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Ultrastructure of the Spermatid of *Caprimulgus europaeus* Linnaeus 1758, the European Nightjar (Aves; Caprimulgidae), With Phylogenetic Implications

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ABSTRACT The sperm of *Caprimulgus europaeus* is typical of other nonpasserines in many respects. Features shared with Paleognathae and Galloanserae are the conical acrosome, shorter than the nucleus; the presence of a perforatorium and endonuclear canal; the presence of a proximal as well as distal centriole; the elongate midpiece with mitochondria grouped around a central axis (here maximally six mitochondria in ~10 tiers); and the presence of a fibrous or amorphous sheath around the principal piece of the axoneme. A major (apomorphic) difference from paleognaths and galloanserans is the short distal centriole, the midpiece being penetrated for most of its length by the axoneme and for only a very short proximal portion by the centriole. Nonpasserines differ from paleognaths in that the latter have a transversely ribbed fibrous sheath, whereas in nonpasserines it is amorphous, as in *Caprimulgus*, or absent. The absence of an annulus is an apomorphic feature of *Caprimulgus*, apodiform, psittaciform, gruiform, and passerine sperm, homoplastic in at least some of these. In contrast to passerines, in *Caprimulgus* the cytoplasmic microtubules in the spermatid are restricted to a transient longitudinal manchette. The structure of the spermatid and spermatozoon is consistent with placement of the Caprimulgidae near the Psittacidae, but is less supportive of close proximity to the Apodidae, from DNA–DNA hybridization and some other analyses. J. Morphol. 000:000–000, 2006.
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The phylogenetic position of the Caprimulgidae is controversial. Sibley and Ahlquist (1990) placed the Caprimulgiformes in a clade of which the other members were the Eurostopodiae (as sister group), and, with increasing distance, Steatornithidae (oilbird) + Nyctibidae (potoos), Batrachostornidae + Podargidae (both frogmouths), Aegothelidae (owlet-nightjars) and Strigidae + Tytonidae (both owls). Sister groups to this assemblage were successively the Musophagidae (mouse birds) and then a clade consisting of the Trochilidae (hummingbirds) and their sister group, the Apodidae (swifts) + Hemiprocnidae (tree swifts). The Caprimulgiformes has frequently been subsumed in the Strigiformes (e.g., Zoonomen) but, as indicated here, this is questionably valid. However, it has been considered that Caprimulgiformes is at least paraphyletic as, in other analyses, the caprimulgiform family Aegothelidae appears to be the sister group of Apodiformes (Mayr, 2002, on osteology; Cracraft et al., 2004, on DNA; Sangster, 2005, on cladistic principles); whether a caprimulgiliform order would be monophyletic if apodiforms were included is unclear (Harshman, 2006). Earlier, in his reweaving of the tapestry, Harshman (1994) had recognized *Caprimulgus* as the sister taxon of the night hawk, *Chordeiles*, a member of the caprimulgid subfamily *Chordeilinae*, ascribed to the Strigiformes, at a considerable distance from the swift *Chaetura*, irrespective of rooting. Mariaux and Braun (1996), analyzing mitochondrial cytochrome b sequences, found a close link between the Caprimulgidae (including *Caprimulgus*) and the Aegothelidae and supported monophyly of the Caprimulgiformes; however, they did not include apodiforms or Strigidae in their study. On the present evidence, we will retain the Caprimulgiformes as a distinct order, while recognizing the nonequivalence of many ordinal avian ranks.

It is remarkable that the sperm of the Strigiformes or, if distinct, the Caprimulgiformes, have almost entirely been neglected as objects of study. We owe to Ballowitz (1888, see Discussion, below) a drawing of a spermatozoon of *Caprimulgus europaeus*, the European nightjar, and to Retzius (1909) a drawing of that of the strigid *Strix aluco*, the tawny owl. Tripepi et al. (1991) have commented on and illustrated the microtubules of the spermatid of *C. europaeus*. The spermatozoon of a swift, *Apus apus*, has been described ultrastructurally by

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Jamieson and Tripepi (2005) and displays little affinity with that of *Caprimulgus* (see Discussion).

Here we describe the ultrastructure of the spermatid and consider the phylogenetic position of the Caprimulgidae as evidenced by the structure of the advanced spermatid of *Caprimulgus europaeus*. The structures here described are sufficiently developed to indicate definitive spermatozoal structure. The account is based on previously unpublished archival micrographs which, although not allowing as comprehensive a description of spermiogenesis as might be desired, provide valuable information on development of the male gamete of this species, which is listed under the Bern Convention Concern Red List.

