Ultrastructure of Imaginal Spermatozoa of Sawflies (Hymenoptera: Symphyta)

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Abstract.—We present the first ultrastructural study of sperm from representatives of three superfamilies of sawflies (Hymenoptera: Symphyta): *Xyela julii* (Xyeloidea), *Cephalcia arvensis* (Pamphiloidea) and *Tremex* sp. (Siricoidea), with particular attention being paid to characters that may be phylogenetically informative. Differences in the location of the centriolar adjunct, particularly in relation to the mitochondrial derivatives, would suggest *Cephalcia* has a better claim than *Xyela* as having sperm that may be representative of a common ancestral form. The centriolar adjunct of *Cephalcia* overlies both mitochondrial derivatives symmetrically, as found in ants and bees, whereas in *Tremex*, which its sperm otherwise closely resemble, the centriolar adjunct is located asymmetrically, abutting a single mitochondrial derivative and thus offsetting the pair of mitochondrial derivatives longitudinally. *Xyela* has radically different sperm in terms of size and both the arrangement and appearance of the organelles, especially acrosomal substructure and lack of an acrosomal rod.

Very little is known about sperm ultrastructure among the Hymenoptera compared with most other insect orders (Phillips 1970; Jamieson 1987; Quicke 1997), and most of the studies that do exist deal largely with common aculeates such as bees and ants (e.g. Dallai and Afzelius. 1990; Wheeler and Krutzsch. 1992). However, an initial study of the spermatozoa of some species (Quicke et al. 1992) revealed a considerable number of ultrastructural features that differ between taxa, raising the possibility that such variation might provide new phylogenetic indicators, as has been possible in many other groups of insects (Jamieson 1987). The phytophagous sawflies (Symphyta) constitute a relatively underived basal grade within the order of Hymenoptera. As such they are important for our understanding of the relationships and development of both the social species of the Aculeata (ants, wasps and bees) and members of the paraphyletic group of the ten or eleven currently recognised, extant superfamilies generally referred to as the 'Parasitica'. This is especially so, since the sister group for the Hymenoptera is not at all certain at present (Whiting et al. 1997), and so it is not possible to make use of outgroup comparison to determine the ancestral sperm morphology of the order (Watrous and Wheeler 1981). Groundplan sperm ultrastructure may therefore be determined best by considering the sperm of those extant taxa (i.e. the sawflies) which represent the most basal hymenopteran lineages (Gibson 1993; Yeates 1995). The only previous work on sawfly sperm ultrastructure (Quicke et al. 1992) presented data for only two of the six symphytan superfamilies, the Tenthredinoidea and the Cephoidea. We have therefore examined sperm ultrastructure, and in particular that of cell organelles, in detail in representatives of three further superfamilies, the Xyeloidea, Pamphiloidea and Siricoidea, leaving only the rare, through interesting, Orussoidea unstudied. Two of the superfamilies examined here, the Xyeloidea represented

by *Xyela julii* (Brébisson) and the Pamphiloidea, represented by the pamphiliid, *Cephalcia arvensis* Panzer have usually been considered to be among the most primitive of sawflies. In contrast, the Siricoidea represented by *Tremex* sp., are close to the origin of the Apocrita (Rasnitsyn 1980, 1988; Heraty *et al.* 1994; Vilhelmsen 1997). The results are discussed in terms of the likely plesiomorphic states for various subcellular features in the Hymenoptera.

MATERIALS AND METHODS

Testes were obtained from adult males of *Xyela julii, Cephalcia arvensis* and *Tremex* sp., which had been maintained on dilute honey solution for a maximum of 3 days. *Xyela* were collected as adults in Silwood Park, Berkshire, U.K., the *Cephalcia* were reared from larvae collected in Italy and the *Tremex* were collected as adults in California and couriered to the U.K. for preparation.

Light microscopy.—Vas deferentia and testes were dissected from living sawflies in insect saline and teased apart on a clean microscope slide. After a few minutes to allow the sperm/spermatodesmata to swim free of the disrupted tissue, the slides were dried on an hot plate at c. 80°C. The smear was then flooded with double-filtered, 0.1% w/w toluidine blue in 1% w/w aqueous sodium borate and stained at 80°C until crystallisation of the stain had started. Following washing in distilled water they were permanently stored dry.

Transmission electron microscopy.—Genitalia were dissected out under 2% glutaraldehyde in phosphate buffered saline (pH 7.2), and fixed for two hours. Tissue was transferred to 2% osmium tetroxide in cacodylate buffer (pH 7.2) for 2 hr. After another buffer wash, tissue pieces were dehydrated to 50% ethanol and then further fixed with saturated uranyl acetate in 50% ethanol prior to complete dehydration, embedding in Epon resin and polymerisation overnight. Silver sections were picked-up on to high resolution grids, stained with uranyl acetate and lead.

RESULTS

Woodwasps of the superfamily Siricoidea are considered to be amongst the most advanced of the sawflies, sharing a number of derived morphological features with the Apocrita (Vilhelmsen 1997). As has been reported previously for other sawflies (Quicke *et al.* 1992), the mature sperm of siricid, *Tremex,* stored within the vas deferens and seminal vesicles are present in spermatodesmata bundles (Fig. 1), though by the time they reach the spermatheca of the females they have broken up completely and only isolated sperm are present (Naito, personal communication). In our preparation of *Tremex* from male seminal vesicles, a small proportion of isolated sperm were also present but it is not clear whether they were the result of spermatodesmata fragmentation upon fixation or whether they indicate a normal pretransfer phenomenon.

