

**SPERMIOGENESIS, SPERM ULTRASTRUCTURE  
AND REPRODUCTIVE TRACT MORPHOLOGY IN  
CICADAS: IMPLICATIONS FOR SYSTEMATIC  
RELATIONSHIPS**

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## ABSTRACT

Sperm structure in five species of cicadine cicadas (*Albanycada albiger*a, *Azanicada zuluensis*, *Platypleura capensis*, *P. hirtipennis* and *Pycna semiclara*) and five species of cicadettine cicadas (*Melampsalta leucoptera*, *Quintilia walkeri*, *Stagira simplex*, *Xosopsaltria thunbergi* and *Monomatapa matoposa*) was investigated by light and electron microscopy. In addition, spermiogenesis in cicadas was described; the information was derived from two cicadettines (*Diceroprocta biconica* and *M. matoposa*) and three cicadines (*Kongota punctigera*, *P. capensis* and *P. semiclara*). Mature spermatozoa of all species investigated are elongate and filiform, consisting of three distinct regions: the head (acrosome and nucleus), mid-piece and tail. All species produce more than one discrete length of nucleated, motile sperm, a form of sperm polymorphism termed polymegaly. Polymegaly is expressed in three ways; sperm have uni-, bi- or trimodal nucleus and tail lengths. Besides the differences in length, there are also notable differences in the size of nuclei. The anterior parts of sperm heads are embedded in an elongate homogenous matrix forming spermatodesmata.

The conical acrosome is deeply invaginated posteriorly, and sits on top of the nucleus. The acrosomal contents are differentiated internally with a tubular substructure and a subacrosomal space. The anterior of the nucleus intrudes into the posterior section of the subacrosomal space. Anteriorly the acrosome is laterally flattened; posteriorly it extends as two tubular processes on either side of the nucleus that gradually decrease in diameter. The homogeneously electron-dense nucleus is pointed anteriorly and is generally cylindrical, although posteriorly there is a lateral invagination that extends part-way along the nucleus. This invagination houses fine granular material of the putative centriolar adjunct which does not form in close proximity to the centriole and hence may not be a true centriolar adjunct. The lamellate disposition of the centriolar adjunct material within the sperm-midpiece of cicadettine cicadas is distinct, and separates these cicadas from their cicadine counterparts in which the centriolar adjunct material is non-lamellate. Vesicle-like elements that are associated with both the posterior nucleus and the centriolar adjunct are also found within the invagination. Immediately posterior of and adjoining the centriolar adjunct is a pair of mitochondrial derivatives that are

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elongated and extend for almost the entire length of the tail. Except for size the architecture of short and long spermatozoa is generally similar in all species. The absence of accessory bodies in cicada sperm suggests that within the Cicadomorpha, the families Cicadidae and Cercopidae are closely related. Only long nuclei were observed in the fertilized eggs of *A. zuluensis* indicating that sperm with long nuclei might be favoured for fertilization.

Spermiogenesis involves: (a) development of the acrosome from a proacrosomal granule; (b) development of the nucleus, characterized by elongation and streamlining with a simultaneous condensation of chromatin; (c) development of the axoneme from the centriole; (d) amalgamation of individual small mitochondria to form elongated mitochondrial derivatives in which cristae are arranged into regularly spaced lamellae; and (f) elimination of cytoplasm. The presence of a manchette, a transient microtubular organelle, which surrounds the acrosome, nucleus and mitochondrial derivatives, is a characteristic feature of spermiogenesis.

The gross morphology of the reproductive tract in both male and female cicadas exhibits an organization similar to that in most oviparous insects. The non-functional spermatheca is the only exceptional feature in the female reproductive tract. Its role has been taken over by the common oviduct which, subsequently, has become modified into a swollen, differentiated structure with a dual role of receiving oocytes from the paired ovaries and storage of spermatozoa. Testis mass varies between cicada species; this variation might be linked to the intensity of sperm competition which has been found to be positively correlated with relative investment in spermatogenesis. Based on the preliminary findings of this study, *K. punctigera*, with its larger testis relative to body size, would be the ideal candidate to show the greatest levels of sperm competition. Accessory glands in both male and female *A. zuluensis*, *D. biconica*, *P. hirtipennis* and *O. quadraticollis* are very long; this character might be of phylogenetic significance. Despite being notoriously refractory spermiocladistics is potentially valuable in systematic and phylogenetic studies of cicadas, especially at the subfamily level.

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## PREFACE

This work is divided into three investigations:

- ❖ Sperm structure in the cicadine and cicadettine cicadas.
- ❖ Spermiogenesis in cicadas.
- ❖ Morphology of the reproductive tracts in cicadas.

Some of the work from the first investigation has been published in *Tissue and Cell*:

Chawanji, A.S., Hodgson, A.N. and Villet, M.H. 2005. Sperm morphology in four species of African platypleurine cicadas (Hemiptera: Cicadomorpha: Cicadidae). *Tissue and Cell*, **37**: 257-267.

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---God bless you all---

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## DECLARATION

This thesis is the result of the author's original work except where acknowledged or specifically stated in the text. It has not been submitted for any other degree or examination at any other university or academic institution.

.....  
Abraham Simbarashe Chawanji  
August 2006

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He who finds a thought that lets us penetrate even a little deeper into the eternal mystery of nature has been granted great grace. He, who in addition, experiences the recognition, sympathy and help of the best minds of his time, has been given almost more happiness than a man can ever bear.

Albert Einstein (1879-1955)

# **CHAPTER ONE**

## **GENERAL INTRODUCTION**

## 1.1. THE SIGNIFICANCE OF SPERM MORPHOLOGY

In recent years the value of comparative spermatology in studies of animal taxonomy and phylogeny has become clear. Spermatozoa are amongst the most diverse of cell types, with an external morphology and ultrastructure that often provides reliable clues for distinguishing taxa and examining phylogenetic relationships (Dallai, 1979; Jamieson, 1987; 1991; Dallai and Afzelius, 1990; 1995; Jamieson *et al.* 1995; 1999; Carpuccio *et al.*, 1995; Dallai *et al.*, 2001b; Lino-Neto and Dolder, 2001). It appears that every animal species has spermatozoa with unique characteristics. For example, spermatozoa from two animal species may be similar in shape but are never quite identical (e.g. Hodgson and Bernard, 1988). This is because species-specificity resides not only in the genetic material, which the spermatozoon carries in its nucleus, but is also imprinted in the morphology of the cell itself (Baccetti and Afzelius, 1976).

The phylogeny of the cicadas is currently littered with problems of taxonomic placement and assignment of species to tribes and genera. Extensive studies, which may involve a combined scrutiny of morphological and molecular data, are necessary to resolve these taxonomic problems. For example, the morphology of the spermatozoa in cicadas has received very little attention. This is unfortunate, considering the potential of sperm characters in the construction of animal phylogenies. The rapid and divergent nature of sperm evolution, as with genitalia, is attributed to intense selection on sperm morphology (Joly *et al.*, 1989; Simmons, 2001). In situations where sperm compete for access to ova, any trait of an individual sperm that enhances its success in fertilization over its competitors (be it swimming speed, longevity, or ability to fasten to and penetrate the egg) will be favoured in the male possessing it (Simmons, 2001). In essence, sperm competition is now

widely recognized as a powerful form of sexual selection (Sivinski, 1980; 1984; Birkhead and Møller, 1998; Simmons, 2001), and therefore able to influence sperm morphology.

## **1.2. SPERM MORPHOLOGY**

The main constituents of most fertilizing sperm cells are (a) a nucleus (b) an acrosome (c) mitochondria and (d) a flagellum. A general type of sperm architecture, the “ect-aquasperm”, occurs among those animal species that have retained the primitive mode of releasing spermatozoa into water (Baccetti and Afzelius, 1976; Rouse and Jamieson, 1987; Baccetti, 1998). Ect-aquasperm are small cells, each having a conical acrosome, a short and usually round or cylindrical nucleus, a mid-piece comprised of a ring of spherical mitochondria surrounding two centrioles, the proximal and distal, and a single simple flagellum with a 9 + 2 arrangement of microtubules (Baccetti and Afzelius, 1976; Rouse and Jamieson, 1987; Healy, 1995). This cell type is quite distinct from any somatic cell and remarkably, is produced by quite unrelated animals like bivalves, cnidarians, horseshoe crabs, oysters, polychaetes, sea urchins and the lancelet (Baccetti and Afzelius, 1976). It appears that “ect-aquasperm” has undergone neither a marked development nor a reduction during metazoan development and presumably the same genomes are responsible for the formation of this cell type wherever it occurs (Baccetti and Afzelius, 1976).

Across the animal kingdom there are some major variations in sperm shape, size, length and ultrastructure. The greatest diversity is found in the invertebrates, especially among the insects (Sivinski, 1984; Jamieson *et al.*, 1999; Simmons, 2001). Most higher insects have long and filiform spermatozoa with the following typical features: (a) a generally slender shape with an extremely elongated head; (b) a 1-, 2- or 3-layered acrosomal complex; and

(c) an exceedingly long tail comprising a pair of mitochondrial derivatives, which flank a central axoneme. Each derivative, usually with a paracrystalline core, is a polymer, having developed from a fusion of several individual mitochondria. Generally, the axoneme has a 9 + 9 + 2 arrangement of microtubules i.e. 9 singlet peripheral accessory tubules, 9 doublets (or singlets near the distal end) and 2 central microtubules (Baccetti, 1998). In some insect species, however, the spermatozoon may be conical, barrel-shaped, ribbon-like, spiny, thumbtack-like, fusiform, lenticular, amoeboidal, discoidal (Jamieson, 1987) or even hook-headed (Baccetti, 1987). In the majority of insects, any one of the main constituents of sperm may be absent or dominant (Jamieson *et al.*, 1999; Simmons, 2001). For example, phasmid spermatozoa lack mitochondria (Baccetti *et al.*, 1973b), whereas mitochondria constitute about 80% of the sperm volume in the water bug *Notonecta glauca* (Afzelius *et al.*, 1976). Some caddisflies (order Trichoptera) have aflagellate spermatozoa (Dallai and Afzelius, 1994; 1995), yet there are a hundred flagella in the sperm of the termite *Mastotermes darwiniensis* (Baccetti and Dallai, 1978a; 1978b). In some species sperm are relatively short while in others they may reach gargantuan proportions; the sperm of the termite, *Reticulitermes lucifugus* are just 1.7µm in length (Baccetti *et al.*, 1981) but in *Drosophila bifurca* the 58 000 µm-long sperm is 35 times the length of the male producing it (Pitnick *et al.*, 1995). Finally, sperm cells associate in pairs in some insect species like the water beetle, *Dysticus marginalis* (Jamieson, 1987) and the primitive zygentoman, *Tricholepidion gertshii* (Dallai *et al.*, 2001b), while in some locusts (Szöllösi, 1974), fishflies (Hayashi, 1998) and cicadas (Chawanji *et al.*, 2005) hundreds of sperm are bound by their heads in a single homogenous extracellular matrix, forming spermatodesmata.

The sperm head consists of an acrosome, derived from the Golgi apparatus, and a condensed nucleus. In most insect species the acrosome is located at the apical end of the sperm head. However, in some species such as nepomorph Hemiptera, the acrosome lies along the sperm head (Lee and Lee, 1992). The acrosome in most insect species has a conical or rod-like shape although in some groups more complicated types are seen. For example the acrosome has a wine-glass shape in several Neuroptera (Afzelius and Dallai, 1979), in the Tettigoniidae (Orthoptera) it is shaped like an arrowhead (Baccetti *et al.*, 1970) and in some saldid bugs (Hemiptera) it is a large flattened disc (Afzelius *et al.*, 1985).

For the majority of insects the sperm nucleus is usually elongated, spindle-shaped, pointed anteriorly, and truncated posteriorly (Baccetti, 1998). Generally the mature spermatozoon nucleus is of the same electron density throughout, an appearance attained by chromatin condensation during spermiogenesis. However, in certain species, the degree of chromatin condensation is variable and consequently the chromatin appears as a conglomerate of coarse granular subunits (Baccetti, 1998).

The sperm mid-piece is the region connecting the head and the flagellum of the spermatozoon. In the majority of insect sperm this region contains the centriole and the centriolar adjunct. In insects the immature sperm has a centriole with a typical configuration of microtubular triplets in a cartwheel array (Baccetti, 1998). However, in fully mature sperm the centriole consists of doublets rather than triplets of microtubules (Baccetti, 1998). The centriolar adjunct is a dense cylinder with a granular structure that

surrounds the centriolar region and the beginning of the axoneme. The origin and function of the centriolar adjunct is still unknown (Baccetti, 1998).

The sperm tail consists of a long axoneme, which is a complex of microtubules. It provides the motility necessary to propel the sperm to the site of fertilization and to ensure that it is appropriately orientated to penetrate the coatings of the ovum (Fawcett, 1994). Normally it is flanked by two kinds of accessory structures (mitochondria and accessory bodies) usually derived from mitochondrial transformations or originating from the Golgi complex.

In the majority of primitive insects the mitochondria, similar to their somatic counterparts, have a conventional structure and appear to be the result of a confluence of several smaller mitochondria (Baccetti and Afzelius, 1976). Such mitochondria are a typical feature in Collembola and Diplura (Baccetti, 1998). However, in higher insects the mitochondria undergo complicated transformations during spermiogenesis, culminating in the formation of two elongated, crystalline structures, termed mitochondrial derivatives, which flank the axoneme (Baccetti, 1998).

Mitochondrial derivatives can be distinguished from their equivalents in somatic cells in three respects: (a) they are very long and in some species they are the dominant part of the spermatozoon with a length of several millimeters (Afzelius *et al.*, 1976; Mazzini, 1976; Pitnick *et al.*, 1995); (b) the mitochondrial cristae tend to be regularly aligned and orientated perpendicularly to the longitudinal axis of each derivative (Phillips, 1974); and (c) the mitochondrial matrix contains a conspicuous crystalline material, termed crystallomitin, which may occupy most or part of the mitochondrial space (Jamieson *et al.*,

1999). Crystallomitin, a typical insect protein, has appeared during apterygote evolution and is absent from Odonata (dragonflies), Ephemeroptera (mayflies) and Isoptera (termites), but primarily present in all of the other pterygote insects having a chondriome in their spermatozoa (Baccetti, 1998).

Besides the mitochondrial derivatives, the axoneme is flanked by at least one, and frequently two, elongated accessory bodies. In some primitive insects with normal mitochondria, the accessory bodies flank the mitochondria. This has been found in some Ephemeroptera and Thysanoptera (thrips). In other insects the structured bodies flank the mitochondrial derivatives and the axoneme ends up embedded in the flagellum. Such an arrangement has been found in Odonata, Ephemeroptera, most Hemiptera (bugs) and most neuropteroid orders (Baccetti, 1998). To summarize, the main components of sperm cells in insects are present in most insects but tend to be modified in several groups in line with the specific requirements for each particular species. In some groups other sperm components have been lost e.g. there is no acrosome in the proturan *Eosentomon transitorium* (Baccetti *et al.*, 1973c) and the nucleus is absent in the apyrene spermatozoa of Lepidoptera (Phillips, 1971).

### **1.3. SPERM POLYMORPHISM IN INSECTS**

In most animal groups, the testes in fertile males usually produce spermatozoa of uniform structure (Parker, 1982). However, in some taxa, sperm morphology can vary within a species (e.g. Hodgson, 1999), a phenomenon known as sperm polymorphism. Sperm polymorphism can include variation in sperm length and structure (Jamieson *et al.*, 1999). The regular production of multiple morphological types of sperm in a common testis has

been described in a wide variety of invertebrates including gastropods (Hodgson, 1999), spiders (Rosati *et al.*, 1970), centipedes (Jamieson, 1986), and insects (Sivinski, 1980). For example, some species of vinegar flies (Diptera: Drosophilidae) and stalk-eyed flies (Diptera: Diopsidae) produce two discrete lengths of nucleated sperm, a form of sperm polymorphism termed polymegaly (Snook *et al.*, 1994; Pasini *et al.*, 1996; Presgraves *et al.*, 1999).

Sperm polymorphism occurs throughout the order Lepidoptera, with males producing nucleated (eupyrene) and non-nucleated (apyrene) sperm (Silberglied *et al.*, 1984; Jamieson, 1987; Friedlander, 1997). Despite being incapable of fertilizing eggs apyrene sperm usually predominate in the ejaculate (Silberglied *et al.*, 1984). They also appear to have some adaptive value; Sahara and Kawamura (2002) have demonstrated them to be indispensable for fertilization in the silkworm *Bombyx mori*. The production of sperm that vary in chromosome complement has been reported in pentatomid stinkbugs (Schrader, 1960), where polymegaly is widespread in the subfamily Pentatominae (tribes Pentatomini, Halyini, Discocephalini and Edesini). This group comprises about four fifths of the 3 000 species of stinkbugs in the subfamily (Swallow and Wilkinson, 2002). Gigantic sperm have also been recorded in some coleopteran families especially Carabidae (Fain-Maurel, 1966; Swallow and Wilkinson, 2002). Giant sperm are always hyperpyrene and include diploid, tetraploid and higher orders of polyploidy. The nuclei are voluminous and in some cases can be bi- or multi-nucleate (Swallow and Wilkinson, 2002). From the hymenopteran wasp, *Dahlbominus fuscipennis*, five different sperm types where the nucleus forms either a left or right-handed helix have been recorded (Lee and Wilkes, 1965). It is only recently that polymorphic sperm have been reported in cicadas; the Japanese polyneurine cicada,

*Graptosaltria nigrofuscata* produces two distinct sizes of spermatozoa, termed long and short sperm (Kubo-Irie *et al.*, 2003). The authors also examined fertilized eggs and concluded that only long sperm fertilized eggs. However, the role of short sperm was never explained.

Some authors, e.g. Silberglied *et al.*, (1984), Cook and Gage (1995) proposed that apyrene sperm in the Lepidoptera are cheap fillers and delay further mating by the females. This was demonstrated in the moth, *Plodia interpunctella* and the butterfly, *Pieris rapae*, where males increased the proportion of eupyrene sperm within the ejaculate when mating with nonvirgin females (Cook and Gage, 1995; Cook and Wedell, 1996). Schrader (1960) suggested that heteroploid sperm in the stinkbugs, which are not used in fertilization, might provide additional nutrients, especially nucleoproteins, to the developing egg. A number of authors have suggested that production of polymorphic sperm may be linked to sperm competition (e.g. Joly *et al.*, 1989; Cook and Gage, 1995; Snook, 1998, Simmons, 2001; Swallow and Wilkinson, 2002). There is evidence to support this idea in some taxa but not in all. As a result the function of polymorphic sperm in insects still remains uncertain.

#### **1.4. SPERM COMPETITION IN INSECTS**

Darwin (1859) defined sexual selection as the struggle for existence in the face of adversity from other organisms and external conditions. Essentially, this is a struggle where one sex (usually the male) endeavours to perpetuate his genes by winning the approval of a female to mate with her (Singh *et al.*, 2002). Failure to win mating rights results in few or no progeny. Consequently, the male's genes are not inherited and thus gene diversity in the alleles diminishes (Singh *et al.*, 2002). For most male insects, the optimal strategy is not

necessarily to mate with as many females as possible, but to fertilize as many eggs as possible (Singh *et al.*, 2002). Sexual selection via sperm competition has culminated in a rapid and divergent evolution of traits that function in sperm competition and its avoidance (Simmons, 2001). Sperm competition (Parker, 1970), in which the sperm from more than one male competes directly for access to a female's ova, is widely recognized as a form of sexual selection and a potent force in the evolution of sperm characteristics (Parker, 1982; Smith, 1984; Birkhead and Møller, 1998).

In many animals, testis size has been shown to be associated with reproductive success and to be positively correlated to sperm production and transfer rates (Gage, 1994; 1995; Birkhead and Møller, 1998; Simmons *et al.*, 1999b; Pitnick and Miller, 2000). For example, a clear relationship between testis size and breeding system has been demonstrated in *Drosophila* (Pitnick, 1996), some fish (Stockley *et al.*, 1997) and frogs (Jennions and Passmore, 1993). The females of these species are all promiscuous and the males have larger testes for their body size than do males of monogamous species. These differences in testis size are attributed to sperm competition which predicts an increase in a male's expenditure on the ejaculate with an increase in the probability of a female remating with another male (Harvey and May, 1989; Parker *et al.*, 1997).

When females are polyandrous, selection is expected to exert a strong evolutionary pressure on those characteristics that allow males to remove the sperm stored by females from previous matings, and/or to avoid sperm removal by males that encounter the female after mating (Parker, 1984; Birkhead and Møller, 1998; Danielsson, 1998; Simmons, 2001; Singh *et al.*, 2002). Mechanical sperm removal has been well documented in the Odonata

(Waage, 1986; Michiels and Dhondt, 1988; Siva-Jothy and Tsubaki, 1989; Cordero and Miller, 1992). For example, the specially adapted aedeagus of the damselfly *Calopteryx maculata* has a dual function; the transfer of sperm to the female and removal of sperm deposited from earlier matings (Waage, 1979). Behavioral, physiological and morphological characteristics of the male, as well as the competitive properties of the sperm itself, are all subject to selection via sperm competition (Danielsson, 1998; Simmons, 2001; Simmons *et al.*, 1999a; Singh *et al.*, 2002). Examples of such characteristics include (a) a tendency to remain with the female after copulation (Gilchrist and Partridge, 2000); (b) use of mating plugs (Parker, 1970; Polak *et al.*, 2001); (c) chemical or physical characteristics of the ejaculate that enhances the success of an individual male in gaining and protecting fertilizations (Eberhard, 1996); and (d) genitalic structures that deliver sperm closer to the site of fertilization or manipulate previously stored sperm of rivals (Birkhead and Møller, 1998; Danielsson, 1998; Simmons, 2001; Singh *et al.*, 2002).

In the majority of insects, variation in reproductive success is common and male-male competition is extended beyond the point of copulation. This is attributed to the promiscuity of female insects and their capacity for sperm storage within the spermathecae (Parker, 1970; Bangham *et al.*, 2003). Consequently, for the majority of male insects, events that occur both before and after mating are important because they affect reproductive success (Bangham *et al.*, 2002). For example, in some insect species, males compete physically for access to females, and large male body size is often associated with success during pre-copulatory competition (Alcock, 1996; Wedell and Cook, 1998). In those insects using sound communication, male competition often takes the form of direct

or indirect acoustic aggression (Sueur, 2003). Rivalry sound production has been reported in several insects such as coleopterans (Alexander *et al.*, 1969), some orthopterans (Loher and Dambach, 1989), and some hemipterans (Claridge, 1985; Heady *et al.*, 1986) especially some species of cicadas like *Pycna semiclara* (Villet *et al.*, 2003) and *Tibicina tomentosa* (Seuer, 2003). A complex form of male competition in which a courting male acoustically jams the calls of a potentially interloping rival, thus preventing the female from responding to the interloper has been discovered in periodical cicadas (Cooley and Marshall, 2001).

Traditionally, sperm competition has been regarded primarily as an intrasexual conflict, with females being an inert arena in which the conflict occurs (Hellriegel and Bernasconi, 2000; Singh *et al.*, 2002). However, experimental evidence has shown that the manipulating role of females may be substantial and can have a dramatic effect on the outcome of sperm competition (Eberhard, 1996; Parker, 1998). Post-copulatory events that may affect the reproductive success of male insects include female cryptic preferences (Sivinski, 1980; Eberhard, 1996; Birkhead *et al.*, 1993). Cryptic female choice is the ability of females to bias fertilization success of the males that copulate with and inseminate them (Eberhard, 1996).

Female insects may influence the outcome of sperm competition through the anatomy of their reproductive tracts (Bangham *et al.*, 2003). Reproductive tracts in most female insects are complex (Eberhard, 1996) and growing evidence indicates that, after sperm transfer and prior to fertilization, female reproductive tracts can manipulate, expel, digest or nurture sperm in storage (Eberhard, 1996; Simmons and Siva-Jothy, 1998). Female insects thus determine the “rules of the game” by which males compete for fertilization (Eberhard,

1996; Miller and Pitnick, 2002; Bangham *et al.*, 2003). Patterns of correlated evolution between sperm length and certain dimensions of the female reproductive tract have been identified in a number of taxa such as ptiliid beetles (Dybas and Dybas, 1981), fleas (Rothschild, 1991), *Drosophila* (Hihara and Kurukawa, 1987; Pitnick *et al.*, 1999), stalk-eyed flies (Presgraves *et al.*, 1999) and birds (Briskie *et al.* 1997). Collectively these studies strongly implicate postcopulatory sexual selection mediated by a component of female cryptic choice.

### **1.5. CICADAS, THE INSECT SINGERS**

The cicadas (Hemiptera: Cicadomorpha) are a diverse group of insects with over 2 000 species catalogued worldwide (Metcalf, 1963a; 1963b; Duffels and van der Laan, 1985; Villet, 1999a; 1999b). They are best known for the characteristic song of each species produced by male stridulation during pair formation and courtship. These loud incessant buzzing sounds, a familiar background sound during the hottest times of day in many places in Africa and in the tropics, are the loudest songs so far measured from any insect (Young, 1990). The songs are primarily for finding mates, but they may also be used for aggregation and repelling predators (e.g. Villet, 1988). Because these signals are generally species-specific (females are only attracted to the calls of conspecific males, (Claridge, 1985; Villet, 1988; 1989; Gogala and Trilar, 2004), the songs are useful in taxonomy (Villet, 1987; Villet, 1988; Simões *et al.*, 2000; Cooley, 2001) and have also been implicated in sexual selection (Seuer and Aubin, 2002). The majority of the larger cicada species are arboreal, and they are often gregarious which might lead to sexual competition and lekking (Villet, pers. comm).

## 1.6. CLASSIFICATION AND PHYLOGENY OF CICADAS

Based on current phylogeny, Bourgoin and Campbell (2002) recognize five hemipteran suborders: (a) Fulgoromorpha, (b) Cicadomorpha, (c) Coleorrhyncha, (d) Heteroptera and (e) Stenorrhyncha. The first four (collectively termed Euhemiptera) are sister groups to the Stenorrhyncha. The hemipteran infraorder Cicadomorpha comprises the superfamilies Cicadoidea (cicadas), Cercopoidea (spittlebugs and froghoppers) and Membracoidea (leafhoppers and treehoppers) (Cryan, 2005). Morphological characters unique to the Cicadoidea include (a) three ocelli (just two in other superfamilies), (b) membranous front wings (in other families they are sometimes thickened) and (c) hindlegs that are elongate, slender and not saltatorial (Richards and Davies, 1977; Moulds, 1990). The often very loud songs or sounds of the cicadas are different from the quiet, low intensity calls of the other Cicadomorpha (Claridge, 1985).

The higher classification of the Cicadoidea is currently in a state of flux and is in need of revision. Three families were listed by Metcalf (1963a, 1963b) whereas Duffels and van der Laan (1985) listed a total of 6 families: Tettigarctidae, Cicadidae, Tibicinidae, Tettigadidae, Plautillidae and Platypediidae. Currently there is little agreement on the number of cicada families and many authors have listed between two and four families (Evans, 1963; Hayashi, 1984; Boulard, 1988; 1997; Moulds, 1990; 2004; 2005; Duffels, 1993; Chou *et al.*, 1997; Heath, 1999; Lee and Hayashi, 2003). For example, Chou *et al.* (1997) classifies cicadas under two families, Cicadidae and Tettigarctidae, with 6 subfamilies in the former. In the catalogue by Duffels and van der Laan (1985), the genus *Lyristes* is placed under the tribe Lyristini, but Boulard (1988) argues that *Lyristes* should be placed in the platypleurine subtribe Cryptotympanaria. He also got rid of the families

Plautillidae and Platypediidae. More recently Moulds (2005) has transferred *Tibicina* Amyot, the type genus of the subfamily Tibicininae, to the subfamily Tettigadinae. He has also transferred the American genus *Magiccada* Davis, previously of the tribe Tibicinini to the Taphurini.

