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Spermiogenesis in the cattle egret (*Bubulcus ibis*)

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ABSTRACT

Avian species comprise more than half of all vertebrates yet there is a dearth of information regarding spermatid development in this class of animals. This report of spermiogenesis in the cattle egret, *Bubulcus ibis*, is the first in the order Pelecaniformes. Five sexually mature and reproductively active male cattle egrets were captured in the wild, humanely euthanized, the reproductive organs dissected out, and tissues from the testes routinely prepared for transmission electron microscopy. Twelve steps of spermatid development, using the step-wise system, were determined. Acrosomogenesis in the egret results in a relatively short, solid, bullet-shaped acrosome that ends bluntly anteriorly and flat posteriorly or basally. The nucleus displays remarkable morphological changes, with the anterior end of the mature spermatid becoming flat, lacking a rostrum and an endonuclear canal. A perforatorium does not develop. It is noteworthy that a longitudinal, but not a circular, manchette develops during spermiogenesis in this bird. The proximal centriole is attached to the nucleus, at the implantation fossa, by means of well-formed, electron dense struts of material. An amorphous fibrous sheath develops in the principal piece. The interesting development and peculiar features of the acrosome and nucleus, as well as the absence of the perforatorium and circular manchette in the spermatozoon of the cattle egret, may be of phylogenetic significance.

1. Introduction

Spermiogenesis, a major process of spermatogenesis, provides vital information on the function of the seminiferous tubules in health and disease (Aire, 2003). Spermiogenesis in birds has been studied in a few and mostly domesticated non-passerine species such as the rooster (Nagano, 1962; McIntosh and Porter, 1967; Tingari, 1973), Japanese quail (Lin and Jones, 1993), ostrich (Soley, 1994, 1996, 1997), turkey (Aire, 2003) and rhea (Phillips and Asa, 1989). This process has been studied in only a few passerine birds such as the house sparrow (Góes and Dolder, 2002), masked weaver (Aire and Ozegbe, 2012), common finch (Kondo et al., 1988) and Carib grackle (Aire et al., 2019). Studies on the development of avian spermatozoa are therefore remarkably few when compared to those of mammals (Jamieson et al., 2006). Furthermore, reports of spermiogenesis in wild non-passerine birds are even fewer and fragmentary (Jamieson, 2007).

This study will complement the previous report on the ultrastructure of the sperm of the cattle egret (Roopnarine et al., 2020), and will help to shed some light on the development of certain structural features of the sperm of this bird that are different from those of most non-passerine birds. In this study, the stepwise system is adopted, as used in only a few reports such as in the rooster (Nagano, 1962; Gunawardana and Scott, 1977), rhea (Phillips and Asa, 1989), Japanese quail (Yamamoto et al., 1967; Lin and Jones, 1993), turkey (*Meleagris gallopavo*) (Aire, 2003) and House sparrow (*Passer domesticus*) (Góes and Dolder, 2002).

The purpose of this study is to examine spermiogenesis in a wild non-passerine bird, the cattle egret (*Bubulcus ibis*), of the order Pelecaniformes, with a view to contributing to knowledge and understanding of this process in birds, in general, and especially in wild species. To our knowledge there are no reports on spermiogenesis in the order Pelecaniformes. The cattle egret is known for its close association with livestock and wildlife (Heatwole, 1965), and for its global habitation (Brown et al., 1982). Local livestock farmers strongly believe that cattle egrets feed on ticks and flies.

2. Materials and methods

Tissues from the testes were collected from five adult and sexually-

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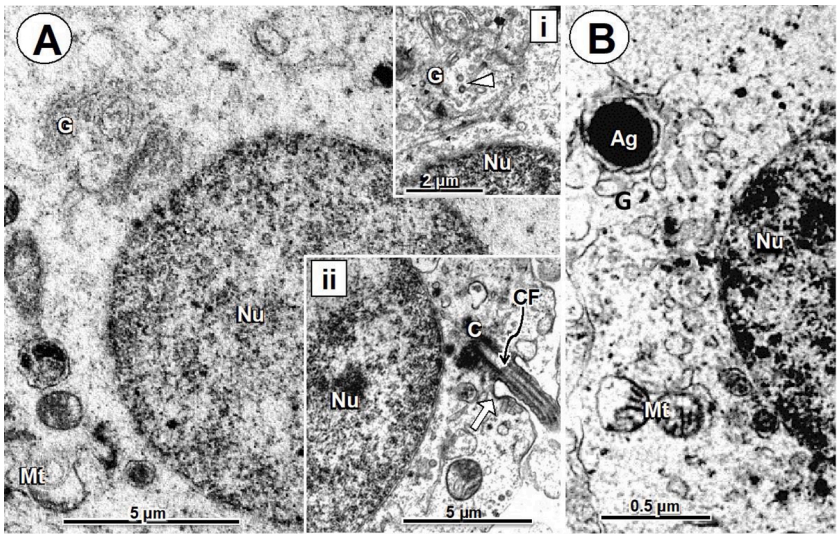

Fig. 1. Step 1 spermatids. **A**. Numerous mitochondria (Mt) and a large Golgi complex (G) lie close to the nucleus (Nu); electron dense proacrosomal granules (arrowhead) in **Fig. A, inset i**. **Inset ii** shows the centriolar complex (C), of a late phase of step 1 spermatid, lying freely in the cytoplasm, close to the cell membrane (white arrow); centrio-flagellar shelf (CF). **B. Step 2 spermatid**. The large, electron-dense proacrosomal granule (Ag) is surrounded by cisternae of the Golgi complex (G); nucleus (Nu), mitochondria (Mt).

active, Cattle egrets, *Bubulcus ibis*, which were humanely caught in the wild, with the permission of the Ministry of Agriculture of Grenada, and approval by the Institutional Animal Care and Use Committee (Approval #, IACUC-18,006-R) of the St. George's University. After being captured by local trappers, with the aid of mist nets and loop-rope traps, the birds were euthanized by an overdose of inhalant isoflurane, or an intracoelomic injection of pentobarbitol. The thoraco-abdominal cavity was opened quickly, and in each of three birds, a *25*-gauge catheter was inserted into the left ventricle or aorta for intravascular perfusion (Aire, 1979), using heparinized physiological saline to flush the blood out of the vessels before perfusion with the fixative. This was followed by the fixative, 3% glutaraldehyde buffered in 0.1 M phosphate buffer, at pH 7.4, for transmission electron microscopy. Tissues obtained from the remaining two birds were fixed by immersion in the same fixative. The tissue samples from all the birds were then immersed in 2% osmium tetroxide, buffered with 0.2 M s-collidine for 1 h. Dehydration with increasing concentrations of ethanol preceded embedding in Epon 812. Survey sections, 1 µm thick, were cut and stained in toluidine blue. Ultrathin sections, 70 nm thick, were cut from appropriate areas and placed on copper grids, stained in uranyl acetate for 30 min, and lead citrate for 10 min. The sections were examined in a Phillips CM 10 transmission electron microscope operated at 80kv.

