Sperm structure of the Cattle egret (*Bubulcus ibis)*.

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Abstract

It remains a major concern that sperm structure has continued to be poorly investigated and reported in avian species. To our knowledge, sperm structure in the order Pelecaniforme has not been reported. Although McFarlane (1963) reported the study of spermatozoa in two genera and two species of the family Ardeidae, he did not provide an account, or the names of the species examined. The present report on the sperm structure of the cattle egret, *Bubulcus ibis*, is, thus, the first in the order Pelecaniformes (this bird has been placed variably under the order Ciconiiformes, or the order Pelecaniformes). Five sexually mature and reproductively active male cattle egrets were obtained from the wild, humanely euthanized, the reproductive organs dissected out, and tissues from the ducti deferentia were prepared for transmission electron microscopy. The sperm structure of this bird is generally similar to that described for most non-passerine birds. However, the acrosome is a short, conical or bullet-shaped, blunt-ending organelle that lacks a perforatorium. The base of the acrosome is flat and makes contact with the nucleus along, a correspondingly flat plane. The nucleus, thus, ends anteriorly in a flat plane devoid of a concavity or a rostrum, and an endonuclear canal. The acrosomal and nuclear features of this bird are, therefore, main deviations from the situation in the non-passerine clade of birds.

Keywords - Acrosomal variations, cattle egret (*Bubulcus ibis*), spermatozoa, ultrastructure, wild non-passerine, Pelecaniforme.

1. Introduction

Studies on the male reproductive biology of birds are grossly lacking, for a class of vertebrates that constitutes more than half of all vertebrates (Aire, 2007; Jamieson, 2007), and particularly, the reports on sperm structure are very few, and mainly on domesticated species of birds. Sperm structure is now regarded as important in avian phylogeny (Jamieson, 2007).

However, ultrastructural studies of avian spermatozoa have mainly focused on domesticated, non-passerine birds (Jamieson, 2007), in orders such as galliformes, anseriformes, struthioniformes, as well as a few other exotic birds, including the budgerigar (Humphreys, 1975), jacana (Saita *et al.,* 1983), white-naped crane (Phillips *et al*., 1987), turtle-dove (Mattei *et al.,* 1972), and the cuckoo (Saita *et al.,* 1982). The spermatozoa of these birds are generally of the sauropsid type (Humphreys, 1972), characterised by a small acrosome, a perforatorium, a well-defined midpiece containing two centrioles, and a long, mobile flagellum (Humphreys, 1972; Baccetti *et al.,* 1980; Aire, 2007; Jamieson, 2007). However, some important differences occur in the structure of spermatozoa among several orders, some of which are of phylogenetic relevance.

It is remarkable that no information is available on sperm structure and its development in the cattle egret (*Bubulcus ibis)*, which is commonly found in Africa, India, Australia, Spain and Japan (Brown *et al.,* 1982), and closely associated with domestic livestock, such as cattle, goats and sheep (Heatwole, 1965). This study reports structural features of the spermatozoon of this bird, with special emphasis on observed deviations from the typical sperm structure in non-passerine birds.

2. Materials and Methods

Tissues from the ductus deferens were obtained from five, adult, sexually-active, male Cattle egrets, which were humanely trapped 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-18006- R) of the St. George's University. After being captured by means of mist nets and loop-rope traps, they were humanely euthanized by an overdose of isoflurane anaesthetic 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 rinse the blood 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 0.2M s-collidine for 1 hour. 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 minutes, and then, lead citrate for 10 minutes. The sections were subsequently examined in a Jeol 100 CX transmission electron microscope, operated at 80kV. For light microscopy, semen was quickly and gently milked out of the ducti deferentia and smears were made on microscopic glass slides. The smears were air-dried, routinely stained, using Romanowsky (Diff-Quick) stain, and examined with an Olympus BX43 light microscope, using 100x oil immersion lens. Measurements of sperm length (total length and length of the acrosome, nucleus, midpiece and

flagellum) were performed using the Olympus software tool accompanying the microscope's camera. For sperm length, smaller, straight segments were measured and summated.

