on the zona (Pl. 3, Fig. 9), but most recovered from the largest follicles immediately prior to ovulation were naked. As seen by phase-contrast microscopy, spermatozoa which had not yet penetrated oocytes were always associated with the zona pellucida by their acrosomal surface. By contrast, spermatozoa trapped in the mucoid deposited on the zona surface after fertilization were randomly orientated (Pl. 4, Fig. 11). The localized association between acrosomal surface and oolemma following zona penetration was often highlighted where pressure from the cover glass forced an intact segment of oolemma with fusing spermatozoon through the large hole it had made in the flimsy zona (Pl. 4, Figs 12 and 13). Decondensation of the sperm nucleus began immediately the head was within the ooplasm, before incorporation of the sperm tail (Pl. 4, Fig. 13). In doing so, it lost its ordered shape and the two posterior arms of the nucleus became twisted laterally (Pl. 4, Figs 13 and 14). Once the sperm tail had come to lie within the vitellus, bulging of ooplasm through the penetration site was uncommon, but by then there was generally some mucoid around the zona.

The early phase of entry and final incorporation of the fertilizing spermatozoon was followed by electron microscopy. The relatively large hole in the marsupial zona pellucida created by penetration (Pl. 5, Fig. 15) persisted after incorporation was complete, and was eventually sealed by mucoid deposited on its surface by the oviduct. An early stage of partial incorporation is shown in Pl. 5, Fig. 15; the head and mid-piece of the fertilizing spermatozoon are within the ooplasm but the annulus lies at the oolemma and its principal and end pieces still project through the perforated zona. Decondensation of the chromatin has begun, the ultrastructure of the mitochondria is disrupted, but no sperm-related membranes lie near the decondensing nucleus. Sperm heads (in different ova) are shown at various stages of early incorporation and decondensation in Pl. 3, Fig. 10, Pl. 5, Fig. 15 and Pl. 7, Fig. 20. Although the plasmalemma or the inner acrosomal membrane of the sperm head were never present within the ooplasm, some plasmalemma of the sperm tail was incorporated. Plasma membrane of the posterior mid-piece, which has characteristic longitudinal parallel scallops (Pl. 6, Fig. 17) underlain by fine filaments (Pl. 6, Fig. 18) (Olson, 1980; Temple-Smith & Bedford, 1980), remained attached to the annulus and lay in a disordered fashion around the disrupted anterior portion of the tail (Pl. 6, Fig. 16). Distal to the annulus, principal piece plasma membrane persisted for some time after incorporation of the sperm tail; and the double membrane (Pl. 6, Fig. 19) suggests that some oolemma is sequestered into the ooplasm.

Sperm heads actually penetrating the zona pellucida and the initial phase of fusion with the oolemma have not yet been observed in the electron microscope. However, 4 oocytes had holes in the zona, without an associated spermatozoon, that were identical to those present at the site of penetration in fertilized ova. Also, a second hole without an associated spermatozoon was found in 2 ova fertilized *in vitro* in which sperm incorporation was complete (Table 1). There was disruption of the oocyte cortex at the site of these zona holes and it seems most likely that gamete fusion had begun, but that the unsupported spermatozoon was lost from the egg surface during processing. This conclusion is supported by the discovery of an intact spermatozoon free in the Epon a short distance from one such egg and of one or more spermatozoa in the thin mucoid deposit adjacent to the gap in the zona in 3 others. 'Penetration' holes were never seen in the zona pellucida of oocytes not exposed to spermatozoa.

The cortical granules of opossum eggs lacked internal ultrastructure, and were lost at

fertilization. Shortly after incorporation of the whole spermatozoon the second polar body was shed, but even before completion of this, a substantial mucoid layer was deposited on the zona surface (Pl. 7, Fig. 20). No supplementary spermatozoa were seen in the perivitelline space (Pl. 7, Fig. 20; Pl. 8, Fig. 21), suggesting an efficient zona block to polyspermy in the opossum. However, spermatozoa were generally absent also from the zona surface. The few occasionally trapped in the inner mucoid coat were always separated from the zona by at least a thin layer of mucoid (Pl. 8, Fig. 21).

## Discussion

Fertilization in the opossum, a marsupial mammal, differs in several respects from the mode in eutherian mammals. Marsupial eggs are ovulated without a granulosa cell vestment, the spermatozoon creates a relatively large gap rather than a narrow slit-like opening in the zona pellucida, and the typically monospermic fusion with the oolemma appears to occur by way of the inner acrosomal membrane. The latter in particular provides a major contrast with the complicated fusion–phagocytosis pattern that characterizes fertilization in eutherians (Bedford & Cooper, 1978).