3. Results

In the description of spermatid differentiation, the step wise method (Aire, 2003) has been employed.

Step 1. This step follows the meiotic division of the secondary spermatocyte into four spermatids, whose nuclei are round and have moderately dense scattered clumps of chromatin within the nucleoplasm and attached marginally to the nuclear membrane (Fig. 1A). The cell displays a well-formed Golgi complex accompanied by numerous mitochondria, at the putative anterior pole of the nucleus. The Golgi complex may contain small, round, dense, vesicles (Fig. 1A, inset i). Rough endoplasmic reticulum (RER) and a few lysosomes are scattered throughout the cytoplasm. The centriolar complex comprises both the proximal and distal centrioles which are arranged in the typical fashion and lie midway between the nucleus and cell membrane, toward the end of this step (Fig. 1A, inset ii). A shelf of electron-dense, transversely orientated material (centrio-flagellar shelf) forms the boundary between the distal centriole and the extracellular flagellum, and from where the axoneme extends (Fig. 1A, inset ii). The annulus is poorly developed.

Step 2. The nucleus still displays scattered clumps of heterochromatin. The discrete dense vesicles in step 1 have coalesced to form a large, electron-dense proacrosomal granule (Ag) in the Golgi complex;

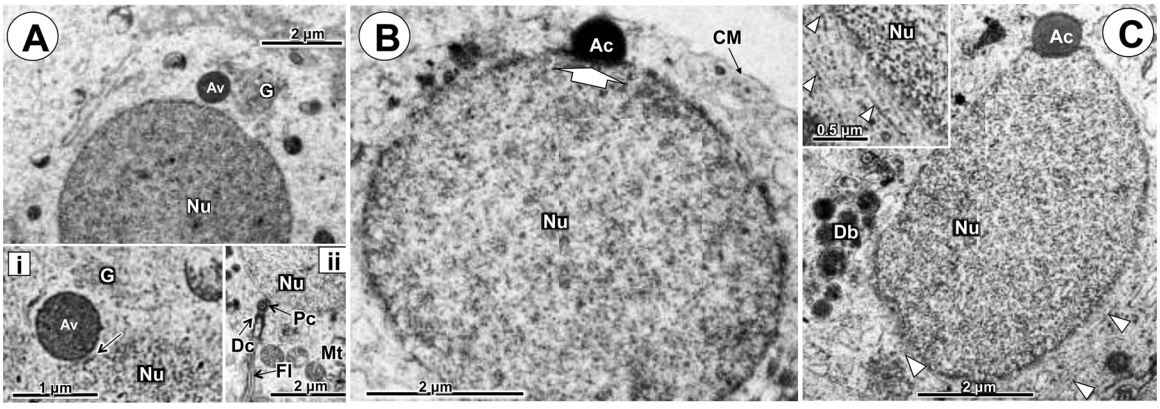

Fig. 2. Step 3 spermatids. **A**. The acrosomal vesicle (Av) lies close to the nucleus (Nu) or just slightly contacts the nuclear membrane in **inset i** (black arrow); Golgi complex (G). **Inset ii** shows that the proximal centriole (Pc) and distal centriole (Dc) lie very close to the nucleus (Nu); flagellum (Fl), mitochondria (Mt). **B. Step 4 spermatid**. The acrosome (Ac) makes contact with the nucleus (Nu) at a thickened interface of the nuclear membrane (broad arrow). The nucleus moves close to the cell membrane (CM). **C. Step 5 spermatids**. The nucleus (Nu) begins to elongate and the acrosome (Ac) indents the nucleus slightly. A layer of longitudinally orientated microtubules (white arrowheads) invests the nucleus (**Main figure & Inset**). Dense bodies (Db).

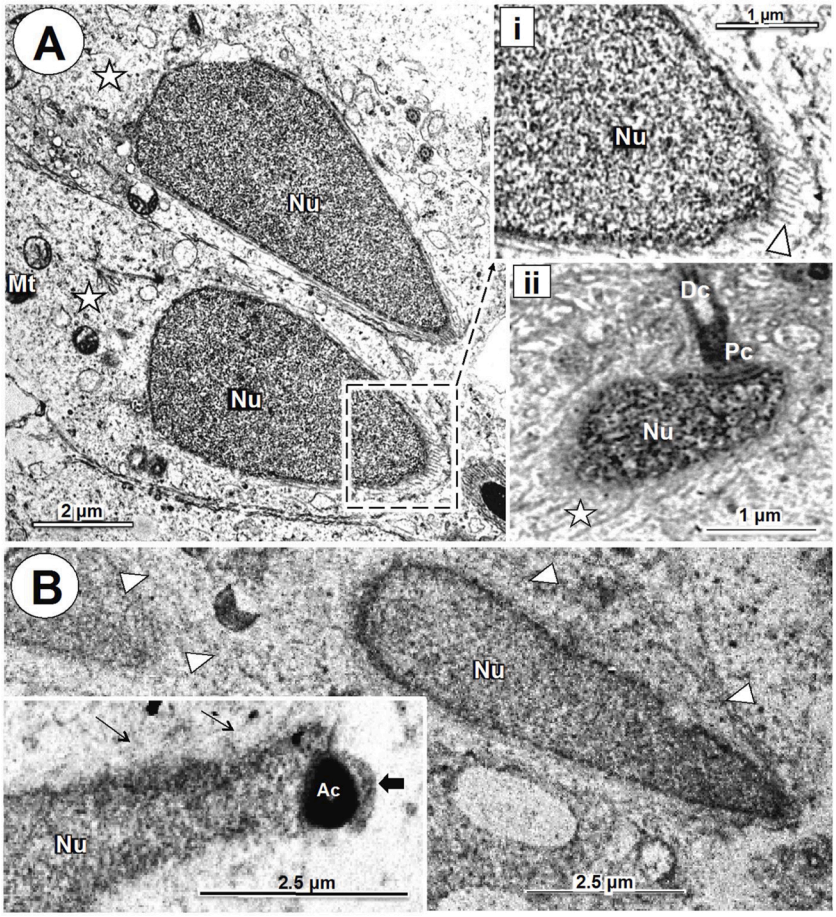

Fig. 3. Step 6 spermatids. **A**. The spermatid nucleus (Nu) is pyramidal or conical in shape. Most of the cytoplasm (star) lies caudal to the nucleus; mitochondria (Mt). **Inset i** shows the longitudinal manchette (white arrowhead) surrounding the nucleus (Nu). **Inset ii** shows the centriolar complex, comprising the proximal (Pc) and distal centriole (Dc); longitudinal manchette (star). **B**. Late step 6 spermatid, with a further elongated nucleus (Nu); cell membrane (arrowheads). **Inset,** the acrosome (Ac) attaches at the apex of the nuclear pyramid at a straight, flat, interface; longitudinal manchette (black arrows). Sertoli cell cytoplasm (black block arrow) is surrounding the acrosome.

the proacrosomal granule is surrounded by cisternae of the Golgi complex. Mitochondria are randomly placed throughout the cytosol, in moderate numbers (Fig. 1B).