3. Results

The structure of the egret spermatozoon (Fig.1, A & B), models that of the sauropsida clade, being highly elongated. It measures 71.38 µm ± 4.28 µm in length. The gently curved, sickle-shaped head, comprises the relatively short, conical acrosome, which makes contact with the anterior end of the nucleus. The midpiece is short, cylindrical in outline and wider in diameter than the head, anteriorly, and the principal piece of the tail, posteriorly. The principal piece is slender, long, and decreases gradually in diameter proximo-distally until it terminates at its junction with the short end piece (Fig.1, A & B).

3.1. The acrosome

The head of the spermatozoon is curved gently and sickle-shaped. The acrosome is a relatively short, conical, blunt-ending, organelle. It is 0.82 µm ± 0.11 µm in length, and 0.43 µm ± 0.08 µm in width, at its posterior end or base. Its maximum width, at its base, is greater than that of the apex of the nucleus with which it makes contact at a horizontal, flat plane (Fig. 2, Insets i & ii). It tapers toward the free, blunt-tipped, anterior end or apex, and it is filled with a homogenous material of moderate electron density. An occasional, electron-lucent, vacuole-like space is observed within this substance (Fig. 2, Insets i & ii).

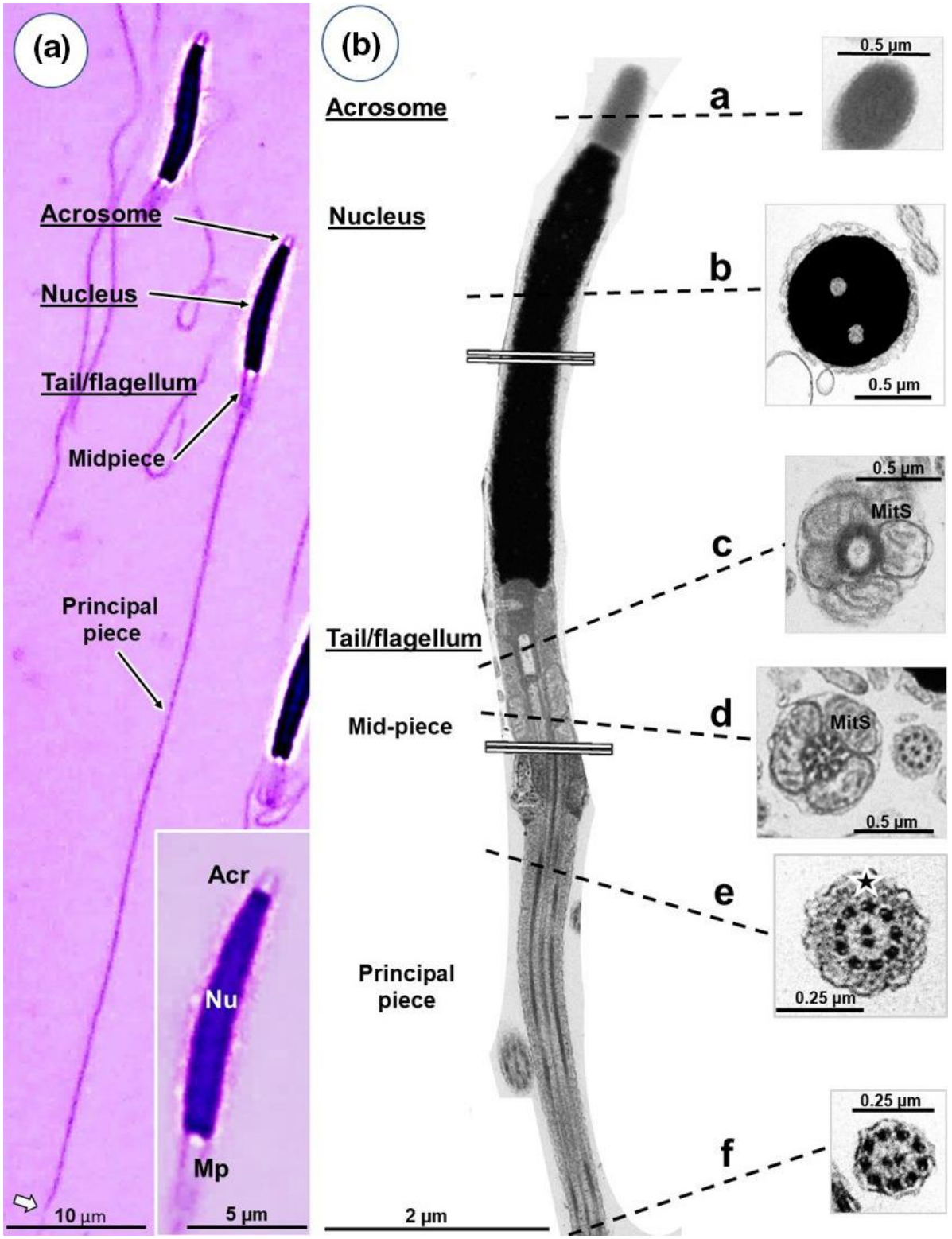

FIGURE 1. (a) Light micrograph of the egret spermatozoon, showing the acrosome, nucleus, midpiece, flagellum, and the endpiece (white blocked arrow). (b) An electron micrograph of a longitudinal section of the egret spermatozoon. (Inset a) transverse section (T.S.) of the acrosome, (inset b) T.S. of the nucleus, (inset c) T.S. of the distal centriole, (inset d) T.S. of post-centriolar part of the midpiece, (inset e) T. S. of the initial part of the principal piece of the flagellum, star = amorphous substance, and (inset f) T.S. of the middle part of the principal piece of the flagellum