The current conservative approach (Moulds, 2005) is to classify the Cicadoidea into two families, Cicadidae and Tettigarctidae with the former divided into three subfamilies, Cicadinae, Cicadettinae and Tettigadinae (Figure 1.1). This convention will be followed in this thesis. The large family Cicadidae is widely distributed throughout the world but is especially abundant and diverse in the tropics and subtropics (Metcalf, 1963a; 1963b; Duffels and van der Laan, 1985; Moulds, 1990). This family contains all the typical cicadas and includes all genera and species that produce air-borne sound using well-developed tymbal muscles and an enlarged abdomen acting as a resonating chamber (Moulds, 1990).

Cicadinae males possess tymbal covers that completely or partly cover the tymbal cavities. In Cicadettinae males, tymbal covers are absent and the tymbals are completely exposed (Moulds, 1990). Cicadinae are currently divided into 20 tribes and 9 subtribes and Cicadettinae are divided into 12 tribes (Duffels and van der Laan, 1985; Moulds, 1999). Mating syndromes are often different between species from different tribes. For example, most platypleurine species are sedentary and will stridulate from the same tree for days to attract females (Villet *et al.*, 2003). In some species these calls will also attract other males within the vicinity, resulting in the formation of choruses (Villet, 1988). In contrast, the males of the small-bodied tettigomyiine cicadas flit rapidly between perches in their search

for sedentary females that generally emit no readily perceivable signals (Villet and van Noort, 1999). Their calls; a variety of clicks, churrs, buzzes and croaks, are quieter than those of the platypleurines.

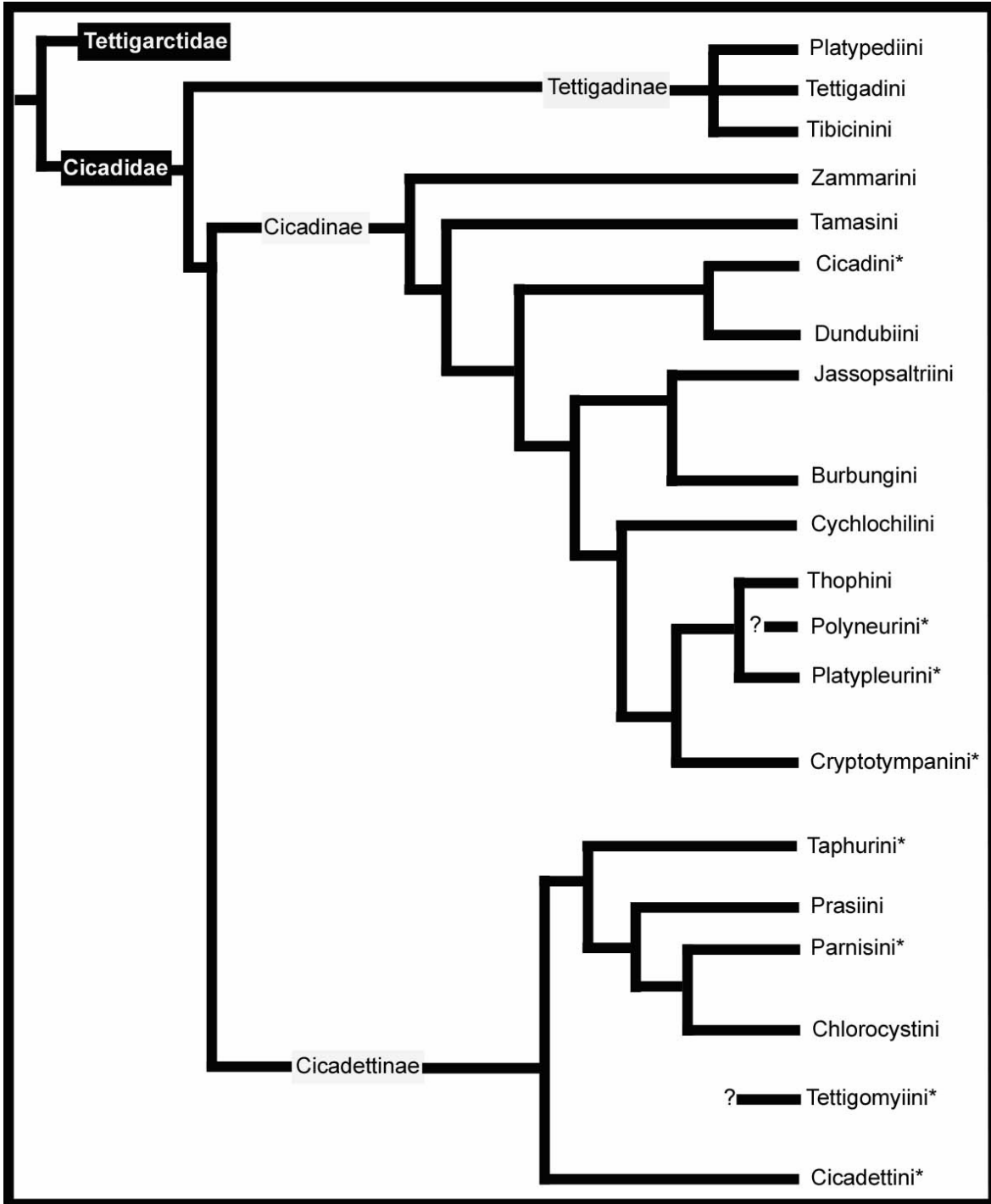


Figure 1.1. Phylogeny of some Australian cicada taxa, based on Moulds (2004, 2005). \* = tribes containing species for which sperm morphology has been published.

## 1.7: OUTLINE OF THE STUDY

Sperm polymorphism is an intriguing phenomenon that has been well documented in some invertebrates including gastropods, centipedes, spiders and insects such as cicadas. It is not clear why species should produce polymorphic sperm especially if some of the morphs are infertile. This would be tantamount to wasting resources. Although sperm morphology appears to be a sensitive indicator of fertilization biology (Baccetti and Afzelius, 1976), it has not been easy to explain sperm polymorphism in relation to fertilization biology in a number of species. The need to find out more about this bizarre phenomenon within the Cicadinae and Cicadettinae provided a major impetus for this investigation. There is a spectrum of reproductive behaviours within the 2 000 cicada species documented to date (Myers, 1929; Alexander and Moore, 1958; Doolan, 1981; Williams and Simon, 1995; Villet and van Noort, 1999; Sueur, 2002; 2003; Sueur and Aubin, 2003; Villet *et al.*, 2003). For example, in some species like *Oxypleura quadraticollis* and *Pycna semiclara* the males make incessant loud buzzing broadcasts from stationary perches to entice receptive females within the vicinity (Villet *et al.*, 2003). The females will then approach the males. In other species like *Quintilia carinata* the males do not stridulate from a fixed position; instead they constantly change their positions in their search for mates. The males of some species like *P. semiclara* are gregarious and the calling signal attracts conspecific males to small aggregations where they produce choruses (Villet, 1988; Villet *et al.*, 2003) while the males of other species like *Azanicada zuluensis* display satellite behaviour where some males remain silent in the vicinity of calling males so as to attract females (Villet, pers. comm.).

Numerous spermiocladists (Dallai, 1979; Jamieson, 1987; 1991; Dallai and Afzelius, 1990; 1995; Carpucino *et al.*, 1995; Jamieson *et al.* 1995; 1999; Lino-Neto and Dolder, 2001)

have recognized the rapid and divergent nature of spermatozoon evolution and its potential in the construction of animal phylogenies. The use of sperm characteristics in the study of cicada phylogeny has never been attempted and sperm morphology is known in a few species only. The aims of this study were to (1) compare published information on sperm morphology in *Cicada orni*, *Lyristes plebejus* and *Graptosaltria nigrofuscata* with some cicadettine and cicadine species in an attempt to get valuable morphological characters for use in cladistic analysis; (2) examine the morphology of the male and female reproductive systems in selected species of cicadas in order to note any variations in shape, position, number and size of individual organs; (3) investigate spermathecal shape in selected cicada species, which is often linked to reproductive biology; and (4) investigate fertilized eggs to identify the sperm morphs responsible for fertilization.

In Chapter 2 variability in sperm morphology within a tribe (Platypleurini) of the Cicadinae is investigated. Five species examined were: *Albanycada albigera*, *Azanicada zuluensis*, *Platypleura capensis*, *P. hirtipennis* and *Pycna semiclara*. In Chapter 3, variation in sperm morphology between four tribes (Cicadettini; Parnisini; Taphurini and Tettigomyini) of the Cicadettinae is described. Some stages of spermiogenesis in cicadas are described in Chapter 4, information being derived from a platypleurine, *Kongota punctigera* and two cicadettines: *Diceroprocta biconica* and *Monomatapa matoposa*. Histochemistry of the spermatodesm is also described briefly in Chapter 4 and a contribution is made to establishing its functional significance. The morphology and morphometrics of male and female reproductive tracts in cicadas are described and compared in Chapter 5, the information being derived from *Azanicada zuluensis*, *D. biconica*, *Platypleura hirtipennis* and *Oxypleura quadraticollis*. The identity of sperm morphs involved in fertilization in the

cicada, *Azanicada zuluensis* was investigated and is also presented in Chapter 5. In Chapter 6 conclusions on the significance of sperm morphology in the phylogeny of cicadas are made, with suggestions on changes to the current classification. Future studies to elaborate more on the findings of this project are also suggested.

Key questions of interest were:

- What variation in sperm morphology is there within a species?
- What variation in sperm morphology is there within a tribe e.g. Platyleurini?
- What variation in sperm morphology is there between tribes e.g. Cicadettini and Taphurini?
- Does sperm morphology support current phylogeny?
- Is there sperm of more than one type within a single ejaculate?

## **CHAPTER TWO**

# **SPERM MORPHOLOGY IN THE CICADINAE, TRIBE PLATYPLEURINI**

## 2.1. INTRODUCTION:

In conformity with current literature, the Cicadinae are divided into a hierarchy of ranks known as subtribes and tribes (Duffels and van der Laan, 1985, Moulds, 1990; 1999; 2005; Boulard, 1997; Chou *et al.*, 1997). However, there is little agreement on the systematic positions of certain (sub) tribes or on the assignment of genera to tribes, a symptom of the current state of flux in the classification of the entire Cicadoidea (Boulard, 1988; Moulds, 1990; 1999; 2005; Duffels, 1993; Duffels and van der Laan, 1985; Chou *et al.*, 1997; Lee, 2005).

The most recent catalogue of the Cicadoidea (Duffels and van der Laan, 1985) which is a supplement to Metcalf's catalogue (Metcalf, 1963a; 1963b) recognizes twenty tribes and nine subtribes from the Cicadinae. Among the tribes are Platypleurini, Zammarini, Polyneurini, Cyclochilini, Lyristini, Dundubiini and Cicadini (Fig. 1.1). Platypleurine cicadas are a diverse tribe of large, attractively ornate, tree-dwelling cicadas occurring throughout the Afrotropical region and southern Asia to Japan (Metcalf, 1963a; 1963b; Duffels and van der Laan, 1985).

In several animals sperm ultrastructure has been shown to provide valuable characters for phylogenetic analysis. Examples include insects (Jamieson *et al.*, 1995; 1999), reptiles (Oliver *et al.*, 1996), fish (Jamieson, 1987) and frogs (Lee and Jamieson, 1993; Scheltinga and Jamieson, 2003). Dallai *et al.* (2004) suggest that comparative spermatology is a branch on its own in biology. Of the more than 2 000 species of cicadas described to date, the sperm structure of only three cicadine species, *Cicada orni*

(Linnaeus, 1758) from the tribe Cicadini (Folliot and Maillet, 1970), *Lyristes plebejus* (Dlabola, 1958) from the tribe Lyristini (Folliot and Maillet, 1970) and *Graptosaltria nigrofuscata* (Hagiwara, 1953) from the tribe Polyneurini (Kubo-Irie *et al.*, 2003) have been described. According to Folliot and Maillet (1970), the Cicadidae have a conventional motile spermatozoon with a 9 + 9 + 2 axoneme, peripheral singlets with 16 protofilaments, an acrosome complex that lacks a perforatorium and very large crystalline mitochondrial derivatives. Kubo-Irie *et al.* (2003) found similar results upon examination of sperm morphology in the Japanese polyneurine cicada, *G. nigrofuscata*. Although the descriptions were not that detailed, they revealed that *G. nigrofuscata* produces two distinct sizes of spermatozoa which they termed long and short sperm. Furthermore, they determined that fertilized eggs contained long sperm, although their sample was too small to rule out the involvement of short sperm too.

If sperm morphology is to be of any value in exploring systematic and phylogenetic relationships of cicadas, and in fertilization biology studies, comparative information from a greater number of taxa is required. The information generated from such studies may provide an alternative source of useful characters that may be important in resolving the current plethora of problems associated with recognition of tribes, placement of certain tribes and (sub) tribes and assignment of some genera to tribes.

The present study presents details on the sperm structure in five species of African cicadas from the tribe Platycleurini. Included in this tribe are several genera that show fairly high degrees of endemism (Villet, 1999b; Villet and van Noort, 1999). They are

phylogenetically compact and display interesting signaling behaviour that could be important in creating situations where males have to compete for mates. Platypleurine cicadas have a “females search” mating syndrome where males make continuous, long-range acoustical advertisements from stationary perches and females approach them (Villet and van Noort, 1999). Advertisement signals reveal the location of the signaler to predators, while searching involves travel-related exposure risk (Burk, 1982).

One motivation for this chapter was to assess variation within a tribe, to put variation between tribes into perspective. Choosing the platypleurines was helpful because they are available locally. The aims of this study were to elucidate and compare sperm structure in five species of platypleurine cicadas *Albanycada albigera* (Walker, 1850), *Azanicada zuluensis* (Villet, 1989), *Platypleura capensis* (Linnaeus, 1764), *P. hirtipennis* (Germar, 1834) and *Pycna semiclara* (Germar, 1834) and to determine whether they have sperm polymorphism. The five species were chosen because they have different mate-attracting behaviours (e.g. Villet *et al.*, 2003) that could result in different degrees of reproductive competition (Villet, 1986).

## **2.2. MATERIALS and METHODS**

### **2.2.1. MATERIALS**

Male cicadas of *Albanycada albigera*, *Azanicada zuluensis*, *Platypleura capensis* and *P. hirtipennis* were collected in the Grahamstown (33° 18' S 26° 32'E) and Kasouga (33° 38' S 26° 44'E) areas of the Eastern Cape, South Africa. *Pycna semiclara* males were collected in the Thomas Baines Nature Reserve (33° 23' S 26° 29'E), in the Eastern Cape.

### 2.2.2. LIGHT MICROSCOPY

Five males of each species were dissected under a binocular microscope in 2 % saline solution and spermatozoa were recovered from the testes and seminal vesicles. Sperm samples were spread evenly on a microscope slide coated with gelatin and chrome alum and a drop of glycerol was added before drying in an oven overnight at 60°C. Slides were then placed in a methanol/acetic acid (75:25 v/v) fixative for four minutes and rinsed in saline solution for one minute. The slides were then placed in 1 % bisbenzimidazole Hoechst 33258, a cell-permeable adenine-cytosine binding fluorescent dye used to stain DNA (Sakaluk and O'Day, 1984) for one minute. Under a fluorescence microscope, nuclei and mitochondria that have been stained with Hoechst 33258 are very conspicuous and can be measured easily. The slides were then rinsed in three changes of saline before a drop of glycerol/PBS (9:1 v/v) was added to the cells and covered with a coverslip. Slides were examined with an Olympus BX-61 epifluorescence microscope at a wavelength of 343 nm. Digital images of 50-100 sperm per male were randomly captured from each specimen. Sperm heads were measured from these images using the analySIS Soft Imaging System programme ([www.softimaging.net](http://www.softimaging.net)). Scatter diagrams were generated to show the relationship between sperm nucleus length and tail length using Statistica version 6.1. Frequencies of different length classes of sperm within and between species were analyzed by chi-square tests. Measurements are reported as mean  $\pm$  standard error.

### **2.2.3. SCANNING ELECTRON MICROSCOPY**

Spermatozoa of each species were attached to gelatin-coated coverslips in 2 % saline solution and fixed overnight with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7) at 4°C, dehydrated in a graded series of acetone, critical-point dried and sputter-coated with gold. The sperm were then examined and photographed in a JEOL 840A scanning electron microscope at 12 kV.

### **2.2.4. TRANSMISSION ELECTRON MICROSCOPY**

Small portions of testes and seminal vesicles from five animals of each species were fixed overnight at 4 °C in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The material was then postfixed in 1 % osmium tetroxide in phosphate buffer for 90 minutes at room temperature, dehydrated in ethanol and embedded in Epon 812 resin. Ultrathin sections (silver-gold) were cut using a diamond knife and collected on 300 mesh copper grids before staining with uranyl acetate and lead citrate. Sections were examined and photographed with a JEOL 1210 transmission electron microscope at 120 kV. Photographs were scanned and dimensions of the centriolar adjuncts, mitochondrial derivatives and nuclei were measured using the AnalySIS Soft Imaging System programme ([www. softimaging.net](http://www.softimaging.net)).

## 2.3. RESULTS

### 2.3.1. Size Heteromorphism

Each of the five species of cicadas has motile spermatozoa that are elongated and thread-like, with slender, needle-like heads, and long tails that taper posteriorly (Figs. 2.1A, B, C, D, 2.2A, B). Total sperm lengths vary considerably within and between species. This variation is attributed to the different lengths of the sperm nuclei and tails (Figs. 2.3A, B, 2.4A, B, 2.5). In all species except *Azanicada zuluensis* and *Pycna semiclara* there is evidence of three different modes of sperm tail lengths and two modes of sperm nucleus lengths. These have been categorized here as short, intermediate and long. The majority of sperm have long heads and short tails (Figs. 2.3A, B, 2.4A, B, 2.5). There is no correlation between nucleus and flagellum length in four of the five species. However, there is a strong negative correlation ( $r = 0.8$ ) between sperm nucleus and tail length in *P. semiclara*.

Three modes of sperm tail lengths are found in *Albanycada albigera*; these are 74, 130 and 220  $\mu\text{m}$ . Nucleus dimensions have a trimodal distribution too, with modal lengths of 43, 27 and 13  $\mu\text{m}$  (Fig. 2.3A). Sixty percent of sperm heads have longer nuclei, whilst sperm with short and intermediate nuclei are found in similar abundances. In all five *A. albigera* males there were no significant differences between observed and expected frequencies of nuclei and tail length classes ( $\chi^2 < 13.28$ ,  $P > 0.01$ ). The tail length distribution of *Platypleura capensis* (Fig. 2.4A), with three modal classes of 74, 130 and 182  $\mu\text{m}$ , is similar to that in *P. hirtipennis* (Fig. 2.4B). However, nucleus lengths in both species are bimodal, with modes of 33 and 14  $\mu\text{m}$  in *P. capensis* and 33 and 18  $\mu\text{m}$  in *P.*

*hirtipennis*. In all five *P. capensis* males there were no significant differences between observed and expected frequencies of nucleus length classes ( $\chi^2$  values  $< 13.28$ ,  $P > 0.01$ ). However, frequencies of tail length classes differ between individuals ( $\chi^2$  values  $> 13.28$ ,  $P < 0.01$ ). Frequencies of intermediate and long nuclei in *P. hirtipennis* are similar in all five males, this is the same for intermediate and long tail classes (see Appendix 2). Frequencies of short nuclei and tail classes are not the same in the five males ( $\chi^2$  values  $> 13.28$ ,  $P < 0.01$ ). In *Azanicada zuluensis*, both nucleus and tail lengths have a bimodal distribution pattern (nucleus modes = 32 and 17  $\mu\text{m}$ ; tail modes = 80 and 210  $\mu\text{m}$ ). Tail lengths, unlike the nucleus lengths, are quite discrete (Fig. 2.3B). Sperm with intermediate tail lengths are almost absent in this cicada. In all five males the frequencies of nuclei length classes are similar ( $\chi^2$  values  $< 13.28$ ,  $P > 0.01$ ), unlike the tail length classes which depart from homogeneity ( $\chi^2$  values  $> 13.28$ ,  $P < 0.01$ ).

In *P. semiclara*, two distinct modes (66 and 93 $\mu\text{m}$ ) of sperm tail length are present (Fig. 2.5). Sperm nuclei appear to fall into a single major class with a modal value of 38  $\mu\text{m}$ , although the distribution of the values shows a markedly skewed pattern. Frequencies of nucleus length classes are similar in all five *P. semiclara* males ( $\chi^2$  values  $< 13.28$ ,  $P > 0.01$ ); frequencies of tail length classes are however, not homogenous ( $\chi^2$  values  $> 13.28$ ,  $P < 0.01$ ). Modal classes of sperm nucleus and tail lengths together with the respective correlation coefficients in each of the five cicada species are summarized in Table 2.1. Frequencies of tail length classes depart from homogeneity and show a statistically significant relationship across species ( $\chi^2$  values  $> 13.28$ ,  $P < 0.01$ ). This departure from homogeneity is also evident in the frequencies of short and intermediate nuclei classes.

The distribution of long nuclei across species is similar ( $\chi^2$  value < 13.28,  $P > 0.01$ ). Within a genus, sperm tail and nucleus length appear to be similar as shown by *P. capensis* and *P. hirtipennis*. Nevertheless, some minor differences in sperm dimensions between genera were evident. For example, sperm nucleus dimensions of *A. zuluensis* are similar to those of *P. capensis* and *P. hirtipennis*, yet there are differences in tail length dimensions. Unlike in the two species mentioned above, there are only two modal classes of sperm length in *A. zuluensis* and the range between them is much wider than in *P. capensis* and *P. hirtipennis*. Three modal classes of sperm tail length are found in *A. albigera*, just like in *P. capensis* and *P. hirtipennis*. However, the range between the medium and large modal classes is much wider in *A. albigera* than in *P. capensis* and *P. hirtipennis*. Sperm nucleus lengths in *A. albigera* are dissimilar to those in *A. zuluensis*, *P. capensis* and *P. hirtipennis* in having three modal classes. The small and intermediate modal classes in *A. albigera* are similar to those found in these three species; the only difference is in the last class (43  $\mu\text{m}$ ). Sperm length dimensions in *P. semiclara* are however, different from the rest of the species. There is only one modal class of sperm nucleus length unlike in the other four species; the range in tail length is also much narrower. Sperm tail lengths are evidently longer in *A. albigera* and *A. zuluensis* than in the other three species; about 6-8 % of sperm in these species have both a long nucleus and a long tail. Such sperm have the longest dimensions, often exceeding 250  $\mu\text{m}$  in total length. In *A. zuluensis*, *P. capensis*, *P. hirtipennis* and *P. semiclara* the majority of sperm (60 – 80%) have long heads and short tails (Figs. 2.3A, B, 2.4A, B, 2.5).

Table 2.1. Modal classes and correlation coefficients (r) of nucleus length versus tail lengths in the sperm of five Cicadinae species. Data obtained from light microscopy.

Species	Modal classes			Modal classes			r
	Nucleus length ( $\mu\text{m}$ )			Tail length ( $\mu\text{m}$ )			
<i>A. albigera</i>	13	27	43	74	130	220	-0.40
<i>A. zuluensis</i>	17	32		80		210	-0.46
<i>P. capensis</i>	14	33		74	130	182	-0.36
<i>P. hirtipennis</i>	18	33		74	130	182	-0.60
<i>P. semiclara</i>		38		66	93		-0.80

### 2.3.2. Shape Polymorphism

Although the majority of sperm heads have rectilinear nuclei, small proportions (approximately 2 %) of heads in all five species have twisted nuclei (Fig. 2.6A, B). In addition, a small proportion of sperm in all species (approximately 5 %) have two tails instead of the conventional single flagellum (Fig. 2.6C, D). Such biflagellate sperm are all uninucleate.

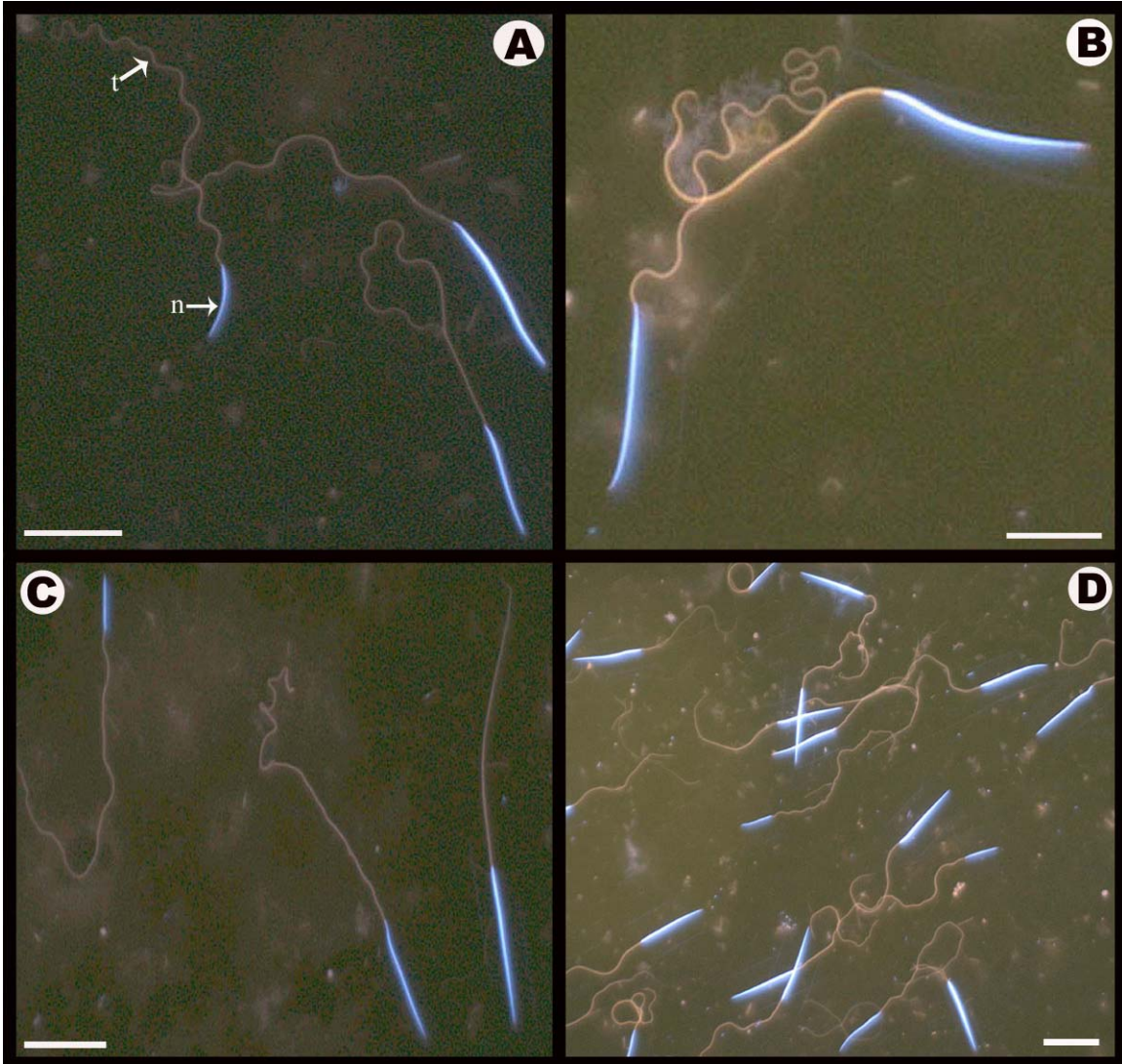


Figure 2.1. Cicada sperm stained with Hoechst 33258 and examined with a fluorescence microscope at 343 nm. A: *Albanycada albigera* sperm with long and short heads. n, nucleus; t, tail. Bar = 25  $\mu\text{m}$ . B: *Platypleura capensis* sperm with long heads. Bar = 25  $\mu\text{m}$ . C: *Azanicada zuluensis* sperm with intermediate and short heads. Bar = 25  $\mu\text{m}$ . D: *Platypleura hirtipennis* sperm with long and short heads. Bar = 25  $\mu\text{m}$ .

