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OSCINE SPERMATOZOA: A LIGHT- AND ELECTRON-MICROSCOPY STUDY

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The remarkable work of Retzius (1909) detailed not only many of the structural peculiarities of avian spermatozoa, but also their organization within the testes. In addition, and importantly, it showed the unique and bizarre nature of oscine (Passeriformes) spermatozoa, with their helical "membranes" and their precise arrangement within the testes. With this strong beginning in avian spermatology, which includes both the early work of Ballowitz (1886, 1888, 1913) and Retzius (1909, 1911, 1912), as well as a number of investigations on the sperm of fowl (summarized by McFarlane 1963), it is surprising that so little has been done to reveal the ultrastructure of oscine spermatozoa and to study further the arrangement of sperm within the testes.

Several electron-microscopy studies (Masuda 1958, Yasuzumi 1956) have attempted to demonstrate the nature of the helical "membrane," but their findings revealed only a "fibrous" character, described as being similar to the structure of middle-piece mitochondria. Nicander (1970) briefly mentioned, but did not figure, a microtubule complex in spermatids of the Zebra Finch (*Poephila guttata*).

McFarlane studied (principally by light-microscopy) sperm from many species of birds from various groups, in an attempt to ascertain whether they had any taxonomic significance. Except among oscines, most avian spermatozoa are very similar to one another; their structure has been described by McFarlane (1963) and many other authors (cited by him) who studied the sperm of fowl. Within the Tyranni the sperm are basically similar to the primitive type but are slightly coiled (McFarlane 1963), so it is only within the oscines that the unique helical "membrane" is present. Fawcett et al. (1971), in their study of spermiogenesis in the Zebra Finch, described the complex corkscrew-shaped sperm head. Our emphasis is on the shaping of the nucleus and acrosome. We present information on spermatozoa from eight oscines with both light- and electron-microscopy to reveal their comparative ultrastructure and some features of their arrangement within the testis.

MATERIALS AND METHODS

The species studied were: American Robin (*Turdus migratorius*), Cardinal (*Cardinalis cardinalis*), Rufous-sided Towhee (*Pipilo erythrophthalmus*), Red-eyed Vireo (*Vireo olivaceus*), White-throated Sparrow (*Zonotrichia albicollis*), Summer Tanager (*Piranga rubra*), Tufted Titmouse (*Parus bicolor*) and Blue Jay (*Cyanocitta cristata*). For comparative purposes, we examined spermatozoa from a piciform, the Red-bellied Woodpecker (*Melanerpes carolinus*). The birds were collected in the vicinity of Chapel Hill, North Carolina. Their testes were dissected out into warm 0.9% saline solution for examination by light-microscopy and for fixation. Sperm from the ductus deferens were not examined.

MICROSCOPY

Sperm from teased bits of testicular tissue in saline were studied with phase contrast optics using a Zeiss Photomicroscope I. Stained smears were examined with bright-field lighting and all light micrographs were made with the Zeiss instrument. The electron micrographs were made with a Zeiss 9S2 microscope.