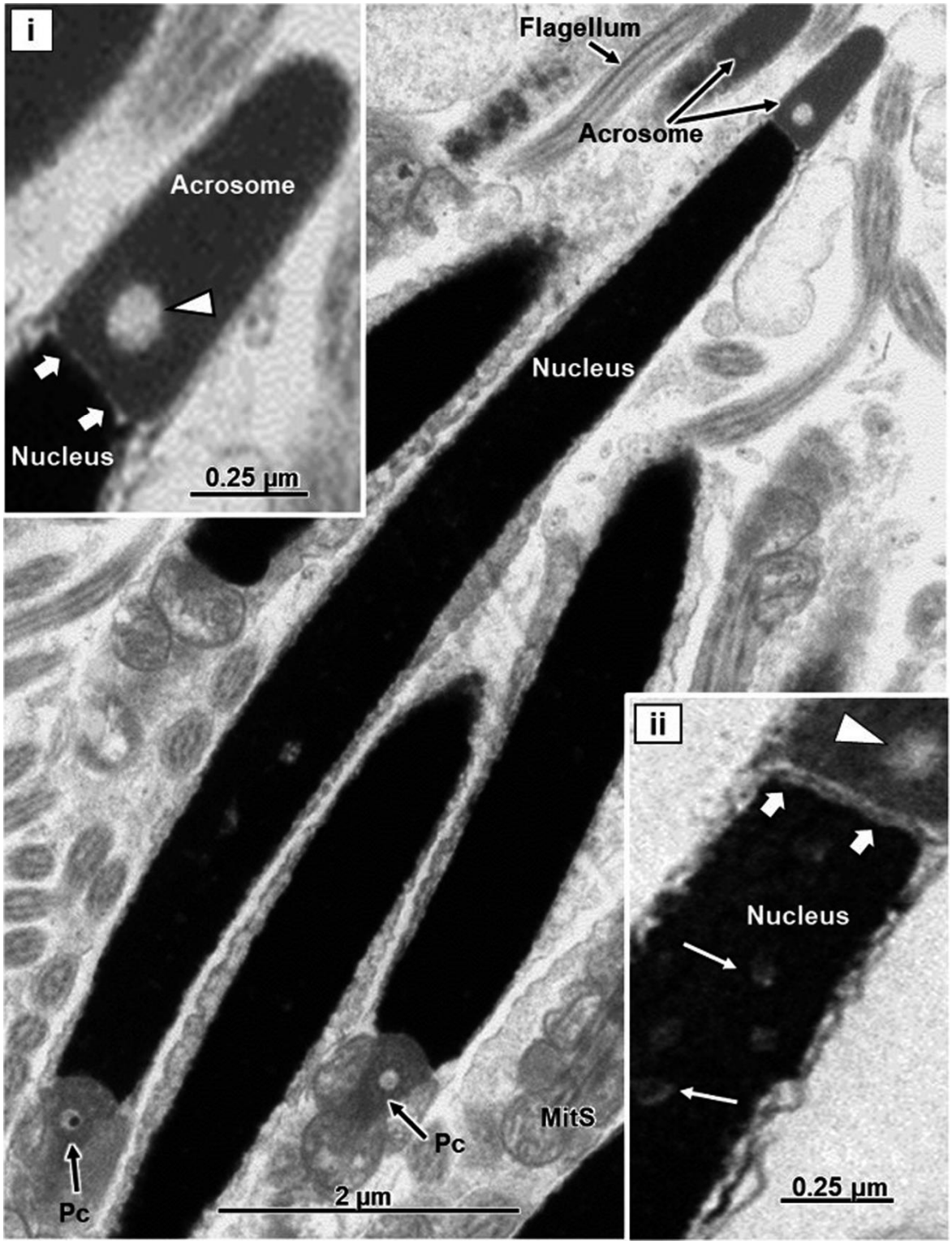

FIGURE 2. Ultrastructure of longitudinal sections of the heads of spermatozoa showing the acrosome, nucleus, and the proximal centriole (Pc). (Inset i) shows the flat, uniform, interface between the acrosome and nucleus (white blocked arrows); white arrowhead = electron-lucent vacuolar space in the acrosome. (Inset ii) shows randomly scattered electron-lucent spaces between dense chromatin granules within the nucleus (white arrows); white blocked arrows = the flat acrosomal-nuclear interface; white arrowhead = vacuolar space in the acrosome

3.2. The nucleus

The nucleus is relatively long and cylindrical and spans 9.81 µm ± 0.64 µm in length and 1.31 µm ± 0.38 µm in width, at its widest, central part. It is gently curved and filled with strongly electron-dense, compact, large chromatin granules, with interspersed areas of electron-lucency in the nucleoplasm (Fig. 2, Inset ii). The anterior end of the nucleus is flat and narrower than the main body of the nucleus, which gradually increases in diameter caudally. From its mid-portion, it gradually narrows, once again, towards its posterior end (Fig. 2). The base of the nucleus, which is narrower than the anterior end of the mid-piece, is moderately concave and constitutes the implantation fossa (Fig. 3 A).

3.3. The centriolar complex and the midpiece

The centriolar complex comprises the proximal and distal centrioles, with the proximal centriole connecting to the nucleus at the implantation fossa (Fig. 3, A & B). Both centrioles display the characteristic structural configuration. The proximal centriole attaches to the nucleus by means of a plate of an electron-dense material on its anterior axial surface. Several, electron-dense struts of material extend from this plate toward another homogenous, electron-dense, thick, curved plate, akin to the basal plate of mammals, but not as well defined as the capitulum in mammals (Fig. 3 B, Inset i). The latter attaches to the concave base of the nucleus. The lumen of the proximal centriole is either devoid of any material or may contain some amorphous moderately electron-dense material. The anterior portion of the distal centriole has thick walls enclosing an open space or cavity that occasionally contains amorphous material (Fig. 3, A & B). Distal to this is a transverse shelf of moderate electron-density

and from which the two central axial fibres originate. This “centrio-flagellar shelf” marks the end of the distal centriole and the beginning of the axoneme (Fig. 3, A & B).