Step 3. The de-condensation of nuclear chromatin begins, conferring on the nucleus a general granularity. The relatively euchromatic nucleus maintains its round to oval shape. The acrosomal vesicle is free from the Golgi complex, and moves close to, or makes tenuous contact with the nucleus, at a thickened junction (contact site) of the nuclear membrane. Later in this step, the centriolar complex makes contact with the nucleus in the same region as the acrosomal vesicle (Fig. 2A).

Step 4. The anterior aspect of the spermatid nucleus, indicated by the location of the acrosomal vesicle, is positioned close to the cell membrane (Fig. 2B). The nuclear chromatin is nearly completely decondensed or euchromatic. The homogeneously electron dense acrosomal vesicle appears pyramidal or elongated, depending on the angle of section. The base of the acrosomal vesicle is broader, and makes firm contact at a thickened interface with the nucleus at its anterior pole (Fig. 2B).

Step 5. The acrosomal vesicle, now regarded as the acrosome, appears round or oval in shape, and indents the now pointed end of the nucleus, at an electron-dense interface. The now euchromatic nucleus commences an elongation phase, becoming pear-shaped. Longitudinally orientated microtubules begin to appear and invest the nucleus (Fig. 2C) forming the longitudinal manchette. Numerous dense bodies, resembling lysosomes, aggregate close to the nucleus. Mitochondria are few and scattered (Fig. 2C).

Step 6. The spermatid nucleus is now pyramidal or conical in outline and contains homogenous, finely granular chromatin, which becomes more electron-dense (Fig. 3A). The acrosome makes contact with the nucleus along a flat plane, at the apex of the pyramid (Fig. 3B), and it is surrounded by Sertoli cell cytoplasm. The nucleus is enclosed in a sleeve of longitudinal microtubules, the longitudinal manchette. The centriolar complex is well-established, and both centrioles continue to lie at right angle to each other (Fig. 3A, inset ii). Round to oval mitochondria migrate posteriorly, in relation to the spermatid nucleus. Most of the cell cytoplasm now trails behind the nucleus (Fig. 3A). There are many vesicular bodies, lysosomes and smooth endoplasmic reticulum in the caudal region of the cytoplasm. At the end of this step, the nucleus is more elongated, and the longitudinal manchette is well developed (Fig. 3A and B).

Step 7. In comparison to that in step 6, the nucleus is more electron-dense and markedly longer with a rounded base which tapers anteriorly towards a narrow apex. The small conical acrosome forms a cap over the apex of the nucleus, as in step 6. The chromatin of the nucleus condenses into dense, granular material, with some electron-lucent areas, the so-called nuclear vacuoles, containing granuloflocculent material. Mitochondria, short strands of rough endoplasmic reticulum, and a few dense bodies are scattered within an abundance of smooth endoplasmic reticulum in the cytoplasm (Fig. 4). The centriolar complex and flagellum are as in step 6, with the latter remaining slender and a little wavy in outline. The manchette is better developed and more prominent anteriorly than posteriorly (Fig. 4).

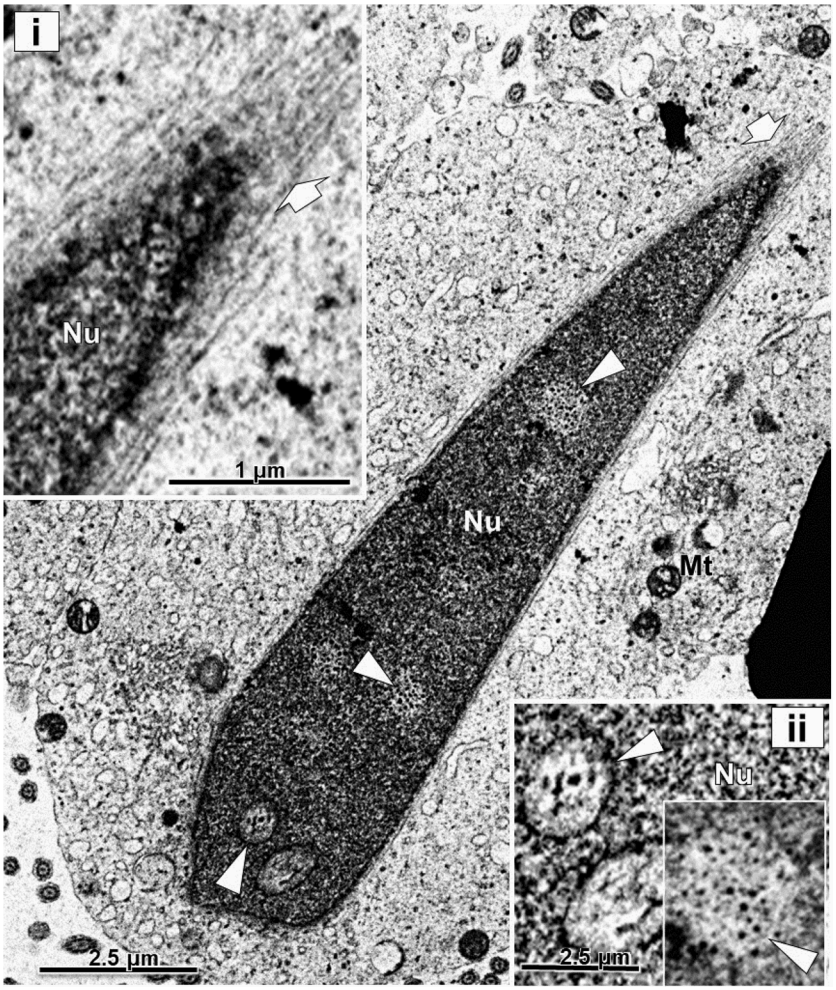

Fig. 4. Step 7 spermatids. The spermatid nucleus appears more electron-dense and elongated than in step 6. The chromatin is dense and granular, with some areas of incomplete condensation (white arrowheads in the main figure and **inset ii** which is a higher power view of two separate electron-lucent areas); longitudinal manchette (white block arrow), nucleus (Nu), mitochondria (Mt). **Inset i** shows the well-developed longitudinal manchette (white block arrow); nucleus (Nu).