The sperm heads are inserted throughout the fairly electron-dense and elongate cap of the spermatodesmata, with those sperm located more centrally being inserted more anteriorly (Figs 1, 2). As a result, many different levels of sperm are evident in a single transverse section of each spermatodesmata (Fig. 2). It is therefore possible, in the same transverse section, to locate adjacent sperm sectioned through acrosome and acrosomal rod (perforatorium), through the nucleus, the basal body with centriolar adjunct, and through the axoneme with mitochondrial derivatives. Also, in transverse section, the acrosome is clearly seen to have a membrane around both the outside and around the invaginated portion of the structure (Fig. 4c). Between the acrosomal membrane and the plasma membrane is an electron dense region extending from the acrosomal membrane (Fig. 4c, *arrowheads*). This may be comparable to the material reported to surround the acrosome in other sperma-

Fig. 1.   Longitudinal section of a spermatodesmatum in the imaginal testes of the sawfly, *Tremex* (Siricoidea).
A, acrosome; C, centriolar adjunct; M, mitochondrial derivative; N, nucleus; R, acrosomal rod; X, axoneme.
Scale bar = 1.0 μm.
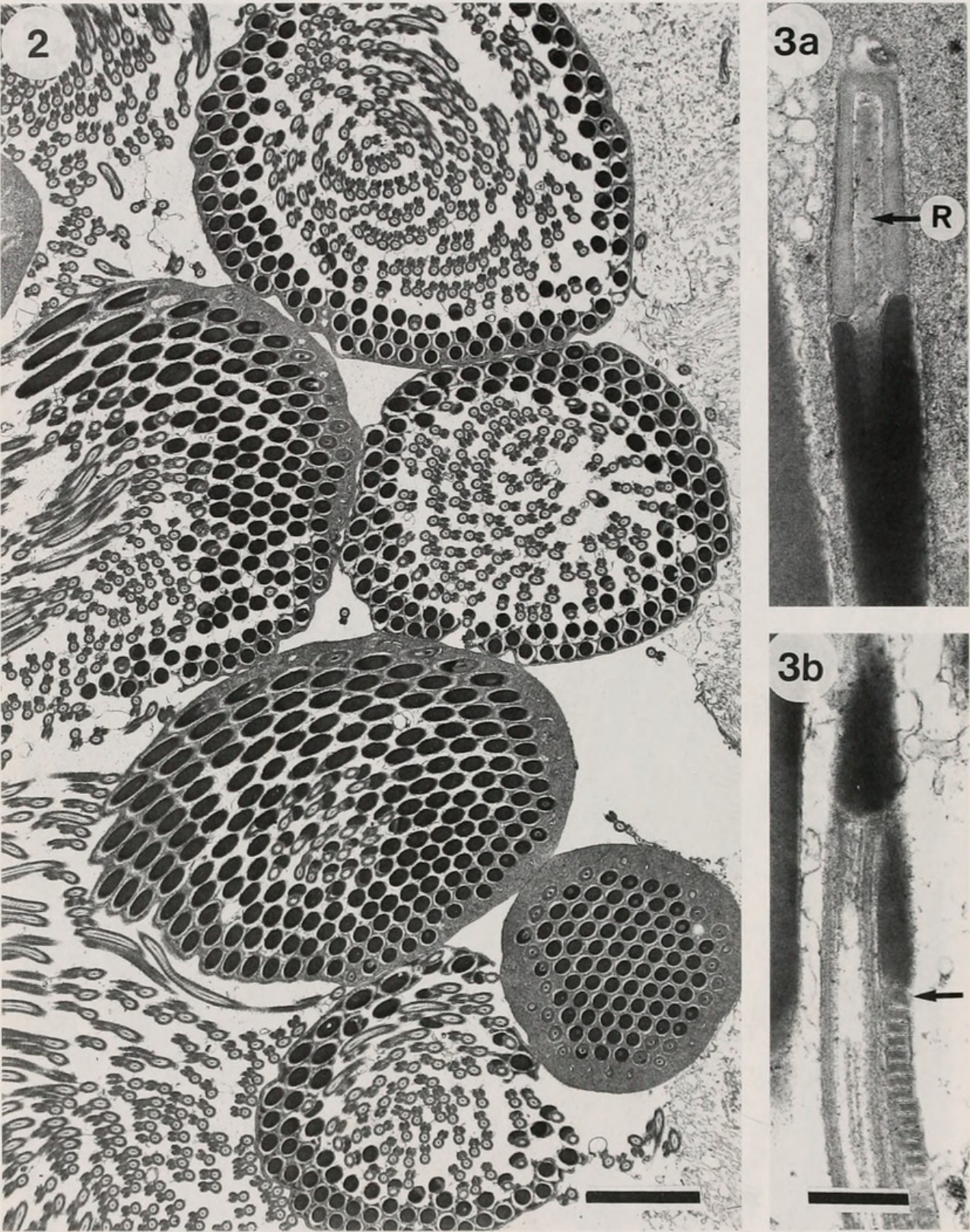
Figs. 2–3.   Features of spermatodesmata and sperm in the imaginal testes of the sawfly, *Tremex* (Siricoidea). 2, transverse sections through several spermatodesmata at different levels showing that the more centrally located spermatozoa have their heads inserted more anteriorly; 3, nuclear-associated organelles showing in 3a, the insertion of the acrosomal rod (R) into the anterior of the nucleus (note also the small anterior sac at the head of the acrosome), and 3b, the position of the centriolar adjunct (arrowed) in relation to the nucleus and striated mitochondrial derivative. Scale bars: 2 = 2.0 μm; 3 = 0.5 μm.