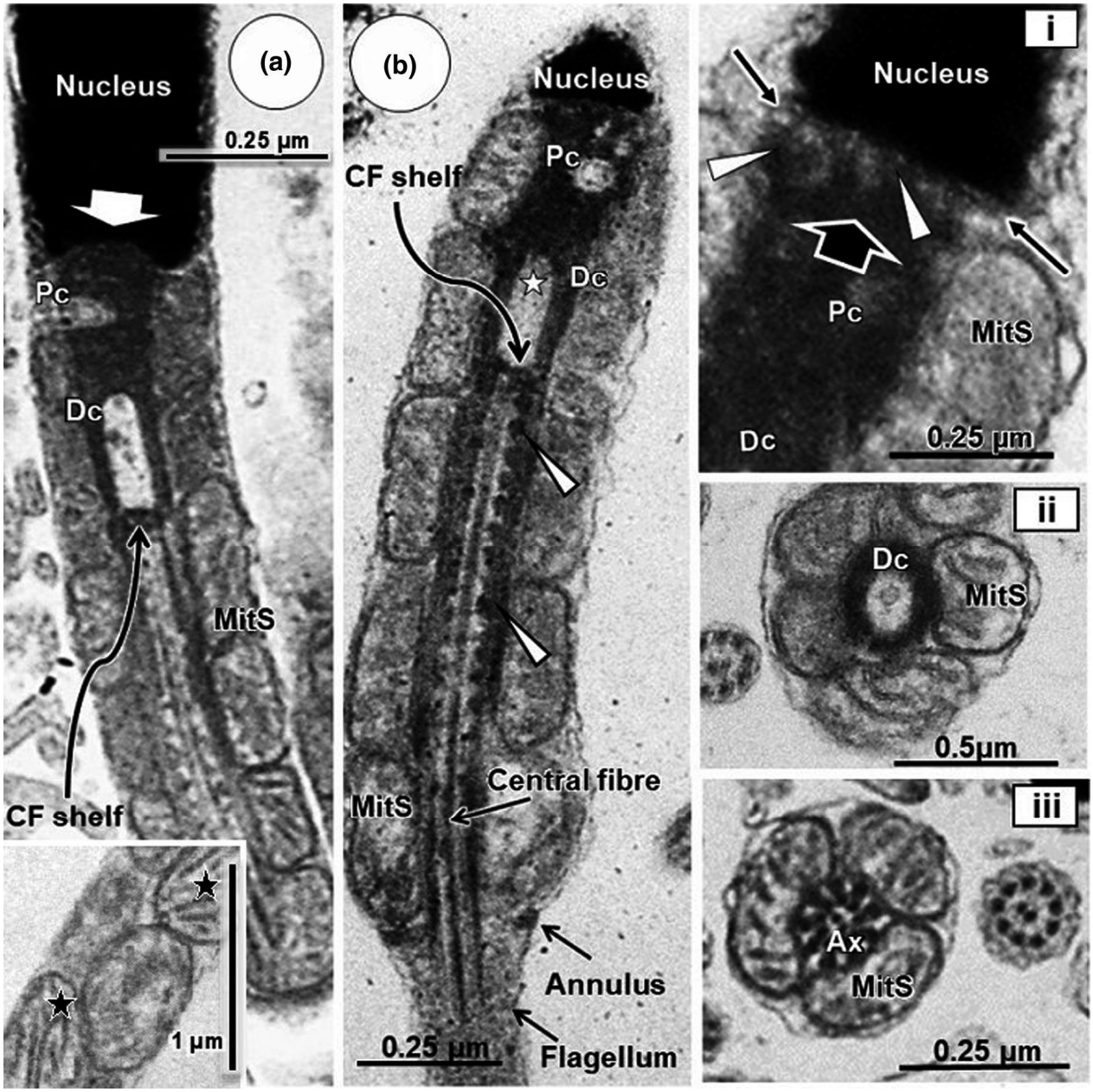

FIGURE 3. (a) Shows the concave implantation fossa of the nucleus (broad arrow), proximal centriole (Pc), distal centriole (Dc), mitochondrial sheath (MitS), and the centrio-flagellar shelf (CF); the inset shows the mostly longitudinal cristae (star) of the mitochondria. (b) Shows the cavity (star) of the distal centriole, mitochondrial sheath (MitS), post-centriolar part of the midpiece (arrow heads), centrio-flagellar shelf (CF), the annulus and flagellum. (Inset i) shows thin struts (white arrow heads) linking the electron-dense plate (black broad arrow) on the anterior surface of the proximal centriole (Pc), and the moderately thick, electron-dense, curved plate (black arrows) adjoining the nuclear fossa. (Insets ii and iii) are transverse sections of the midpiece, (ii) at the level of the distal centriole (DC), and (iii) at the post-centriolar part of the midpiece; Ax, axoneme