Step 8. The nucleus continues to elongate but has become cylindrical in shape. The nuclear chromatin condenses into coarse, round, and electron-dense granules (Fig. 5A and B). The acrosome is conical, with a smoothly blunt apex. Its base is flat and makes contact with an equally flat anterior end of the nucleus. Therefore, the junction between the spermatid nucleus and acrosome is approximately flat, rather than concave. The longitudinal manchette arises from the anterior end of the nucleus, at the level of this interface (Fig. 5B). The neck is formed by the proximal centriole attached to a curved, electron-dense plate that fits into the implantation fossa of the nucleus (see Fig. 7 B for greater detail). Most of the cell cytoplasm trails behind the nucleus, and the longitudinal manchette is well developed and extends caudally beyond the nucleus into the midpiece region. The latter is short, comprising the proximal and distal centrioles arranged typically, perpendicular to each other (Fig. 5C). The annulus remains poorly developed. The immediate post-midpiece segment of the principal piece displays a thicker and greater diameter than in the earlier spermatids due to accumulation of relatively amorphous material between the plasmalemma and the axoneme, signalling the commencement of the formation of an amorphous sheath (Fig. 5C).

Step 9. The cylindrical nucleus becomes gently curved, anteriorly, and the chromatin remains highly electron-dense and granular (Fig. 6). Mitochondria are found alongside the nucleus and in the trailing cytoplasm. The conically shaped acrosome becomes smoothly rounded anteriorly and attached by its base, to the anterior end of the nucleus (Fig. 6, inset i). The manchette, in transverse section, displays, in the main, a single row of microtubules around the nucleus (Fig. 6, inset ii).

Step 10. The nucleus has elongated further with a greater curvature in its anterior half. Its diameter is further reduced, compared to the previous spermatids. The nuclear chromatin has condensed considerably into large, round to oval granules, interspersed with numerous electron-lucent areas (Fig. 7 A). The nucleus is heavily invested by the longitudinal manchette which, in transverse sections, shows several microtubules arranged in multiple layers, and extending beyond the nucleus and midpiece into the trailing cytoplasm of the spermatid (Fig. 7A and B). During the late phase of this step, the longitudinal manchette begins to disintegrate. The centriolar complex is as has been described in step 8, but it is described more fully in this step in a more favourable section of the spermatid. The centriolar complex assumes its final and complete configuration. The neck lies between the proximal centriole and the base of the nucleus. Thus, the proximal centriole anchors the complex to the nucleus by means of an electron-dense plate

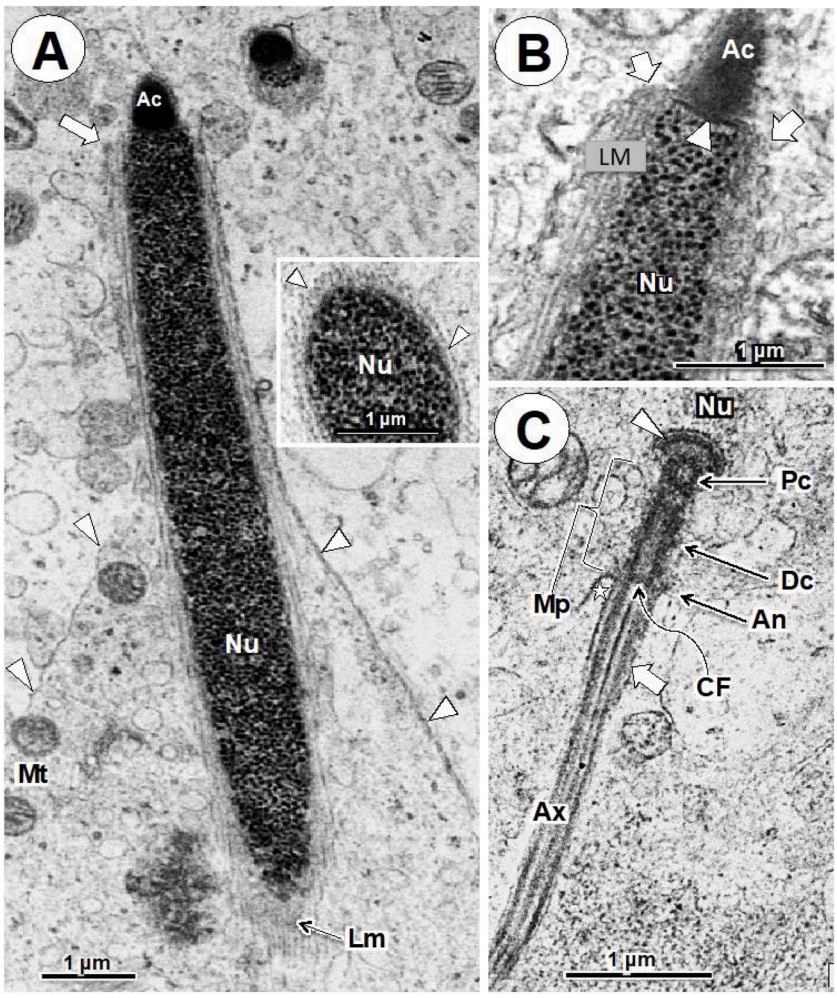

Fig. 5. Step 8 spermatids. **A**. The spermatid nucleus (Nu) is further elongated, with most of the cytoplasm trailing behind; acrosome (Ac), mitochondria (Mt), cell membrane (white arrow heads); white block arrow indicates the origin of the longitudinal manchette (Lm). **Inset** shows the now coarse, dense, chromatin granules and the longitudinal manchette (white arrow heads). **B.** Note the flat acrosomal-nuclear interface (white arrowhead) and the longitudinal manchette (white block arrows) forming at the level of that junction. **C.** The centriolar complex arranged in the typical fashion; centriolar shelf (CF), distal centriole (Dc), proximal centriole (Pc) attached to an electron dense plate (white arrowhead) at the implantation fossa of the nucleus (Nu); annulus (An), midpiece (Mp), flagellar canal (star), axoneme (Ax), developing amorphous sheath (white block arrow).

arising from the anterior surface of the proximal centriole, and from which strut-like electron-dense projections extend anteriorly to a thick, electron-dense, curved plate that fits into the implantation fossa of the nucleus (Fig. 7 B). The distal centriole forms most of the length of the midpiece, extending from the proximal centriole to the annulus, at the junction between the flagellum and cell membrane (Fig. 7B). The distal centriole has thick walls enclosing a lumen that is either clear or occasionally containing amorphous material. At the junction between it and the cell membrane, there is shelf of moderately dense material from which the double central axial fibres arise (Fig. 7B).