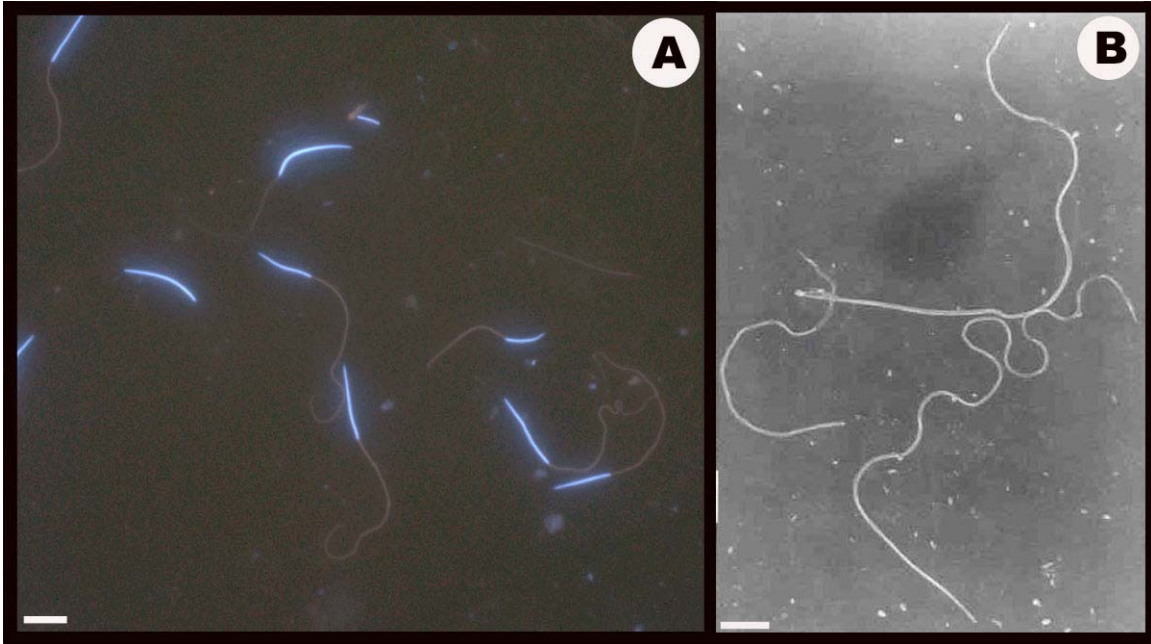


Figure 2.2. A: *Pycna semiclara* sperm with long and short nuclei stained with Hoechst 33258 and examined with a fluorescence microscope at 343 nm. Bar = 20  $\mu\text{m}$ . B: Long and short *Albanycada albigera* sperm as seen by scanning electron microscopy. Bar = 10  $\mu\text{m}$ .

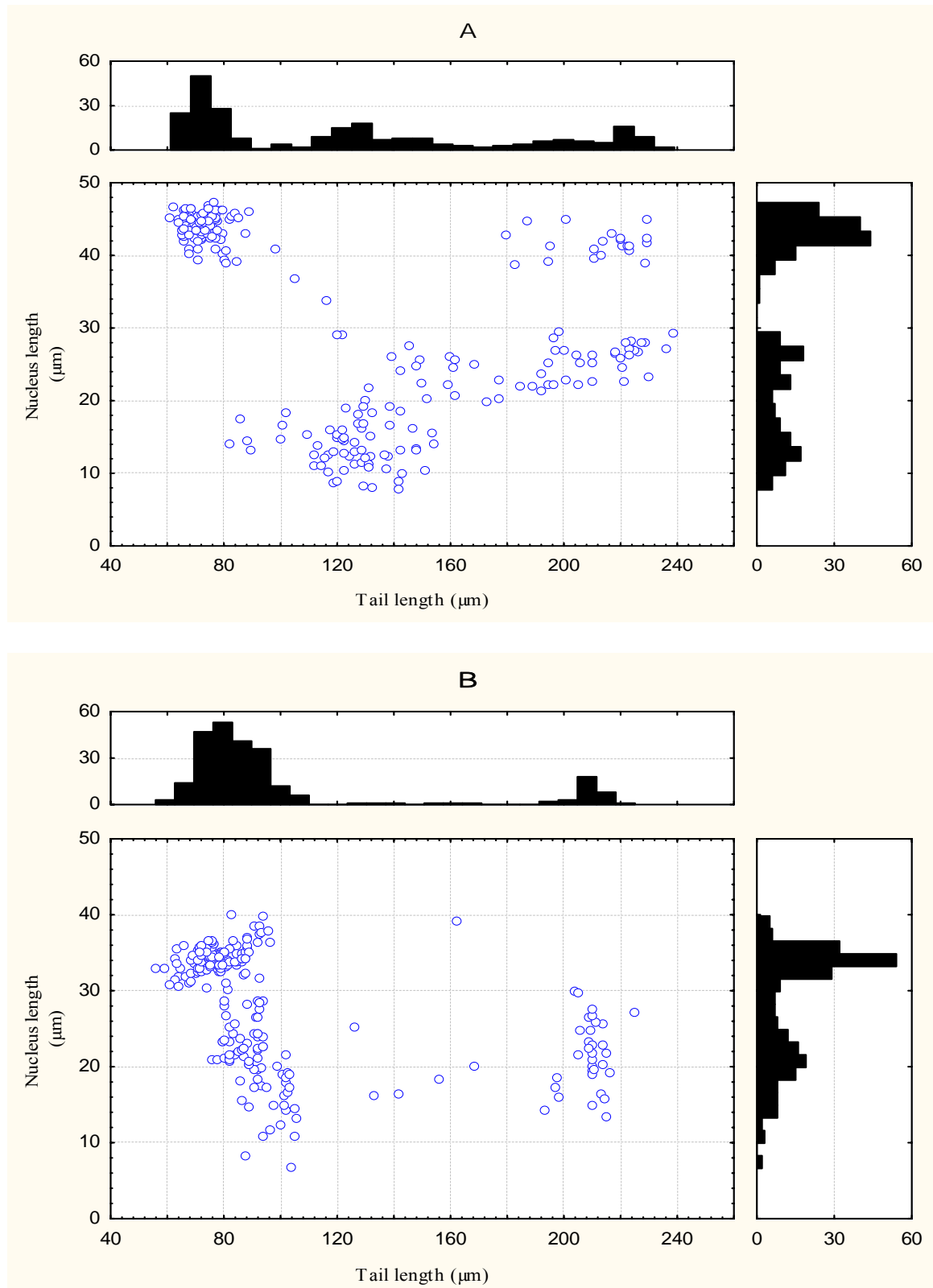


Figure 2.3. Scatter-plots of nucleus lengths versus tail lengths and size-frequency histograms of nucleus lengths and tail lengths. Data pooled from five individuals per species. A: *Albanycada albigera*; B: *Azanicada zuluensis*.

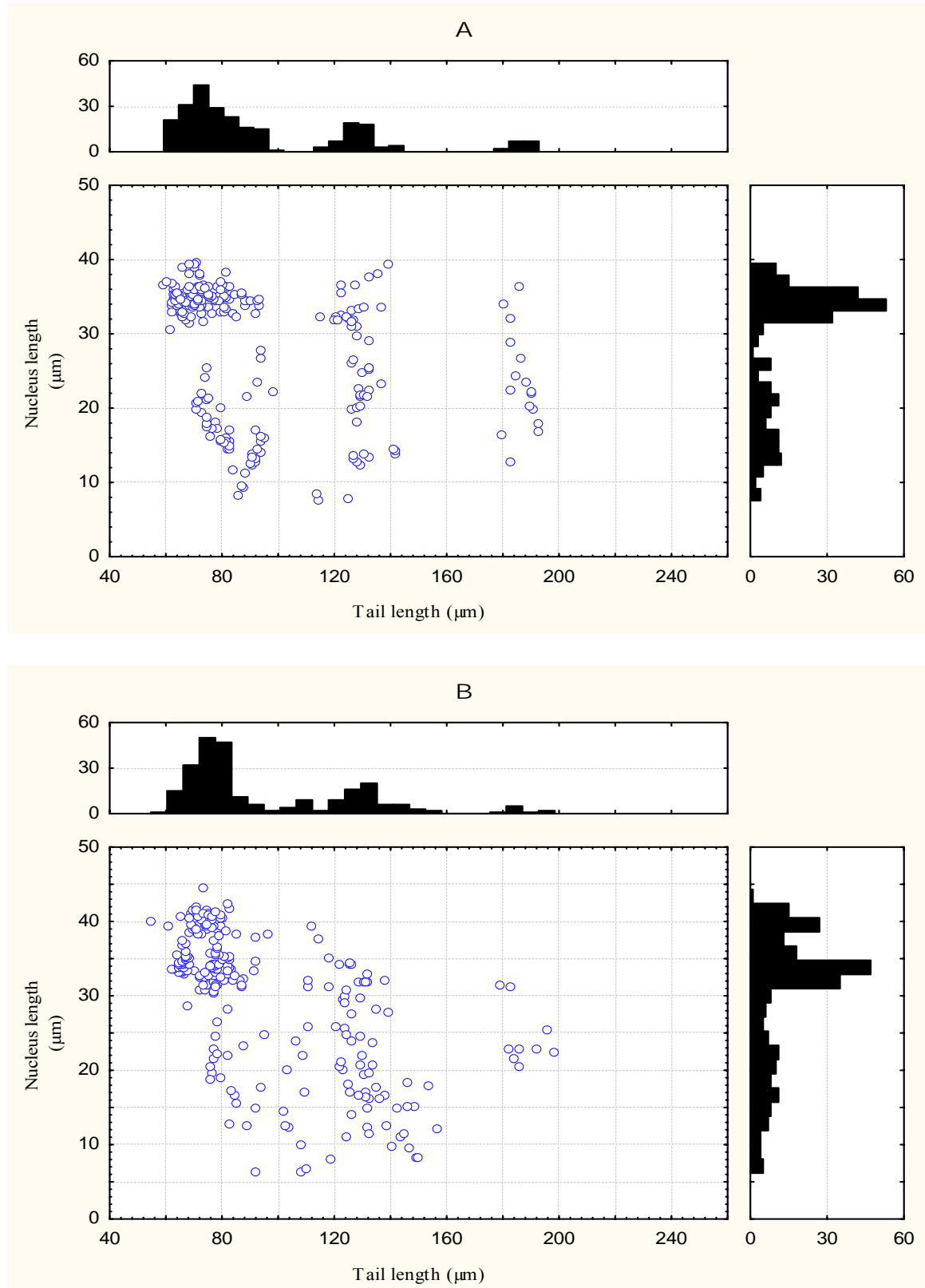


Figure 2.4. Scatter-plots of nucleus lengths versus tail lengths and size-frequency histograms of nucleus lengths and tail lengths. Data pooled from five individuals per species. A: *Platypleura capensis*; B: *P. hirtipennis*.

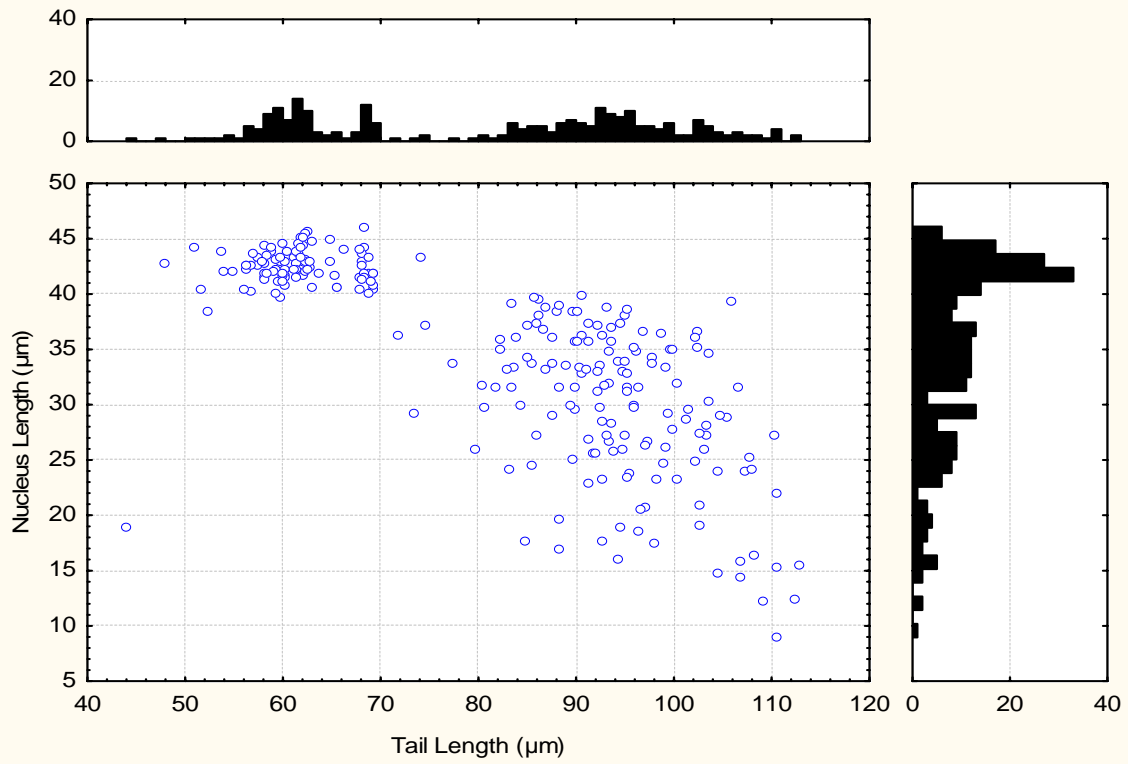


Fig. 2.5. Scatter-plot of nucleus length versus tail length and size-frequency histograms of nucleus lengths and tail lengths in *Pycna semiclara*. Data pooled from five individuals.

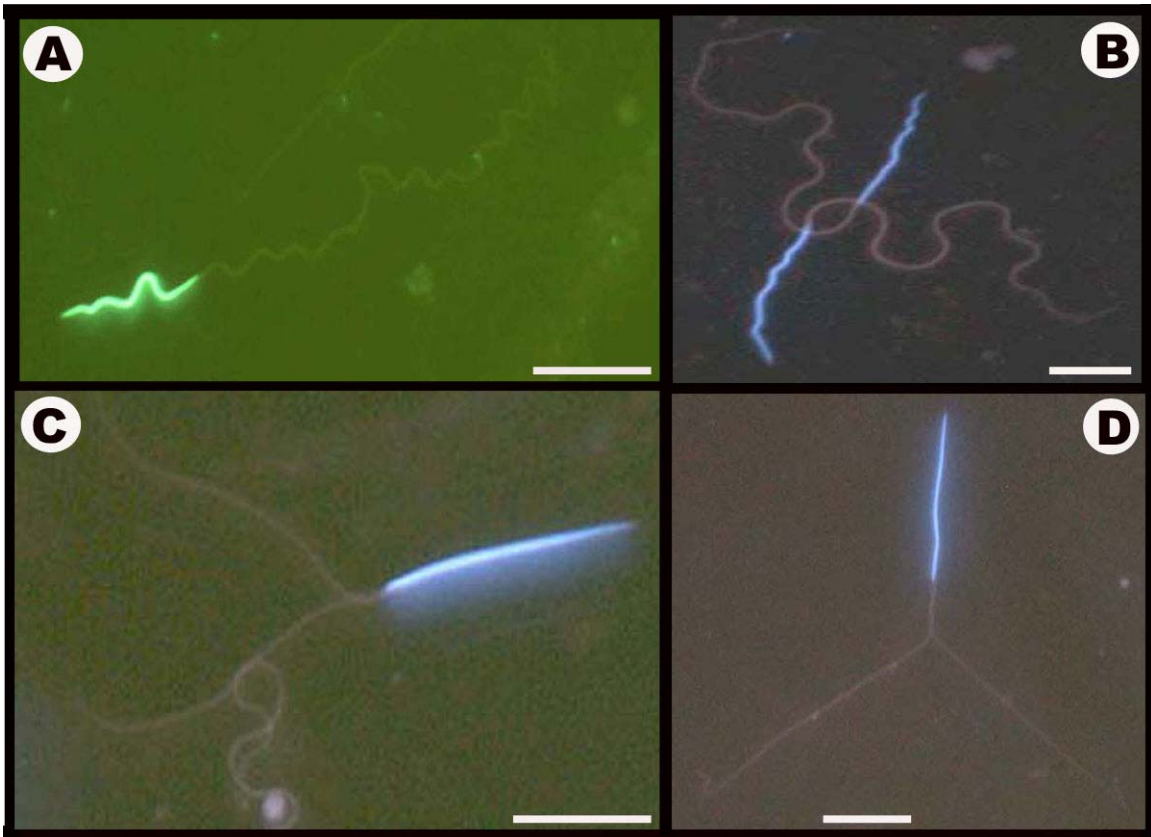


Figure 2.6. A: Spermatozoon of *Platypleura capensis*. Fluorescence image of a sperm cell with a twisted nucleus. Bar = 20  $\mu\text{m}$ . B: Spermatozoa of *Pycna semiclara*. Fluorescence image of sperm cells with twisted nuclei. Bar = 20  $\mu\text{m}$ . C: Spermatozoon of *P. hirtipennis*. Fluorescence image of a biflagellate spermatozoon. All such sperm were uninucleate. Bar = 20  $\mu\text{m}$ . D: Spermatozoon of *P. semiclara*. Fluorescence image of a biflagellate spermatozoon. Bar = 20  $\mu\text{m}$ .

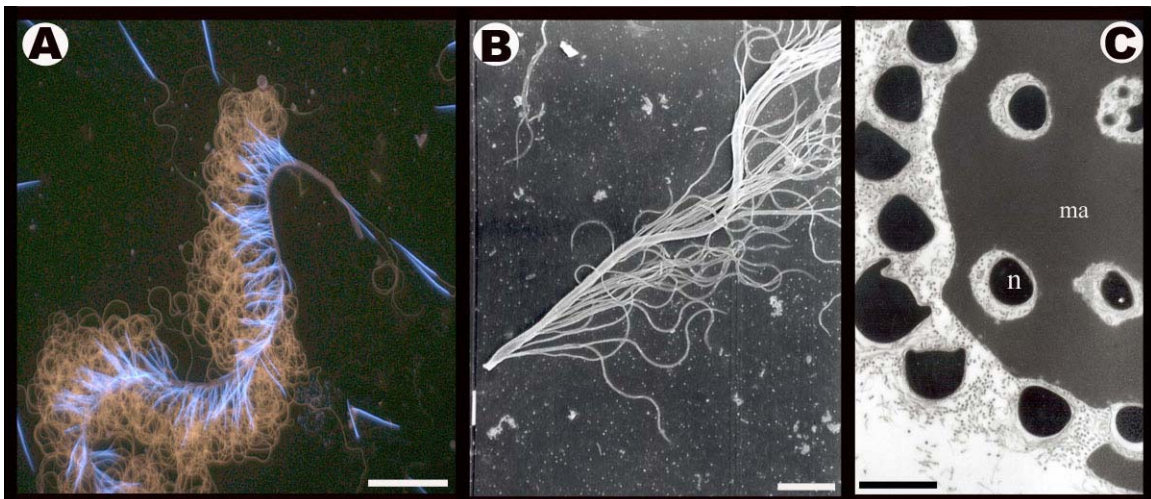


Figure 2.7. A: Fluorescence image (wavelength 343 nm) of a sperm bundle of *Azanicada zuluensis* stained with Hoechst 33258. Spermatozoa are released in bundles with their anterior ends embedded in a matrix forming a spermatodesm. Bar = 50  $\mu\text{m}$ . B: Sperm bundle of *A. zuluensis*, shown by scanning electron microscopy. Bar = 20  $\mu\text{m}$ . C: Transverse section through

a sperm bundle of *Platypleura capensis* showing the nuclei (n) and the homogenous electron-dense matrix (ma). Bar = 500  $\mu\text{m}$ .

### 2.3.3. Ultrastructure

The morphological features exhibited by spermatozoa of different lengths within or between the species *Albanycada albigera*, *Azanicada zuluensis*, *Platypleura capensis*, *P. hirtipennis* and *Pycna semiclara* are sufficiently similar to allow only a single ultrastructural description. In all species spermatozoa are released in bundles with their anterior ends embedded in an elongate homogenous electron-dense matrix forming a spermatodesm (Fig. 2.7A, B, C).

### 2.3.4. Head Region

The head region consists of a nucleus and an acrosome. The anteriorly positioned acrosome (see Table 2.2 for dimensions) is conical in shape, deeply invaginated posteriorly and sits on top of the nucleus (Fig. 2.8A, B, C). It comprises two regions: the acrosome proper and an inner subacrosomal space (Fig. 2.8D, E, F). The contents of the acrosome have a tubular substructure (Fig. 2.8B, D, E, F, G). The anterior tip of the nucleus intrudes into the subacrosomal space (Fig. 2.8A, B, F). Posteriorly the acrosome extends as two tubular extensions positioned on both sides of the anterior section of the nucleus and which gradually decrease in diameter (Fig. 2.8G, H, I).

Differentially sized nuclei (see Table 2.3 for diameters) are conspicuous in cross-sectional profiles across seminal vesicles. Some nuclei have a small diameter while others have a larger diameter (Fig. 2.11E). In all species the homogeneously electron-dense nucleus (Fig. 2.9A, B) is pointed anteriorly (Fig. 2.8A, B). Anteriorly the nucleus forms a slender cone and is circular in cross-section (Fig. 2.8F); towards the posterior limit of the acrosomal complex the nucleus becomes bilaterally concave (Fig. 2.8G, H),

the degree of concavity gradually increasing posteriorly. Adjacent to the base of the acrosomal complex the bilateral concavity of the nucleus changes to a roundish profile (Fig. 2.9C) before the nucleus becomes laterally flattened and then invaginated (Fig. 2.9D, E). Initially, the invagination is devoid of material; however, the posterior segment of each invagination houses fine granular material of the centriolar adjunct and the anterior section of the mitochondrial derivatives (Fig. 2.9B, F, G).

Table 2.2. Mean (nm)  $\pm$  s.e. of tip width, base width and length of the acrosome for Cicadinae sperm. Data obtained from TEM.

Species	Tip width			Base width			Length		
	mean	s.e.	n	mean	s.e.	n	mean	s.e.	n
<i>A. albigera</i>	53.37	2.02	3	490.98	4.25	3	1 347.14	12.81	3
<i>A. zuluensis</i>	36.99	1.92	4	459.11	2.21	3	966.91	23.97	3
<i>P. capensis</i>	34.95	0.81	3	460.81	13.71	3	1 186.07	2.65	3
<i>P. hirtipennis</i>	37.65	2.31	3	412.18	1.06	3	178.27	20.55	3
<i>P. semiclara</i>	43.79	0.74	3	459.24	14.33	3	199.14	7.63	3

Table 2.3. Means (nm)  $\pm$  s.e. of long and short nucleus diameter for Cicadinae sperm. Data obtained from TEM.

Species	Large nucleus diameter			Small nucleus diameter		
	Mean	s.e.	n	mean	s.e.	n
<i>A. albigera</i>	600.11	14.36	5	224.31	12.59	5
<i>A. zuluensis</i>	557.27	11.82	5	212.64	11.92	6
<i>P. capensis</i>	549.76	4.29	5	208.43	12.45	6
<i>P. hirtipennis</i>	558.75	5.78	5	220.64	14.67	5
<i>P. semiclara</i>	528.42	18.31	5	205.74	10.26	6

The centriolar adjunct (Figs. 2.9B, F, 2.10A, C) is located anterior to the mitochondrial derivatives which it adjoins. In longitudinal sections, the centriolar adjunct can be seen to be elongated and composed of electron-dense material. In addition, vesicle-like structures of about 88 nm in diameter (Fig. 2.10A, B) can be seen in some sections where they are arranged in an ordered array. These structures are located between the centriolar adjunct and the nucleus.

### 2.3.5. Mid-Piece Region

The neck region of the mid-piece contains a centriole-like organelle (Fig. 2.10C, D), about 254 nm in diameter and approximately 662 nm in length, that lies posterior to the nucleus. This organelle consists of a ring of microtubule doublets and accessory microtubules rather than triplets. The axoneme (Fig. 2.10C, E), which extends for the entire length of the tail, emerges from the centriole-like organelle and has a 9 (peripheral singlets) + 9 (intermediate doublets) + 2 (central singlets) pattern of microtubules. The diameter of the axoneme, about 254 nm, was similar in all species. A pair of mitochondrial derivatives, which extend along most of the tail, are positioned lateral to the axoneme and posterior to the centriolar adjunct (Fig. 2.10A, C, E, G). Each derivative contains an electron-dense crystallized region and cristae at the periphery (Fig. 2.10E). Mitochondrial derivatives with different diameters (presented in Table 2.4) are present and appear to be related to the size of the sperm nucleus diameter. In longitudinal sections (Fig. 2.10H) the derivative has a striated appearance, the striations having a periodicity of approximately 43 nm.

Table 2.4. Mean diameter of large and small mitochondria for Cicadinae sperm.