In view of the fact that molecular analyses have produced widely conflicting phylogenies (see above and references in Jamieson and Tripepi, 2005), morphological characters assume special significance in testing the validity of different phylogenetic hypotheses. Spermatozoa offer a valuable set of such characters.

MATERIALS AND METHODS

An adult male of *Caprimulgus europaeus* Linnaeus 1758 was captured, under permit, near Rogliano, a small town of the Sila Piccola Mountain, Calabria, Italy, on 28 May 1986. The bird was euthanized with chloroform and dissected; small testicular blocks were fixed for 2 h in 3% glutaraldehyde in 0.1 M phosphate buffer (PB). After two washes in PB the samples were postfixed in 1% osmium tetroxide in the same buffer for 2 h. After dehydration through a graded ethanol series, the specimens, prestained in uranyl acetate, were embedded in epon-araldite and sectioned with an ultramicrotome (Leica Ultracut UCT, Rockleigh, NJ). Ultrathin sections, stained with lead citrate, were observed and photographed with a Hitachi HU-12A electron microscope.

RESULTS

Stages of Spermiogenesis

Avian spermiogenesis has been divided into eight stages by Soley and Groenwald (1999) and 12 steps by Aire (2003). While such subdivision is unquestionably of value, spermiogenesis in *Caprimulgus europaeus* is here described in the context of two phases: the isodiametric and the elongating spermatid.

Isodiametric spermatids. The young spermatid (Figs. 1A,B,D, 2A,B) has an approximately isodiametric nucleus and cell body. Spermatids are interconnected by cytoplasmic bridges (Fig. 1A,B), each a zonula collaris sensu Jamieson (1981), with a dense outer coat. The chromatin is coarsely granular. Small mitochondria are scattered in the cytoplasm (Figs. 1A,B, 2A). Further features are described under the various components below.

Elongating spermatids. As the spermatid and its nucleus elongate, the chromatin becomes coarsely granular (Figs. 2C, 3E, 4A,C) and in the late spermatid has become strongly condensed and electron dense (Fig. 4D). Nuclear elongation is accompanied by growth of a longitudinal manchette of microtubules in the surrounding cytoplasm. No circular manchette is present. Further features are described under the various components below.

Development of components

Acrosome. The acrosome consists of the acrosome vesicle and associated material of a presumed perforatorial nature. The vesicle surmounts the anterior pole of the elongating, condensing nucleus (Fig. 3A–F). At an early stage (Fig. 3A) the vesicle is approximately as long as wide and has the form of a dome with a basal rim; at this stage there is no subacrosomal space nor a recognizable perforatorium but a density at the vesicle–nuclear junction appears to be the precursor of the latter. Later (Fig. 3B), a large subacrosomal space develops and the vesicle forms a dome over this. Varying degrees of condensation of dense material at the nuclear surface at the base of the subacrosomal space are seen, resulting in compaction to form an electron-dense, stout, spine-like structure, the putative perforatorium. This penetrates a short distance into the tip of the nucleus within an endonuclear canal (Fig. 3C) and protrudes into the subacrosomal space (Fig. 3D). In transverse section, the acrosome vesicle forms a thick ring, with moderately electron-dense contents, around the tip of the nucleus with its central endonuclear canal and perforatorium (Fig. 3F). Subsequently (Fig. 3E), the acrosome vesicle elongates and assumes a conical form; the perforatorium becomes less electron dense and the portion within the endonuclear canal is considerably longer than the ill-defined portion in the now conical subacrosomal space.

Nucleus. In the isodiametric spermatid the chromatin is finely granular and moderately electron dense, but somewhat more densely packed at the nuclear envelope (Figs. 1A,B,D, 2A,B). In the elongating spermatid the chromatin is at first only slightly more coarsely granular (Figs. 3A–D, 4C) but later forms fewer, coarse, electron-dense granules that are not more closely packed peripherally (Figs. 2C, 3E, 4A). At maturity, as seen in the advanced spermatid, the nucleus is cylindrical with strongly condensed, homogeneous, electron-dense chromatin (Fig. 4D).