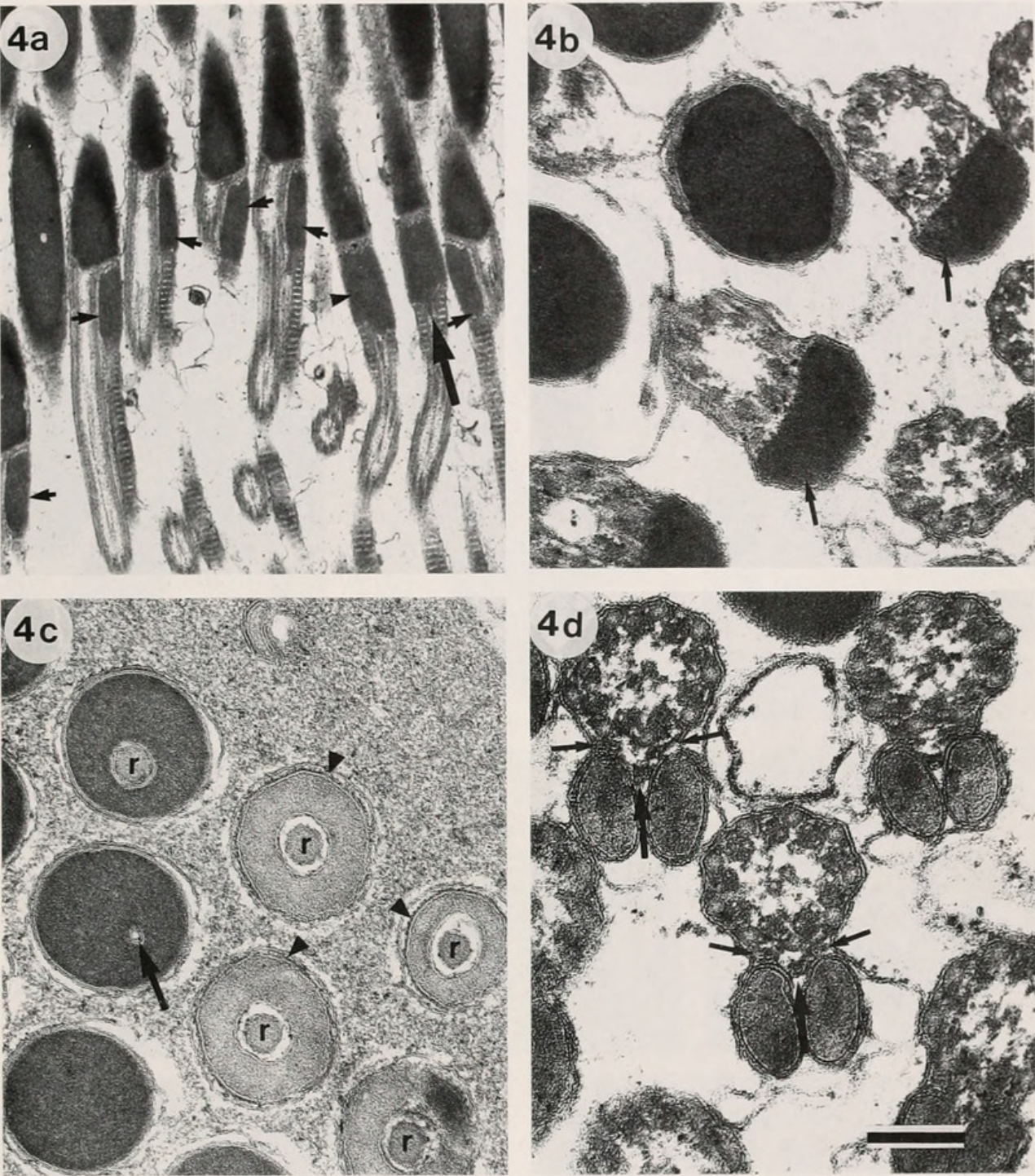Fig. 4. Organelles of *Tremex* sperm (Siricoidea) seen in tranverse and oblique section: 4a, showing the centriolar adjuncts (small arrows) of a number of spermatozoa, and in the section indicated by the large arrow the centriolar adjunct can be seen to make contact with both mitochondrial derivatives; 4b, section at the level of the centriolar adjunct (arrowed) where it occupies all the extra-axonemal area; 4c, sections through anterior of nucleus and acrosome showing the acrosomal rod (r) fitting tightly into the nucleus but loosely in the sub-acrosomal space (note: the clear membranes surrounding acrosome and nucleus but not the rod; granular