Species	Diameter (nm) of large mitochondria			Diameter (nm) of small mitochondria		
	mean	s.e.	n	mean	s.e.	n
<i>A. albigera</i>	218.85	5.54	7	101.24	3.71	10
<i>A. zuluensis</i>	207.10	3.33	6	96.28	4.78	9
<i>P. capensis</i>	217.21	2.27	5	102.86	5.49	8
<i>P. hirtipennis</i>	213.33	3.31	8	100.61	5.29	10
<i>P. semiclara</i>	213.85	2.51	6	97.22	2.17	9

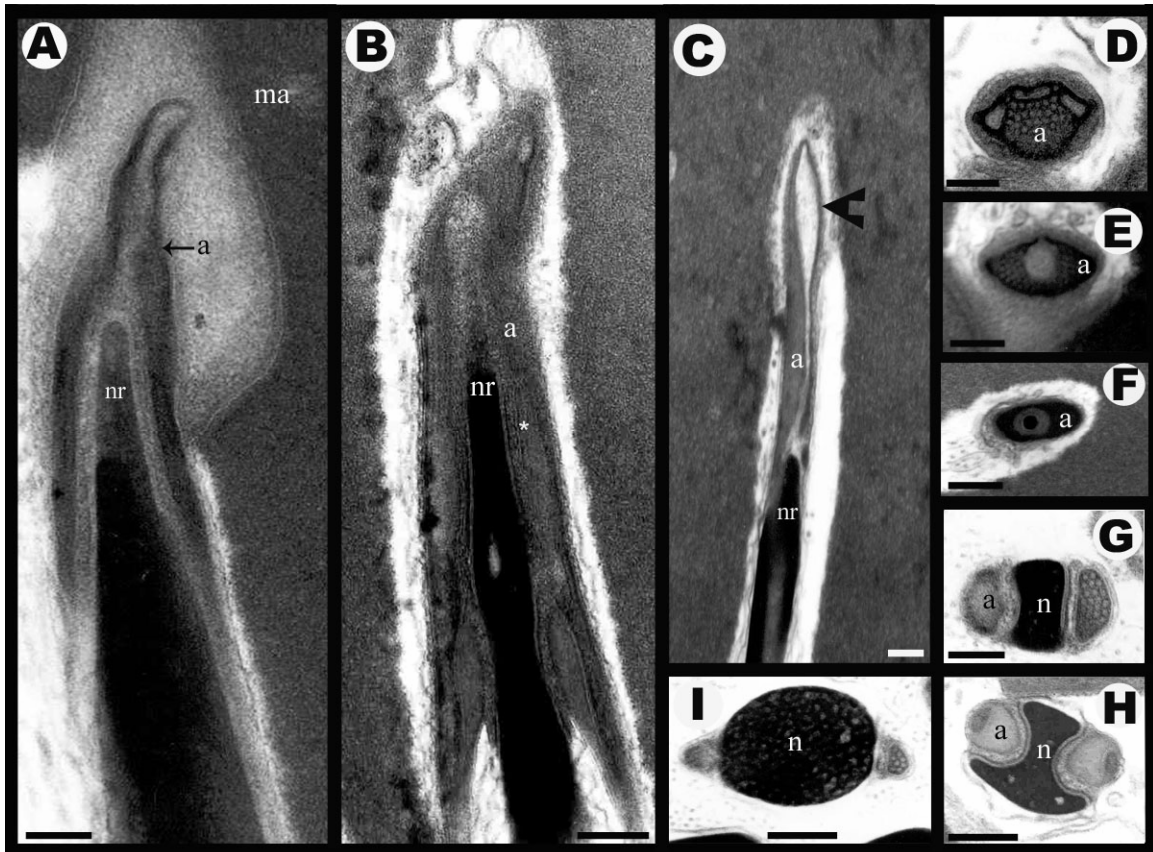


Figure 2.8. A: *Albanycada albigera* sperm. TEM. Longitudinal section through the acrosome and nucleus. The acrosome (a) completely encloses the anterior rostrum of the nucleus (nr). Note the homogenous matrix (ma). Bar = 100 nm. B: Longitudinal section through the sperm head of *Pycna semiclara*. The acrosome (a) encloses the rostrum of the nucleus (nr). Note the tubular elements (\*) within the acrosome. Bar = 100 nm. C: *Platycleura capensis* sperm. Longitudinal section through the sperm head to show the subacrosomal space (arrowhead), acrosome (a) and the rostrum of the nucleus (nr). Bar = 100 nm. D: Transverse section through the anterior tip of the acrosome in *A. albigera* showing the tubular substructure of the acrosome (a). Bar = 100 nm. E; Spermatozoon of *A. albigera*. Transverse section through the acrosome to show the acrosome (a) and the central sub-acrosomal space. Bar = 100 nm. F: Spermatozoon of *A. albigera*. Transverse section through the sperm head to show the subacrosomal space and the acrosome (a) surrounding it. The rostrum of the nucleus at the centre is within the sub-acrosomal space. Bar = 100 nm. G: Transverse section through the sperm head to show the nucleus (n) and the two extensions of the acrosome (a) flanking it. Note the tubular appearance of the acrosome. Bar = 100 nm. H: Transverse section towards the base of the acrosome in the sperm of *A. albigera*. Here the nucleus (n) is bilaterally concave and is still flanked laterally by extensions of the acrosome (a). Bar = 100 nm. I: Transverse section at the posterior level of the acrosome in the sperm of *A. albigera*. Here the nucleus (n) is now spherical and the extensions of the acrosome have decreased in diameter. Bar = 100 nm.

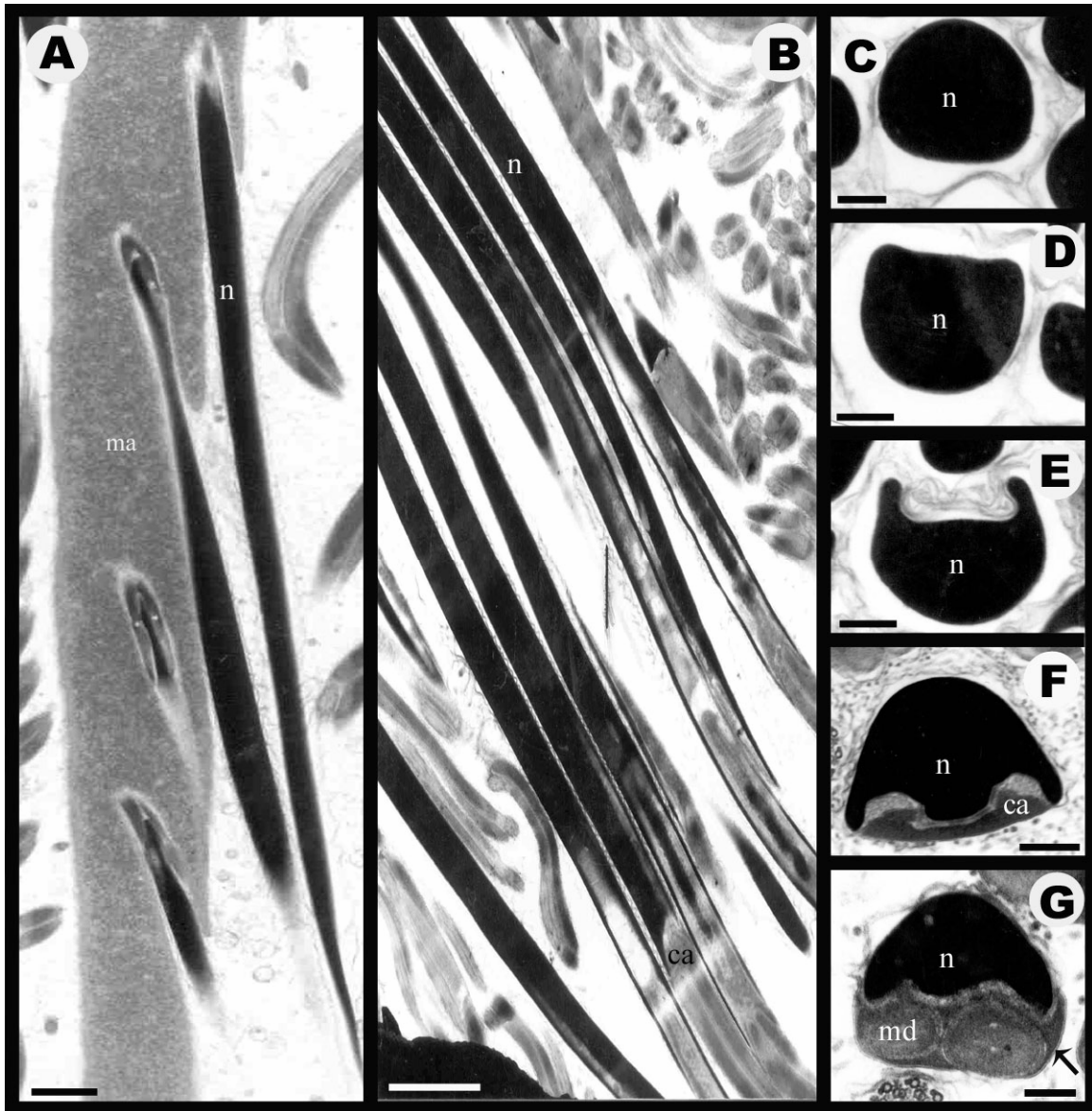


Figure 2.9. A: Spermatozoon of *Platyleura hirtipennis*. Longitudinal section through the anterior part of the nucleus. The nucleus (n) is elongate and the entire acrosome together with a small portion of the nucleus is embedded in a homogenous matrix. Bar = 1  $\mu$ m. B: Longitudinal section through the posterior part of the sperm nucleus (n) and anterior mid-piece of *P. hirtipennis* showing the nuclear grooves housing the centriolar adjunct (ca) and the anterior ends of the two mitochondrial derivatives. Bar = 1  $\mu$ m. C: Transverse section of the nucleus through a region adjacent to the acrosomal base in the sperm of *P. hirtipennis*. There the nucleus (n) has a cylindrical shape. Bar = 200 nm. D: Spermatozoon of *P. hirtipennis*. Transverse section of the nucleus at a region further from the posterior end of the acrosome. The cylindrical nucleus (n) is now shaped like a semi-circle. Bar = 200 nm. E: Transverse section of the nucleus through part of the mid-region of the sperm head of *P. hirtipennis*. Here the nucleus (n) is laterally invaginated. Bar = 200 nm. F: Transverse section of a more posterior region of the sperm nucleus of *P. hirtipennis* showing the centriolar adjunct (ca) housed in the nuclear groove. Bar = 200 nm. G: Transverse section at the nuclear base in the sperm of *P. hirtipennis*. The anterior ends of the mitochondrial derivatives (md) protrude into the material of the centriolar adjunct (arrow). The nucleus (n) has become semi-elliptical. Bar = 100 nm.

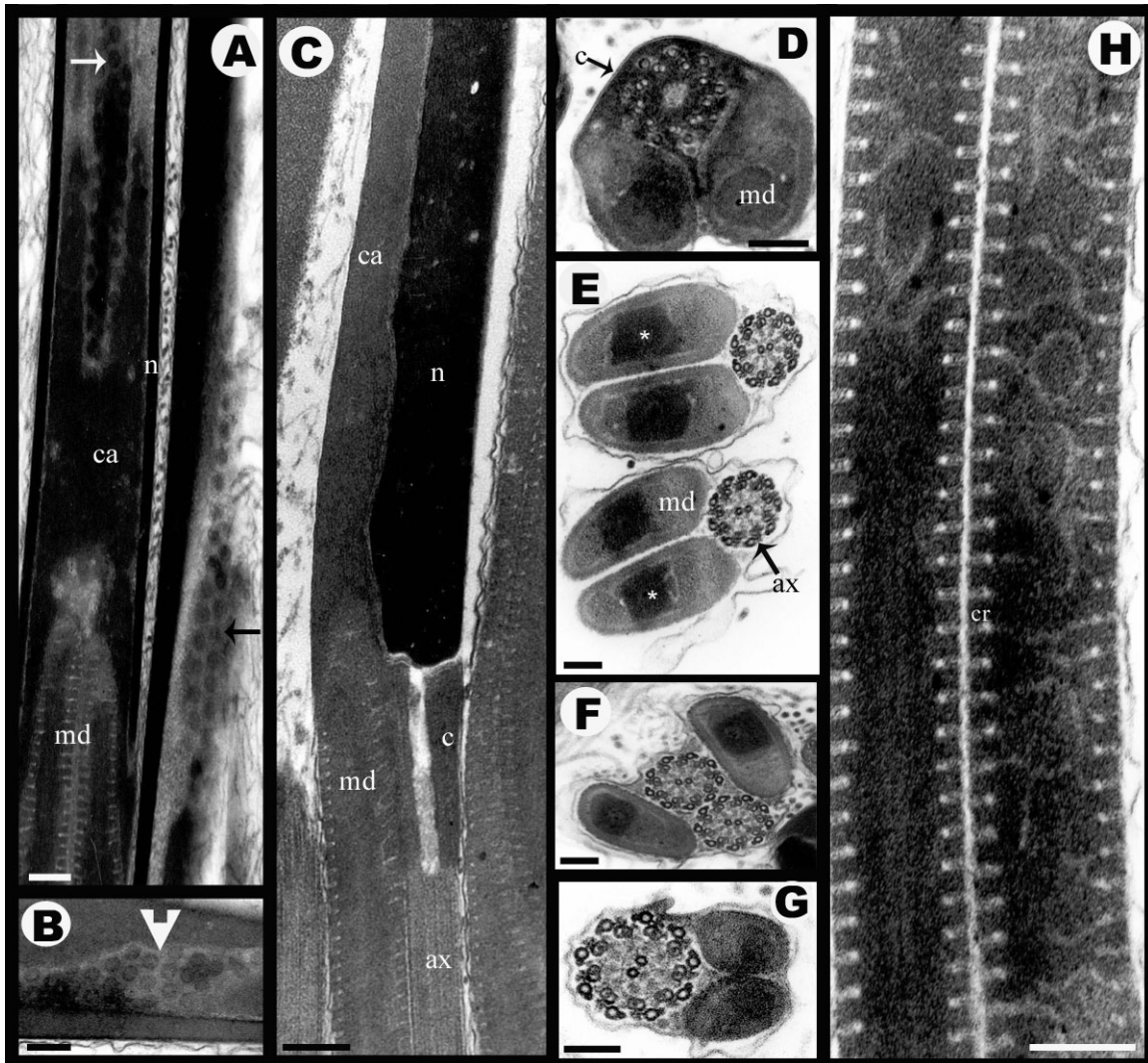


Figure 2.10. A: Spermatozoon of *Platycleps hirtipennis*. Longitudinal section through part of the nucleus-flagellum region showing the centriolar adjunct (ca), nucleus (n) and the mitochondrial derivatives (md). The centriolar adjunct is composed of electron-dense material. Note the vesicular-like structures (arrows) located between the posterior segment of the nucleus and the centriolar adjunct. Bar = 200 nm. B: Longitudinal section through the sperm mid-piece of *P. hirtipennis* showing the vesicle-like structures (arrowhead). Bar = 200 nm. C: Longitudinal section through the nucleus-flagellum transition region in the sperm of *P. hirtipennis*. Posterior to the nucleus (n) lies a centriole-like organelle (c) from where the axoneme (ax) emerges. The two mitochondrial derivatives, (md) are positioned lateral to the axoneme and posterior to the elongated centriolar adjunct (ca). Bar = 200 nm. D: Transverse section through the neck region in the sperm of *Pycna semiclara*. The centriole-like organelle, (c), consists of a ring of doublets rather than triplet microtubules. md, mitochondrial derivative. Bar = 100 nm. E: Transverse section through sperm tails of *P. hirtipennis* to show the 9 + 9 + 2 axoneme (ax) flanked by the two mitochondrial derivatives (md). Note the crystallized regions (\*) in each derivative. Bar = 100 nm. F: Spermatozoon of *P. hirtipennis*. Biflagellate sperm with two axonemes. Bar = 100 nm. G: Transverse section at the posterior level of the sperm of *P. semiclara*. The mitochondrial derivatives have decreased in diameter. Bar = 100 nm. H: Spermatozoon of *P. capensis*. Longitudinal section through the mitochondrial derivatives (md). The peripheral cristae (cr) in

each derivative are arranged in regular patterns perpendicular to the longitudinal axis of the derivative. Bar = 100 nm.

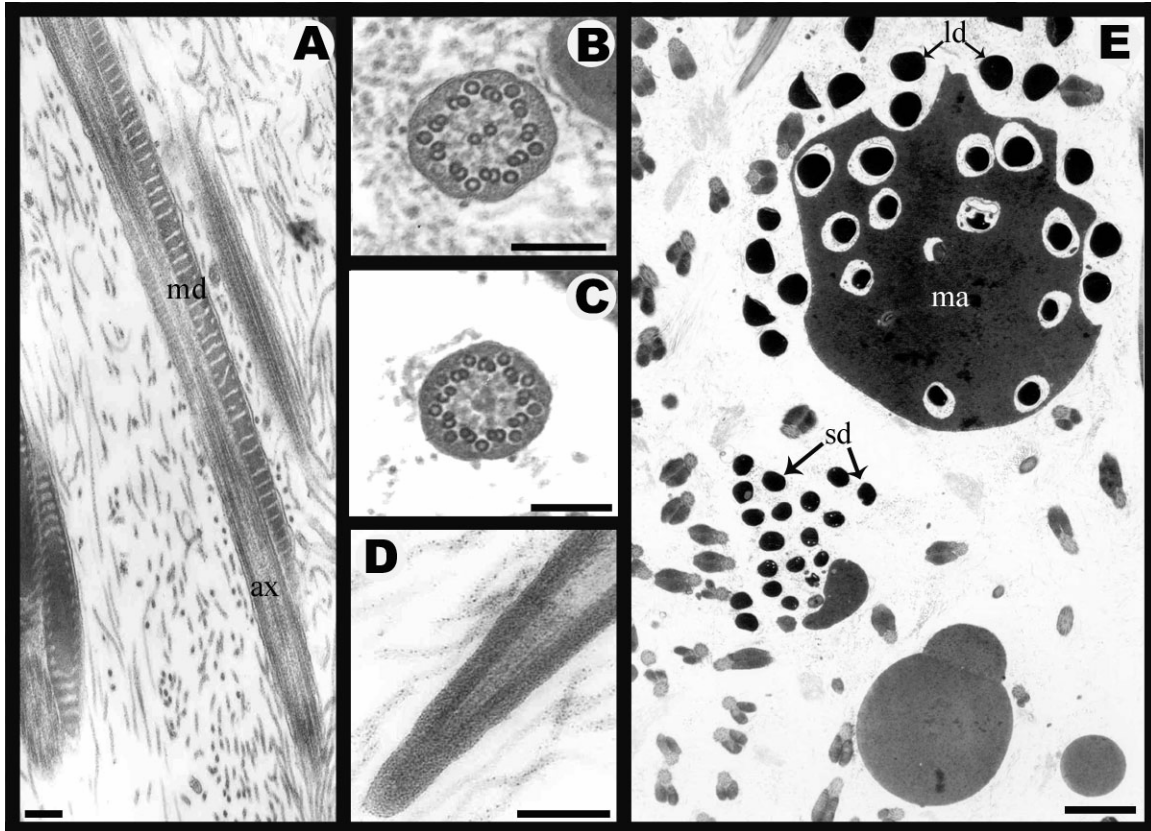


Figure 2.11. A: Spermatozoon of *Azanicada zuluensis* sectioned longitudinally along part of the sperm tail. The two mitochondrial derivatives (md) do not flank the axoneme (ax) along the entire length of the sperm tail. Bar = 200 nm. B: Transverse section at the posterior level of the sperm tail of *A. zuluensis* to show the microtubules in the axoneme. Bar = 100 nm. C: Transverse section at the terminal end of the sperm of *Albanycada albigera*. Here the central microtubules of the axoneme have been lost. Bar = 100 nm. D: Spermatozoon of *A. albigera*. Longitudinal section at the terminal end of the sperm tail showing the narrowing of the axoneme. Bar = 200 nm. E: *Pycna semiclara*. Low magnification of a cross-sectional profile through a seminal vesicle. One profile shows sperm nuclei embedded in a homogeneous matrix (ma). There are differences in the size of the sperm nucleus; in some the diameter is small (sd) while in others the diameter is larger (ld). Bar = 1  $\mu$ m

### 2.3.6. Endpiece Region

In *Albanycada albigera* approximately 900 nm of axoneme extends beyond the posterior limits of the two mitochondrial derivatives, narrows to a point at the terminal end, and disrupts the normal 9 + 9 + 2 pattern of microtubules (Fig. 2.11A, B, C, D). Other species could not be measured but they have the same arrangement.

## 2.4. DISCUSSION

The sperm of the platypleurine cicadas described in the present study have a number of morphological features that are common to the Cicadomorpha (Cicadoidea, Cercopoidea and Membracoidea) and Fulgoromorpha described to date (Folliot and Maillet, 1970; Jamieson *et al.*, 1999; Kubo-Irie *et al.*, 2003). Similarities include (a) more than one length of nucleated motile spermatozoa; (b) a cylindrical, bilaterally symmetrical nucleus; (c) the absence of a perforatorium; (d) a centriolar region with a centriole-like organelle consisting of microtubular doublets and their accessories rather than triplets; (e) vesicle-like elements in an ordered array that are associated with both the nucleus and centriolar adjunct and housed in the nuclear invaginations; (f) a centriolar adjunct that is located in front of the mitochondrial derivatives; (g) two crystalline mitochondrial derivatives that are lateral in position and extend along the axoneme; (h) a single axoneme with a 9 + 9 + 2 arrangement of microtubules; and (f) sperm that are grouped together to form spermatodesmata. The sperm mid-pieces of the Cicadidae and Cercopidae lack accessory bodies, suggesting that within the Cicadomorpha, these two families are more closely related. Such a relationship between the cicadas and spit bugs is supported by current molecular phylogeny (Campbell *et al.*, 1995; Bourgoin and Campbell, 2002; Cryan, 2005).

It is difficult to compare the results of the present study with those of previous investigations on cicadas and cercopids (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003) because sperm morphologies in these insects were not described completely. For example, these studies lacked details of the internal structure of the acrosome, axoneme

and centriolar adjunct. However, according to Folliot and Maillet (1970) and Kubo-Irie *et al.* (2003) the acrosomes in *Lyristes plebejus*, *Cicada orni* and *Graptosaltria nigrofuscata*, whilst anterior in position, are not located at the very tip of the nucleus because there is an anteaerosomal bleb that is as long as the acrosome itself. In this study the acrosomes of the five species of platypleurine cicadas were positioned at the tip of the nucleus and the anteaerosomal bleb was absent. The presence of nuclear pores and their complexes at the base of the nucleus was reported by Folliot and Maillet (1970). From the present study it is not clear whether the vesicular structures housed within the nuclear invaginations and associated with both the nucleus and centriolar adjunct are indeed nuclear pore complexes. Further detailed ultrastructural work is needed to decide this.

Two previously undescribed features, the presence of some biflagellate sperm and some helical/strepsiform sperm heads were found in the platypleurine cicadas. Biflagellate and binucleate sperm have been described in the primitive zygentoman insect *Tricholepidion gertshii* (Dallai *et al.*, 2001b). The sperm cells of this insect are released as individuals from the testes and pairing occurs in the deferent ducts. The spermatozoon thus formed has two acrosomes, two nuclei and two separate tails (Dallai *et al.*, 2001b). There is no evidence of two nuclei or two acrosomes in the five cicada species studied, hence there is no sperm pairing. Abnormal spermatozoa that are biflagellate but uninucleated are found in many animals, including insects and result from the failure of the second meiotic division (e.g. Gonzales *et al.*, 1998; Callaini *et al.*, 1999). The way they are formed is unrelated to the existence of normal biflagellate spermatozoa in some insect species

which result from unorthodox but normal meiotic divisions (e.g. Friedlander and Wahrman, 1971). In these species only two and not four spermatozoa are produced. The presence of biflagellate, uninucleate sperm in the five cicada species would be of interest for further analysis; it is well known that insects have only a single axoneme because the centriole does not replicate before the second meiotic division (Gonzales *et al.*, 1998; Callaini *et al.*, 1999). Occasional biflagellarity is not unique to the Cicadidae or other auchenorrhynchs; it occurs more extensively and regularly in other hemipteroid orders which are closely related to cicadas (Jamieson *et al.*, 1999). Nevertheless, it is difficult to understand systematic relationships based on studies concerning abnormal spermatogenesis.

Kubo-Irie *et al.* (2003) have previously described two distinct sizes of spermatozoa in *G. nigrofuscata*. They found a significant correlation between nuclear length and total sperm length. In the present study there were no significant correlations ( $P > 0.05$ ) between these parameters (Figs. 2.3A, B, 2.4A, B, 2.5). Sperm with long nuclei could have either short or long tails, although the majority possessed long heads with short tails. The higher frequency of long nuclei might indicate that such sperm are favoured in fertilizations. The dimensions of sperm heads and tails in *G. nigrofuscata* are considerably shorter than those from the five platypleurine cicadas examined in this study.

The functional significance of sperm polymorphism remains unclear (Snook, 1998; Swallow and Wilkinson, 2002). Pasini *et al.* (1996) found that although *Drosophila*

*subobscura* males produce two classes of motile spermatozoa that differ in total length and nucleus length, there was no difference in the amount of DNA contained in each sperm morph. This implies different packaging of DNA in the sperm nucleus. To date no attempt has been made to quantify the DNA in cicada sperm morphs. Hopefully, this will be done in the future. The variation in total sperm length has been of particular interest, as comparative studies on diverse taxa have found positive relationships between sperm size and the risk of sperm competition (Pitnick *et al.*, 2003). Collectively, these studies strongly implicate postcopulatory sexual selection mediated by a component of female cryptic choice.

In *Drosophila obscura* (Snook *et al.*, 1994) and *G. nigrofusca* (Kubo-Irie *et al.*, 2003), sperm possessing long nuclei and long tails fertilized all eggs. However, nothing was mentioned about the role of short sperm that are also produced in abundance. Such a high proportion of short sperm might indicate that they have functional significance and are not aberrations such as sperm with helical nuclei or those that are biflagellate. Snook and Karr (1998) demonstrated that in some drosophilids both long and short spermatozoa fertilize the eggs although the short ones do that only after the long ones have already been used and depleted. This example possibly supports the idea that long sperm generate more power and are therefore able to swim faster than short sperm during the race to reach the fertilizing site (Katz and Drobnis, 1990). In extreme sperm dimorphism e.g. in snails and moths, not only size varies among the morphs but also sperm structure and DNA content. In addition, only the haploid morph can fertilize the egg, the other morph is generally anucleate or lacks most of the nuclear DNA (e.g. Buckland-Nicks,

1998). Variation in sperm size may represent different provisioning of gametes by males as a form of parental investment. Sperm with longer tails have longer mitochondrial derivatives. These mitochondrial derivatives might be important as nutritive resources to the zygote or might function in cytoplasmic inheritance (Perotti, 1973; Afzelius *et al.*, 1976, Sivinski, 1984).

From this study the high percentage of sperm with long heads and short tails in the five species of cicadas probably indicates that nuclear length may be more critical to successful fertilization. Theoretically, sperm with short tails might also provide these nutritive resources, although the quantity would probably be less. Such investments by the male may increase future fecundity. This would be important to insects like cicadas that have a characteristic short adult life span and yet spend years underground as nymphs.

In conclusion, sperm morphology was found to be very similar in the five species of platypleurine cicadas studied although there is some variation in the dimensions of the sperm nuclei and tails. Both the sperm nucleus and tail can be very long in *A. albigera*. The dimensions of the nucleus in *A. zuluensis*, *P. capensis* and *P. hirtipennis* are basically similar; however tails are longer in the former. Sperm dimensions in *P. semiclara* are unrelated to those of the other four species; this may signify the weak affiliations between these species. Further spermatological comparisons between and within genera would be useful to decipher the importance of sperm dimensions in exploring species relatedness.

## **CHAPTER THREE**

### **SPERM MORPHOLOGY IN THE CICADETTINAE, TRIBES CICADETTINI, TETTIGOMYIINI, PARNISINI AND TAPHURINI**

### 3.1. INTRODUCTION:

The subfamily Cicadettinae is characterized by the presence of entirely covered tymbals and tymbal cavities. Currently, this subfamily is divided into 12 tribes including Cicadettini, Chlorocystini, Parnisini, Tibicinini, Taphurini, and Tettigomyiini (Duffels and van der Laan, 1985). The cosmopolitan Cicadettini, comprising about 25 genera and 368 species are distributed across five continents and many islands in the South Pacific. Representatives of this tribe have been found in Australia, New Caledonia, New Zealand, North America, South Africa, Europe and Asia (Arensburger *et al.*, 2004). The Taphurini, comprising about 29 genera and 122 species, are found in Africa, Asia, Australia and New Zealand. The Tibicinini, with 12 genera and 114 species, are mostly common in Asia, Australia, North and South America and Europe. Included in this tribe is the cosmopolitan red cicada, *Tibicina haematodes*. The tribes Parnisini and Chlorocystini, which are closely affiliated, form a major group of cicadas in the West Pacific region (Duffels, 1988; 1990). This group of about 49 genera and 170 species is also found in Australia, Africa, Brazil and Ecuador. The Tettigomyiini, another small tribe, is found in Africa (Villet, 1997).

As stated in Chapter 1, the appraisal of sperm morphology in exploring the systematics, phylogeny and fertilization biology in cicadas requires comparative information from a greater number of taxa. Sperm morphology in the Cicadettinae has never been described and yet these cicadas (comprising both small and large species) form an important component of the cicada fauna worldwide.