Midpiece. In the isodiametric spermatid, mitochondria are scattered in the cytoplasm around the whole periphery of the nucleus (Figs. 1A,B, 2A) but in the young elongating spermatid some begin to be associated with the distal centriole (Fig. 4C). As the cytoplasmic canal around the proximal centriole contracts in a caudal direction, the mitochondria group around the proximal region of the axoneme

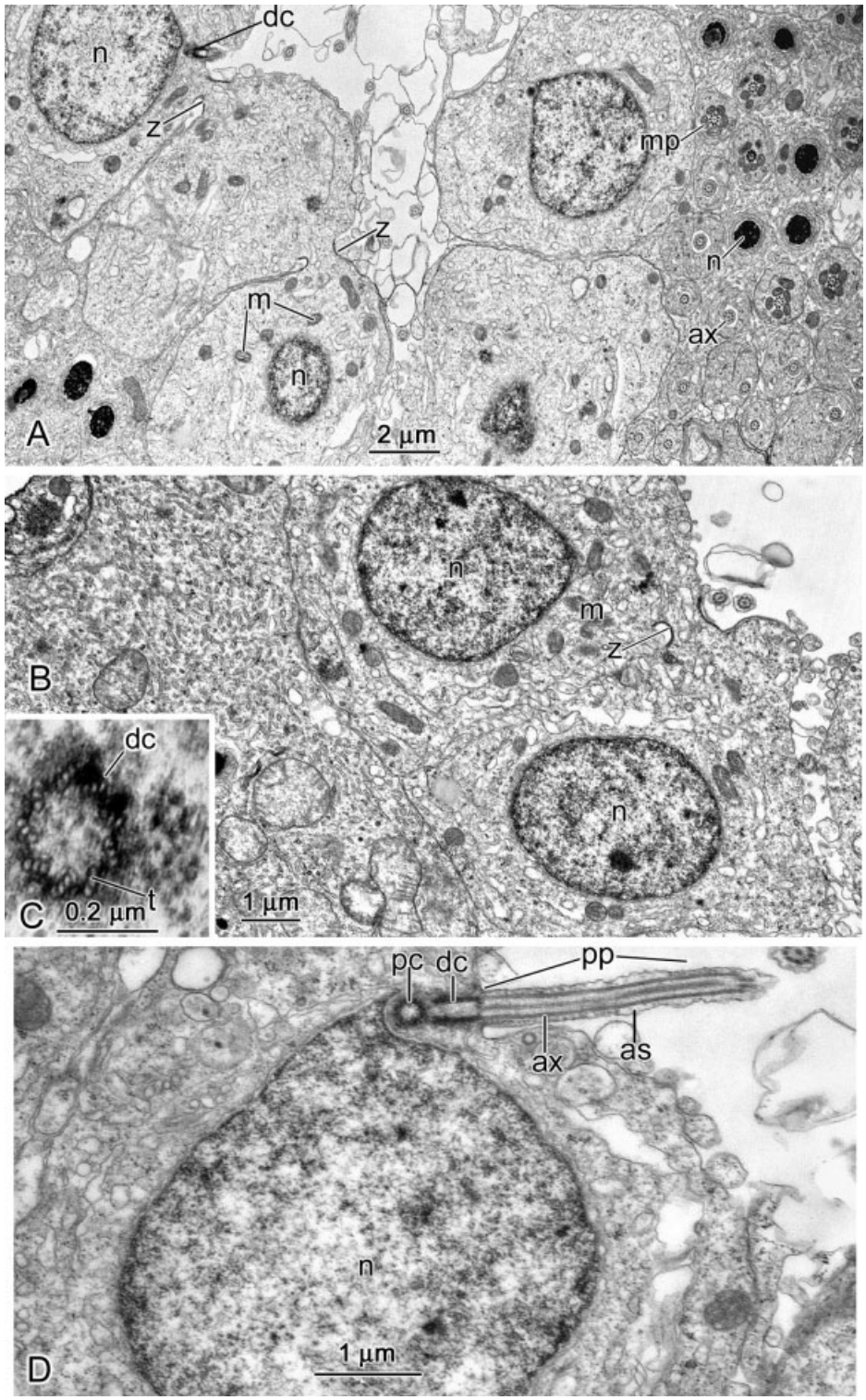

Fig. 1. *Caprimulgus europaeus*. TEM. **A:** Left, isodiametric spermatids. Three spermatids are shown interconnected by cytoplasmic bridges. The centrioles and base of the flagellum are sectioned in the spermatid at top left. Far right, elongating spermatids. The mitochondria, previously scattered, have assembled around the axoneme to form the midpiece and the chromatin of the nucleus has condensed. **B:** Two isodiametric spermatids conjoined by a cytoplasmic bridge with dense coat. **C:** Transverse section of a distal centriole, showing nine triplets of microtubules. **D:** An isodiametric spermatid sectioned longitudinally through the axoneme. The proximal and distal centrioles are mutually at right angles. The distal centriole is short and, at this stage, is not penetrated by the central axonemal singlets. Mitochondria have not yet assembled to form the midpiece. An amorphous sheath surrounded the axoneme and defines the principal piece. as, amorphous sheath; ax, axoneme; dc, distal centriole; m, mitochondria; mp, midpiece; n, nucleus; pc, proximal centriole; pp, principal piece; z, zonula collaris (cytoplasmic bridge).