material between acrosomal and plasma membranes (arrowheads); putative nuclear 'pore' (large arrow)); 4d, axoneme with small deltoid bodies (small arrows) and central rod (large arrows) (note also mitochondrial derivatives with distinct membrane and internal structure). Scale bar: 4a = 0.66 μm; 4b,c = 0.25 μm; 4d = 0.2 μm.
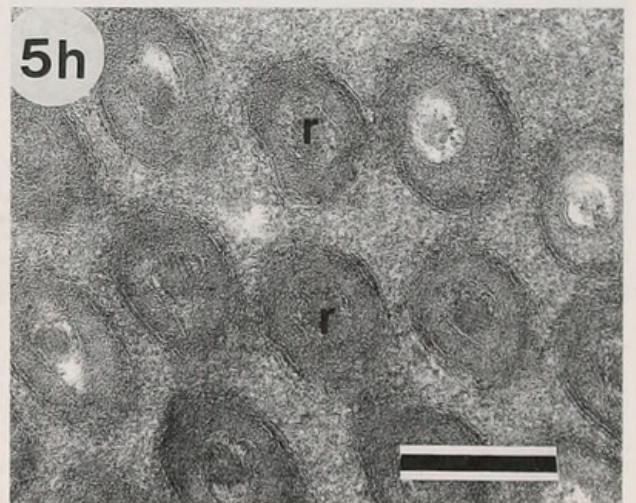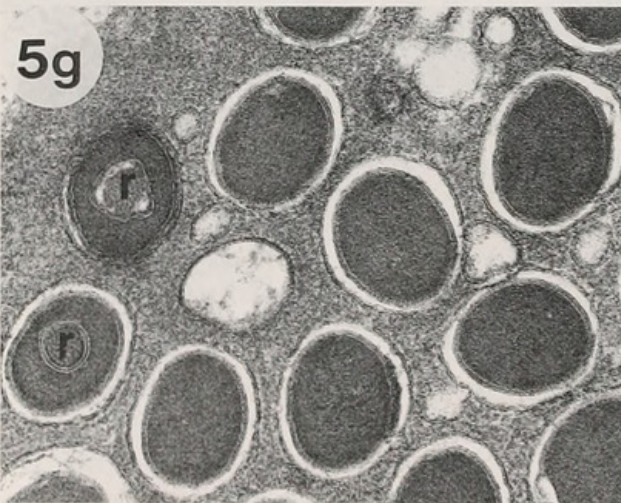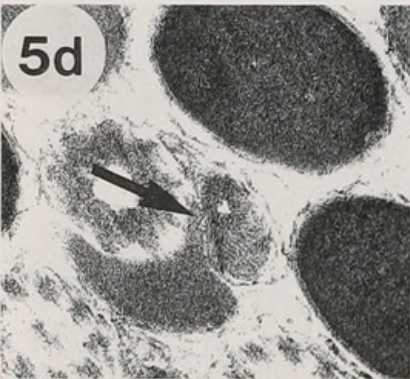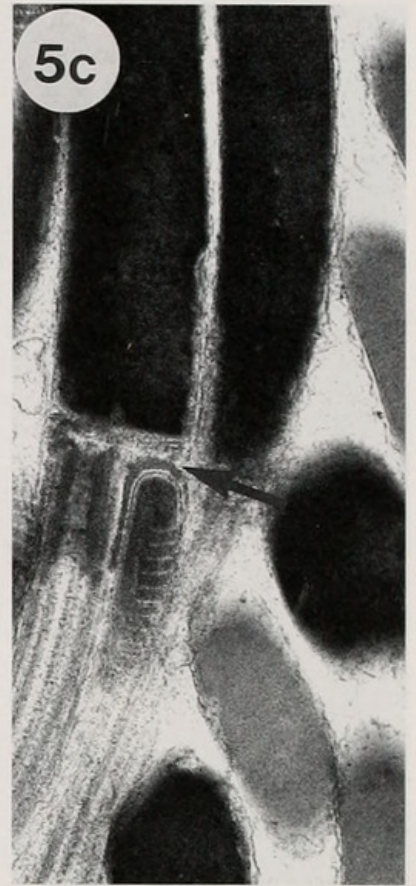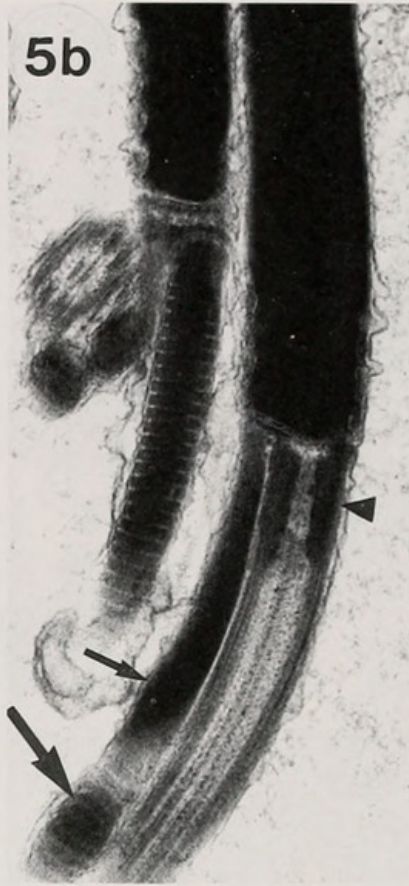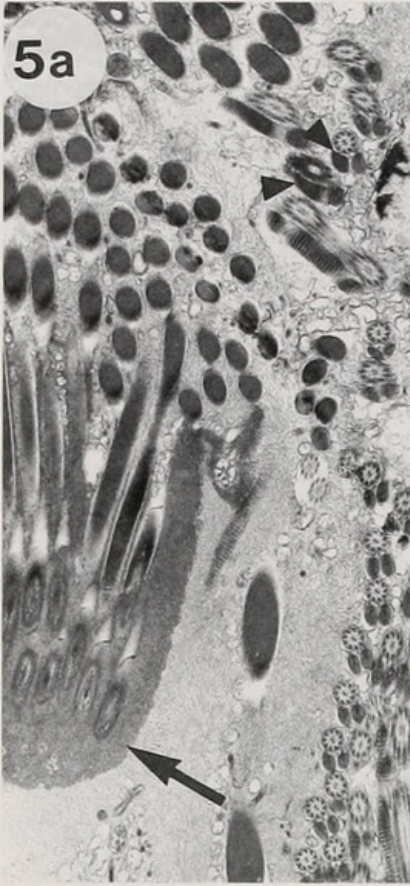
tozoa (Quicke *et al.* 1992), although the latter structures are larger and have a sublayered appearance in at least some taxa.