Step 11. The longitudinal manchette is lost and the gently curved head is capped by a bullet-shaped acrosome (Fig. 8A and B, inset i). The Sertoli cell tenuously holds on to the acrosome (Fig. 8A). A single, round or oval, electron-lucent area or spot, first observed in step 9, is frequently seen located centrally toward the base of the acrosome (Fig. 8B, inset i). The nuclear chromatin is almost homogenously condensed, except for a few scattered electron-lucent spaces (Fig. 8B, inset ii). Most of the mitochondria are aggregated around the midpiece, between the nuclear base and the annulus (Fig. 8A and B).

Step 12. The spermatid is mature structurally (Fig.10), and only slips of Sertoli cell cytoplasm invest it (Fig. 9A and B). The acrosomal-nuclear junction remains straight, and both the nucleus and acrosome are distinct separate structures, sharing only a common narrow, electron-lucent interface and cell membrane. The nuclear chromatin is fully condensed except for a few scattered electron-lucent spots in the nucleoplasm. The mitochondrial sheath has formed. Sleeves of Sertoli cells hold on tenuously to the spermatid which is almost free in the seminiferous tubular lumen (Figs. 9A and B and 10).

4. Discussion

Spermiogenesis in the cattle egret is generally similar to that of other non-passerine birds, but there are some notable differences. The process of acrosomal development in the egret from steps 1–5 of spermiogenesis follows the pattern seen in most non-passerine birds, such as the rooster (Gunawardana and Scott, 1977), quail (Lin and Jones, 1993), ostrich (Soley, 1996), and turkey (Aire, 2003). Thereafter, the egret acrosome transforms from a round or oval structure to a smooth, bullet-shaped organelle with a bluntly rounded apex and a wider, flat, base. However, the acrosome commences its development in the Golgi complex from a proacrosomal vesicle that is membrane-bound and uniformly homogenous, as has been described for most non-passerine birds (Aire, 2014). The acrosomal vesicle makes only a shallow groove on the nucleus at the contact site, and soon afterwards spreads sideways over a flat, straight modification of the nuclear membrane. As the acrosome develops further, it becomes conical in shape, displaying a rounded, and

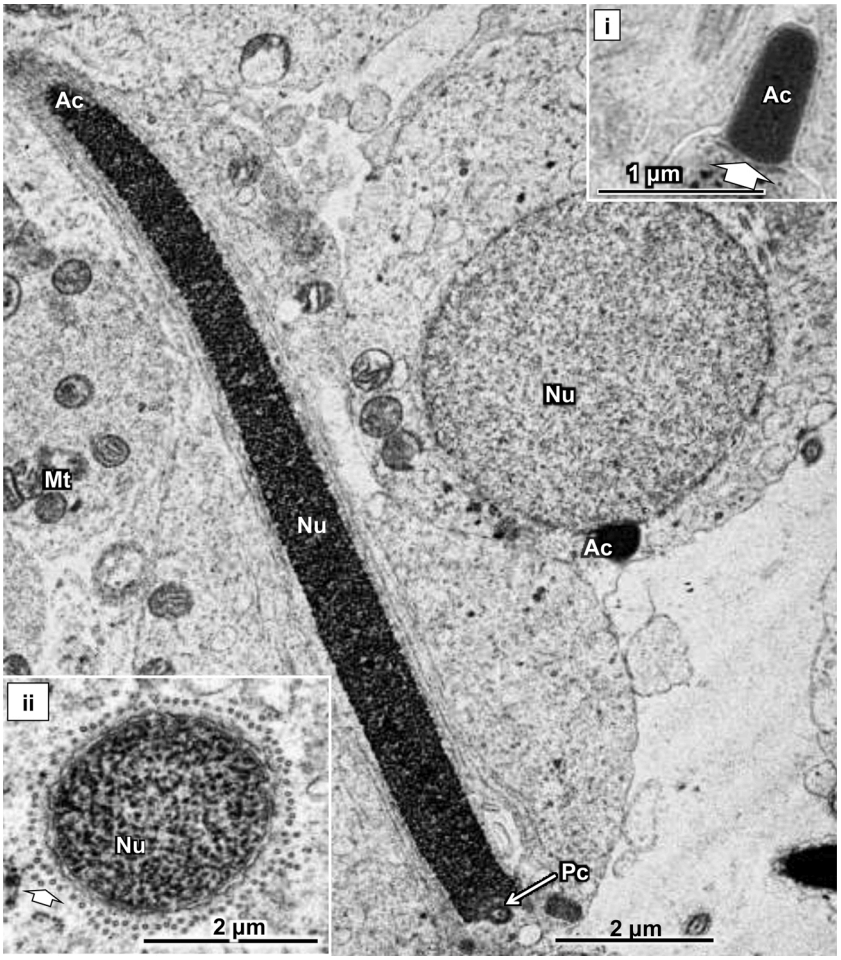

Fig. 6. Step 9 spermatids. The nucleus (Nu) has a slight curvature. Mitochondria (Mt) are found alongside and caudal to the nucleus; acrosome (Ac), proximal centriole (Pc). **Inset i** shows the flat acrosomal-nuclear junction (white block arrow); acrosome (Ac). **Inset ii** is a transverse section of the nucleus (Nu) with the ensleeving longitudinal manchette (white broad arrow).

blunt anterior end, and a flat base. Thus, the acrosome neither overlaps nor indents the anterior part of the nucleus, as occurs in most non-passerine birds; rather, it makes contact with the nucleus at a narrow, *en face*, flat or straight, interface. An anterior nuclear concavity is therefore not formed as in the common swift, *Apus apus* (Jamieson, 2007), woodpecker, *Melanerpes carolinus* (Henley et al., 1978), jacana, *Jacana jacana* (Saita et al., 1983), and the white-naped crane, *Grus vipio* (Phillips et al., 1987). It is clear that the acrosome of the egret is not a hollow, conical organelle that overlies a subacrosomal cone (Jamieson, 2007), which, itself, is an invagination of the subacrosomal space. The acrosome of the egret sperm may not be accurately referred to as an acrosomal complex because it is structurally simpler than that of most non-passerine birds (McIntosh and Porter, 1967; Okamura and Nishiyama, 1976; Gunawardana and Scott, 1977; Lin and Jones, 1993; Soley, 1997; Aire, 2003; du Plessis, 2012; Aire, 2014), in enclosing no subacrosomal cone and in lacking a perforatorium. The significance of the occurrence of an electron-lucent spot in the basal region of the acrosome, first observed in the step 9 spermatid, and retained in the spermatozoon (Roopnarine et al., 2020), is unknown. It appears to be a normal feature of the cattle egret spermatozoon.