(Figs. 2D, 4A,B). A maximum number of six mitochondria has been observed at one level (Fig. 4A, top left).

Centrioles. Each spermatid has a proximal and distal centriole, mutually perpendicular; the distal centriole is continuous with the flagellar axoneme, as seen in the isodiametric spermatid (Figs. 1A,D, 2A,B) and the elongating spermatid (Fig. 4C). The centrioles have the normal configuration of nine triplets of microtubules, here illustrated for the distal centriole (Fig. 1C) and proximal centriole (Fig. 2B). In the isodiametric spermatid this centriole comes to lie in an indentation of the nucleus (Figs.

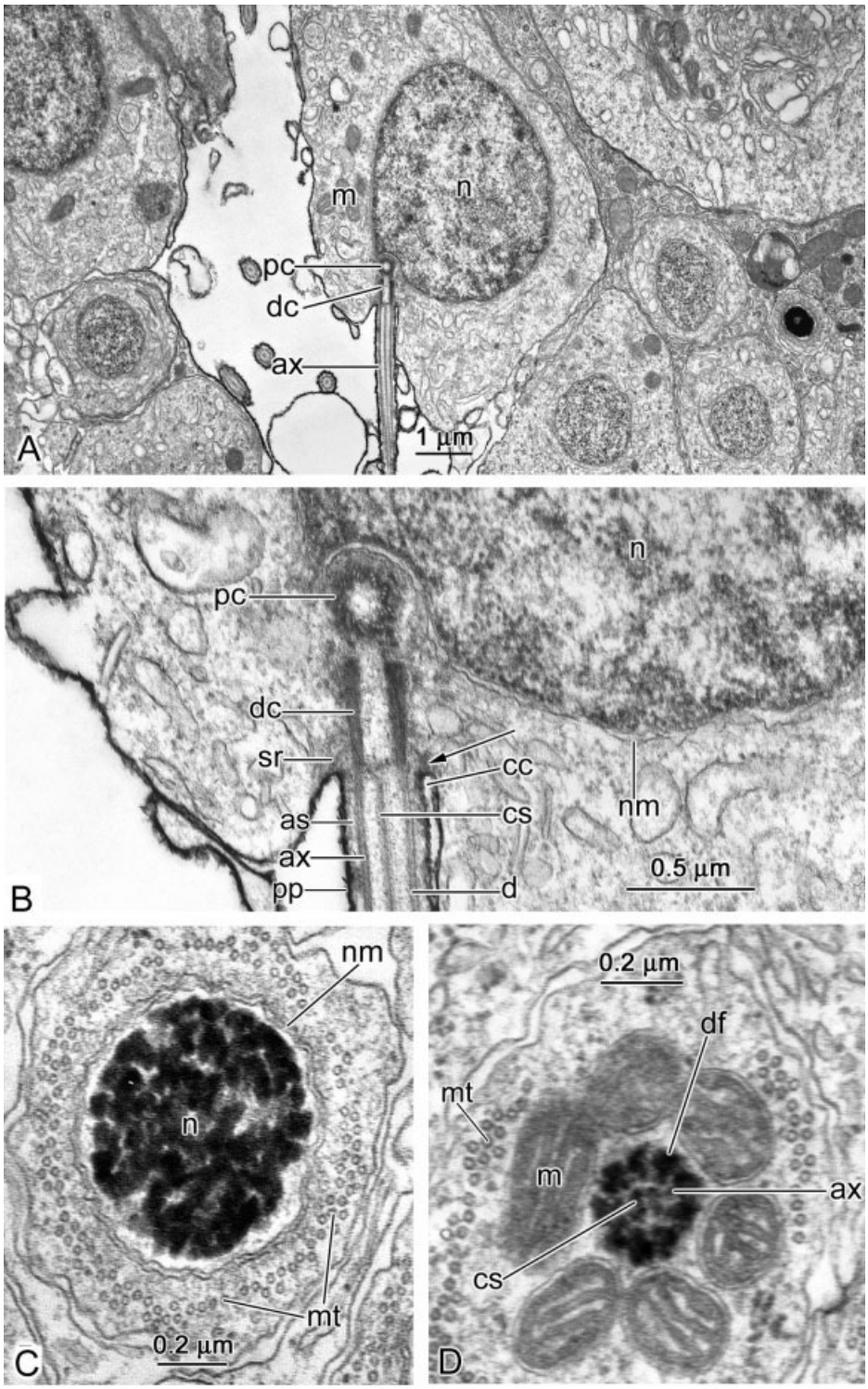

Fig. 2. *Caprimulgus europaeus*. TEM. **A:** A further isodiametric spermatid sectioned longitudinally through the axoneme, confirming the short condition of the distal centriole compared with that in Galloanserae. Mitochondria are still scattered in the cytoplasm around the uncondensed nucleus. **B:** Detail of the same spermatid. Satellite rays are seen at the base of the distal centriole. The base of the flagellum is indented into the spermatid to form the transient cytoplasmic canal. As indicated by the arrow, there is no definite annulus. At this stage the central singlets of the axoneme do not penetrate the lumen of the distal centriole. **C:** Transverse section (TS) of the nucleus, with condensing chromatin, of the elongating spermatid. Longitudinal microtubules of