SECTIONED MATERIAL

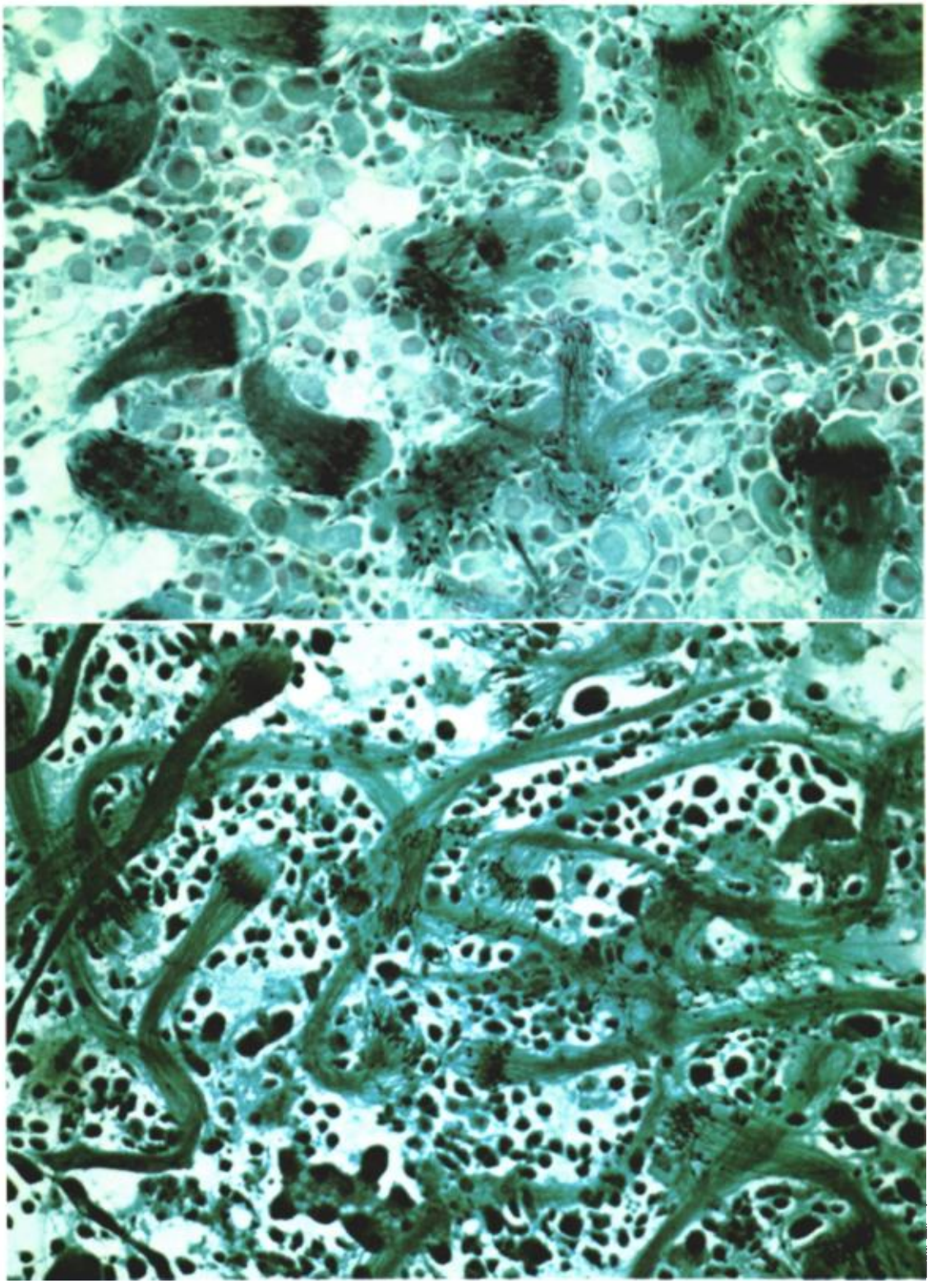
Minced bits of testis were fixed in a 4.5% glutaraldehyde buffered with 0.2 M cacodylate buffer for 2–4 h in the cold; they were post-treated with cacodylate-buffered 2% osmium tetroxide for 1½ h, dehydrated through an ethanol series and propylene oxide, and embedded in an Epon-Araldite mixture. Sections were cut on a Sorvall MT-2 ultramicrotome; they were picked up on uncoated 200-mesh copper grids, and were doubly stained with uranyl acetate and lead citrate.

NEGATIVE STAINING

Small pieces of testis were teased with sharp needles and the contents mixed with 1% aqueous phosphotungstic acid (PTA), pH 6.8, according to the method described by Henley (1970) and Henley and Costello (1973). PTA-treatment durations ranged from 6 to 42 min. The technique of negative staining with phosphotungstic acid involves considerable maceration of biological material, so that the components of a cell such as a spermatozoon may be partially digested away, to varying degrees (see Henley 1970).