In longitudinal section (Fig. 3), the acrosome of *Tremex* can be seen to have a large sub-acrosomal space (Fig. 3a) which is partly occupied by the acrosomal rod. This rod extends into the nucleus for almost the same length again as it does into the acrosome. Unlike the nucleus and acrosome, the rod is not membrane bound, but where the rod is inserted into the nucleus there is no surrounding space, giving the impression that the rod is being held by the nucleus. The plasma membrane surrounding the acrosome extends slightly anteriorly to produce a small extra-acrosomal space. A membrane bound area found within the nucleus (Fig. 4c, *arrow*) may represent a longitudinally running pore. The nucleus (Fig. 3b) is abutted posteriorly by the axoneme at the level of the latter's basal body, where the axoneme lacks the central pair of microtubules. A large centriolar adjunct is present, and this in turn contacts the mitochondrial derivatives which have very clearly defined membrane bound cristae (Fig. 3b, *arrow*). There is at least one membrane separating the centriolar adjunct from the nuclear membrane. The exact arrangement of the centriolar adjunct and nucleus, and in particular, how the centriolar adjunct contacts the nucleus, is not always obvious. In many insect spermatozoa this has given rise to confused interpretations of the

structure, even to suggestions that the centriolar adjunct is absent. From the present study, the relationship becomes clear at higher magnification where several spermatozoa lie in close proximity (Fig. 4). The centriolar adjunct (Fig. 4a, *arrows*) contacts two equally sized mitochondrial derivatives, and can be seen to extend some way in between the two, forming what appear in transverse sections, tail-like structures (Fig. 4a, *large arrow*). The mitochondrial derivatives are thus intimately connected to the centriolar adjunct. In transverse section, certainly at the level of the basal body, this produces an arrangement where the centriolar adjunct and basal body occupy most of the area of section; an appearance which could be mistakenly interpreted as there being an extension of the nucleus overlying the axoneme (Fig. 4b).

Although the acrosome is smaller in diameter than the nucleus, there is not a great discrepancy. The axoneme itself has the 9+9+2 arrangement (Fig. 4d) common to Hymenoptera; 9 outer single accessory tubules, 9 doublets and 2 central single microtubules. Intertubular material is abundant with radial spokes (Afzelius rays) and indications of the inner and outer dynein arms. Two deltoid bodies, (also referred to as triangular rods, (Lensky *et al.* 1979) are present, but they are not large. Between the two bodies, and the two mitochondrial derivatives (Fig. 4d) is a single central rod, as previously report-

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Fig. 5. Cell ultrastructure of *Cephalcia* sperm (Pamphiloidea): 5a, spermatadesmata with surrounding cap material (arrowed); 5b, centriolar adjunct (small arrow) abuts nucleus at membranous complex, extending beyond the level of the basal body (arrowhead) to abut a mitochondrial derivative (large arrow); 5c, showing that the centriolar adjunct does not overly both mitochondrial derivatives with one of the two derivatives (arrowed) abutting the nucleus at the region of the membranous complex; 5d, transverse section at level of basal body showing that the centriolar adjunct extends to partially enclose the parallel mitochondrial derivative (arrowed); 5e, transverse section through axoneme showing only a single mitochondrial derivative posteriorly near the tail piece; 5f, transverse section through midregion of axoneme with two mitochondrial derivatives (note distinct sub-structure at periphery of each mitochondrial derivative); 5g, transverse sections showing acrosomal rod (r) insertions into the nucleus; 5h, transverse sections showing insertion of acrosomal rod (r) into the acrosome. Scale bar: a = 1.3 μm; b = 0.5 μm; c, d = 0.42 μm; e = 0.3 μm; f = 0.27 μm; g = 0.7 μm; h = 0.57 μm.
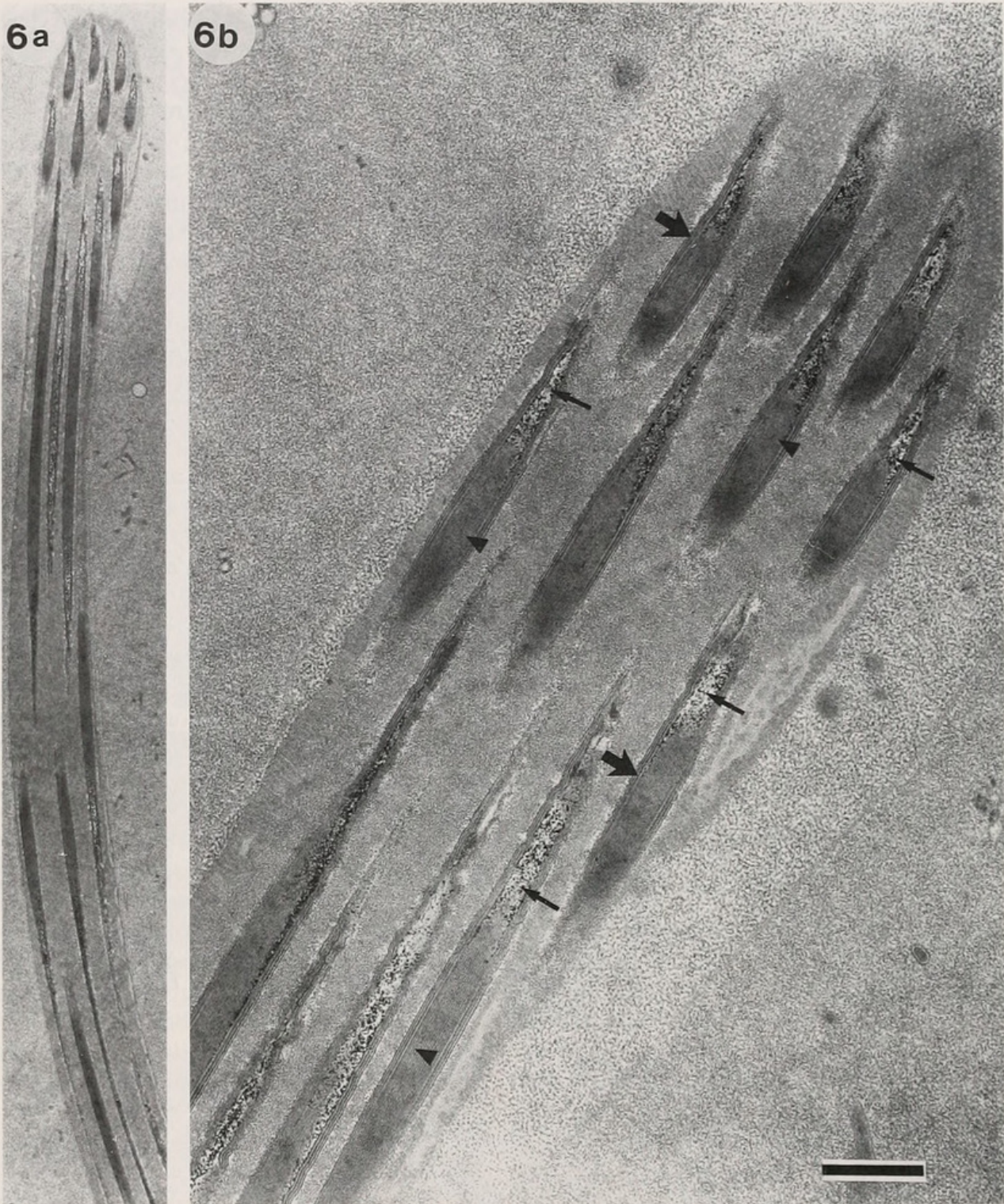
Fig. 6.   Spermatatodesmata of *Xyela* (Xyeloidea): 6a, showing the extended length of the spermatodesmatal cap; 6b, showing the distinctive arrangement of the acrosome at the region of insertion into the cap with particulate material (small arrows), distinct periodicity in the core material (arrowheads; appears like longitudinal striations), and multilayered membrane coat to the acrosome (large arrows). Scale bar: a = 2.5 μm; b = 0.66 μm.