the manchette surround the nuclear membrane but no circularly running microtubules are present. **D:** TS of the midpiece of an advanced elongating spermatid. Six mitochondria have assembled around the proximal axoneme to form the midpiece, within which the axonemal doublets are accompanied by dense fibers. Dense structures of unknown nature accompany the central singlets of the axoneme at this level. Microtubules of the manchette are still present. as, amorphous sheath; ax, axoneme; cc, cytoplasmic canal; cs, central dense structures; d, doublets of the axoneme; dc, distal centriole; m, mitochondrion; mt, longitudinal microtubules of the manchette; n, nucleus; nm, nuclear membrane; pc, proximal centriole; pp, principal piece; sr, satellite ray.

1D, 2A,B). Both centrioles are short. The lumen of the distal centriole is not, initially, penetrated by the central singlets of the axoneme (Figs. 1D, 2A,B) but in the young elongating spermatid it is penetrated by these singlets (Fig. 4C).

Axoneme. The axoneme forms an extension of the distal centriole (Figs. 1A,D, 2A,B, 4C). In transverse section, the axoneme has the typical "9 + 2" arrangement of microtubules: nine peripheral doublets and two central singlets, the doublets each consisting of a complete A microtubule, bearing two dynein arms, to which is conjoined an incomplete B microtubule (Fig. 4A,B,E). In the isodiametric and young elongating spermatid the axoneme is proxi-