SMEARS

Smears of teased testicular tissue and spermatozoa were prepared on #1 coverslips and were fixed while wet by exposure to formalin vapor for periods of 30



Bundles of sperm in smear from testes of American Robin (*Turdus migratorius*), upper, and Rufous-sided Towhee (*Pipilo erythrophthalmus*), lower, by phase contrast microscopy. Stain: Feulgen and Light Green. Approximately 320 ×. Note the relatively long length of the towhee sperm bundles.

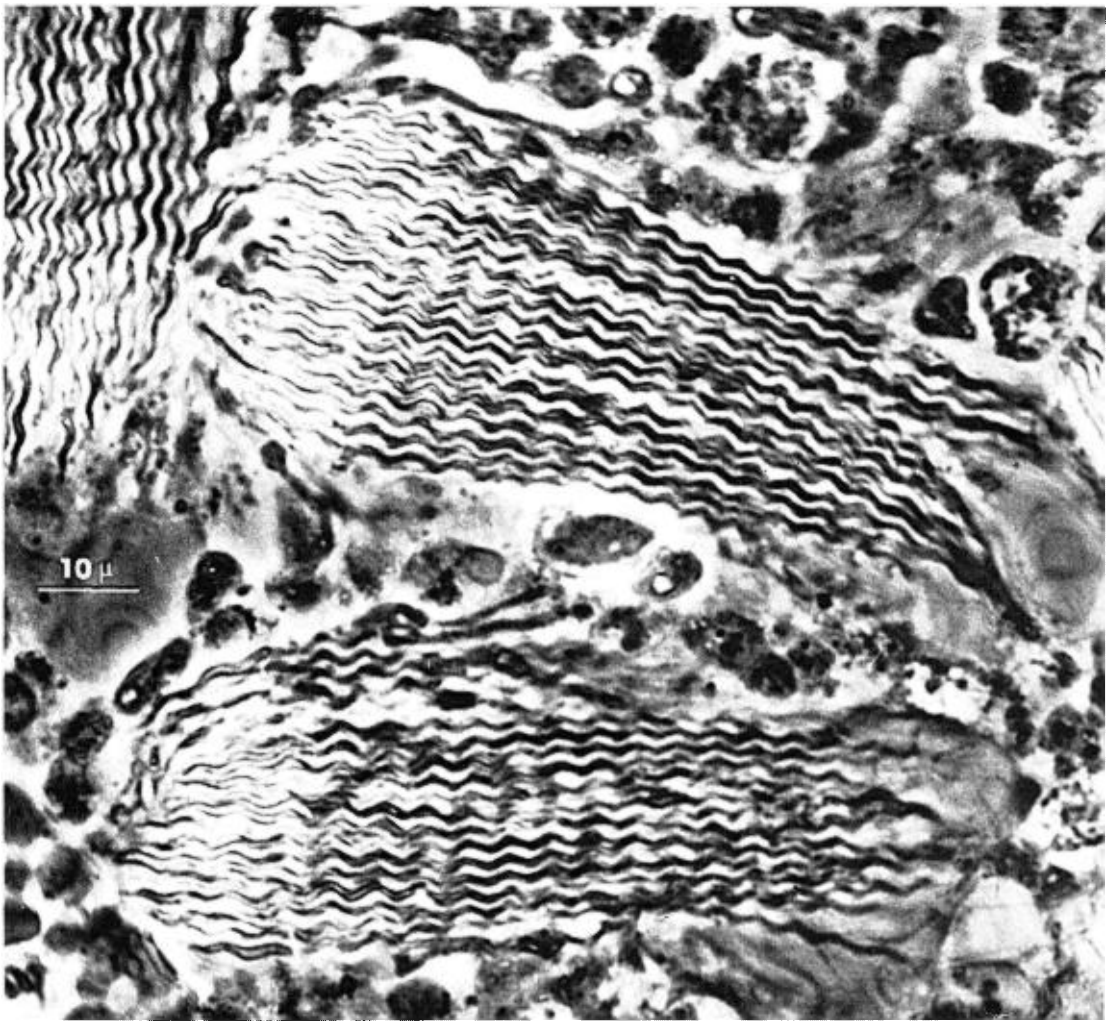


FIGURE 1. Phase contrast light micrograph of several bundles of oscine spermatozoa in the living condition. The nuclei are the bright regions to the left. Note the very precise register along the lengths of the bundle components. American Robin.