As in most birds, the nucleus of the egret spermatid undergoes several transformational configurations as it evolves, but in the cattle egret, the elongating spermatid nucleus does not show the wavy, irregular outline as seen, for example, in the spermatids of the turkey (Aire, 2003), domestic fowl (Gunawardana and Scott, 1977), Japanese quail (Yamamoto et al., 1967), guinea fowl (Aire et al., 1980), ostrich (Soley, 1997) or the crested tinamou (Asa et al., 1986), which feature is probably due to differential subnucleolemmal chromatin condensation, and concomitant constriction of the nucleus (Soley, 1997; Aire, 2007, 2014). These constrictions have irregularly scattered groups of microtubules associated with them (Okamura and Nishiyama, 1976; Lin and Jones, 1993; Soley, 1997; Aire, 2003; du Plessis, 2012), and the microtubular groups are probably the precursors of the circular manchette found in spermatids of these species of birds (see below). Rather, in the cattle egret, the nuclear shape changes from oval in step 5, to conical in step 6, to elongated conical, in step 7 (in the latter two steps the nucleus remains smooth surfaced), and finally gently curved, sickle-shaped in later developmental stages. This is another departure from the usual developmental process of the spermatid in non-passerine birds. As the longitudinal manchette becomes fully established, the maturing nucleus assumes a gently curved, cylindrical shape. As indicated above, the anterior end of the nucleus is flat and does not possess a rostrum, an endonuclear cavity, or perforatorium that are commonly found in most non-passerine birds (Roopnarine et al., 2020). The lack of

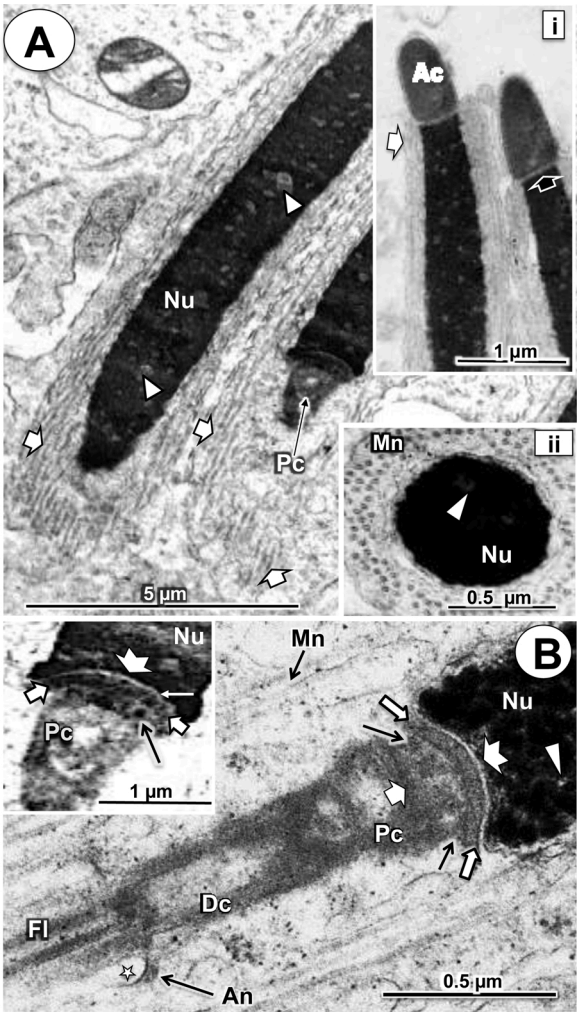

Fig. 7. Step 10 spermatids. **A**. Electron lucent spaces (white arrowheads) lie between the electron dense, tightly packed, large and round chromatin granules; longitudinal manchette (white block arrows); nucleus (Nu), proximal centriole (Pc). **Inset i** shows the longitudinal manchette (white block arrow) and the flat acrosomal-nuclear junction (black block arrow); acrosome (Ac). **Inset ii** is a transverse section of the nucleus (Nu) showing an area of electron-lucence (white arrowhead) and the longitudinal manchette (Mn). **B** and **inset** show the electron dense plate (white block arrow) on the anterior surface of the proximal centriole (Pc); electron dense struts (black arrows) arise from the dense plate and attach anteriorly to a thick, curved, dense, band of material (white, longer, block arrows), which lies adjacent to the implantation fossa (white notched arrow); distal centriole (Dc), flagellum (Fl), flagellar canal (star), annulus (An), nucleus (Nu), electron-lucent areas (white arrowhead). The manchette (Mn) has begun to disintegrate.

a nuclear rostrum and an endonuclear cavity are characteristics shared by the cattle egret with the Common swift, *Apus apus* (Jamieson, 2007), Red-billed woodpecker, *Melanerpes carolinus* (Henley et al., 1978), and jacana, *Jacana jacana* (Saita et al., 1983). Roopnarine et al. (2020) have briefly reviewed the spermatozoa of these species.

Granulofilamentous condensation of chromatin occurs at step 5, where it condenses into dense, rod-like or round chromatin in step 6. These granules become denser, larger, and more numerous, with each subsequent step. It is not clear why chromatin condensation in the egret commences uniformly throughout the nucleus, with no focal or scattered patches of heterochromatin, from step 5 to the mature spermatid, unlike in the ostrich (Soley, 1997) and guinea fowl (Aire et al., 1980). The commencement of granular condensation of the entire nuclear chromatin appears to be contemporaneous with the beginning of elongation of the nucleus and the establishment of the longitudinal manchette, in step 5.