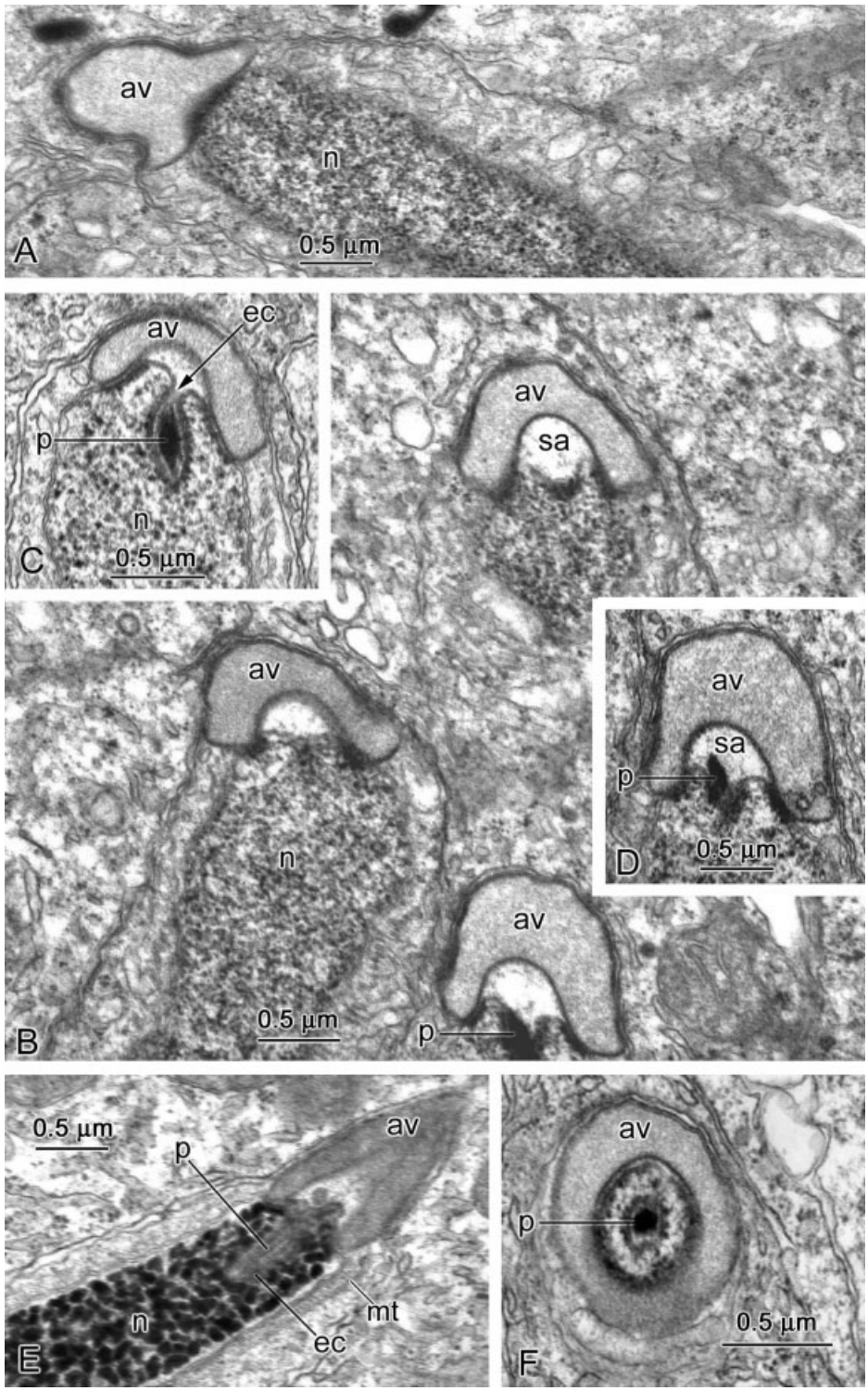

Fig. 3. *Caprimulgus europaeus*. TEM. Elongating spermatids showing the form of the developing acrosome. **A:** Longitudinal section (LS) of the acrosome vesicle and adjacent elongating nucleus. At this stage the acrosome vesicle has a simple, cowl-like shape. **B:** LS of acrosomes of three elongating spermatids. A subacrosomal space has developed and (lower acrosome) subacrosomal material is forming a putative perforatorium that projects into the tip of the nucleus. **C:** The perforatorium lies in an endonuclear canal. **D:** The dense perforatorium projects into the subacrosomal space. **E:** LS of a more mature acrosome. The perforatorium lies in the endonuclear canal and extends somewhat amorphously into the subacrosomal space. The acrosome vesicle is assuming a conical form. **F:** Transverse section of an advanced acrosome showing the base of the acrosome vesicle enclosing the tip of the nucleus with its endonuclear canal and perforatorium. av, acrosome vesicle; ec, endonuclear canal; mt, microtubules of manchette; n, nucleus; p, putative perforatorium; sa, subacrosomal space.

mally embedded in the cytoplasm, from which it is separated by the transient cytoplasmic canal (Figs. 2B, 4B). This canal is eliminated by the caudal growth of the midpiece. Within the midpiece, a dense fiber is attached to the outer aspect of each of the nine doublets (Figs. 2D, 4A,B). The free axoneme lacks dense fibers and is surrounded by a weakly developed amorphous sheath, defining the principal piece (Figs. 1D, 2A,B, 4B,C,E).

DISCUSSION

By light microscopy, the sperm of *Caprimulgus europaeus* appears in a drawing (fig. 85 in Ballowitz,