The present study presents details on the sperm structure in five species of cicadettine cicadas from different tribes (Table 3.1). Reproductive behaviour in most cicadettine cicadas is often different from cicadine cicadas (Villet *et al.*, 1999). Male cicadettines do not stridulate from a fixed position; instead they constantly change their positions in their search for receptive females (Villet and van Noort, 1999). The aims of this study, therefore, were to elucidate and compare sperm structure at a tribal level by studying five species from four tribes of cicadettine cicadas, and to determine whether they have sperm polymorphism.

## **3.2. MATERIALS AND METHODS**

### **3.2.1. MATERIALS**

Male cicadas of *Melampsalta leucoptera* (Izzard, 1958), *Stagira simplex* (Germar, 1834) *Xosopsaltria thunbergi* (Metcalf, 1955) and *Quintilia walkeri* (China, 1925) were collected in the vicinity of Grahamstown (33° 18' S 26° 32'E). Males of *Monomatapa matoposa* (Boulard, 1980) were collected in Malilangwe Private Game Reserve, Zimbabwe (20° 58' S 31° 47' E).

### **3.2.2. LIGHT MICROSCOPY**

Five males of each species were dissected under a binocular microscope in 2% saline solution and spermatozoa were recovered from the testes and seminal vesicles. Sperm samples were spread evenly on a microscope slide coated with gelatin and chrome alum and a drop of glycerol was added before drying in an oven overnight at 60°C. Slides were then placed in a methanol/acetic acid (75:25 v/v) fixative for 4 minutes and rinsed in

saline solution for one minute. The slides were then placed in 1% bisbenzimidazole Hoechst 33258, a cell-permeable adenine-cytosine binding fluorescent dye used to stain DNA (Sakaluk and O'Day, 1984) for one minute. The slides were then rinsed in three changes of saline before a drop of glycerol/PBS (9:1 v/v) was added to the cells and covered with a coverslip. Slides were examined with an Olympus BX-61 epifluorescence microscope at a wavelength of 343 nm. Digital images of 50-100 sperm per male were captured from each specimen. Sperm heads were measured from these images using the analySIS Soft Imaging System programme ([www.softimaging.net](http://www.softimaging.net)). Scatter diagrams were generated to show the relationship (if any) between sperm nucleus length and tail length using Statistica version 6.1 and correlation coefficients were calculated. Frequencies of different length classes of sperm within and between species were analyzed by chi-square tests. Measurements are reported as mean  $\pm$  standard error.

Table 3.1. List of cicadettine cicadas examined for sperm morphology.

Species	Subfamily	Tribe	Location
<i>Melampsalta leucoptera</i>	Cicadettinae	Cicadettini	Grahamstown, South Africa
<i>Xosopsaltria thunbergi</i>	Cicadettinae	Tettigomyiini	Grahamstown, South Africa
<i>Stagira simplex</i>	Cicadettinae	Tettigomyiini	Grahamstown, South Africa
<i>Quintilia walkeri</i>	Cicadettinae	Parnisini	Grahamstown, South Africa
<i>Monomatapa matoposa</i>	Cicadettinae	Taphurini	Malilangwe Game Reserve, Zimbabwe

### 3.2.3. SCANNING ELECTRON MICROSCOPY

Spermatozoa of each species were attached to gelatin-coated coverslips in 2 % saline solution and fixed overnight with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7)

at 4°C, dehydrated in a graded series of acetone, critical-point dried and sputter-coated with gold. The sperm were then examined and photographed in a JEOL 840A scanning electron microscope at 12 kV.

#### **3.2.4. TRANSMISSION ELECTRON MICROSCOPY**

Testes and seminal vesicles from at least two individuals of each species were dissected in 2 % saline solution before fixation overnight at 4°C in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After washing in 0.1M phosphate buffer, tissues were postfixed in 1 % osmium tetroxide in 0.1M phosphate buffer for 90 minutes at room temperature, dehydrated in ethanol and embedded in Epon 812 resin. Ultrathin sections (silver-gold) were cut using a diamond knife and collected on 300 mesh copper grids before staining with uranyl acetate and lead citrate. Sections were examined and photographed with a JEOL 1210 transmission electron microscope at 120kV. Photographs were scanned and dimensions of the centriolar adjuncts, mitochondrial derivatives and nuclei were measured using the AnalySIS Soft Imaging System programme ([www. softimaging.net](http://www.softimaging.net)).

### **3.3. RESULTS**

#### **3.3.1. Light Microscopy**

Mature spermatozoa of the five species are elongate and filiform, consisting of three distinct regions: the head (acrosome and nucleus), midpiece and tail (Fig. 3.1A, B, C, D, E). Polymegaly is expressed in three ways; sperm have uni-, bi- or trimodal nucleus and

tail lengths. Besides the differences in length, there are also notable differences in the size of nuclei. In all species except *Quintilia walkeri*, approximately 20% of sperm have voluminous nuclei; with diameters being almost double that of the other sperm (Fig. 3.5A, B). Voluminous nuclei are not only restricted to the short-headed sperm; they are also present in long-headed sperm.

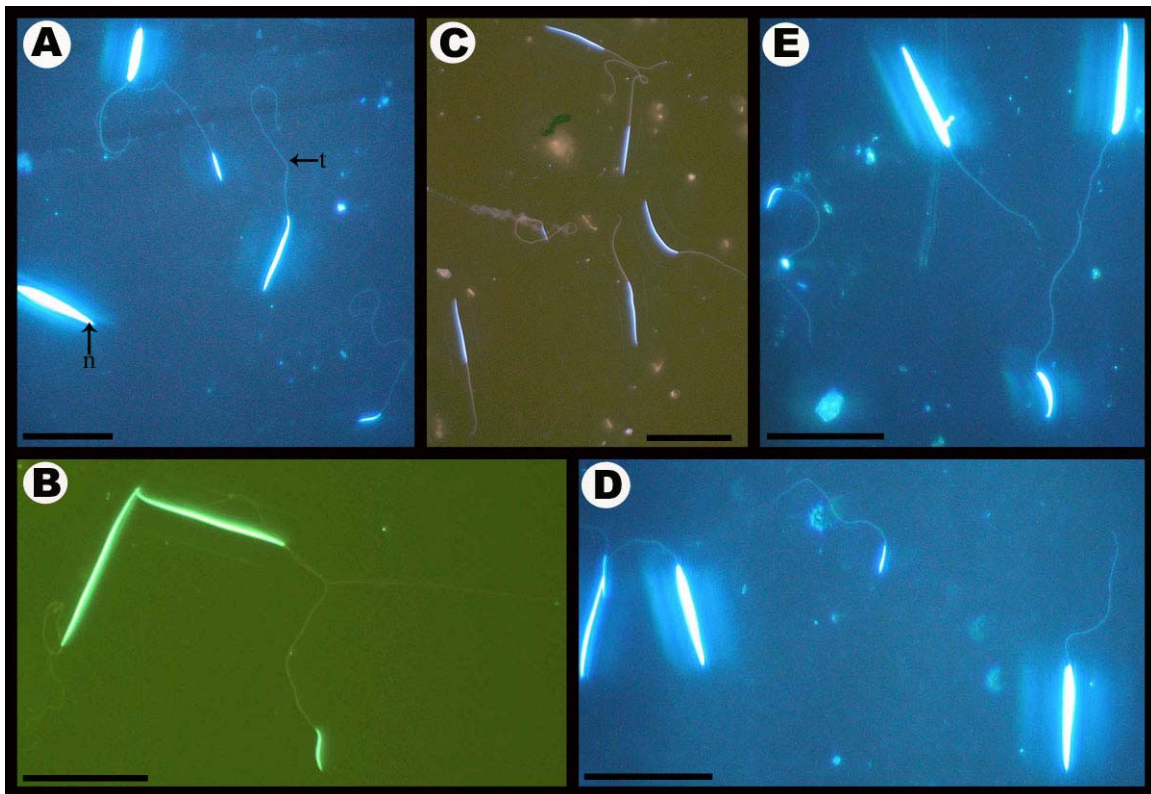


Figure 3.1. Long and short sperm of cicadettine cicadas stained with Hoechst 33258 and examined with a fluorescence microscope at 343 nm. A: *Melampsalta leucoptera*. n, nucleus; t, sperm tail. Bar = 50  $\mu\text{m}$ . B: *Monomatapa matoposa*. Bar = 50  $\mu\text{m}$ . C: *Quintilia walkeri*. Bar = 50  $\mu\text{m}$ . D: *Stagira simplex*. Bar = 50  $\mu\text{m}$ . E: *Xosopsaltria thunbergi*. Bar = 50  $\mu\text{m}$ .

Table 3.2. Modal classes and correlation coefficients (r) of nucleus length versus tail lengths in the sperm of five cicadettine cicadas. Data obtained from light microscopy.

Species	Modal classes		Modal classes		r
	Nucleus length (μm)		Tail length (μm)		
<i>M. leucoptera</i>	16	48	67	122	-0.81
<i>S. simplex</i>	10	40	68	113	-0.59
<i>X. thunbergi</i>	12	20	47	94	-0.60
<i>M. matoposa</i>	26	65		112	-0.25
<i>Q. walkeri</i>	21	39	48	98	-0.68

Table 3.2 summarizes the modal classes of the sperm nucleus and tail lengths, together with the respective correlation coefficients in each of the five cicada species. In *Melampsalta leucoptera*, two modes (67 and 122 μm) of sperm tail length are present. The tail length values of the shorter modal class are markedly asymmetrical (Fig. 3.2A). Sperm nucleus lengths fall into two major classes, with modes of 16 and 48 μm. There is a strong negative correlation between sperm nucleus and tail lengths ( $r = -0.81$ ). Sperm with longer nuclei generally have shorter tails, while sperm with shorter nuclei have generally longer tails (Fig. 3.2A). The distribution of sperm dimensions in *M. leucoptera*, analyzed by chi-square tests, indicates that the frequencies of short nuclei and short tail classes differ between individuals ( $\chi^2 > 13.28$ ,  $P < 0.01$ ). The rest of the frequencies are similar (see Appendix 5). Tail lengths in *Quintilia walkeri* fall into two major classes, with modes of 48 and 98 μm (Fig. 3.2B). The values of the short modal class are markedly asymmetrical. There are two modes for the nucleus length (21 and 39 μm) and the values of the short class are markedly skewed. There is a modest correlation ( $r = -0.68$ ) between sperm nucleus and tail length. In all five individuals the frequencies of nuclei and tail length classes are similar ( $\chi^2 < 13.28$ ,  $P > 0.01$ ).

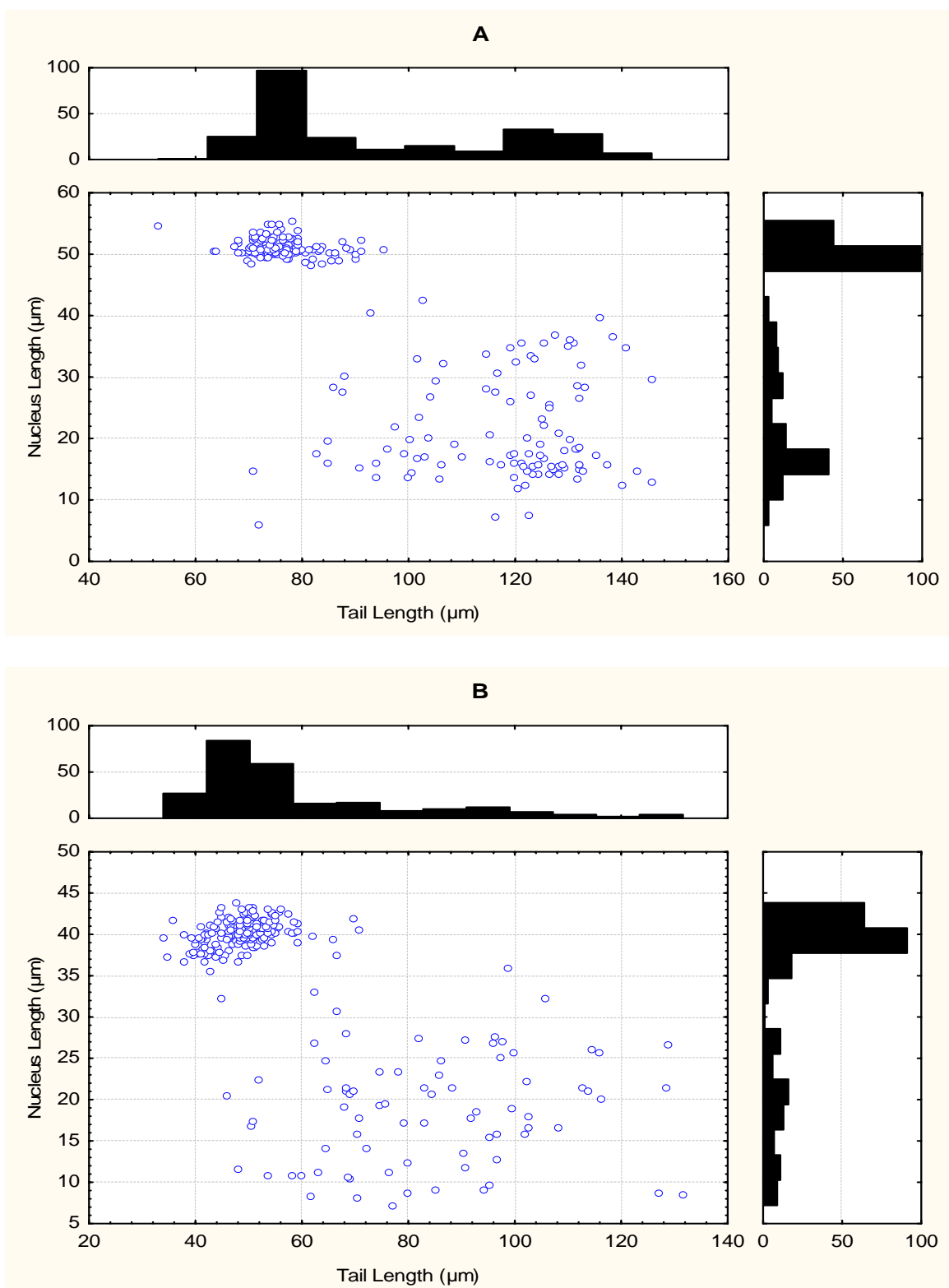


Figure 3.2. Scatter-plots of nucleus length versus tail length and size-frequency histograms. A: *Melampsalta leucoptera*; B: *Quintilia walkeri*.

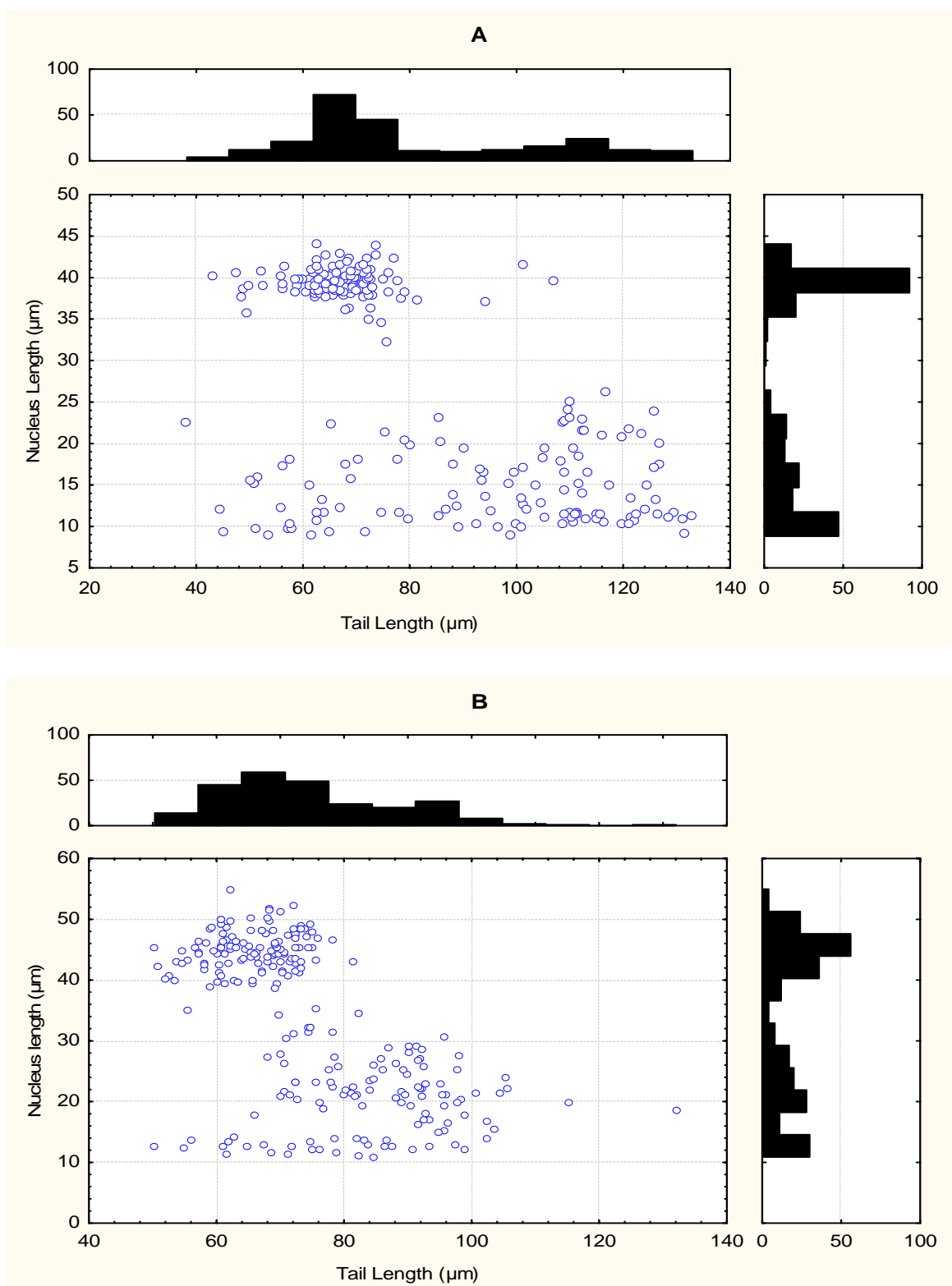


Figure 3.3. Scatter-plots of nucleus length versus tail length and size-frequency histograms. A: *Stagira simplex*; B: *Xosopsaltria thunbergi*.

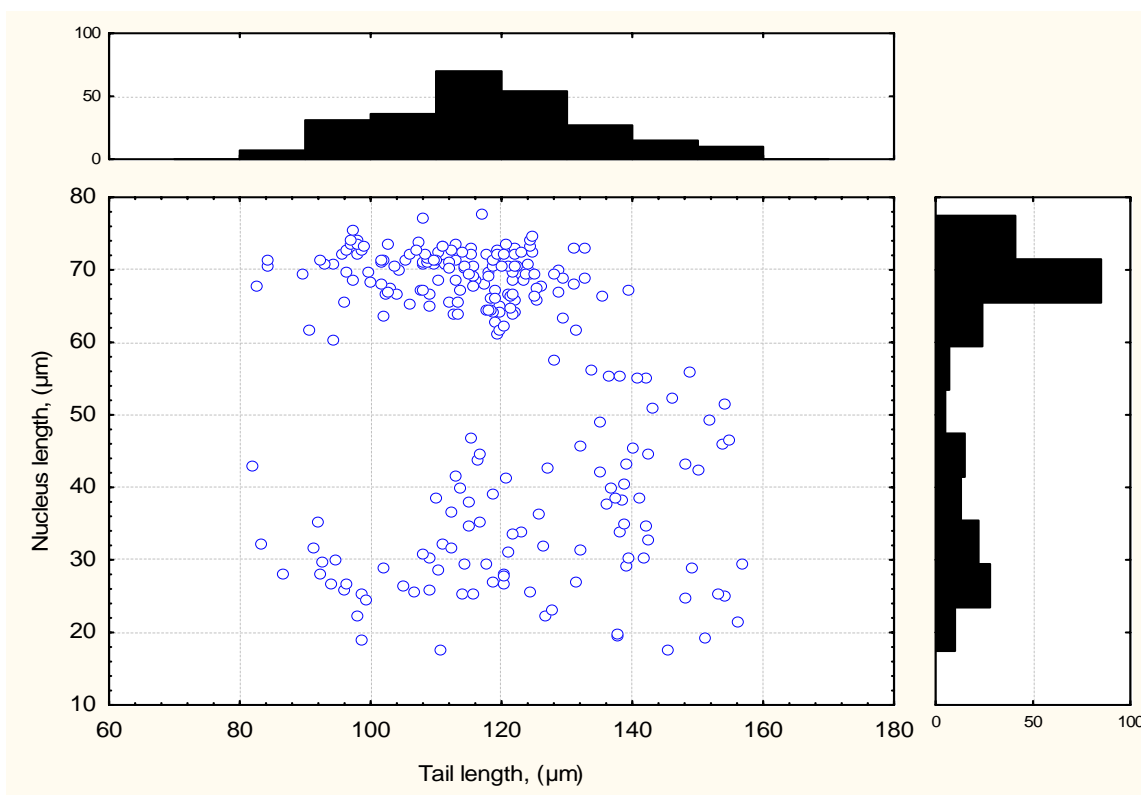


Figure 3.4. Scatter-plot of nucleus length versus tail length and size-frequency histograms in *Monomatapa matoposa*.

*Stagira simplex* has two modes each (68 and 113  $\mu\text{m}$ ; 10 and 40  $\mu\text{m}$ ) of tail and nucleus length respectively (Fig. 3.3A). Sperm nucleus length is moderately correlated ( $r = -0.59$ ) to tail length. Long-headed sperm generally have short tails while short-headed sperm have longer tails. Frequencies of sperm nuclei and tail length classes are similar in all five individuals ( $\chi^2 < 13.28$ ,  $P > 0.01$ , see Appendix 5). *Xosopsaltria thunbergi* (Fig. 3.3B) has two modes of tail length (67 and 94  $\mu\text{m}$ ) and three modes of nucleus lengths (12, 20 and 47  $\mu\text{m}$ ). The correlation between sperm nucleus and tail lengths in this species is also modest ( $r = -0.6$ ). The frequencies of the short nucleus class differ between five *X. thunbergi* males but the intermediate and long nuclei classes are similar (see Appendix 5). In *Monomatapa matoposa*, a single mode (112 $\mu\text{m}$ ) of tail length is present

although the distribution of the values is slightly asymmetrical (Fig. 3.4). There are two major classes of sperm nucleus lengths, with modes of 26 and 65  $\mu\text{m}$ . A weak correlation ( $r = -0.25$ ) exists between nucleus and tail lengths in this species. Frequencies of long nuclei and all tail length classes are similar in *M. matoposa* males (see Appendix 5).

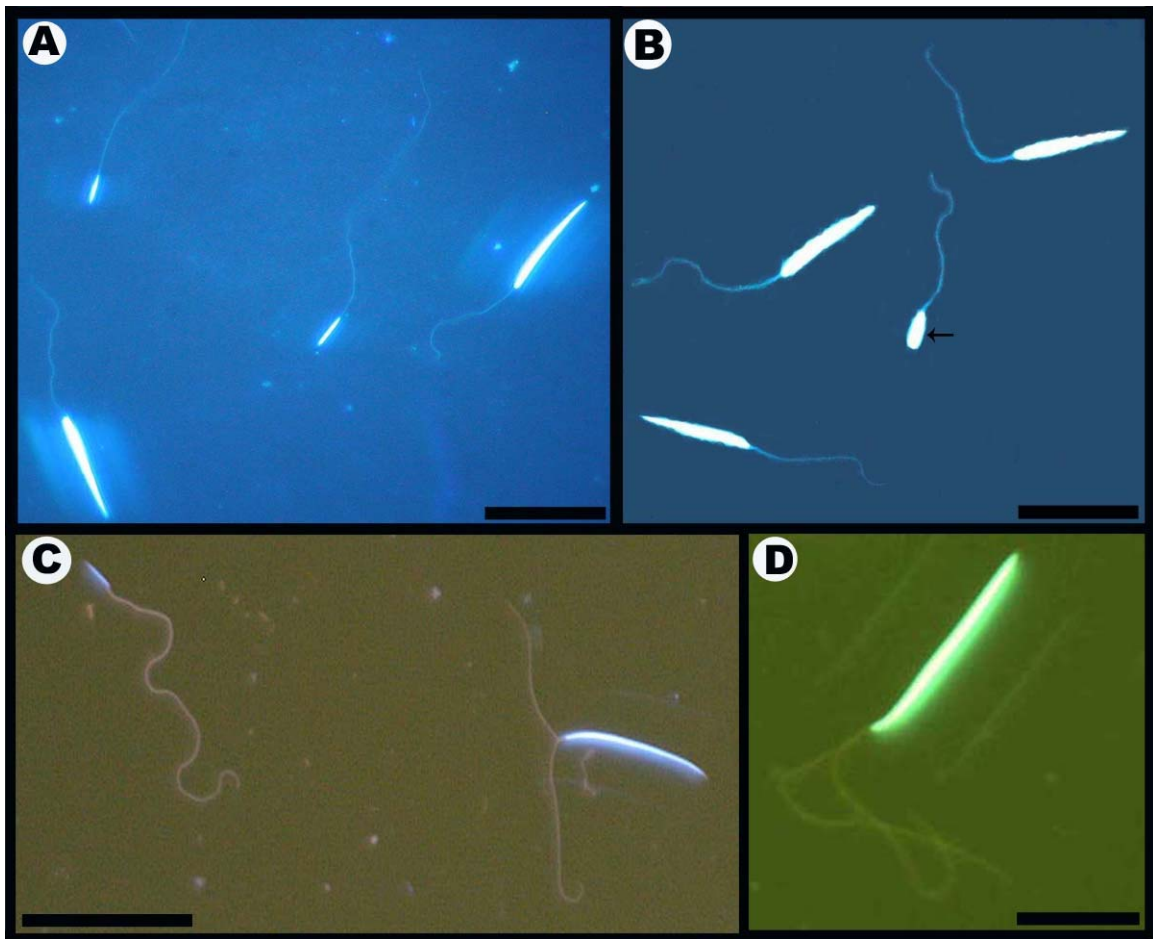


Figure 3.5. A: Sperm of *Melampsalta leucoptera*. Fluorescence images of voluminous nuclei. Unlike the short-headed sperm, the long-headed sperm have voluminous nuclei. Bar = 50  $\mu\text{m}$ . B: Sperm of *Xosopsaltria thunbergi*. Fluorescence images of sperm with voluminous nuclei. Note that both long and short-headed sperm (arrow) have voluminous nuclei. Bar = 50  $\mu\text{m}$ . C: Sperm of *Quintilia walkeri*. Fluorescence images of a biflagellate and a short-headed spermatozoa. All such sperm were uninucleate. Bar = 40  $\mu\text{m}$ . D: Sperm of *Monomatapa matoposa*. Fluorescence image of a biflagellate spermatozoon. Bar = 35  $\mu\text{m}$ .

The frequency of short nuclei differ between these five cicadettines ( $\chi^2 = 21.63$ ,  $P < 0.01$ ) just like the tail length classes (see Appendix 6). The frequencies of long nuclei are, however, similar across species. The most frequently occurring values for nucleus length of long-headed sperm in *M. leucoptera*, *Q. walkeri*, *S. simplex* and *X. thunbergi* are generally similar. In contrast, the modal values of the nucleus lengths (especially long-headed sperm) in *M. matoposa* are larger, almost twice those from the other four tibicinines. Analysis of the scatter plots of nucleus length against tail length and size-frequency histograms of nucleus and tail length show three patterns. One nucleus length and two tail lengths classes characterize the first pattern, seen in *M. matoposa*. Two length classes of both the nucleus and tail are evident in the second pattern; these are evident in *Q. walkeri*. The third pattern found in *M. leucoptera*, *S. simplex* and *X. thunbergi*, has two length classes of both the nucleus and tail but with no allometry within sperm morphs. Approximately 5 % of sperm in all species possess two tails instead of the conventional single flagellum (Fig. 3.5C, D). Such biflagellate sperm are all uninucleate.