In most non-passerine birds, a circular manchette (CM) is evident in elongating spermatids, from about step 6 of spermatogenesis until it disappears, for example, in the Galloanserae (McIntosh and Porter, 1967; Okamura and Nishiyama, 1976; Gunawardana and Scott, 1977; Lin and Jones, 1993; Aire, 2003, 2014) and ratites (Soley, 1997; du Plessis, 2012). The CM is poorly developed in *Columba* species (Fawcett, 1971; Mattei et al., 1972) and the Cuckoo, *Crotophaga ani* (Saita et al., 1982), but in the egret it is absent, as also in the Swift, *Apus apus* and the Nightjar, *Caprimulgus europaeus* (Tripepi et al., 1991; Jamieson and Tripepi, 2005). The role of the CM in nuclear shaping, during spermiogenesis, has been equivocal. Several investigators, McIntosh and Porter (1967); Okamura and Nishiyama (1976); Gunawardana and Scott (1977); Lin and Jones (1993), and Soley (1997) believe that the circular manchette is definitely involved in nuclear shaping, while Fawcett (1971); Phillips (1976) and Phillips et al. (1987), strongly contest this assertion. It is clear from our observations that the CM is not needed in nuclear morphogenesis, at least specifically, in the cattle egret. Saita et al. (1982) are also of the opinion that this manchette plays no role in nuclear morphogenesis in the cuckoo. This may be why the evolving nuclear shape in the egret is conical between steps 5 and 7 and continues to elongate in this form until step 8 when it becomes cylindrically shaped, by means of forces not yet clearly understood. It is not known if and how the absence of the CM affects nuclear shaping, but it is noteworthy that the earlier series of elongating spermatids of the egret have pyramidal or conical shapes, of varying lengths, not commonly reported for other birds. The absence of the CM in the earlier series of spermatids may be responsible for the regularly smooth, albeit conical, nuclear shapes of the evolving egret spermatid, as opposed to the varying, irregular, shapes of the nucleus in the turkey (Aire, 2003), domestic fowl (Gunawardana and Scott, 1977), Japanese quail (Lin and Jones, 1993), guinea fowl (Aire et al., 1980), the ostrich (Soley, 1997) or the crested tinamou (Asa et al., 1986). Irregular nuclear profiles during early spermiogenesis have been attributed to differential subnucleolemmal chromatin condensation, and concomitant constriction of the nucleus, possibly by clumps of microtubules during the formation of the CM (Soley, 1997; Aire, 2014). However, from step 5 until step 11, the egret spermatid displays a robust longitudinal manchette (LM), which disappears thereafter. Most non-passerine birds, including those discussed above, display the LM at later steps of spermiogenesis. Phillips (1976); Myles and Hepler (1982), and Russell et al. (1991) have shown that experimental disruption of the structure of the manchette caused deformities in nuclear shapes in certain species of animals. Aire (2014) has reviewed the presence and possible functions of both the circular and longitudinal manchette in birds.

The development of the neck and midpiece of the spermatozoon in the egret is similar in most particulars to that described for several non-passerine birds (Aire, 2007). The structure of the neck is similar to the pattern in non-passerine birds, but appears a little more robust in the cattle egret, than in some non-passerine birds, such as the turkey (Aire, 2003), in some species of parrots (Jamieson et al., 1995), and Japanese quail (Aire, 2014). In the cattle egret, a capitulum-like plate of electron dense material lies between the nuclear fossa and the struts of electron dense material, akin to the connecting piece of mammals, running from a thickened plate on the anterior surface of the proximal centriole (Roopnarine et al., 2020). However, among birds, ratites such as the rhea (Phillips and Asa, 1989), ostrich (Soley, 1994) and emu (du

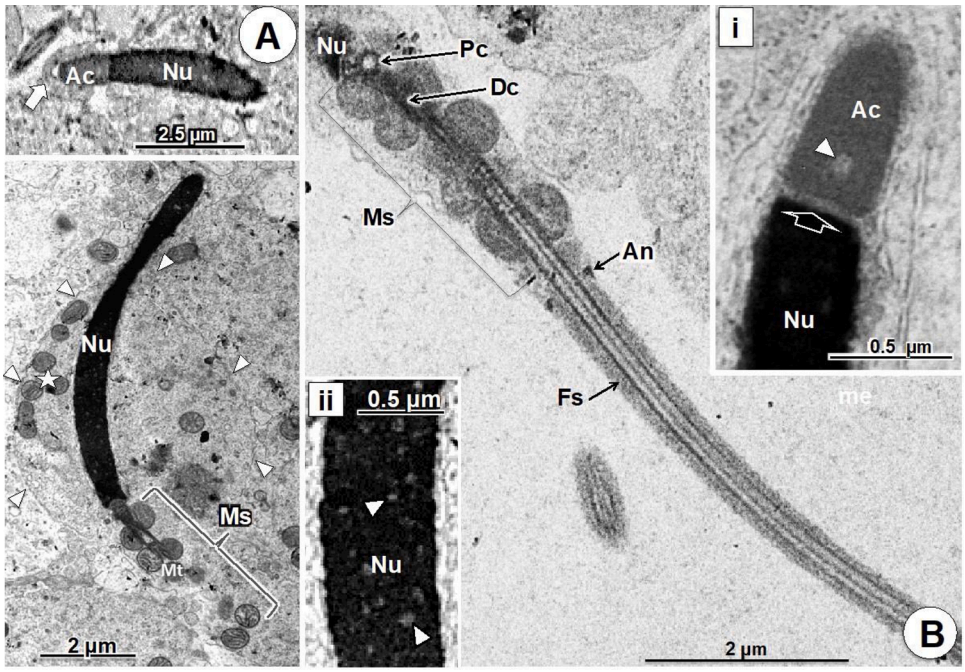

Fig. 8. Step 11 spermatids. **A.** The nuclear chromatin is homogeneously denser; the nucleus (Nu) is gently curved. The longitudinal manchette is absent. Mitochondria (Mt) aggregate around the midpiece forming the mitochondrial sheath (Ms); cell membrane (arrow heads), uncommitted mitochondria (star). The **inset** shows a slip of Sertoli cell cytoplasm (white block arrow) tenuously holding on to the acrosome (Ac); nucleus (Nu). **B.** Mitochondria aggregate around the midpiece forming the mitochondrial sheath (Ms); nucleus (Nu), proximal centriole (Pc), distal centriole (Dc), annulus (An), fibrous sheath (Fs). **Inset i** shows an electron-lucent area (arrow head) within the homogenous, moderately dense, substance of the acrosome (Ac); flat acrosomal-nuclear junction (broad arrow), nucleus (Nu). **Inset ii** shows electron lucent spaces (white arrowheads) within the nucleus (Nu).