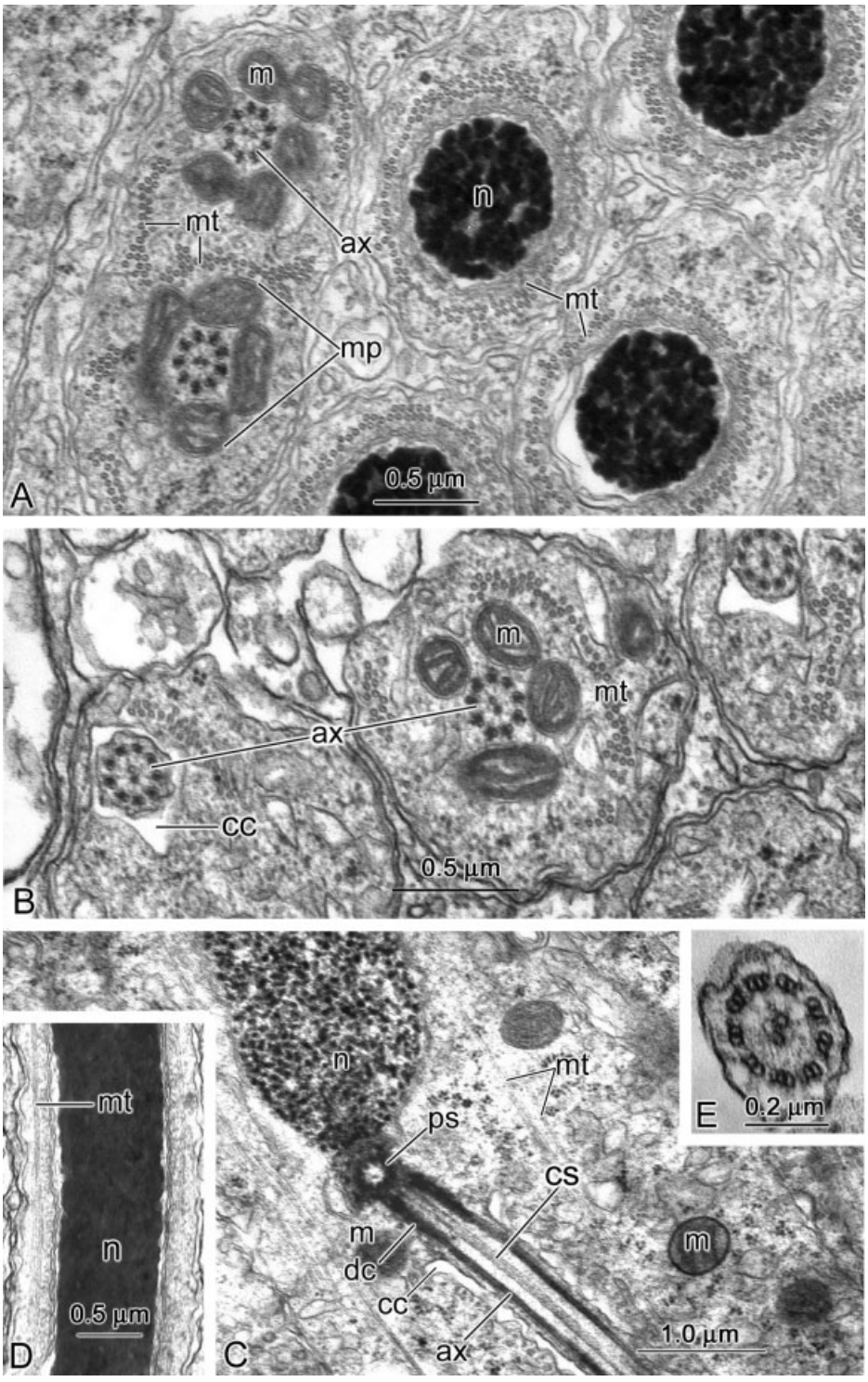

Fig. 4. *Caprimulgus europaeus*. TEM. **A:** Transverse sections (TS) of midpieces and nuclei of advanced elongating spermatids. Four to six mitochondria surround the proximal axoneme as the midpiece. Dense fibers associated with the axonemal doublets are small in these sections. The chromatin of the nuclei has become clumped as condensation proceeds. Longitudinal but no circularly running cytoplasmic microtubules are present. **B:** TS of elongating spermatids. Left through the transient cytoplasmic canal and contained axoneme. Center right: TS through a developing midpiece that has assembled only four mitochondria at this level. **C:** Longitudinal section (LS) of an elongating spermatid, showing proximal and distal centrioles and the axoneme surrounded basally by the transient cytoplasmic canal. Mitochondria are beginning to assemble to form the midpiece. The central axonemal singlets have penetrated the distal centriole. **D:** LS of nucleus that is fully condensed but is still accompanied by the manchette. **E:** TS of axoneme with weakly developed amorphous sheath. ax, axoneme; cc, cytoplasmic canal; cs, central singlets of axoneme; dc, distal centriole; m, mitochondrion; mp, midpiece; mt, microtubules of manchette; n, nucleus; ps, proximal centriole.

1888) to have a conical acrosome tapering evenly from the much longer but fairly stout, curved nucleus. In the legend for his figure 86 the head is described as "an der Spitze deutlich das kleine blasse Spitzen-stueck zeigend." This pale point is consistent with the conical form of the maturing acrosome observed here (Fig. 3E). It is in marked contrast to the button-like acrosome of the other, supposedly coordinal strigiform, the tawny owl (Retzius, 1909). The *Caprimulgus* midpiece, about half the length of the nucleus, has some 10 tiers of mitochondria; the free axoneme is about the same length as the head plus midpiece (see Ballowitz, 1888).