min to 24 h. A routine Feulgen procedure was carried out, with hydrolysis of the smears in 1 N HCl at room temperature for 5 min and at 60°C for 10 min. The preparations were treated with Schiff reagent for 30 min, followed by two 3-min changes of sulfurous acid; they were then washed in running tap water for 5 min, dehydrated through an ethanol series, counterstained in 0.5% light green and mounted in Permount.

OBSERVATIONS AND DISCUSSION

STUDIES BY LIGHT-MICROSCOPY

All of the oscine spermatozoa we examined have the following features in common:

(1) Syncytial spermatids are common, with from two to ten or more nuclei present in an undivided mass of cytoplasm. Both odd and even numbers of nuclei per syncytium have been seen.

(2) Very late spermatids and mature spermatozoa occur in bundles with varying numbers of components. This important observation is undoubtedly associated with the syncytial state. The spermatozoa in these bundles are in precise and striking register with one another (Figs. 1 and 2) in the mature condition, but late spermatids are noticeably shorter in length and their nuclei occur at varying levels along the bundle. In Feulgen preparations, the spermatid nuclei are noticeably less intensely stained than those of mature spermatozoa in the same bundle.

(3) At the beginning of the breeding season (early April in Chapel Hill), the spermatogonia and spermatocytes showed great uniformity and synchrony of development, but later this synchrony was lost and all stages, from gonial cells to mature spermatozoa, were present. The spermatogonia and spermatocytes were characterized by the presence of

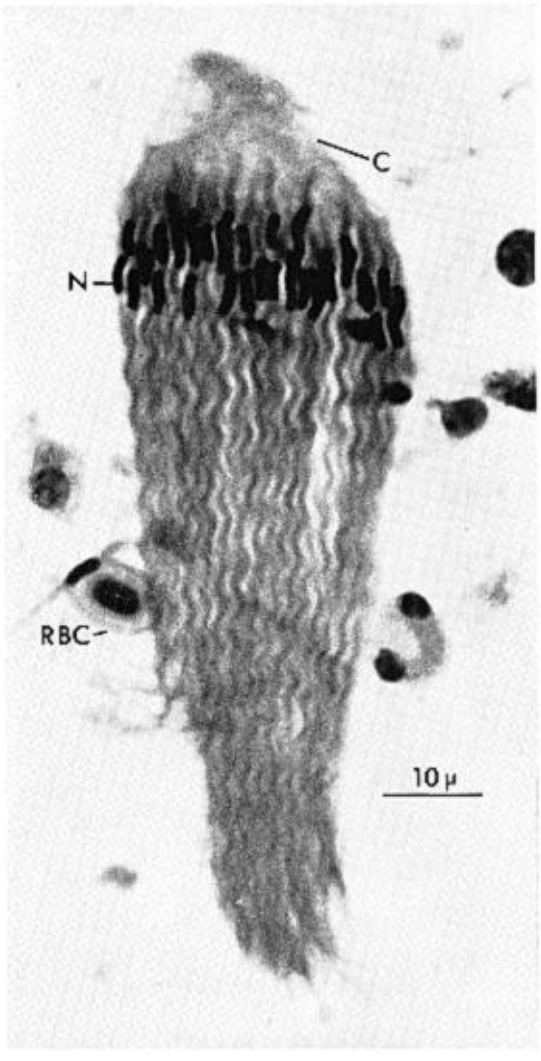


FIGURE 2. Bright-field light micrograph of a single oscine sperm bundle stained by the Feulgen method. The nuclei (N) are still embedded in a common cytoplasmic mass (C), and the remaining parts of the spermatozoa are in sharp register with one another. RBC, red blood corpuscle. American Robin.

many small chromosomes, arranged rather irregularly on mitotic and meiotic spindles. Such chromosomes were usually only moderately Feulgen-positive. The morphogenetic stages of spermiogenesis were very similar in all the oscines.