The midpiece averages 3.26 µm ± 0.35 µm in length and 0.74 µm ± 0.14 µm in width, across its widest part, which coincides with its center. It is wider than the head, anteriorly and the principal piece, posteriorly. The midpiece displays five to six rows of mitochondria longitudinally, containing three to five mitochondria in transverse sections (15-30 in total), and articulated to form a helix around the proximal centriole, distal centriole and the initial part of the axoneme (Fig. 3, A & B). The mitochondria appear as oval, round, elongated or sometimes, flattened rectangles. The cristae are mostly longitudinally arranged in an electron-dense matrix (Fig. 3, A & B). A few mitochondria may appear to have slightly oblique or transverse cristae. A moderately developed, triangular annulus demarcates the end of the midpiece from the rest of the tail (Fig. 3 B).

3.4. The principal piece and end piece of the flagellum

The flagellum or tail forms the longest segment, being 62.89 µm ± 1.98 µm long and comprising 83.81 ± 1.81 % of the entire sperm length. Immediately distal to the annulus, the diameter of the principal piece narrows proximo-distally, throughout its length (Fig. 4). It consists of the typical 9 + 2 microtubular arrangement (Fig. 4, Inset ii). The axoneme is surrounded by an amorphous fibrous sheath, which is thickest at the beginning of the principal piece, and gradually tapers posteriorly, becoming very thin towards, and terminating at, the end-piece (Fig. 4, Insets, ii & iii). The surface of this sheath is corrugated, and its contents is amorphous (Fig. 4, Inset i). The endpiece is short and thin, and its axonemal configuration is disrupted (Fig. 4, Inset iv).