ed in ant spermatozoa (Wheeler *et al.* 1990). A tail piece is found where the axoneme has no associated mitochondrial derivatives.

Of the more basally derived sawfly superfamilies investigated, the Pamphiloidea (*Cephalcia*) represents a slightly more advanced evolutionary lineage than the Xyeloidea although it was once included in the same family. The spermatozoa of *Cephalcia* are arranged in spermatodesmata (Fig. 5a) and have heads (nucleus plus acrosome) approximately 28μm long, and tail, 75μm long. Ultrastructurally, they are very similar to those of *Tremex*, especially in terms of the size of the acrosomal rod, its position within the subacrosomal space (Fig. 5h), and its insertion into the nucleus (Fig. 5g). The most noticeable difference between the two is in the position of the centriolar adjunct. In *Cephalcia* the centriolar adjunct can be seen to run parallel to one to the pair of mitochondrial derivatives (Fig. 5b), rather than overlying both as in *Tremex*, as is evident in the region where one of the mitochondrial derivatives is found to abut the nucleus (Fig. 5c, *arrow*). For part of its length (at the level of the basal body) the centriolar adjunct contacts and even partially encloses the single mitochondrial derivative that lies parallel to it (Fig. 5d, *large arrow*). Possibly as a result of this arrangement, a region occurs at the posterior part of the sperm, where there is only a single mitochondrial derivative lying next to the axoneme (Fig 5e); here there is also only a single deltoid body, as opposed to the two found in normal section (Fig. 5f). Similarly at the level of the centriolar adjunct the single mitochondrial derivative has only a single deltoid body. The axoneme is again similar to that of *Tremex* in the arrangement of elements. The ray material is particularly evident, with Afzelius rays having distinct spoke heads, and with distinct electron-opaque granules between the peripheral singlets (Bairati and Baccetti 1965).

Xyela spermatozoa, although present in spermatodesmata (Fig. 6), differ in a number of ways from those of both other sawflies described here as well as from the tenthredinoids described by Quicke *et al.* (1992). The spermatozoa are extremely long with the head (=nucleus plus acrosome) being approximately 60μm long, and the tail 150 μm long (Fig. 6a). This elongation compared with sperm of other sawflies, at all levels, viz. the acrosome, nucleus and tail, is also apparent in longitudinal section. At the anterior end the acrosomes can be seen to be asymmetrical and pointed, containing two types of material: an irregularly granular material and a core material that has an almost crystalline periodicity, aligned parallel to the long axis of the spermatozoa. In transverse section (Fig. 7) the most prominent of the features is an enlargement of one of the mitochondrial derivatives to a diameter greater than that of the axoneme (Fig. 7a), with a concomitant enlargement of that mitochondrial derivative's deltoid body (Fig. 7a, *right arrowhead*). This displaces the other mitochondrial derivative which, together with its deltoid body, now occupies an area approximately equivalent to the other, larger, deltoid body alone.