### **3.3.2. Transmission Electron Microscopy**

In all species, spermatozoa are aggregated into organized bundles with their heads embedded in a homogenous matrix to form spermatodesmata (Fig. 3.6A, B, C). Individual sperm separate from the bundle to swim in the fluid once exposed to 2 % saline solution. Except for size, the architecture of short and long spermatozoa is generally similar in all species.

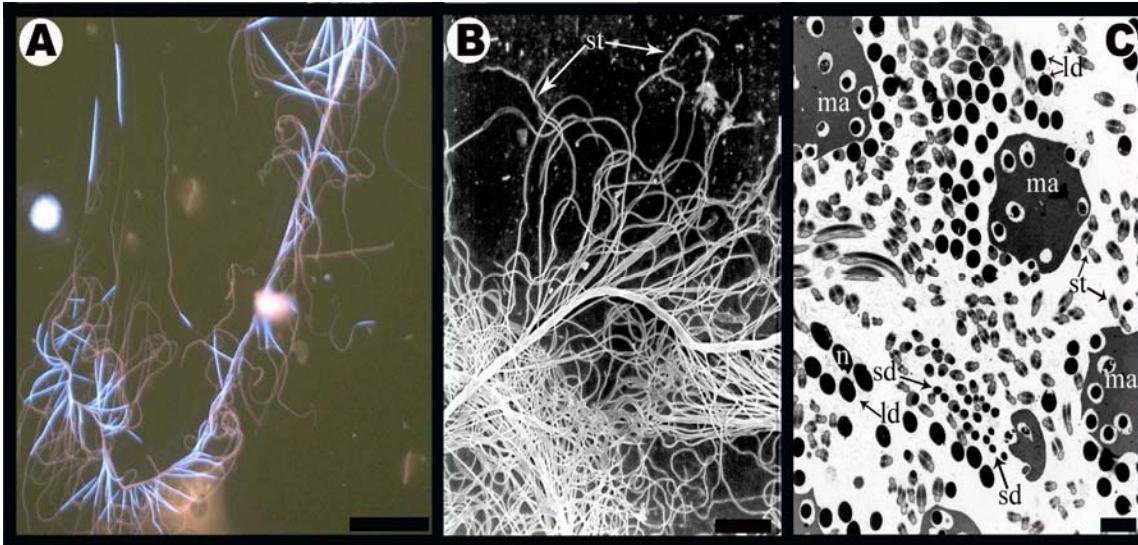


Figure 3.6. A. Fluorescence image (wavelength 343 nm) of a sperm bundle from *Quintilia walkeri* stained with Hoechst 33258. Spermatozoa are released in bundles with their anterior ends embedded in a matrix to form a spermatodesm. Bar = 50  $\mu\text{m}$ . B: Scanning electron micrograph of a spermatodesm from *Q. walkeri*. The sperm tails (st) in the spermatodesm are free. Bar = 20  $\mu\text{m}$ . C: *Melampsalta leucoptera*. Low magnification of a cross-section through a seminal vesicle to show some homogenous matrices (ma) in the lumen showing sperm nuclei (n) and tails (st). The nucleus diameter in some sperm heads is large (ld) yet in others it is small (sd). Bar = 1  $\mu\text{m}$ .

### 3.3.2.1. Tribe Parnisini (*Quintilia walkeri*)

#### Head Region (acrosome and nucleus)

The acrosome (see Table 3.3 for dimensions) is conical in shape and deeply invaginated posteriorly (Fig. 3.7A, B) resulting in a distinct subacrosomal space (Fig. 3.7C, D). The acrosomal contents are differentiated internally with a tubular substructure (Fig. 3.7C, D, E). The anterior of the nucleus intrudes into the posterior section of the subacrosomal space which appears hexagonal in cross-sections (Fig. 3.7D). Anteriorly the acrosome is laterally flattened; posteriorly it extends on either side of the nucleus as two tubular processes that gradually decrease in diameter (Fig. 3.7F, G, H).

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The elongated nucleus is tapered anteriorly to form a rostrum (Fig. 3.7A, B). In cross-section the nuclear rostrum appears circular (Fig. 3.7D). Towards the posterior limit of the acrosomal complex the nucleus undergoes a transformation in shape and becomes semi-elliptic. The transition between the rostrum and the main body of the nucleus is abrupt and cross-sections across this region show the nucleus to be flanked by two acrosomal processes (Fig. 3.7E). Further posteriorly the nucleus becomes bilaterally concave and then rounded (Fig. 3.7F, G, H and 3.8B). The rounded profile delimits the extent of the acrosomal complex. The average diameter of the large nucleus in this region was  $526.22 \pm 7.68 \mu\text{m}$  ( $n = 5$ ). Further towards its posterior limit the nucleus becomes laterally invaginated (Figs. 3.8C and D). The average depth of the lateral invagination was 20.68 % of the sperm nucleus diameter (Table 3.5). The invagination disappears posteriorly and the nucleus becomes circular in cross section (Fig. 3.8E). Basally the nucleus assumes a semi-elliptic profile (Fig. 3.8F). Two sizes of nuclei are evident in several cross-sectional profiles of the seminal vesicles (Fig. 3.7I, J). These nuclei (see Table 3.4 for dimensions) are clumped together according to size.

### **Mid-piece and Tail Region**

A conspicuous centriolar adjunct (Fig. 3.8D) is associated with the nucleus in the sperm mid-piece of *Q. walkeri*. This structure, with electron-dense material, lies parallel to the nucleus within the lateral invaginations. In longitudinal sections the material of the centriolar adjunct is organized as an array of dense lamellae (Fig. 3.8G). Vesicle-like elements are also evident in the sperm mid-piece and are associated with both the nucleus and centriolar adjunct. Further posteriorly, the dense lamellae organization of the

centriolar adjunct material gives way to a conspicuous arrangement of microtubular-like elements (Fig. 3.8E). Posterior to the base of the nucleus is a centriole-like organelle from which the axoneme emerges (Fig. 3.8A, H). This marks the tail region of the sperm, which is formed by an axoneme, and two flanking mitochondrial derivatives in which cristae are arranged in an orderly array (Fig. 3.8I). There are two sizes of mitochondrial derivatives (Table 3.6); the small ones (Fig. 3.8K) appear to be associated with the small nuclei and the large ones with the large nuclei. The anterior ends of each derivative penetrate into the centriolar adjunct material (Fig. 3.8F). The mitochondrial derivatives are oval in cross-section and a crystalline core in each derivative is evident (Fig. 3.8J).

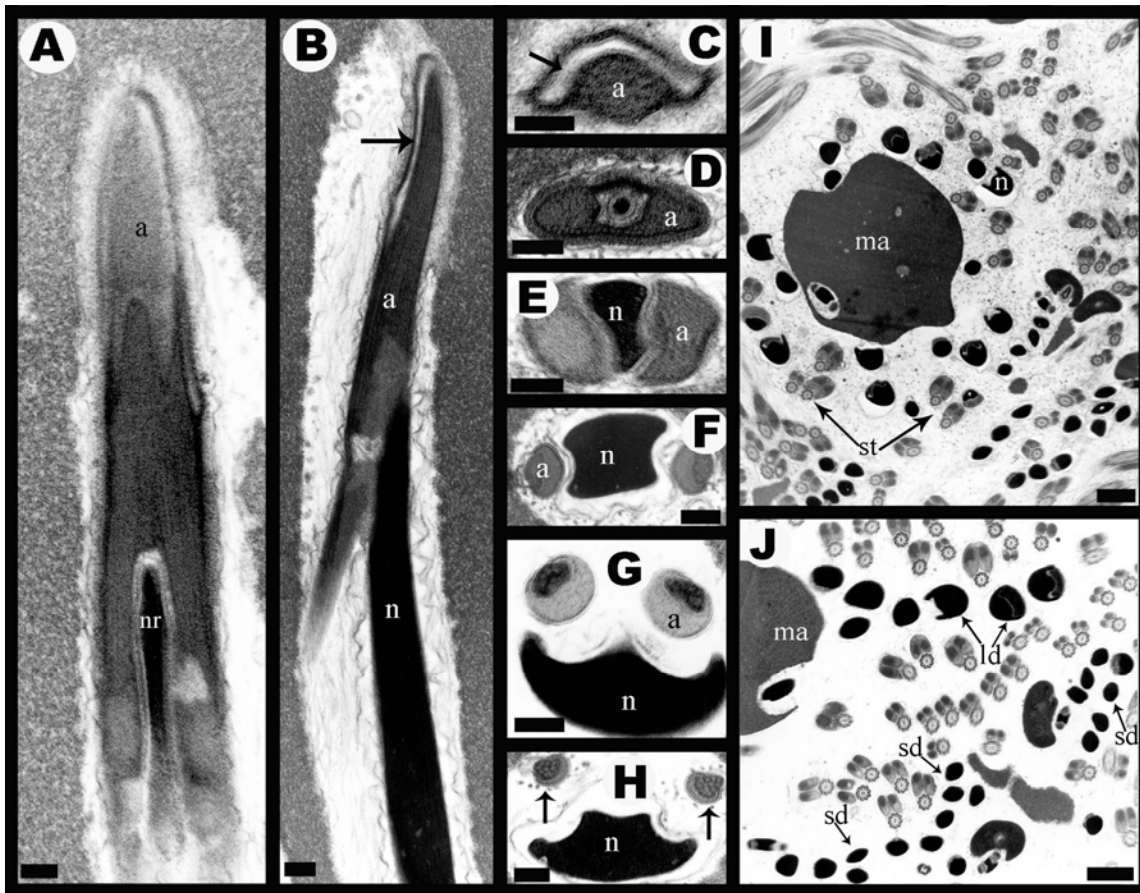


Figure 3.7. *Quintilia walkeri* sperm. TEM. A: Longitudinal section through the sperm head showing the apical acrosome (a) and the elongated rostrum of the nucleus (nr). The tubular substructure of the acrosome is conspicuous. Bar = 100 nm. B: Longitudinal section through the sperm head showing the nucleus (n), acrosome (a) and the subacrosomal space (arrow). Bar = 100 nm. C: A transverse section through the anterior tip of the acrosome (a) to show its tubular substructure. arrow, subacrosomal space. Bar = 100 nm. D: A transverse section through the acrosome (a) and the rostrum of the nucleus. The anterior part of the nuclear rostrum is completely surrounded by the subacrosomal space. Note the hexagonal shape formed by the subacrosomal space. Bar = 100 nm. E: A profile of the sperm head sectioned transversely through the acrosome (a) and nucleus (n). Bar = 100 nm. F: A more posterior profile of the sperm head sectioned transversely through the acrosome (a) and nucleus (n). Bar = 100 nm. G: A cross-sectional profile of the sperm head through a region in close proximity to the base of the acrosome. Here the nucleus (n) is semi-elliptic. Bar = 100 nm. H: A cross-sectional profile of the sperm head through the caudal region of the acrosome. Here extensions of the acrosome (arrows), which form a progressively narrowing sheath, flank the nucleus (n). Bar = 100 nm. I: Low magnification of a transverse-section through a spermatodesm. Several sperm nuclei (n) are free of the homogenous matrix (ma). st, sperm tails. Bar = 500 nm. J: Low magnification of several cross-sectional profiles at various levels through the sperm and spermatodesms. Note the differentially- sized sperm nuclei; some nuclei have small diameters (sd) and others have larger diameters (ld). ma, homogenous matrix. Bar = 500 nm.

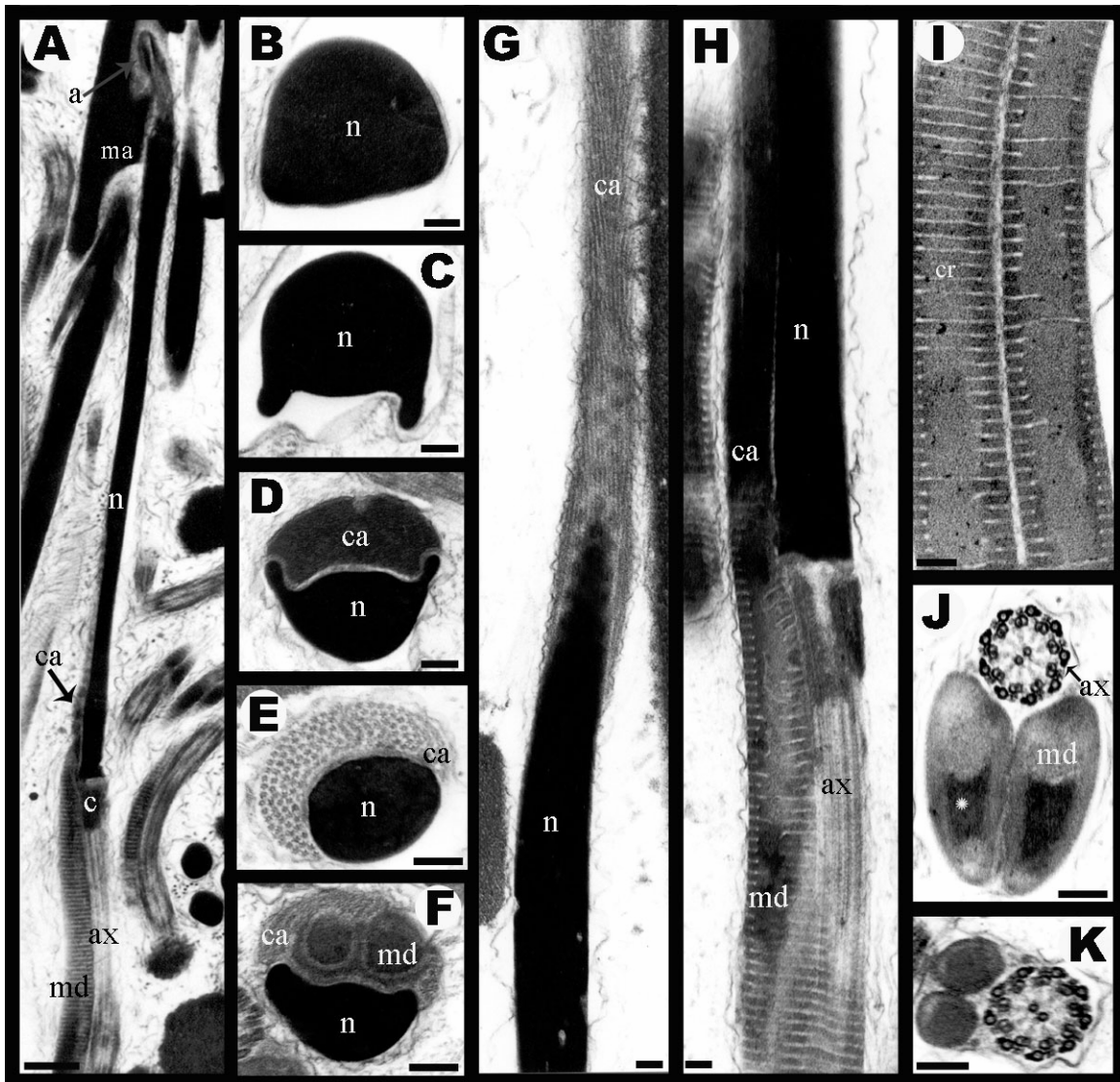


Figure 3.8. *Quintilia walkeri* sperm. TEM. A: Low magnification of a longitudinal section through the head, mid-piece and anterior axoneme of short-headed sperm. The entire acrosome (a) and part of the nucleus (n) are embedded in the homogenous matrix (ma). The centriolar adjunct (ca), positioned along the posterior region of the nucleus, is anterior to the mitochondrial derivatives (md). Posterior to the nucleus is a centriole-like organelle (c) from where the axoneme (ax) emerges. Bar = 500 nm. B: Transverse section through the anterior region of the nucleus but posterior to the acrosome. Here the nucleus (n) is rounded. Bar = 100 nm. C: A transverse section from about the mid-region of the nucleus. In this region, the deep invagination of the nucleus (n) is devoid of material. Bar = 100 nm. D: A transverse section through a more posterior region of the nucleus. In this region, the invagination is filled with material of the centriolar adjunct (ca). Bar = 100 nm. E: A transverse section through a region in close proximity to the base of the nucleus. Here the nucleus (n) is oval. Note the tubular substructure of the centriolar adjunct (ca). Bar = 100 nm. F: Transverse section at the base of the nucleus. Here the nucleus (n) is elliptical and the anterior ends of the two mitochondrial derivatives (md) are embedded into the material of the centriolar adjunct (ca). Bar = 100 nm. G: Oblique section through the middle region of the nucleus (n) and the centriolar adjunct (ca). Bar = 100 nm. H:

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Longitudinal section through the nucleus-flagellum transition region showing the nucleus (n), centriolar adjunct (ca), axoneme (ax) and mitochondrial derivatives (md). Bar = 100 nm. I: Longitudinal section through the mitochondrial derivatives. Note the orderly array of the mitochondrial cristae (cr). Bar = 200 nm. J: Transverse section through the tail to show the axoneme (ax) and the two mitochondrial derivatives (md). Note the crystallized matrix \* of each derivative. Bar = 100 nm. K: A cross-sectional profile through the posterior tail region of short sperm. Note the small size of each derivative. Bar = 100 nm.

### 3.3.2.2. Tribe Cicadettini (*Melampsalta leucoptera*)

#### Head Region (acrosome and nucleus)

The acrosome (see Table 3.3 for dimensions) is laterally flattened and is located anterior to the nucleus (Fig. 3.9A, B, C). Posteriorly, it is invaginated to form a subacrosomal space which is lateral in position towards the anterior of the acrosome. The acrosomal contents are not homogenous in appearance but consist of an electron-dense tubular substructure (Fig. 3.9C, D). The subacrosomal space surrounds the anterior of the nucleus (Fig. 3.9E). Anteriorly, the acrosome is narrow and laterally flattened into a roughly oval apex; posteriorly it widens, gradually forming two narrow tubular processes (Fig. 3.9A to G), which flank the nucleus. These acrosomal processes gradually decrease in diameter posteriorly, finally terminating in a region where the nucleus becomes ellipsoidal to circular in cross-section (Fig. 3.9H, I, J).

The chromatin of the filiform nucleus appears compact and homogenous (Fig. 3.9A, K). Anteriorly the nucleus has a narrow rostrum that is circular in transverse section (Fig. 3.9E). The circular profile of the nucleus in transverse section remains constant up to the base of the rostrum. Towards the posterior limit of the acrosome the nucleus gradually

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becomes bilaterally concave and forms a T- or mushroom-shape when seen in transverse section before it takes on a rounded profile (Fig. 3.9F, G, J). Posteriorly, the nucleus develops a wide lateral invagination, which is initially devoid of material but is distally occupied with material of the centriolar adjunct (Fig. 3.10C, D, E). The average depth of the lateral invagination, expressed as a percentage of the sperm nucleus diameter was 21.46 % (Table 3.5). Towards the base of the nucleus, the lateral invagination gradually narrows and the nucleus, with a decreased diameter, takes on an elliptic cross-section (Fig. 3.10F) before finally terminating at the level where the centriole appears (Fig. 3.11A, B). Spermatozoa with either large ( $590.4 \pm 5.5$  nm) or small ( $202 \pm 4.9$  nm) diameter nuclei are conspicuous in several cross-sectional profiles through seminal vesicles (Fig. 3.6C; Table 3.4). These spermatozoa are clumped and these aggregations are size-based (Fig. 3.6C).

### **Mid-Piece and Tail Region**

The mid-piece consists of a centriolar complex, centriolar adjunct and mitochondria. The centriolar adjunct lies anterior to the centriole (Figs. 3.9K and 3.10A, B, H). Anteriorly, the centriolar adjunct is small (Fig. 3.10D), and is confined to the lateral invagination of the nucleus. Posteriorly, it becomes progressively larger and protrudes from the lateral invagination, eventually completely surrounding the nucleus (Fig. 3.10E, F, G). The material of the centriolar adjunct, which is moderately electron-dense, has a lamella-like substructure (Fig. 3.10A, B, F, G). Vesicle-like elements of approximately 88 nm diameter, that are associated with both the nucleus and centriolar adjunct, are housed in the nuclear invaginations (Fig. 3.11A, D).

The centriole-like organelle and mitochondrial derivatives are located immediately posterior to the nucleus. The centriole-like organelle (Fig. 3.11A, B, C) has a configuration of microtubular doublets and their accessories rather than triplets. The axoneme (Fig. 3.11A, B, F, G) emerges from the centriole and is flanked by two mitochondrial derivatives that extend for most of the tail length (see Table 3.6 for dimensions of mitochondrial derivatives). In each derivative the cristae are regularly aligned, and arranged perpendicular to the longitudinal axis (Fig. 3.11E). Paracrystalline material is present within the centre of each derivative (Fig. 3.11F). Mitochondrial derivatives with different diameters are conspicuous in several cross-section profiles of the seminal vesicles and appear to be associated with particular sperm sizes (Fig. 3.6C). The majority of large-diameter derivatives are always in close proximity to sperm that possess large diameter nuclei. In contrast, those derivatives with smaller diameters (Fig. 3.11G) are correspondingly associated with sperm that have smaller diameter nuclei. The posterior tip of the sperm tail is tapered and consists of a 9 + 9 + 0 microtubular arrangement, having lost the two central singlets (not illustrated). The two derivatives do not flank the axoneme along the entire sperm tail, but terminate in a region close to the posterior segment and expose a small segment of the axoneme.

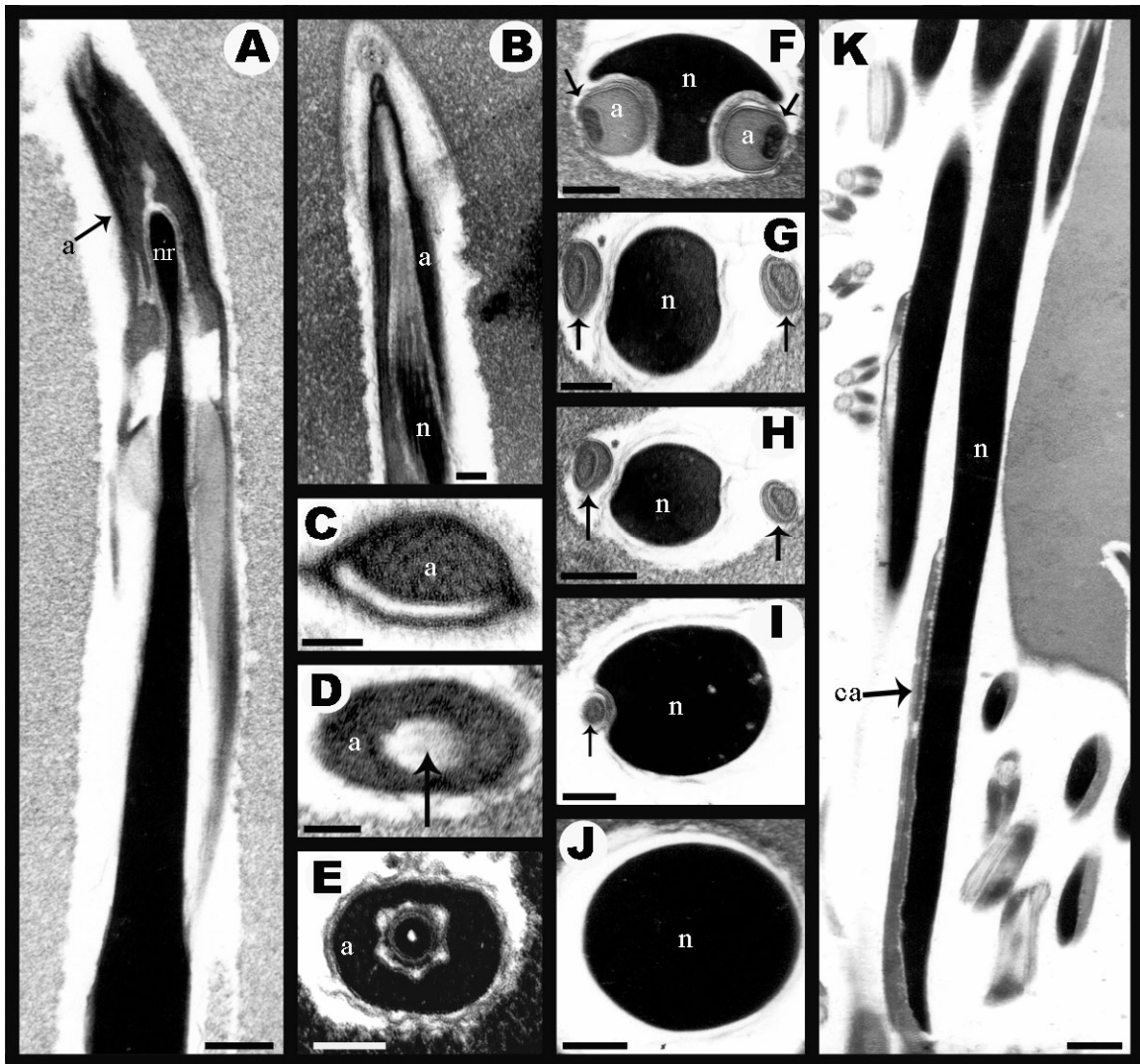


Figure 3.9. *Melampsalta leucoptera* sperm. TEM. A: Longitudinal section through the sperm head showing the apical acrosome (a) and the nucleus. The entire acrosome and the rostrum of the nucleus (nr) are embedded in a homogenous matrix. Bar = 200 nm. B: Longitudinal section through the acrosome showing the subacrosomal space and the surrounding acrosome (a). n, nucleus. Bar = 100 nm. C: A transverse section through the anterior tip of the acrosome. The acrosome (a) has a tubular substructure. Note the laterally positioned subacrosomal space. Bar = 100 nm. D: Cross-section through the acrosome just anterior to the nucleus showing the now central subacrosomal space (arrow) and the acrosome (a) surrounding it. Bar = 100 nm. E: Transverse section through the anterior tip of the sperm nucleus showing the acrosome (a), which surrounds the subacrosomal space. Embedded within the subacrosomal space is the rostrum of the nucleus. Note the hexagonal pattern of the subacrosomal space. Bar = 100 nm. F: Transverse section through the sperm head. The acrosomal processes (arrows), which flank the mushroom-shaped nucleus (n), have an electron-dense medulla and electron-lucent cortex. Bar = 100 nm. G: Transverse section towards the distal region of the acrosome. The nucleus (n) has become ovoid and is still flanked laterally by extensions of the acrosome (arrows). Bar = 100 nm. H: Transverse section through a more distal region of the acrosome. Here the nucleus (n) has become circular in cross-section. Note the two acrosomal processes (arrows). Bar = 100 nm. I:

Transverse section at the posterior limit of the acrosome (arrow). Bar = 100 nm. J: Transverse section through the sperm nucleus. The nucleus (n), without any invagination, is distinctly circular. Bar = 100 nm. K: Longitudinal section showing the elongated nucleus (n) and the centriolar adjunct (ca). Bar = 1  $\mu$ m.