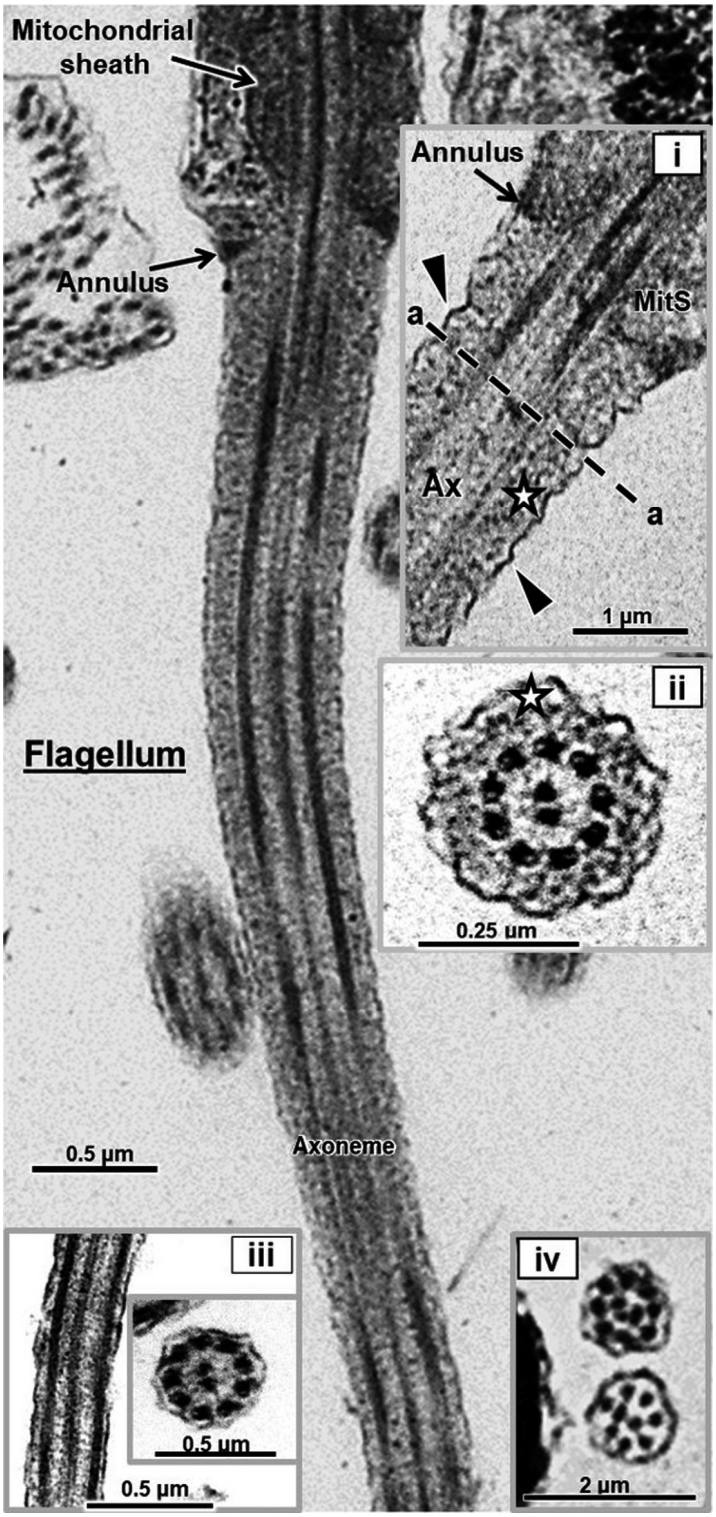

FIGURE 4. Shows aspects of the tail of the egret spermatozoon including, the annulus, axoneme and mitochondrial sheath. (Inset i) shows the amorphous substance (star) exhibiting a corrugated outline (black arrowheads), annulus, axoneme (Ax), and mitochondrial sheath (MitS). (Inset ii) is a transverse section of the flagellum at the broken line (a) of (inset i); star = amorphous substance. (Inset iii) is a transverse section of the posterior region of the flagellum, showing the 9 + 2 microtubular arrangement. (Inset iv) shows transverse sections of the endpiece, displaying the irregularly arranged microtubules surrounded by the plasmalemma

4. Discussion

The spermatozoon of the cattle egret conforms with the general features of the spermatozoon of non-passerine birds, but a number of important variations occur. The scope of this report is limited mainly to the structure of the spermatozoon of the cattle egret, but with the hope that relevant features would be available to phylogeneticists in their continuing evaluation of phylogenesis of birds.

There are no reports available of studies on the ultrastructure of the sperm of any member of this order Pelecaniformes. McFarlane (1963) indicated that he examined the structure of the sperm in two genera and two species of the family Ardeidae, but he did not give any account. However, in the egret, the acrosome is a short, conical, round-tipped, organelle with a homogeneously and moderately electron-lucent content. This matrix displays a vacuole occasionally, but its significance