In *Xyela*, it is not immediately apparent if there is a centriolar adjunct. In some transverse sections, at the position of the smaller mitochondrial derivative/deltoid body a darker structure is present (Fig. 7b, *arrows*). This does not seem to be simply a denser mitochondrial derivative because it lacks a deltoid body and generally the two occur together (in shape it is actually closer to a deltoid body). In longitudinal section (Fig. 7c) a structure abutting on to the smaller mitochondrial derivative can be found. This closely resembles the situation in *Cephalcia*. The structure does not however extend to the nucleus like the other centriolar adjuncts found. Instead, at the region of the basal body, identifiable by the absence of the central pair of mi-
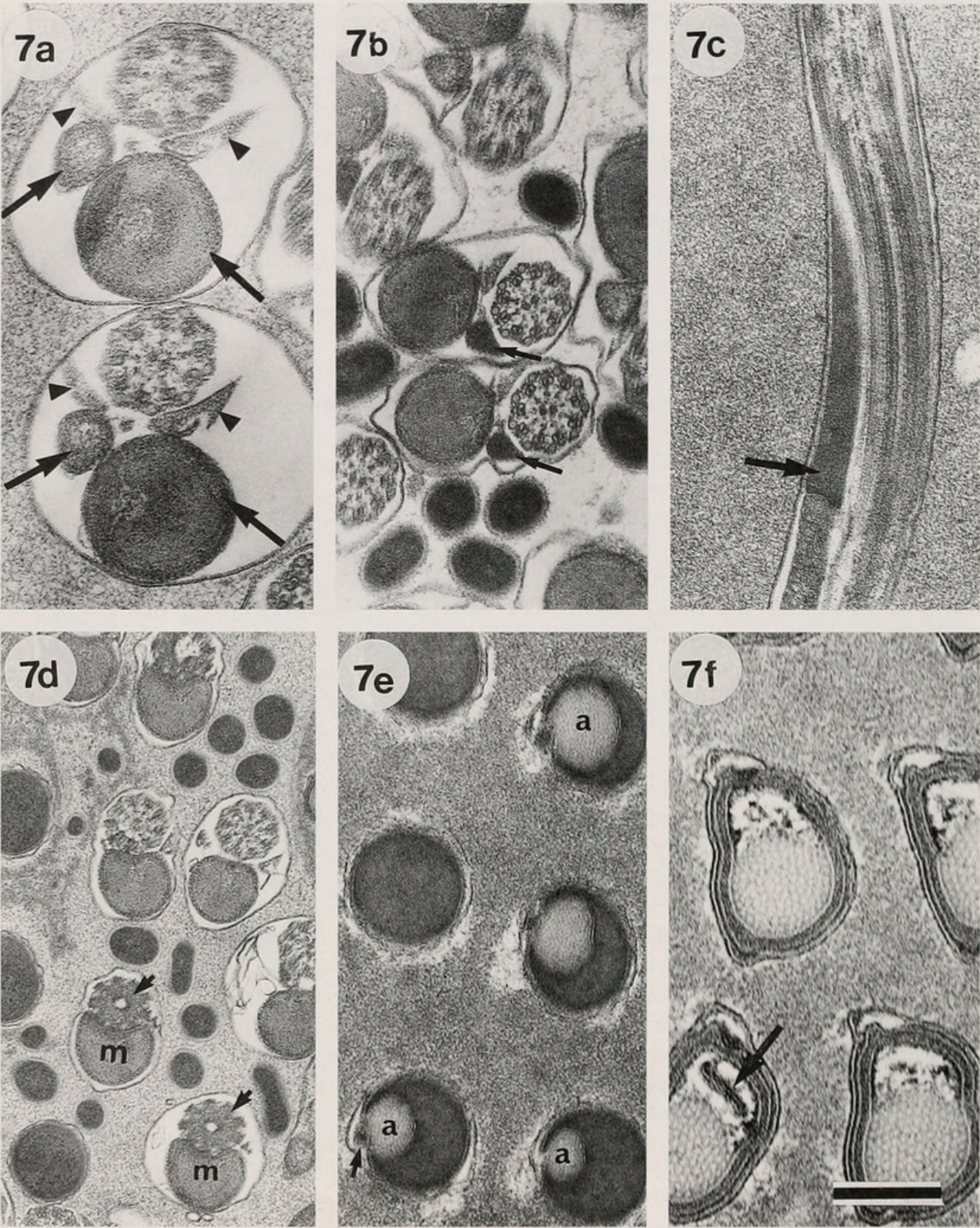
Fig. 7. Organelles of *Xyela* spermatozoa (Xyeloidea): 7a, asymmetric mitochondrial derivatives (arrows) and well-devloped deltoid bodies (arrowheads); 7b, in one region the smaller mitochondrial derivative is replaced by a darker structure similar to a centriolar adjunct (arrows) (note also the numerous, smaller nuclear-like cross sections, smaller than other readily identified nuclei); 7c, longitudinal section showing a centriolar adjunct-like body (arrow) that abuts the smaller mitochondrial derivative; 7d, transverse section at the level of the basal body (arrows) showing that the centriolar adjunct-like organelle and the smaller of the two mitochondrial derivatives are both absent, and that the larger mitochondrial derivative (m) partially covers the basal body; 7e, showing asymmetric insertion of a cone of acrosomal (a) material into the nucleus isolating a

crotubules, the larger mitochondrial derivative wraps round to partially enclose the basal body and the smaller mitochondrial derivative/deltoid body is absent (Fig. 7d). If there is a centriolar adjunct, then how it terminates anteriorly and its relationship with the nucleus remains unclear. The nucleus itself appears similar to those of the other sawflies in density and membrane organisation at the level of the spermatodesmata. However, perhaps in accord with its greater length, there appears to be an area, posterior to its insertion into the cap of the spermatodesmata, that has a relatively smaller diameter and where it is significantly smaller than the tail region with its enlarged mitochondrial derivative (Fig. 7b, d).