(4) Somewhat surprisingly, the testicular sperm were not motile in the 0.9% saline medium.

(5) Bi- or multi-flagellated spermatozoa were common. To our knowledge, oscines are one of the few groups of higher animals in which this occurs. It may well be associated with the syncytial origin of at least some spermatozoa.

Most of the oscine spermatozoa also possessed a so-called "undulating membrane," in a spiral for varying lengths (according to species) along their body, as previously described (see introduction). In contrast, however, the spermatozoa of corvids were reported to lack such a "membrane," the entire body of the spermatozoon being more or less helical (Retzius 1909, McFarlane 1963). Retzius described and figured the mature spermatozoon as lacking a free flagellum or tail in the Hooded Crow (*Corvus corone*), whereas McFarlane pictured it with a free flagellum in the Common Crow (*C. brachyrhynchos*). Our observations of *Cyanocitta* (Fig. 3) agree with those of Retzius (1909). The discrepancy between Retzius's and McFarlane's findings might be due to the possibility that McFarlane actually examined a very late spermatid rather than a mature spermatozoon. A free flagellum does persist until very late in spermiogenesis, according to our observations (Fig. 3), so that the mistake might easily have been made.

Spermatozoa of the woodpecker are markedly different from those of the oscines. They are more generally referable to the type seen in various fowls by Retzius (1909), McFarlane (1963) and others, and seen by us in a duck (unpubl. observ.). The acrosome in such spermatozoa is compact and button-like, rather than tapering and moderately long as in the oscines. In oscines, the nucleus is usually short and compact, but in *Melanerpes* it is much longer and somewhat more slender. The middle-piece in the piciform sperm is likewise elongated and narrow, and is succeeded by a free flagellum. There is no trace of any "undulating membrane." Piciform sperm that we have examined, unlike those of oscines, are motile when isolated in warm 0.9% saline.

Spermatogenesis in *Melanerpes*, as in the oscines, often occurs from a syncytial mass but does not result in the formation of precisely aligned bundles of spermatozoa. Rather, the components are very irregularly arranged, in no set pattern with respect to one another; the general appearance of such groups is very like that seen for the corvids (Fig. 4).

STUDIES BY ELECTRON MICROSCOPE

One of our more striking findings in sectioned material studied by electron-microscopy is that the "undulating membrane" of oscine spermatozoa is, in actuality, a tripartite structure, composed of (1) a straight axoneme, with the usual 9 + 2 pattern of