is unknown. Unlike in most non-passerine birds (Jamieson, 2007), the egret acrosome lacks a perforatorium, and its contact with the anterior end of the nucleus is flat. Therefore, the nucleus of the egret sperm neither has an endonuclear canal nor a rostrum, which are to be found in most non-passerine birds. Although the nucleus of the sperm of the Psittaciformes makes this type of connection with the acrosome (Jamieson, 2007), nevertheless, there is a perforatorium linking the acrosome with the nucleus. There are some other bird species that have acrosomes that are close to the acrosome of the egret in shape and conformation, as well as lacking the perforatorium. For example, the acrosome of the *Apus apus* (Jamieson, 2007) and the woodpecker, *Melanerpes carolinus* (Henley *et al.,* 1978) is a slender, smooth, pointed, cone, but that of the *Jacana jacana* is button-like (Saita *et al*., 1983), while that of the egret is short, conical, and blunt-ending. Other species with the short or round, the so-called button or subspherical, acrosomes are the white-naped crane,

Grus vipio (Phillips *et al*., 1987), and several Charadriiformes (Fawcett, 1971). However, the spermatozoa of these species, including the *Jacana jacana*, have rounded acrosomal bases which fit into anterior, receiving, concavities of the nuclei. The acrosome of the egret, relative to these other species, therefore, appears unique. However, in the egret, as in these other species, the absence of a perforatorium is a noteworthy structural deviation from the paleognaths and galloanserae.