In *Xyela* the interface of the acrosome with the nucleus also appears different. There is no discernible rod. Instead acrosomes, which have a distinct, paracrystaline substructure, contact the nucleus and may even be partially enclosed by it (Fig. 7e). This insertion is displaced to one side, and this asymmetry is also present in the acrosome itself (Fig. 7f). A ridge runs down one side of the acrosome (Fig. 7f, *arrow*). Spermatozoa are orientated within the spermatodesmata so that the ridges all point in the same direction. Interestingly, this is also the same side of the spermatozoon that the acrosome inserts into the anterior of the nucleus, although at this point the acrosomal material appears to have lost the ridge, and the acrosome at this level only shows the core of 'periodic material'. The ridge itself contains the particulate matter. In some areas the granules surround membranes resembling the multilayered coated complex that surrounds the acrosome itself. There is also an extension of the outer layers of this coat to form a small ridge to one side of the acrosome. This position of this smaller ridge is again consistent amongst the spermatozoa.

DISCUSSION

At least with regard to the ultrastructure of the spermatozoa, *Cephalcia* appears to have a better claim than *Xyela* as having sperm that may be representative of a common ancestral form. *Cephalcia* sperm are very similar to those of *Tremex*. It is mainly in the positioning of the centriolar adjunct that *Cephalcia* varies from *Tremex*, having an asymmetric location overlying only one mitochondrial derivative. *Tremex* by comparison has a centriolar adjunct overlying both mitochondrial derivatives. The arrangement of the centriolar adjunct might seem to have possible usage as a phylogenetic indicator. Unfortunately the arrangement of this organelle has often been poorly understood (e.g. Wilkes and Lee 1965), and so it is difficult to draw any conclusion from all previously reported works. From studies of bee sperm Jamieson (1987) concluded that in bees the centriolar adjunct also lies between the nucleus and one of the mitochondrial derivatives. Recently we have described the ultrastructure of the parasitic braconid wasp, *Aleiodes*, which appears to have relatively underived sperm (Quicke *et al.* 1992; Newman and Quicke 1998). The ultrastructure of individual *Aleiodes* spermatozoa closely resembles both *Tremex* and *Cephalcia*. The mitochondrial derivatives are similarly sized and the acrosomal rod is similarly positioned. The centriolar adjunct is, however, asymmetric and hence similar to *Cephalcia*. Given the primitive status proposed for *Cephalcia* this might be considered to be the archetype arrangement retained through evolution.

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small amount of membrane bound nuclear material (arrowed); 7f, showing the periodic appearance of the acrosome within the multilayered coat and membrane material (arrowed). Scale bar: a = 0.27 μm; b, c = 0.5 μm; d = 0.6 μm; e = 0.5 μm; f = 0.25 μm.

The case of *Tremex*, with symmetric centriolar adjunct overlying both mitochondrial derivatives, would have to be considered as an apomorphic development. However, it is not clear at present which of the arrangements of the centriolar adjunct represents the groundplan for either the Hymenoptera as a whole or for any of the major lineages within it, and more careful study is necessary.

As was made clear in the results, the positioning of the centriolar adjunct is not clear in *Xyela*. It appears to be asymmetric, but unlike the arrangement in other sawflies it does not appear to abut the nucleus. *Xyela* has sperm with a structure that is extremely divergent in a number of other ways; the shape and arrangement of the acrosome, the apparent absence of the acrosomal rod and the size difference in the two mitochondrial derivatives. It seems likely that these must represent a response to selective pressures subsequent to the divergence of both the other sawflies and the main body of the order of Hymenoptera.

The presence of an asymmetric centriolar adjunct in *Cephalcia* appears to cause the mitochondrial derivatives to be offset longitudinally, and this may explain why some sections through the posterior part of the spermatozoa have only a single mitochondrial derivative (e.g. Fig. 5e). Where there is only one mitochondrial derivative the deltoid body is also absent suggesting they may be a good marker for mitochondrial derivative identification.

From the present observations, it seems probable that the identification of the coat material surrounding the acrosome in many taxa may be incorrect. In the Hymenoptera, this material has been referred to as extracellular matrix (Quicke *et al.* 1992) and in some insect orders (e.g. Orthoptera) it has been reported that extracellular matrix granules accumulate around the plasma membrane of the acrosome to form an extracellular cap (Szöllösi 1974). However, these structures are often highly complex, with layered or repeated substructure (see for example, Fig. 6b in Quicke *et al.* 1992), and it is not immediately clear how such a structure could be secreted if extracellular; the possibility that they are produced by the epithelia of a deferent duct cannot be excluded. Many plasma membranes possess a glycocalyx which comprises the carbohydrate portion of integral membrane glycoproteins and glycolipids together with associated glycosamminoglycans and proteoglycans, and these carbohydrates extend from the plasma membrane into the extracellular space. Where organelle membranes become glycosilated, as in the case of secretory granules that will eventually fuse with a plasma membrane, the coated face of the membrane that opposes the interior of the organelle is the one that will, upon fusion, face the extracellular space. In the sawflies investigated here, it is clear that the coat lies between two membranes, and is not extracellular as previously reported. An intracellular origin for this structure would at least allow a more conventional, although as yet, completely unrecognised, mechanism for its production.

ACKNOWLEDGMENTS

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