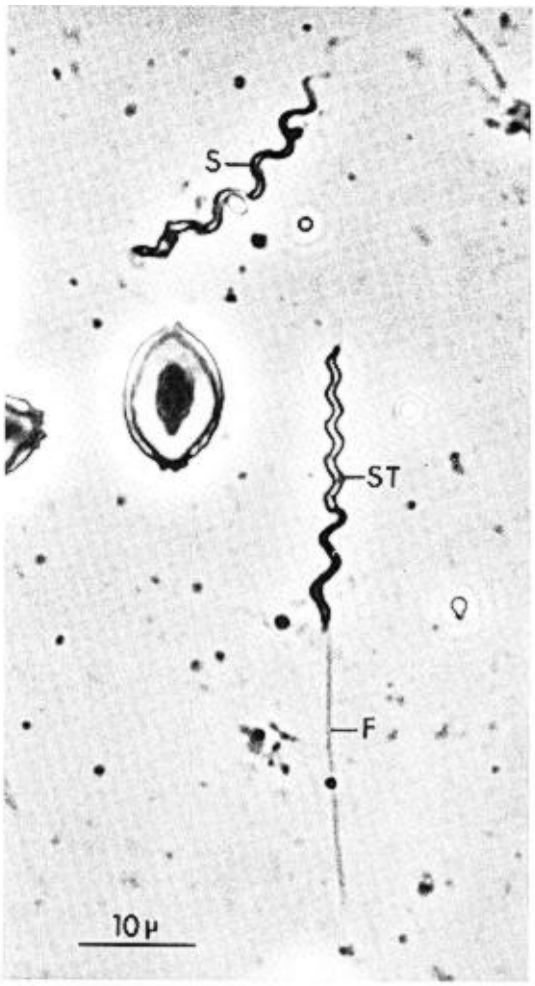


FIGURE 3. Phase contrast light micrograph of mature sperm (S) and late spermatid (ST) with flagellum (F). Blue Jay.

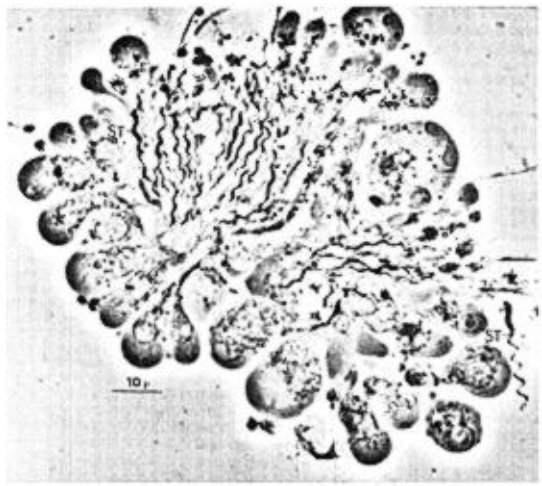


FIGURE 4. Phase contrast light micrograph of a bundle of spermatids, not arranged in any particular orientation with reference to one another. Compare with Fig. 1. Blue Jay.

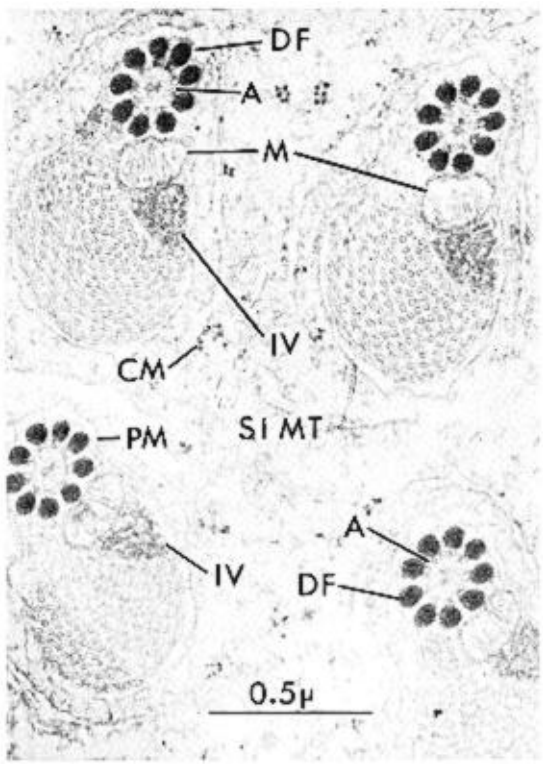


FIGURE 5. Electron micrograph of a transverse section through a bundle of spermatozoa at a level posterior to the region of the nuclei. Each member is invested in a plasma membrane (PM) and each has dense fibers (DF) surrounding a central axoneme (A). There is a single mitochondrion (M) in each and the plane of section through the sheath of singlet microtubules (SI MT) is almost exactly the same for each. Part of the residue of the cytoplasmic mass (CM) in which the bundle originated is peripheral to, and between, the spermatozoa. There is a fourth component (IV), roughly triangular in section, of unknown nature, next to the mitochondrion. American Robin.

microtubules, (2) a mitochondrial sheath with a fibrous component, and (3) a helically disposed longitudinal array of singlet microtubules.