The structure of the spermatozoon of *Caprimulgus europaeus* can be deduced from the description by TEM of the late spermatid given here when considered in conjunction with the light microscope description of the mature spermatozoon by Ballowitz (1888). It is typical of other nonpasserines in many respects. Features shared with Paleognathae (ratites and tinamous) and Galloanserae (e.g., rooster and duck) are the conical acrosome, shorter than the nucleus; the presence of a perforatorium and endonuclear canal; the presence of a proximal as well as distal centriole (recently demonstrated, where previously questioned, for guineafowl by Aire and Soley, 2003); the elongate midpiece with mitochondria grouped around a central axis (here maximally six mitochondria in ~10 tiers); and the presence of a fibrous or amorphous sheath around the axoneme. Most of these features characterize nonpasserines in general. A major (apomorphic) difference from paleognaths and galloanserans is the short distal centriole, the midpiece being penetrated for most of its length by the axoneme and for only a very short proximal portion by the centriole. In paleognaths a long distal centriole penetrates the entire midpiece, a plesiomorphic feature shared with crocodiles (Jamieson, 1999) and the apodiform *Apus apus* (Jamieson and Tripepi, 2005). In the Galloanserae the centriole, although long, does not extend for the entire length of the midpiece. Nonpasserines differ from paleognaths in that the latter have a transversely ribbed fibrous sheath, whereas in nonpasserines it is amorphous, as in *Caprimulgus*, or absent.

In lacking an appreciable annulus, which in reptiles terminates the midpiece, the sperm of *Caprimulgus,* like those of Psittaciformes (Jamieson et al., 1995; Jamieson, 1999), Gruiformes (*Grus vipio*, Phillips et al., 1987), Apodiformes (Jamieson and Tripepi, 2005), and passerines (e.g., Asa and Phillips, 1987; Jamieson, 1999), differ from those of paleognaths (e.g., Baccetti et al., 1991; Soley, 1993) and lower nonpasserines including the mallard duck (Humphreys, 1972; Maretta, 1975), the turkey, chicken, guineafowl (Thurston et al., 1982; Thurston and Hess, 1987; Aire and Soley, 2003; Jamieson, 2006) and Charadriiformes as represented by *Jacana* (Saita et al., 1983). An annulus is basal to paleognaths and these nonpasserines. Absence of the annulus is therefore an apomorphic feature of caprimulgid sperm.

In contrast with paleognaths and nonpasserines, passerines have the acrosome longer than the nucleus (excepting the poorly known suboscines, most Corvida, and see Retzius, 1909, some hirundins); lack a proximal centriole (excepting suboscines); always have a short distal centriole; a single mitochondrial strand along the anterior region (Corvida) or wound for a great distance along the axoneme (Passerida); and lack the fibrous, or amorphous, sheath. Passerines differ further from nonpasserines in possessing, in the spermatid, a "helical membrane," consisting of multiple transverse and longitudinal microtubules forming a thick strand helically coiled around at least the flagellum (e.g., Asa and Phillips, 1987; Jamieson, 2006). In contrast, in *Caprimulgus europaeus* the circular manchette has been lost and only a longitudinal manchette is present in the developing spermatid (Tripepi et al., 1991; this study). A similar arrangement is seen in *Jacana jacana* (Saita et al., 1983) and in the apodiform *Apus apus* (Jamieson and Tripepi, 2005). Tripepi et al. (1991) consider the arrangement of microtubules in *C. europaeus* to be the second above a "reptilian" level.

The caprimulgid spermatid (and clearly the spermatozoon) is closely similar in structure to that of the Psittaciforms. The two taxa are fairly closely related in the DNA–DNA hybridization analyses of Sibley and Ahlquist (1990), although having an unresolved relationship in Cracraft et al. (2004). However, the Apodidae, placed in the same clade as the Caprimulgidae in both analyses, appear from the long distal centriole of the spermatozoon to be far more basal, near the paleognaths, if the centriolar condition is not, as seems unlikely, a reversal (Jamieson and Tripepi, 2005; but see alternative scenario in Jamieson, 2006). Other members of the caprimulgid clade of Sibley and Ahlquist (1990) (Hemiprocnidae through Eurostopodidae) have not been examined for sperm ultrastructure. Although the Trochilidae and Strigidae, in this clade, are listed as having been examined by light microscopy (McFarlane, 1963), no data are given. If aegothelids are the sister group of Apodiformes, it will be interesting to determine whether they share the elongate distal centriole of the latter. This would further distinguish them from the Caprimulgidae.

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