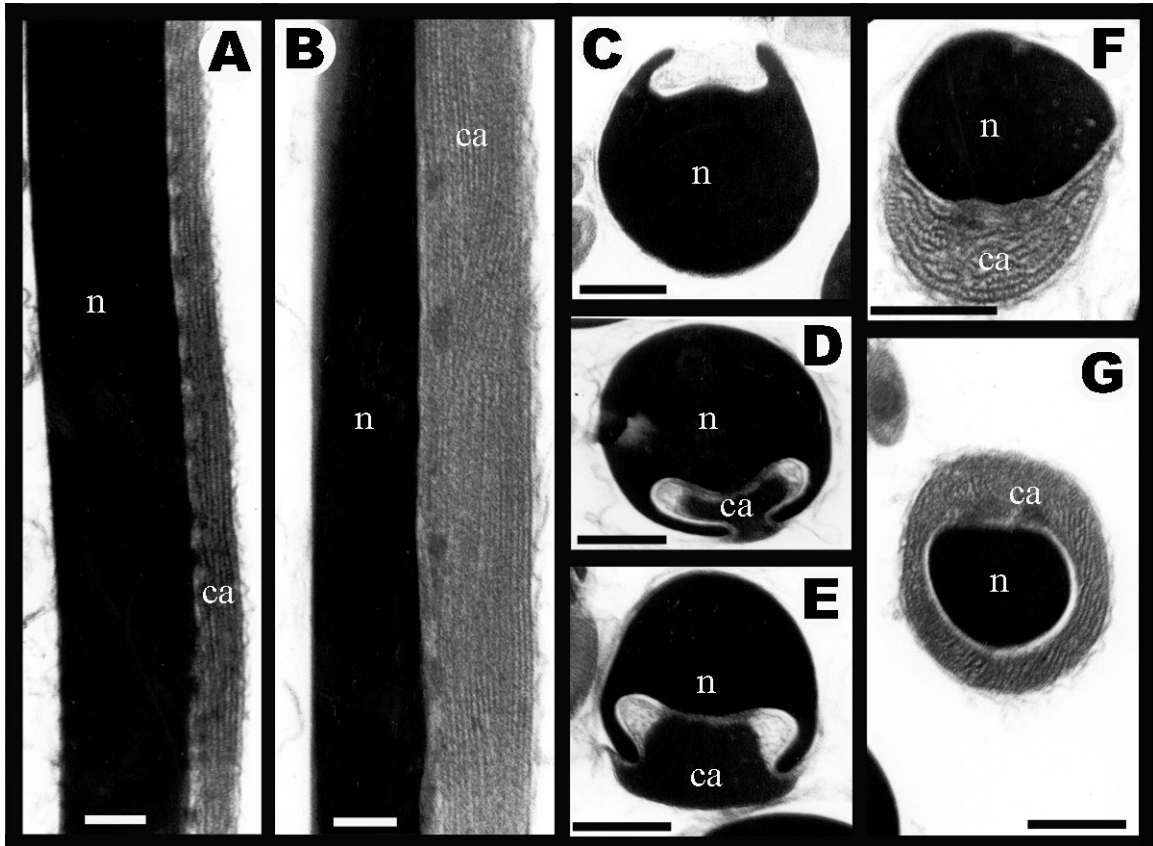


Figure 3.10. *Melampsalta leucoptera* sperm. TEM. A: Longitudinal section showing the nucleus (n) and centriolar adjunct (ca). Bar = 200 nm. B: Higher magnification of a longitudinal section through a more posterior region of the sperm nucleus (n) and the lamellate substructure of the centriolar adjunct (ca). Bar = 200 nm. C: Transverse section of the nucleus through part of its mid-region. Here the nucleus has a lateral groove that is devoid of material. Bar = 200 nm. D: Transverse section through part of the nucleus mid-region showing the centriolar adjunct (ca) housed in the invagination of the nucleus (n). At this stage the centriolar adjunct is small. Bar = 200 nm. E: Transverse section of a more distal region of the sperm nucleus (n). Here the centriolar adjunct (ca) is larger and protrudes from the lateral invagination. Bar = 200 nm. F: Transverse section through a region close to the base of the nucleus (n). The lamella-like substructure of the centriolar adjunct (ca) is conspicuous. Bar = 200 nm. G: Transverse section through the base of the nucleus. In this region the lamellate centriolar adjunct completely surrounds the nucleus (n). Bar = 200 nm.

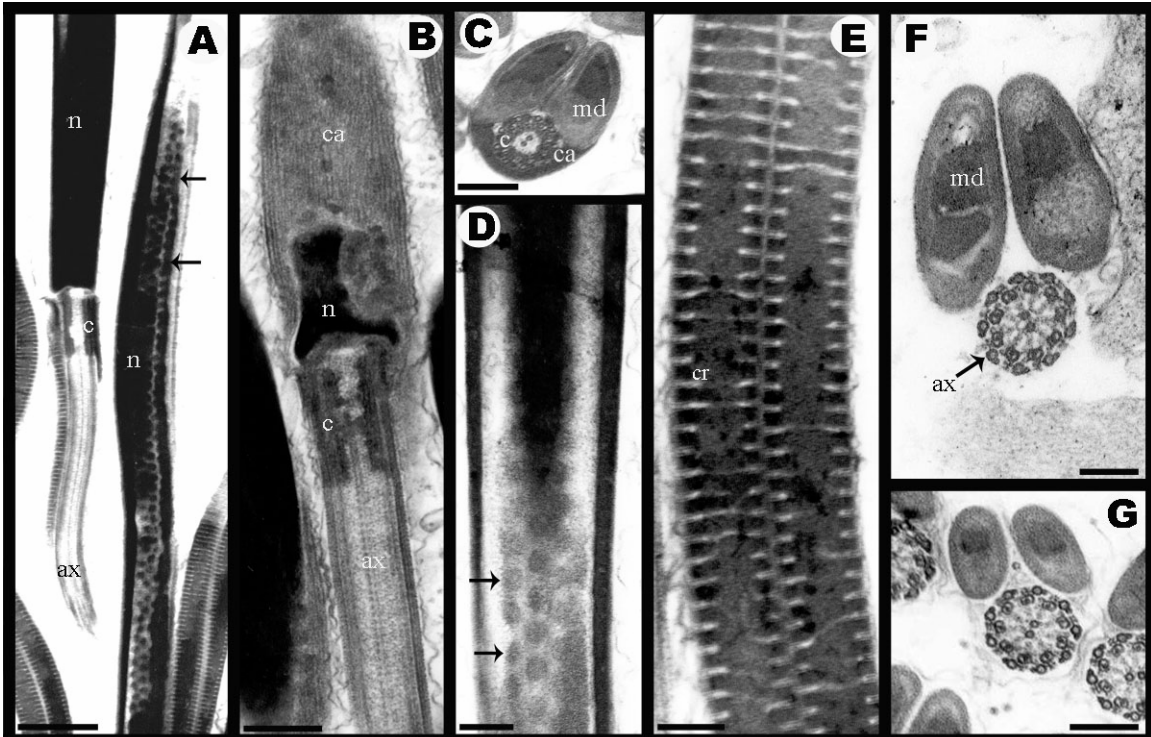


Figure 3.11. *Melampsalta leucoptera* sperm. TEM. A: Two profiles sectioned longitudinally through the nuclear mid-piece region. One profile shows the vesicle-like elements (arrows) that are associated with both the nucleus (n) and centriolar adjunct. The other profile represents a more posterior section and shows the caudal end of the nucleus, the centriole-like organelle (c) and the emerging axoneme (ax). Bar = 500 nm. B: High magnification profile of the nucleus-flagellum transition region. Posterior to the nucleus (n) is a centriole-like organelle (c) from which the axoneme (ax) emerges. Bar = 200 nm. C: Cross-sectional profile through the neck region. The centriole-like organelle (c), which is surrounded by material of the centriolar adjunct (ca), consists of a ring of doublets rather than triplets of microtubules. md, mitochondrial derivatives. Bar = 400 nm. D: Higher magnification profile of the vesicle-like elements (arrows) in the sperm mid-piece. Bar = 100 nm. E: Longitudinal section through the mitochondrial derivatives. In each derivative, the peripheral cristae (cr) are arranged in regular patterns perpendicular to the longitudinal axis of the derivative. Bar = 200 nm. F: Transverse section through the sperm tail showing the 9 + 9 + 2 axoneme (ax) flanked by two mitochondrial derivatives (md). Note the crystallized matrix of each derivative. Bar = 100 nm. G: Transverse section through the short sperm tail. The mitochondrial derivatives are small but the axoneme maintains the same size as long sperm. Bar = 100 nm.

### 3.3.2.3. Tribe Tettigomyini (*Stagira simplex* and *Xosopsaltria thunbergi*)

#### Head Region (acrosome and nucleus)

The morphology of the sperm head of *S. simplex* and *X. thunbergi* is similar to that of *Q. walkeri* and *M. leucoptera*. In both species the conical acrosome (see Table 3.3 for dimensions) has a tubular substructure and a deep subacrosomal invagination (Figs. 3.12A, B, C, 3.13A and 3.14A, B). Anteriorly, the acrosome is tapered; posteriorly it broadens to form two acrosomal processes (Figs. 3.12A, E, F and 3.14E, F, G) that flank the anterior nucleus.

The anterior of the nucleus forms a rostrum, which projects into the subacrosomal space (Figs. 3.12A, D, E and 3.14A, H). In this region the nucleus is circular in transverse section (Figs. 3.12D, E and 3.14D). However, towards the base of the acrosome the elongated nucleus becomes bilaterally concave and mushroom-shaped in transverse section (Figs. 3.12F and 3.14F). Further posteriorly, the nucleus is rounded before becoming laterally invaginated (Figs. 3.13A, B, 3.14I, J and 3.15A). The invaginations (see Table 3.5 for dimensions) are filled with material of the centriolar adjunct. Towards its base the nucleus changes shape again, first becoming circular before taking on a semi-elliptic profile up to its posterior limit (Figs. 3.13C, D and 3.15B). Groups of sperm with nuclei of different sizes are conspicuous in some sections of seminal vesicles (see Table 3.4. for dimensions). Sperm with small diameter nuclei are aggregated in clusters, as are sperm with larger diameter nuclei (Fig. 3.12I).

### **Mid-Piece and Tail Region**

The mid-piece of *S. simplex* and *X. thunbergi* contains the posterior segment of the nucleus, the centriolar adjunct and a centriole-like organelle (Figs. 3.13G and 3.15E). The centriolar adjunct is initially small and is confined to the lateral invagination of the nucleus. Posteriorly, it widens, overlaps from the lateral invagination, eventually partly or completely enclosing the nucleus distally (Figs. 3.13C, E and 3.15B). The material of the centriolar adjunct is structured into a honeycomb arrangement of lamellae (Figs. 3.13C, E, F and 3.15B, D). Several vesicle-like structures, arranged in an orderly fashion, are evident in the mid-piece region (Fig. 3.13H). The single centriole-like structure (Figs. 3.13G, I and 3.15E) is located posterior to the nucleus. The transition between these structures is abrupt and marks the beginning of the sperm tail. Two structures constitute the sperm tail: an axoneme and two mitochondrial derivatives. The axoneme (Figs. 3.13G, J and 3.15E, G), which develops from the centriole, has the typical 9 + 9 + 2 arrangement of microtubules. It is flanked by two mitochondrial derivatives along most of the sperm tail. In both species diameters of the derivatives are not uniform (Table 3.6). Each derivative has a crystalline core and a moderately electron-lucent peripheral region (Figs. 3.13J and 3.15F, G). Extending beyond the posterior limit of each derivative is a short length of axoneme marking the endpiece of the sperm (Fig. 3.13K).

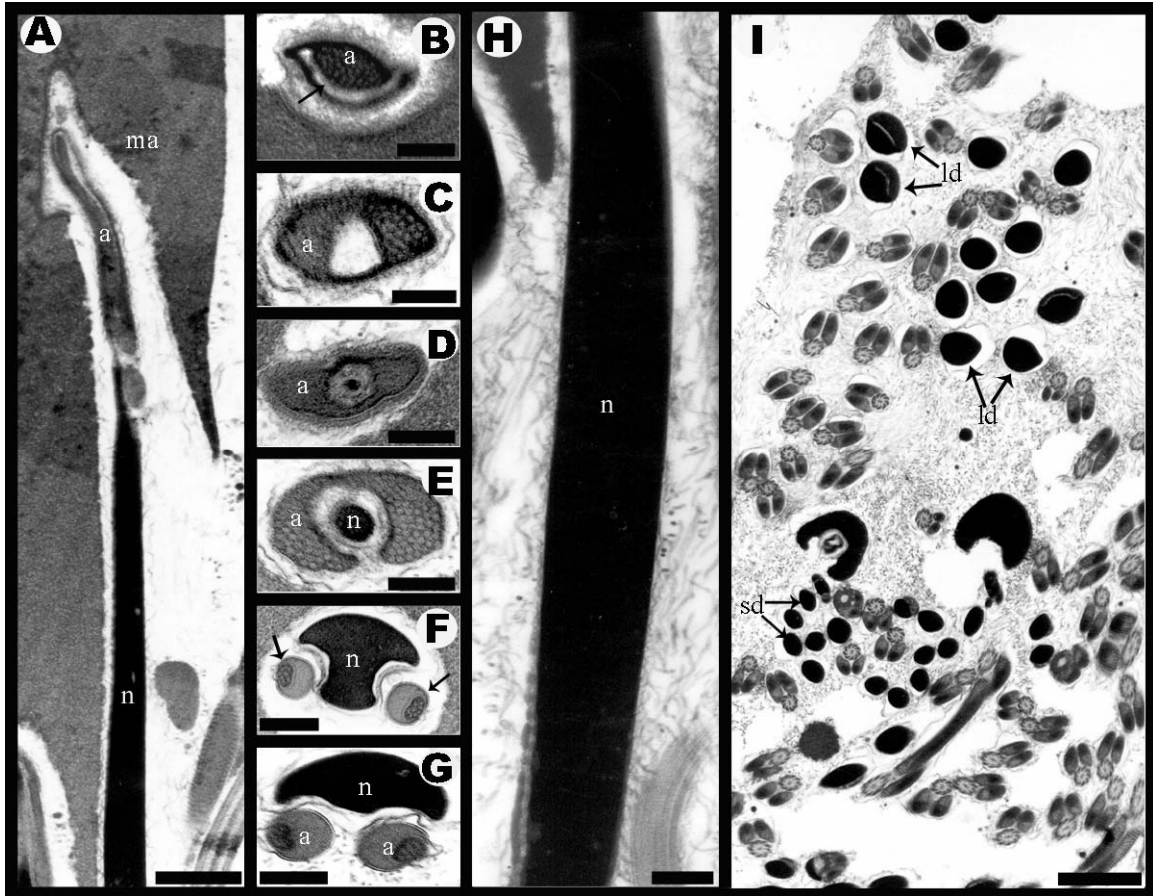


Figure 3.12. *Stagira simplex* sperm. TEM. A: Longitudinal section through the sperm head. The apical acrosome (a), which has a tubular-substructure, completely surrounds the nucleus (n). Note the matrix (ma). Bar = 200 nm. B: A transverse section through the anterior tip of the acrosome to show the tubular substructure of the acrosome (a) and the subacrosomal space (arrow) which forms a pocket. Bar = 100 nm. C: Transverse section through the anterior tip of the acrosome to show the acrosome (a) and the central subacrosomal space. Bar = 100 nm. D: A cross-sectional profile through the acrosome (a) and anterior tip of the nucleus at the centre. The anterior of the nucleus intrudes into the posterior section of the subacrosomal space. Bar = 100 nm. E: A cross-sectional profile through the acrosome and nucleus to show the subacrosomal space and the surrounding acrosome vesicle (a). The anterior of the nucleus (n) protrudes into the subacrosomal space. Bar = 100 nm. F: A transverse section through a more posterior region of the acrosome (a). In this region the mushroom-shaped nucleus (n) is flanked by two tubular processes of the acrosome (arrows). Bar = 100 nm. G: Cross-section through a more distal region of the acrosome. Here the nucleus (n) has become semi-elliptical. Note the two acrosomal processes (a). Bar = 100 nm. H: Longitudinal section through the sperm nucleus. The elongated nucleus (n) is electron dense. Bar = 500 nm. I: Low magnification of several cross-sectional profiles of sperm at various levels in the seminal vesicle. Differently sized nuclei are conspicuous; some have small diameters (sd) and others have larger diameters (ld). Differently sized mitochondrial derivatives are also evident. Bar = 1  $\mu$ m.

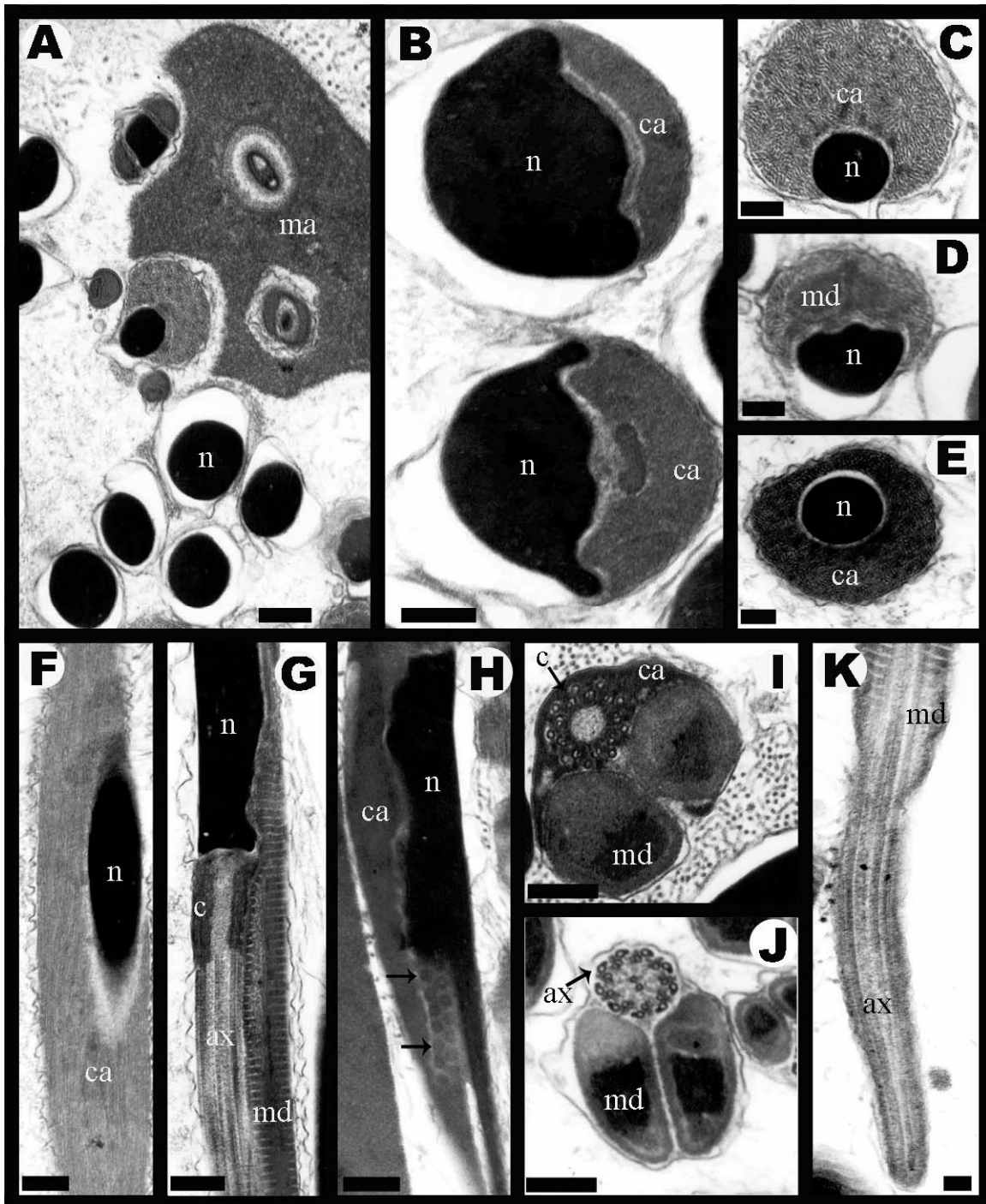


Figure 3.13. *Stagira simplex* sperm. TEM. A: A cross-sectional profile through sperm heads in a sperm bundle showing the nuclei (n) embedded in a homogenous matrix (ma). Bar = 200 nm. B: Two cross-sectional profiles of the nucleus. The centriolar adjunct (ca), initially confined to the area of the lateral invagination of the nucleus (n), gradually broadens until it overlaps the area of the lateral invagination. Bar = 100 nm. C: A transverse section through the posterior region of the nucleus in a sperm with a small nucleus diameter. In this region the centriolar adjunct (ca) partly surrounds the spherical nucleus (n). Bar = 100 nm. D: A transverse section at a more

posterior level of the nucleus in the sperm of *S. simplex* showing the nucleus (n) and the mitochondrial derivatives (md). Bar = 100 nm. E: Transverse section through the base of the nucleus. In this region the lamellate centriolar adjunct (ca) completely surrounds the nucleus (n). Bar = 200 nm. F: Oblique section through the sperm nucleus (n) and centriolar adjunct (ca). Bar = 200 nm. G: A longitudinal section through the nucleus-flagellum transition region showing the nucleus (n), centriole-like organelle (c), axoneme (ax) and mitochondrial derivatives (md). Bar = 200 nm. H: A longitudinal section through the posterior nucleus (n), centriolar adjunct (ca) and the vesicle-like structures (arrows). Bar = 200 nm. I: A transverse section through the neck region. Here the centriole-like organelle (c) is surrounded by material of the centriolar adjunct (ca) and flanked by two mitochondrial derivatives (md). Bar = 200 nm. J: A transverse section through the sperm tail showing the 9 + 9 + 2 axoneme (ax) flanked by two mitochondrial derivatives (md). Note the crystallized matrix of each derivative. Bar = 200 nm. K: A longitudinal section at the terminal end of the sperm tail to show the termination of the mitochondrial derivatives (md) and the narrowing of the axoneme posteriorly (ax). Bar = 200 nm.

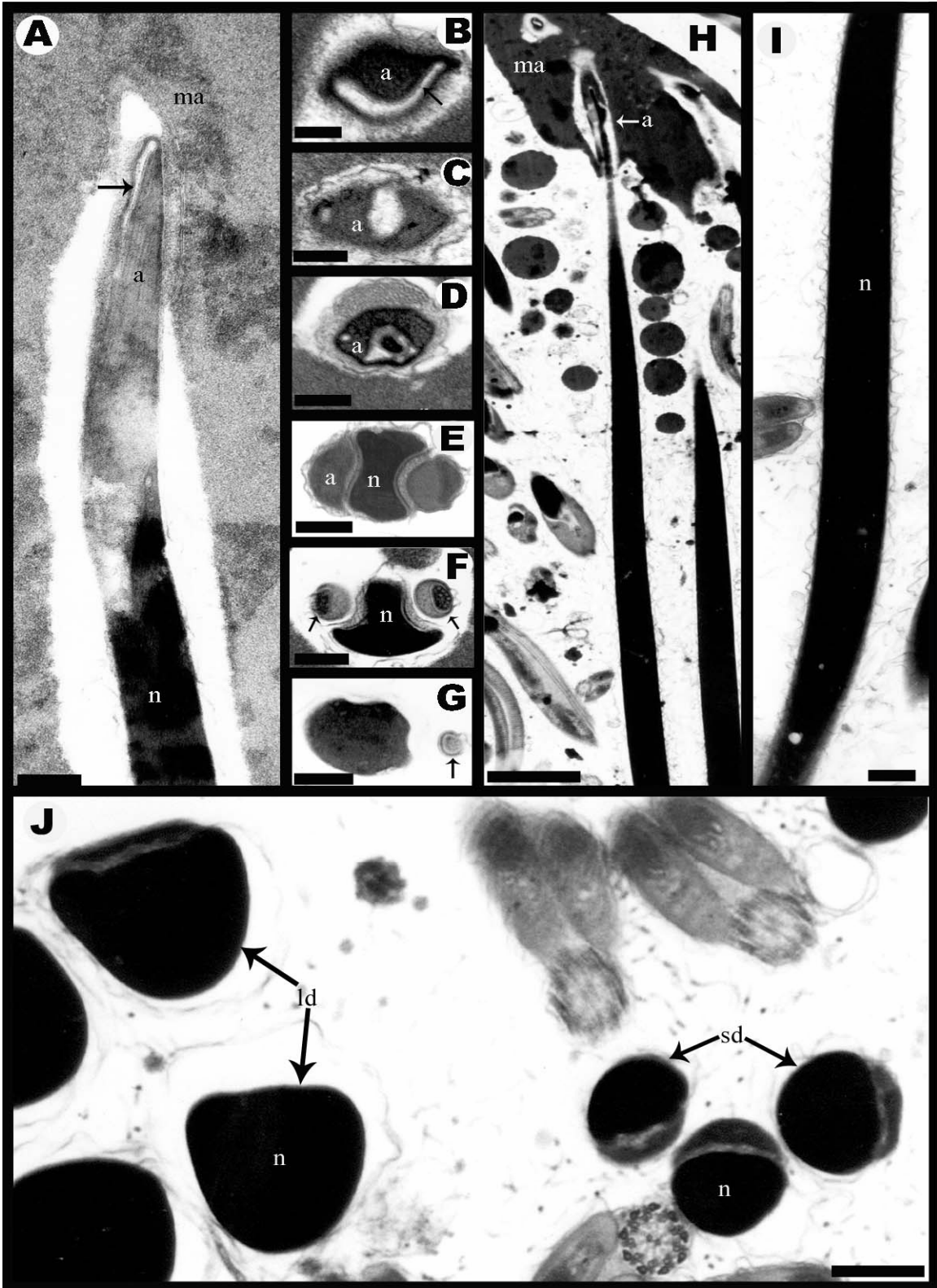


Figure 3.14. *Xosopsaltria thunbergi* sperm. TEM. A: Longitudinal section through part of the sperm head to show the apical acrosome (a), which has a tubular substructure and the nucleus (n).

arrow, subacrosomal space. Bar = 200 nm. B: A transverse section through the anterior tip of the acrosome to show the tubular substructure of the acrosome (a) and the subacrosomal space (arrow) which forms a pocket. Bar = 100 nm. C: Transverse section through the sperm acrosome to show the sub-acrosomal space and the surrounding acrosome (a). Bar = 100 nm. D: Transverse-section through the acrosome showing its characteristic tubular substructure (a) and the subacrosomal space. The rostrum of the nucleus at the centre protrudes into the subacrosomal space. Bar = 100 nm. E: Transverse section towards the base of the sperm acrosome. In this region the nucleus (n) is bilaterally concave and is flanked by extensions of the acrosome (a). Bar = 100 nm. F: A transverse section through part of the sperm head. In this region the concavities of the nucleus (n) that house the two extensions of the acrosome (arrows) have enlarged. Bar = 100 nm. G: Transverse section at the posterior region of the acrosome. One extension of the acrosome (arrow), which still flanks the conical nucleus, is shown at this level. Bar = 100 nm. H: Longitudinal section through the sperm head of short sperm. The acrosome (a) and part of the sperm nucleus (n) are embedded into the homogenous matrix (ma). Bar = 1  $\mu$ m. I: Longitudinal section through the sperm nucleus. The elongated nucleus (n) is uniformly electron dense. Bar = 200 nm. J: Several cross-sectional profiles across sperm showing nuclei of different sizes. Nuclei with small diameters (sd) are clumped together just like those nuclei with large diameters (ld). Bar = 200 nm.