The nucleus of the egret spermatozoon gradually tapers to both ends and is narrower than the flat base of the acrosome, anteriorly, and the beginning of the midpiece, posteriorly. In most non-passerine birds, but not in the egret, a shoulder of the nucleus appears to support the posterior rim of the acrosome, as in the domestic fowl (Jamieson, 2007). The conformation of the nuclear chromatin is generally similar to that described for most birds, in being densely heterochromatic and granular, as in the rhea (Philips and Asa, 1989), ostrich (Soley, 1993), domestic fowl and quail (Jamieson, 2007), drake (Maretta, 1975), Japanese quail (Woolley, 1995), turkey (Aire, 2003), and in certain members of the Psitaciformes, e.g., the cockatiel, *Nymphicus hollandicus* (Jamieson *et al.,* 1995). The basal nuclear fossa or implantation fossa is a moderately developed concavity in the neck, housing the proximal centriole of the centriolar complex and articulating electron-dense materials. The structure is simple, displays certain elements of the system in mammals, albeit rudimentarily, and appears relatively better developed or more robust in the egret than in several members of the Neoaves.

The midpiece of the cattle egret spermatozoon shows no remarkable features relative to other birds. The mitochondrial sheath comprises 15 to 30 mitochondria in the egret, 11-12 in the duck (Simoes *et al.,* 2011), 25-30 in the chicken, turkey and guinea fowl (Lake *et al*., 1968; Marquez and Ogasawara, 1975; Thurston and Hess,

1987; Phillips and Asa, 1989), 20 in the ostrich (Soley, 1993) and 1400 in the Japanese quail (Korn *et al.*, 2000). The distal centriole is short and occupies the anterior one-fifth of the length of the midpiece. In comparison, the distal centriole of ostriches and ducks is long and traverses the entire length of the midpiece (Soley, 1993; Simoes *et al.,* 2011). The distal centriole ends at a transverse shelf, which we refer to as the "centrio-flagellar shelf", and from which the two axial fibrils arise. The microtubular content and organization are as described for the turkey, duck, rooster, Japanese quail and the guinea fowl (Aire and Soley, 2003; Aire, 2007). The midpiece terminates at a small, poorly developed annulus, as in higher non-passerine birds (Jamieson, 2007), but unlike in the paleognaths (Philips and Asa, 1989; Bacetti *et al.,* 1991; Soley, 1993) and several lower non-passerine birds, such as the duck, domestic fowl, turkey, guineafowl, and Japanese quail (Thurston and Hess, 1987; Woolley, 1995; Jamieson, 2007) in which it is well developed.

The principal piece possesses a wide amorphous sheath displaying circular or transverse rings or hoop-like configurations on its external surface. As in those birds that bear this sheath, that of the egret gradually decreases in diameter, posteriorly. In the Mallard, *Anas platyrhynchos*, the amorphous sheath appears to be unique in presenting an inner dense and an outer, less dense portions (Maretta, 1975). In members of the Struthioniformes, the fibrous sheath bears prominent ribs (Soley, 1993; Philips and Asa, 1989). The fibrous sheath is also present in some birds that have short, conical, acrosomes without the perforatorium, such as the *Jacana jacana,* Charadriiformes (Saita *et al.,* 1983), *Apus apus* (Jamieson and Tripepi, 2005), the red-billed woodpecker, *Melanerpes carolinus* (Henley *et al.,* 1978), the cuckoo and the smooth-billed ani, *Crotophaga ani* (Saita *et al*., 1982). But, the sperm of the white necked crane, *Grus vipio*, lacks the amorphous sheath (Philips *et al.*, 1987), as do

members of the Columbriformes (Jamieson, 2007) and Psitaciformes; for example, the Budgerigar, *Melopsittacus undulates*, although possessing a perforatorium, has an acrosome that makes an *en face* articulation with the anterior end of the nucleus (Jamieson *et al.,* 1995).

In conclusion, the structure of the sperm of the cattle egret generally conforms with that of the Galliformes and Anseriformes, or the so-called Galloanserae, and of other members of the Neoaves. The main differences in sperm structure between the egret and most non-passerine birds include a shorter, conical acrosome, with a free, rounded anterior end and a flat base, as well as the absence of a perforatorium, an endonuclear canal and a nuclear rostrum, in the egret.

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Conflicts of interest

There are no conflicts of interest.

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