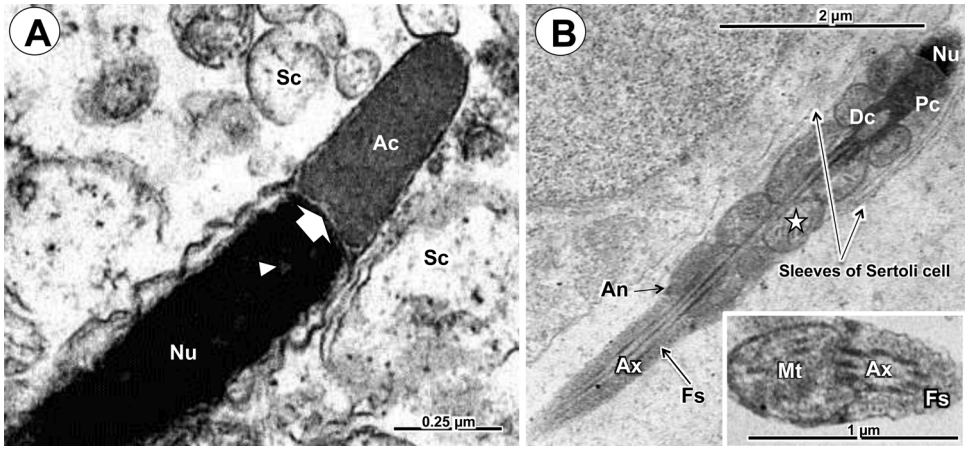

Fig. 9. Step 12 spermatids. **A.** The nucleus and acrosome are separated by a very thin, straight space (broad arrow); acrosome (Ac), nucleus (Nu), Sertoli cell cytoplasm (Sc), electron-lucent space (arrowhead). **B.** Sleeves of Sertoli cell cytoplasm tenuously hold on to the spermatid. The midpiece comprises the neck [between the proximal centriole (Pc) and the implantation fossa of the nucleus (Nu)], the proximal centriole, distal centriole (Dc) and mitochondrial sheath (star), bounded posteriorly by a small annulus (An); axoneme (Ax), fibrous sheath (Fs). The **inset** is a transverse section at the junction of the midpiece and principal piece of the flagellum; mitochondria (Mt), axoneme (Ax), fibrous sheath (Fs).

Plessis, 2012) display the most structurally developed neck. An amorphous sheath is well developed in the egret spermatozoon, as has been seen in several non-passerine birds, except in ratites in which the flagellum exhibits ribs in the form of dense blocks of material (Soley, 1994).

In summary, twelve steps of spermatid development are described for the cattle egret, in which spermiogenesis is generally similar to that in most non-passerine birds, with some remarkable exceptions. As the acrosome develops, it transforms from an oval or button-like figure to a bullet shaped structure, while the nucleus transforms from an oval to, an elongated conical structure which ultimately becomes gently curved. As the spermatid nucleus develops, it fails to exhibit an undulating, irregular outline as seen in the spermatids of other non-passerine orders. Additionally, chromatin condensation in the egret commences uniformly throughout the nucleus, with no focal or scattered patches of subnucleolemmal chromatin condensation. The non-development, and therefore the absence, of a perforatorium, the circular manchette, and a nuclear rostrum, as well as the presence of an approximately flat acrosomal-nuclear junction, are all special features of cattle egret sperm (Roopnarine et al., 2020) resulting from spermatid morphogenesis. Although the focus of this study is on morphogenesis of the spermatozoon of the cattle egret, yet certain striking differences between the egret and other species in different orders are obvious. Most of these structural features are shared, to varying degrees, by certain species of birds, such as the Swift, *Apus apus* and the Nightjar, *Caprimulgus europaeus* (Tripepi et al., 1991; Jamieson and Tripepi, 2005), the jacana, *Jacana jacana* (Saita et al., 1983), the woodpecker, *Melanerpes carolinus* (Henley et al., 1978), and the white-naped crane, *Grus vipio* (Phillips et al., 1987). The absence of a perforatorium in the spermatozoon of the cattle egret and these other species separates them from members of the

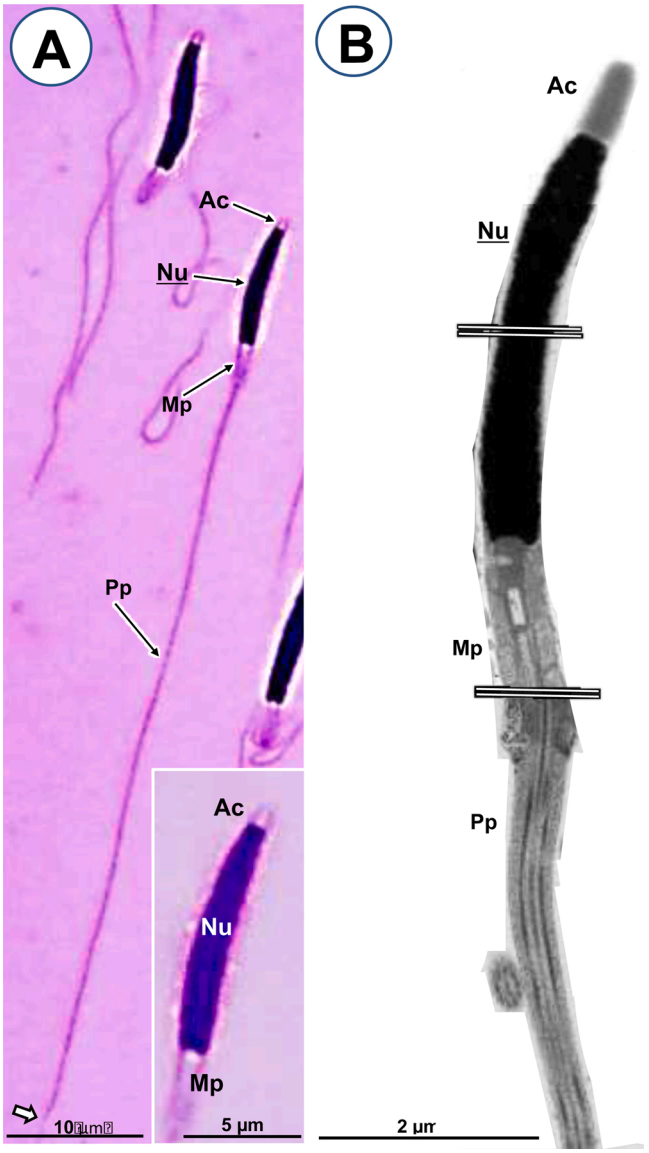

Fig. 10. A. Light micrograph of mature cattle egret spermatozoa showing the acrosome (Ac), nucleus (Nu), midpiece (Mp), principal piece (Pp) and endpiece (white block arrow). **B**. A transmission electron micrograph of a mature cattle egret spermatozoon highlighting the acrosome (Ac), nucleus (Nu), midpiece (Mp) and principal piece (Pp). Modified from Roopnarine, N.H., Gupta, S.K., du Plessis, L., Aire, T.A., 2020. Sperm structure of the cattle egret (*Bubulcus ibis)*. Anat. Histol. Embryol. 00, 1-6 https://doi.org/10.1111/ahe.12586. Reproduced with the permission of Blackwell Verlag GmbH.

Galloanserae. However, they are currently placed in different orders. How close, phylogenetically, are these birds, which share a number of structural features?

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Hong Kong Journal of Occupational Therapy*.

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames. e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Category 1

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Category 2

N. H. Roopnarine, S. K. Gupta, L. du Plessis, T. A. Aire: Drafting the manuscript.

N. H. Roopnarine, S. K. Gupta, L. du Plessis, T. A. Aire: revising the manuscript critically for important intellectual content

Category 3

N. H. Roopnarine, S. K. Gupta, L. du Plessis, T. A. Aire: Approval of the version of the manuscript to be published (the names of all authors must be listed)

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tice.2020.101457.

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