A transverse section through a bundle of late spermatids of a Robin is shown in Fig. 5. The plane of section passes through a portion of the sperm bundle posterior to the level occupied by the nuclei. The very regular alignment of adjacent spermatozoa is shown, with the axonemes, the mitochondria and the microtubules of the sheath of each component cut in almost exactly the same plane of section as its fellows.

Our material also shows the presence of nine dense fibers, peripherally arranged around the axoneme of each tail. These fibers are similar in some respects to those found in mammalian (Fawcett 1965, Fawcett and Phillips 1970) and reptilian (Furieri 1970) spermatozoa, but differ in that they are of

FIGURE 6. Electron micrographs of parts of oscine spermatozoa negatively stained with PTA. The investing plasma membranes have been digested away by action of the negative stain, revealing the three components: straight axoneme (A), helical strand of mitochondria (M) and helical array of singlet microtubules (MT). The black structures (B) surrounding the three on either side are artifacts of negative staining. American Robin.

The lower figure shows the same three components, but in a slightly different orientation with respect to one another. Note the complex interweaving of the microtubules at arrow.

uniform size and rounded to ovoid in shape (see Fig. 3 of Fawcett and Phillips 1970). In mammalian spermatozoa, the dense fibers usually vary in size and shape according to their location with respect to the doublet microtubules of the axoneme and their position along the tail.

Negatively stained oscine spermatozoa reveal the tripartite nature of the "undulating membrane" in a somewhat different fashion. Fig. 6 shows the straight axoneme, which is relatively unaffected by the macerating action of the negative stain. Around it are wound, in helical array, a compact strand of mitochondria and a complex array of singlet microtubules. These microtubules are interwoven with one another in a basketweave-manner (Fig. 6 lower). Retzius (1909:Taf. XXXIII, Figs. 2 and 3), with only the excellence and acuity of his observations with the light microscope, was able to demonstrate precisely the same tripartite structure, although he was, of course, unable to resolve the microtubular nature of one of the components. Nicander (1970), utilizing sectioned material and electron microscopy, stated that, for passerines (p. 51), "The development of the helical character of this sperm type involves a helical bundle of twisted microtubules. . . . This microtubular complex is shed during the passage of the spermatozoa through the ductus deferens. . . ." However, Retzius (1909), found that for the Robin only a portion of what we may now identify as the microtubule sheath was discarded in the terminal stages of spermiogenesis, a small complement being retained in mature sperm. Our observations (unpubl.) confirm the work of Retzius in every detail for the Robin.

The dense fibers surrounding the axoneme appear to be sensitive to the macerating action of the negative stain, for we have never observed them in preparations made by the use of this technique.

In both sectioned and negatively stained material, spermiogenesis and morphology of the mature sperm in the Blue Jay appear to be entirely comparable to these features in other oscines, except for the absence of a free flagellum in the mature sperm.

Electron microscopy of woodpecker spermatozoa reflects the relatively simple organization suggested by light microscopy. Mature spermatozoa possess an acrosome, a densely compact nucleus and a middle-piece composed of mitochondria of coventional morphology and varying number surrounding the tail axoneme (Fig. 7). In such spermatozoa there is no evidence of any singlet microtubules in the sheath arrangement noted for oscines, although during spermiogenesis large numbers of singlet microtubules are present in the syncytium in which the spermatids develop. These microtubules presumably serve some sort of cytoskeletal function during morphogenesis of the spermatozoa, as is known to occur during spermiogenesis of many other animals. In addition to the lack of an "undulating membrane," *Melanerpes* spermatozoa differ from those of oscines in lacking the dense fibers which are peripheral to the axonemal microtubules (Fig. 7).