Sperm pairs separate in the oviduct, and this unpairing is associated with the unexpected appearance of membrane bound vesicles within the matrix of the intact acrosome (Pl. 2, Figs 7 and 8). However, this change is not comparable to the acrosome reaction, the precise character of which is yet to be established for any marsupial. The acrosome of the opossum spermatozoon contains at least four hydrolytic enzymes seen in eutherian spermatozoa, i.e. acrosin, arylsulphatase, hyaluronidase, and *N*-acetylhexosaminidase (Rodger & Young, 1981). The size and appearance of the penetration hole in the opossum zona pellucida (Pl. 5, Fig. 15) is far more suggestive of enzyme action than the very narrow sharp-edged penetration slit seen typically in the eutherian egg. The opossum zona appears more vulnerable than that of the eutherian to enzymic digestion. It is dissolved to extinction (phase-contrast microscopy) in 2–3 sec by 0·1% bovine pancreatic trypsin (Sigma, type 3) in medium 199 (pH 7·2, 37°C) (J. M. Bedford & J. C. Rodger, unpublished), whereas the rabbit zona resists such treatment for 15–20 min (Bedford & Cross, 1978). A crude extract of opossum acrosomes will dissolve the opossum zona in 20 min (J. M. Bedford & J. C. Rodger, unpublished) and is able to disperse the mouse cumulus mass at a rate equivalent to that seen with extracts of comparable numbers of rabbit spermatozoa (Rodger & Young, 1981). Sperm acrosomal enzymes, in particular hyaluronidase, are widely thought to facilitate penetration through the cumulus oophorus (see McRorie & Williams, 1974; Rodger & Young, 1981) and yet ovulated opossum ova lack any such cellular investment, and there is a strong likelihood that the opossum is not a special case in this respect, judging by data from 5 species of Australian marsupials (*Dasyurus*: Hill, 1910; *Sminthopsis*: Godfrey, 1969; *Setonix*: Sharman, 1959; *Pseudocheirus*: Hughes, Thomson & Owen, 1965; *Trichosurus*: Hughes, 1977). Hartman (1919) observed that oviducal ova of *Didelphis* lacked a corona radiata or granulosa cells and Martinez-Esteve (1942) recognized that the cumulus surrounding oocytes in the follicle was unlike that in eutherian mammals, being very thin (1–2 cell layers) and having no corona radiata.

The complete absence of cellular vestment around the marsupial egg suggests that the acrosomal enzymes probably act exclusively in zona penetration. The situation in eutherian mammals is unclear largely as a result of the complex nature of the cellular vestments enclosing the ova of most eutherian mammals. In one shrew, *Elephantulus*, it has been claimed that the cumulus is lost at ovulation (van der Horst & Gillman, 1940), but this has not been confirmed. In the cow and sheep (Dziuk, 1965; Lorton & First, 1979), the oocyte is ovulated with a cumulus mass that largely disperses in 2–6 h so that the fertilizing spermatozoon may need only to penetrate a much reduced cumulus or the corona radiata. Although this might appear to

represent a trend toward the marsupial situation, it is not equivalent since the marsupial spermatozoon need not negotiate any cellular barrier to reach the zona pellucida. Conception in marsupials and eutherians is, in general therefore, different in this respect.

The present observations support the conclusion that the opossum spermatozoon fuses with the oolemma by the inner acrosomal membrane, as is the case for all non-eutherian vertebrates and invertebrates examined so far. This conclusion is based on two observations: (1) the consistent finding by phase-contrast microscopy of spermatozoa adhering firmly to the oolemma by the acrosomal face in otherwise unfertilized eggs (Pl. 4, Fig. 12), and (2) the absence of any sperm or egg membranes around the decondensing sperm head very soon after its incorporation by the vitellus (Pl. 3, Fig. 10). This contrasts with the situation at this stage in eutherian mammals, in which the incorporated sperm head is always shrouded by a complex of inner acrosomal-oolemmal membrane. This manner of gamete fusion and incorporation in eutherians appears now to be quite unique to this single infraclass within the Metazoa.

The presence of a distinct sperm rich region, some distance from the zona, in the mucoid coat and the absence of supplementary spermatozoa from the zona pellucida surface (Pl. 8, Fig. 21) suggest that the arrival of spermatozoa at the site of fertilization in the oviduct and the initial deposition of mucoid are finely regulated. Cortical granule release accompanies fertilization, but whether a strong block to polyspermy is established at the zona in the opossum as a result of this is uncertain. The occasional observation of two zona penetration sites in oocytes incubated *in vitro* with moderate numbers of tubal spermatozoa, and the entrapment of possible supernumerary spermatozoa in the initial layers of mucoid, suggest that the oviducal mucoid may have an important secondary role in the block to polyspermy.

To conclude, marsupials are therian mammals, yet they do not display what is now generally recognized as the 'mammalian' pattern of fertilization. The manner of sperm incorporation by the marsupial egg is essentially comparable to that seen in non-mammalian vertebrates and even invertebrates.

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