McFarlane (1963) noted that the more primitive type of avian spermatozoon showed a "remarkable resemblance to that found among the reptiles," leading to the designation of the type by some authors (notably Ballowitz and Retzius) as "sauropsid." Our findings confirm this to some degree, with the general morphology of acrosome, nucleus and middle-piece of piciform sperm resembling those shown for reptilian spermatozoa (e.g.,

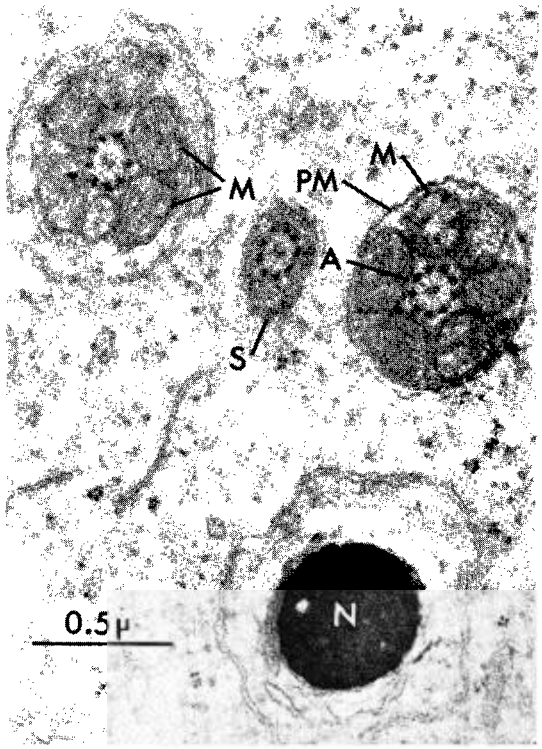


FIGURE 7. Electron micrograph of a transverse section through a group of maturing piciform spermatozoa. The section passes transversely through different levels of the components, in the regions of nucleus (N), and mitochondrial middle-piece (M), and in (S) through the tail posterior to the mitochondrial region. Note the absence of dense fibers between mitochondria and axonemes. Red-bellied Woodpecker.

Furieri 1970). The notable difference, however, is in the complete absence of the nine outer dense fibers in sperm of lower orders of birds that we have examined. These fibers are a conspicuous feature (along with other modifications of the tail) in the reptilian sperm described by Furieri. The functional significance of the dense fibers is still not clear, but Fawcett (1970) summarized the findings that suggest they must play an important role in motility of the tail. Baccetti et al. (1973) isolated and characterized the dense fibers of bull sperm, but the function of these fibers is still unknown.

Fawcett et al. (1971) stated that in the Zebra Finch the singlet microtubules disappear as the spermatids mature. They suggested that the microtubular helix is a transient organelle, homologous with the manchette and participating in spermatid elongation and perhaps in determining the configuration of the mitochondrial sheath. Intranuclear factors are considered to be responsible for shaping the nucleus.

It is important to note that the "undulating membrane" of oscine sperm is in no way truly comparable to the superficially similar structure found in amphibian spermatozoa. Such a true undulating membrane is composed of a more or less elongated fold of plasma membrane, in the margin of which is embedded a typical axonemal complex (Fawcett 1970). The axoneme apparently is the sole source of the undulatory motility typical of the amphibian type of sperm. In the case of oscine sperm, the role of the axoneme is less well defined, as is that of the associated singlet microtubules of the helical sheath.

SUMMARY

Spermatozoa of eight oscines are shown to have a tripartite structure to the so-called "undulating membrane" which is their unique characteristic. The three components are (1) a relatively straight axoneme with the 9 + 2 arrangement of microtubules, (2) a helically wound strand of mitochondria, and (3) a longitudinal array of singlet microtubules, likewise arranged in a helical configuration. Peripheral to the doublets of the axoneme are nine regular dense fibers, similar to those described by others for sperm of mammals and reptiles. Oscine spermatozoa characteristically occur in bundles with the component spermatozoa in very precise register with one another. Under the conditions we used (isolation of testicular tissue in warm 0.9% saline), the sperm were not seen to move. In contrast, the spermatozoa of a woodpecker lack the "undulating membrane" and dense bodies, and are of the simpler type characteristic of lower orders of birds. Piciform sperm occur in groups but these lack the very regular orientation of components noted for the oscines. They were motile under the conditions used.

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