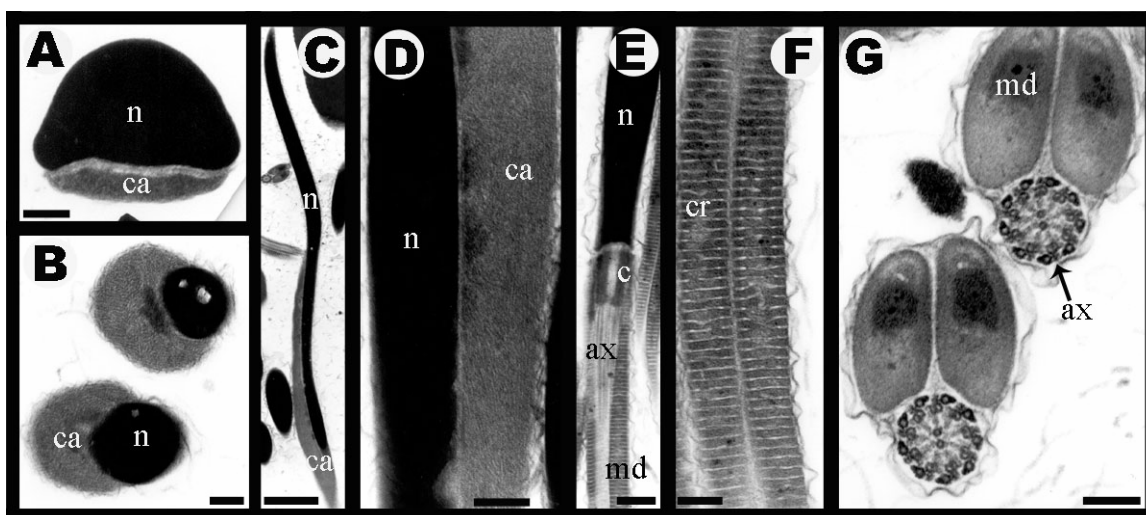


Figure 3.15. *Xosopsaltria thunbergi* sperm. TEM. A: Higher magnification of a cross-section through a more posterior region of the nucleus. The rounded nucleus (n) has a shallow invagination that houses material of the centriolar adjunct (ca). Bar = 150 nm. B: Transverse sections through part of the mid-region of the nucleus (n) showing the semi-elliptic centriolar adjunct (ca). Bar = 100 nm. C: Longitudinal section through the sperm mid-piece showing the nucleus (n) and centriolar adjunct (ca). Bar = 1  $\mu$ m. D: Longitudinal section showing the nucleus (n) and centriolar adjunct (ca). Bar = 100 nm. E: Longitudinal section through the neck region to show the posterior end of the nucleus (n), centriole-like organelle (c), axoneme (ax) and the mitochondrial derivatives (md). Bar = 200 nm. F: Longitudinal section through the mitochondrial derivatives. Note the orderly arrangement of the mitochondrial cristae (cr). Bar = 200 nm. G: Cross-section through the sperm tail to show the 9 + 9 + 2 axoneme (ax) and the mitochondrial derivatives (md). Note the crystallized assortment of the cristae in each derivative. Bar = 100 nm.

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#### 3.3.2.4. Tribe Taphurini (*Monomatapa matoposa*)

##### Head Region (acrosome and nucleus)

The morphology of the head region in the sperm of *Monomatapa matoposa* is generally similar to that of the four cicadettine cicadas previously described. The conical acrosome (see Table 3.3 for dimensions) is deeply invaginated posteriorly and has a characteristic tubular substructure and a subacrosomal space (Fig. 3.16A, B, C, D). Anteriorly the acrosome has a tapered profile; posteriorly it widens and forms two lateral processes, which flank the anterior section of the nucleus (Fig. 3.16E, F, G, H).

The elongated nucleus forms a rostrum at its anterior (Fig. 3.16A, B, C), where it penetrates into the subacrosomal space. A progressive alteration in shape occurs towards the posterior limit of the acrosomal complex where the nucleus takes on a more or less cylindrical outline. The nucleus becomes bilaterally concave and is T-shaped with a narrow stem in transverse section (Fig. 3.16G). Further posteriorly, the stem of the “T” gradually thickens and the two acrosomal extensions become increasingly smaller, resulting in a rounded outline (not illustrated). The average diameter of the nucleus in this region measured  $560.07 \pm 19.38 \mu\text{m}$  (Table 3.4). Further posteriorly, the nucleus becomes laterally invaginated (Fig. 3.16I; see Table 3.5 for dimensions of invagination). These invaginations are initially empty; posteriorly they are occupied by material of the centriolar adjunct (Fig. 3.16I). Towards its base the nucleus becomes semi-elliptical in transverse section (Fig. 3.16J, K).

### Mid-Piece and Tail Region

The sperm mid-piece of *M. matoposa* comprises a centriole-like organelle, a centriolar adjunct and mitochondrial derivatives (Fig. 3.17A, B). The material of the centriolar adjunct is structured as flat longitudinal lamellae, which form a complex labyrinth in cross section (Figs. 3.16I, J and 3.17C). The centriole-like organelle is positioned posterior to the nucleus (Fig. 3.17A, B). Vesicle-like elements are present in the sperm midpiece region (Fig. 3.17D). The axoneme and the two mitochondrial derivatives that flank it constitute the sperm tail (Fig. 3.17E). The derivatives (see Table 3.6 for dimensions), whose anterior ends are inserted into the centriolar adjunct material, are obliquely striated in longitudinal sections (Fig. 3.17E), the striations having a periodicity of about 88 nm. The anterior segments of each derivative embedded differentially within the centriolar adjunct, with one extending further than the other (Fig. 3.16K). In transverse sections a crystalline core surrounded by a moderately electron-dense region is evident (Fig. 3.17F). The standard axonemal arrangement of 9 + 9 + 2 microtubules is present.

Table 3.3. Mean (nm)  $\pm$  s.e. of tip width, base width and length of the acrosome for Cicadettinae sperm. Data obtained from transmission electron microscopy.

Species	Tip width			Base width			Length		
	mean	s.e.	n	mean	s.e.	n	mean	s.e.	n
<i>M. leucoptera</i>	50.07	7.07	3	530.87	17.69	6	2363.33	17.46	3
<i>S. simplex</i>	55.30	1.37	4	352.57	1.71	4	2006.11	8.81	3
<i>X. thunbergi</i>	54.49	1.12	3	358.86	2.1	3	2036.56	4.62	3
<i>M. matoposa</i>	52.33	1.22	3	412.18	1.06	3	1862.03	18.77	3
<i>Q. walkeri</i>	71.59	1.56	3	519.53	2.65	3	2049.07	0.01	3

Table 3.4. Means (nm)  $\pm$  s.e. of long and short nucleus diameter for Cicadettinae sperm. Data obtained from transmission electron microscopy.

Species	Large nucleus diameter			Small nucleus diameter		
	mean	s.e.	n	mean	s.e.	n
<i>M. leucoptera</i>	590.40	5.50	10	202.03	4.9	6
<i>S. simplex</i>	494.17	12.32	7	182.14	3.76	7
<i>X. thunbergi</i>	580.22	9.01	10	198.81	5.37	7
<i>M. matoposa</i>	560.07	19.38	7	255.84	7.44	6
<i>Q. walkeri</i>	526.22	7.68	5	212.83	4.33	6

Table 3.5. Means (nm)  $\pm$  S.E. of width and %depth of nuclear invagination expressed as a percentage of the sperm nucleus diameter for Cicadettinae sperm. Data obtained from transmission electron microscopy.

Species	Invagination width			% Depth of Invagination
	mean	s.e.	n	
<i>M. leucoptera</i>	542.68	6.28	10	21.46
<i>S. simplex</i>	399.18	14.86	5	12.01
<i>X. thunbergi</i>	425.96	4.77	5	7.41
<i>M. matoposa</i>	489.29	9.67	6	23.15
<i>Q. walkeri</i>	332.14	6.21	10	20.68

Table 3.6. Mean diameter of large and small mitochondria for Cicadettinae sperm. Data obtained from transmission electron microscopy.

Species	Diameter (nm) of large mitochondria			Diameter (nm) of small mitochondria		
	mean	s.e.	n	mean	s.e.	n
<i>M. leucoptera</i>	215.50	4.21	5	100.14	4.06	6
<i>S. simplex</i>	208.01	3.81	5	98.54	5.32	5
<i>X. thunbergi</i>	197.14	8.91	6	88.21	4.68	5
<i>M. matoposa</i>	217.30	5.55	6	103.29	4.85	5
<i>Q. walkeri</i>	225.95	1.39	6	107.34	1.62	5

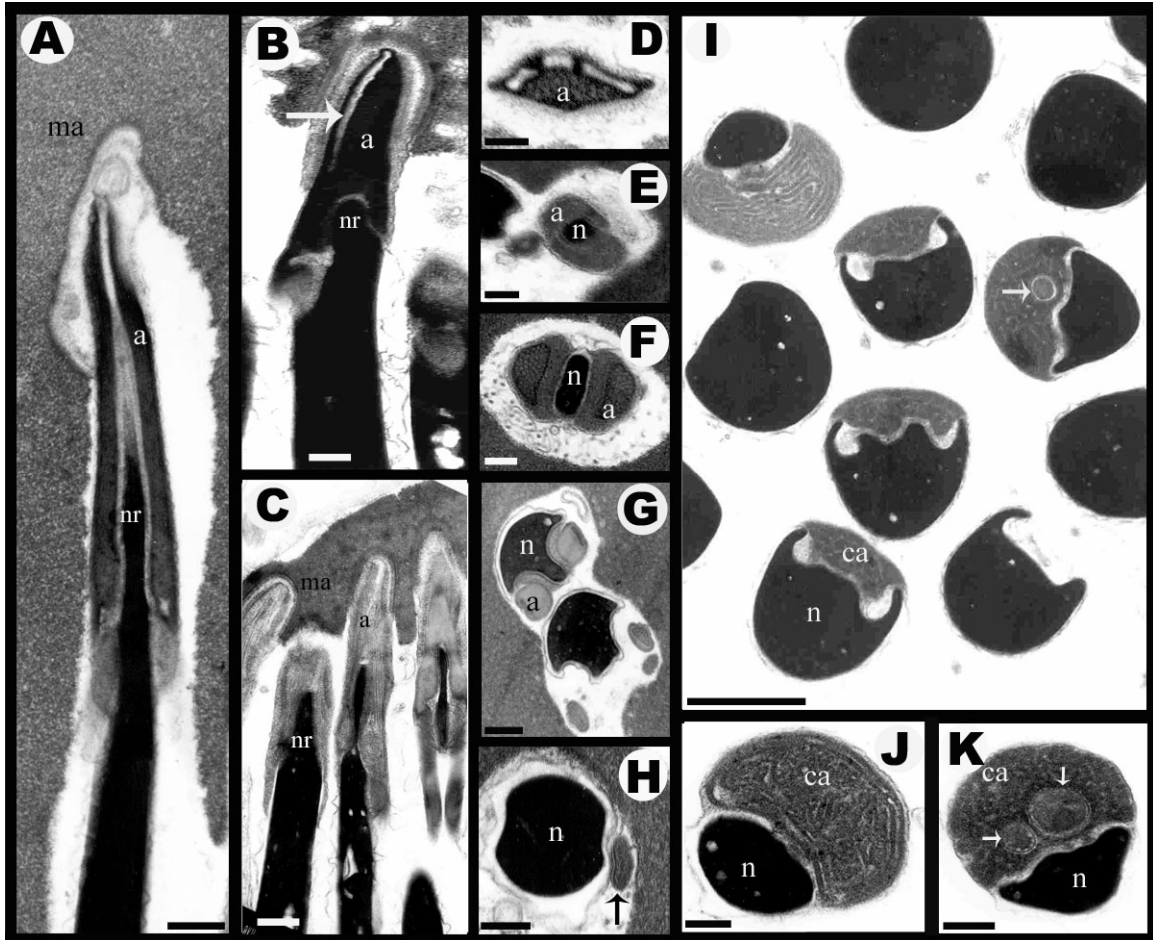


Figure 3.16. *Monomatapa matoposa* sperm. TEM. A: Longitudinal section through the sperm head. The homogenous matrix (ma), rostrum of the nucleus (nr) and the surrounding acrosome (a) are visible. Bar = 200 nm. B: Longitudinal section through the sperm head. The acrosome (a) surrounds the subacrosomal space (arrow) that forms a cap around the rostrum of the nucleus (nr). Bar = 200 nm. C: Three cross-sectional profiles of sperm heads in a homogenous matrix (ma). The rostrum of the nucleus (nr) is completely surrounded by the acrosome (a). Note the orderly array of the microtubule-like elements of the acrosome (a). Bar = 200 nm. D: A transverse section through the anterior tip of the acrosome. Note the subacrosomal space. Bar = 100 nm. E: Transverse section through the acrosome and anterior tip of the nucleus. The nucleus (n) is circular and is completely enclosed by the acrosome. Bar = 100 nm. F: Transverse section through the acrosome and nucleus. Tubular structures are visible within the extensions of the acrosome (a), which flank the nucleus (n). Bar = 100 nm. G: Two profiles of the sperm head sectioned transversely through a region in close proximity to the base of the acrosome. In this region the nucleus (n) is bilaterally concave and is flanked laterally by extensions of the acrosome (a). Bar = 100 nm. H: Transverse section at the posterior region of the acrosome. One extension of the acrosome (arrow), which still flanks the conical nucleus, is shown at this level. Bar = 100 nm. I: A number of cross-sectional profiles of the nuclei at various levels. One profile shows the nucleus without the invagination. Other profiles represent more posterior cross-sections and show the centriolar adjunct (ca). Note the anterior portion of one mitochondrial derivative (arrow) that is embedded in the centriolar adjunct. Bar = 400 nm. J: Transverse section at a region near to the base of the nucleus. The centriolar adjunct (ca) has an elliptical outline and the lamellate

structure is evident. Bar = 200 nm. K: A more posterior transverse section through the distal region of the nucleus. The anterior ends of the mitochondrial derivatives (arrows) protrude into the material of the centriolar adjunct (ca) and appear as two circular structures. Bar = 100 nm.

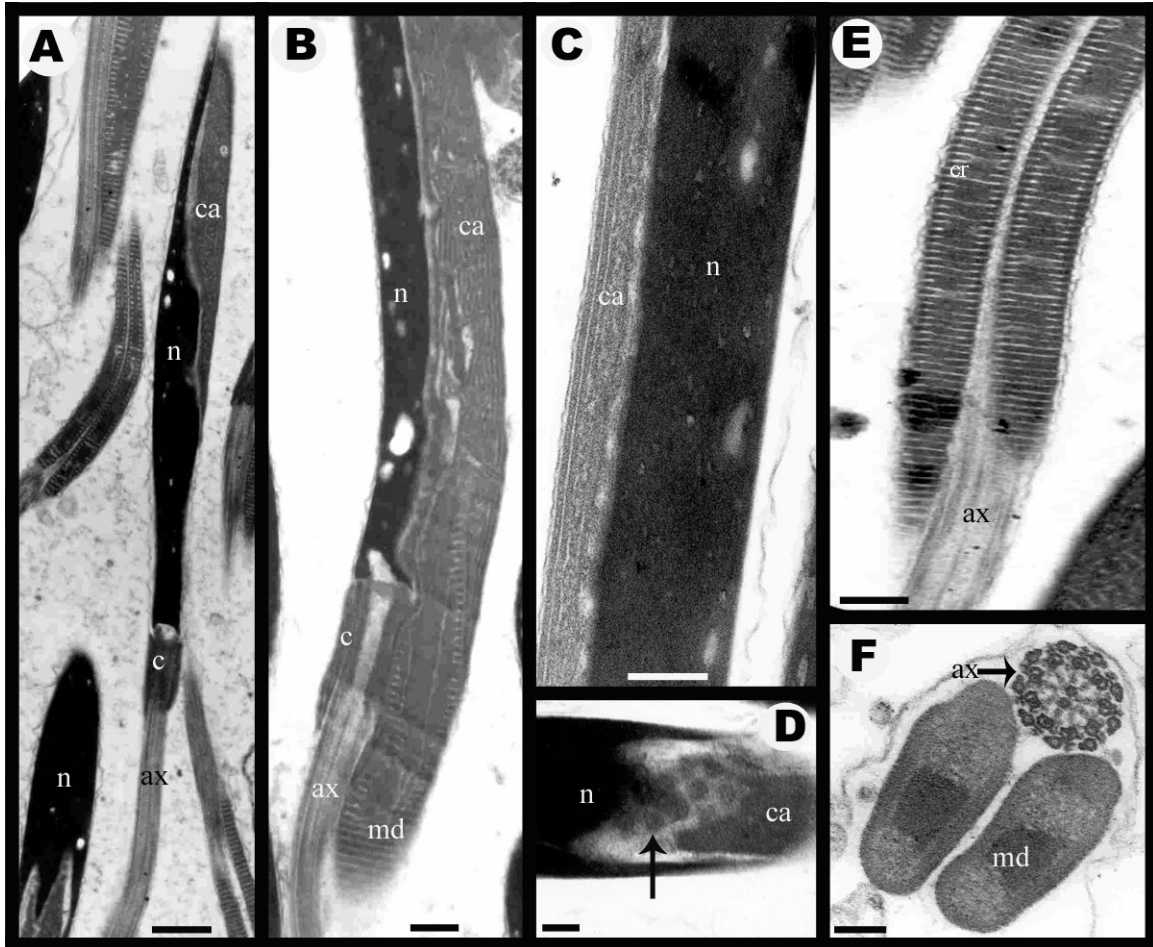


Figure 3.17. *Monomatapa matoposa* sperm. TEM. A: Low magnification of a longitudinal section through the sperm mid-piece to show the nucleus (n), centriolar adjunct (ca), centriole-like organelle (c), and the axoneme (ax). Bar = 500 nm. B: Transverse section through the nucleus-flagellum transition region. The centriole-like organelle (c) lies posterior to the nucleus (n). The axoneme (ax) emerges from the centriole. The two mitochondrial derivatives (md) are positioned lateral to the axoneme and posterior to the elongated centriolar adjunct (ca). Bar = 200 nm. C: Longitudinal section through the posterior part of the sperm nucleus (n) and centriolar adjunct (ca) which is made up of several elongated flattened plates that are stacked together to form a compact structure. Bar = 200 nm. D: Longitudinal section through the mid-piece to show the vesicle-like structures (arrow) that are associated with the nucleus (n) and the centriolar adjunct (ca). Bar = 100 nm. E: Longitudinal section through the sperm tail showing the axoneme (ax) and the crystalline structure of the matrix (cr) within the two mitochondrial derivatives (md). Bar = 200 nm. F: Transverse section through the sperm tail showing the 9 + 9 + 2 axoneme (ax) and the two mitochondrial derivatives (md). Note the crystallized region in each derivative. Bar = 100 nm.

### 3.4. DISCUSSION

The spermatozoa of the cicadettine cicadas described in this study have a number of morphological similarities to the spermatozoa of other cicadas so far studied (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003; Chapter 2). Similarities include: (a) more than one discrete length class of nucleated motile sperm (polymegaly); (b) a conical acrosome with a deep subacrosomal invagination and two posterior acrosomal processes; (c) a filiform, bilaterally symmetrical nucleus that is invaginated postero-laterally; (d) a centriolar region with a centriole-like organelle consisting of microtubular doublets and accessory microtubules rather than triplets; (e) vesicle-like elements associated with both the nucleus and centriolar adjunct and housed in the nuclear invagination; (f) a centriolar adjunct located anterior to the mitochondrial derivatives; (g) two crystalline mitochondrial derivatives positioned laterally; (h) a single axoneme with a 9 + 9 + 2 arrangement of microtubules; (i) aggregation of sperm types in a homogenous matrix to form spermatodesmata; and (j) clumping of large and small nuclei in and around spermatodesmata. Therefore, it is likely that these features will be common to the sperm of other cicadas.

The sperm of the five cicadettine cicadas however, exhibit some differences when compared to the cicadine cicadas with respect to: (a) the length of the nucleus of the long-headed sperm; (b) tail modal lengths; and (c) size and structure of the centriolar adjunct.

Like the platypleurines (Chapter 2) all five species of cicadettine cicadas exhibit polymegaly. This unusual phenomenon has also been reported in *Graptosaltria nigrofuscata* (Kubo-Irie *et al.*, 2003). Therefore, polymegaly seems to be a common occurrence within the Cicadidae. However, the functional significance of polymegaly in cicadas as well as in other insects remains unclear (Snook, 1998; Swallow and Wilkinson, 2002). From the present study the high proportion of sperm with long nuclei in all species may indicate their involvement in fertilization. Yet the abundance of sperm with shorter nuclei is high enough for them to have a functional significance; it would seem a waste of resources for cicadas to produce sperm that have no function. Variation in sperm size may represent differential provisioning of gametes by males as a form of parental investment. It is possible that sperm with shorter nuclei may fertilize some eggs. Pasini *et al.* (1996) found that long and short spermatozoa of *Drosophila subobscura* contain a similar amount of DNA and this may also be true for the different-sized cicada sperm. The amount of DNA in the sperm morphs of cicada sperm, however, is still to be determined.

Two modal classes of both nucleus and tail length were evident in the size frequency histograms of *Melampsalta leucoptera*, *Quintilia walkeri*, *Stagira simplex* and *Xosopsaltria thunbergi*. In *Monomatapa matoposa* two modes of nucleus length and a single mode of tail length were evident. These length frequency distributions are different from those of three of the platypleurine studied (*Albanycada albiger*, *Platypleura capensis* and *P. hirtipennis*) species, where most of the tails have a trimodal distribution, but similar to those of *Azanicada zuluensis* and *Pycna semiclara* (Chapter 2).

Nuclei lengths of long-headed sperm in four of the five cicadettines studied (40-50  $\mu\text{m}$ ) are very similar to those of the platypleurines, *A. albigera*, *P. semiclara* and *G. nigrofuscata*. There is no sufficient evidence to support a relationship between sperm size distribution and tribe. *M. matoposa* long nuclei, with a modal class of 65  $\mu\text{m}$ , are clearly distinct from all the other cicadettines. Provisional results show that the dimensions of long-headed sperm in *Magicicada septendecim* (formerly of the tribe Tibicinini), a periodical cicada from North America, are similar to those in *M. matoposa*. The morphology of the centriolar adjunct in *M. septendecim* is also similar to that in *M. matoposa*, suggesting that these two cicadas may be closely related. Current taxonomy supports the hypothetical affinity between these two species; the genus *Magicicada*, hitherto placed under the tribe Tibicinini, now falls within the tribe Taphurini (Moulds, 2005).

Tail lengths (all modal classes) in the five cicadettine cicadas studied are generally short (48 to 122  $\mu\text{m}$ ). By contrast, tail lengths in the platypleurines, with the exception of *P. semiclara*, are much longer (74 to 240  $\mu\text{m}$ ). An increased tail length may therefore be a characteristic feature of the platypleurines. The shorter tails found in *P. semiclara* might indicate the ancestral state. This is because *P. semiclara*, unlike *A. albigera*, *Azanicada zuluensis*, *Platypleura capensis* and *P. hirtipennis*, is a more basal platypleurine and has presumably undergone minor modification (M. Villet, pers. comm.). The advantages of longer tails in cicada sperm are not known. However, studies of embryogenesis in *Drosophila* (Perotti, 1973; 1975, Karr and Pitnick, 1996; Pitnick and Karr, 1998), and in the neuropteran, *Chrysopa carnea* (Friedländer, 1980), have shown that some products

from the sperm tail contribute to embryogenesis. Longer tails would provide a greater contribution to fertilization and may hence be preferred. This could be the same in cicadas.

Although a centriolar adjunct is present in many insect spermatozoa (Cantacuzène, 1970; Wheeler *et al.*, 1990; Newman and Quicke, 1999; Lino-Neto *et al.*, 2000), its origin and function is still not clear (Jamieson *et al.*, 1999; Dallai *et al.*, 2003). The term centriolar adjunct, coined by Gatenby and Tahmsian (1959), refers to a structure that forms around the centriole, between the nucleus and tail. Such a structure could have a mechanical function; the compact collar may fasten the sperm head and tail together (Baccetti, 1998). The shape, structural organization and position of the centriolar adjunct are different in a large number of insects. For example, it can be reduced, prolonged into a rod and sometimes appears as a w-shaped structure as in Dermaptera (Jamieson *et al.*, 1999), or it can be expanded, obscuring the centriole, as is the case of Mantophasmatodea (Dallai *et al.*, 2003) and Plecoptera (Fausto *et al.*, 2001). It is not clear whether the structure referred to as a centriolar adjunct in cicadas (originally by Folliot and Maillet, 1970) is indeed a true centriolar adjunct. This is because it is located anterior to the mitochondrial derivatives and it appears to have no direct link to the centriole. Nevertheless, the term centriolar adjunct has been retained in this study.

In the five platypleurine cicadas studied (*A. albigera*, *A. zuluensis*, *P. capensis*, *P. hirtipennis* and *P. semiclara*) the centriolar adjunct is elongated, generally confined to the lateral nuclear groove and consists of homogenous moderately electron-dense material

(Chawanji *et al.*, 2005; Chapter 2). By contrast, the centriolar adjunct of the cicadettine cicadas is relatively larger with an elliptic cross-section that protrudes from the nuclear groove, and is lamellate in structure. Caudally, it partly surrounds the nucleus in three of the five cicadettine species examined in the present study. In the platypleurine cicadas and the other Cicadinae species that have been studied (*Lyristes plebejus*, *Cicada orni* and *Graptosaltria nigrofuscata*) there is no evidence of a lamellate structure and the anterior segments of each mitochondrial derivative appear to be located at the same level within the centriolar adjunct (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003). The large centriolar adjunct with its characteristic lamella-like substructure can be considered to be a synapomorphic character that unites the cicadettine cicadas. A centriolar adjunct with similar characteristics has also been found in the sperm of the cicada *Magicicada septendecim* (pers. obs).

In conclusion, this study of five species of cicadettine cicadas along with that of platypleurine cicadas (Chapter 2) has revealed a number of features that appear to be common to cicada sperm. However, a number of important morphological differences exist between the two tribes. These include the structural organization of the centriolar adjunct and dimensions of sperm tails. The enlarged centriolar adjunct, with its characteristic lamella-like substructure, can be considered to be a synapomorphic character in the Cicadettinae and is potentially useful in the separation of these cicadas from the Cicadinae. In addition, the great length of the sperm nucleus of long-headed sperm in *M. matoposa* could be a synapomorphy of this genus and related taphurine and

cicadettine species. Further work with more cicada species should reveal the systematic importance of these characters and their usefulness in phylogenetic analysis.

**CHAPTER FOUR**  
**SPERMIOGENESIS IN CICADAS**

#### 4.1. INTRODUCTION:

The spermatozoon, a highly specialized haploid cell normally with a condensed nucleus, an acrosome derived from the Golgi apparatus, and a complex motile flagellum, is the product of an intricate process of cellular differentiation (Fernandes and Bao, 1999; Fernandes *et al.*, 2001). This process, termed spermatogenesis, involves several phases, namely: cellular proliferation by repeated mitotic divisions, duplication of chromosomes, genetic recombination through crossing over, reduction-division (meiosis) to produce haploid spermatids, and terminal differentiation of the spermatids into spermatozoa (Phillips, 1970; Baccetti, 1972; Guraya, 1987; Fawcett, 1994; Ndiaye *et al.*, 1996; Hess, 1999). Spermatogenesis in insects (unlike in mammals) occurs within hollow cysts, groups of germ cells surrounded by an epithelium (Jamieson *et al.*, 1999). In addition, in several insects (e.g. biting lice, sucking lice, thrips and coccids) the meiotic process of spermatogenesis proceeds in a very aberrant way (see White, 1973; Paccagnini *et al.*, 2006). Furthermore, in some aphids spermatogenesis is abnormal from first anaphase onwards (Pijnacker and Ferwerda, 1982). In most mammals Sertoli cells nurture the developing spermatids for the duration of spermatogenesis until the formation of mature spermatozoa (Guraya, 1987; Fawcett, 1994). However, in insects the developing spermatogenous cells do not remain attached to Sertoli cells.

The first phase of spermatogenesis (mitosis) is often referred to as “proliferation and renewal” of spermatogonia (Hess, 1999). During this phase the diploid spermatogonia, which are situated at the periphery of the seminiferous tubules, multiply mitotically to form primary spermatocytes and also to give rise to new spermatogonial stem cells

(Guraya, 1987; Fawcett, 1994; Hess, 1999). These cells undergo the first meiotic prophase, a complex process characterized by an ordered series of chromosomal rearrangements which are accompanied by molecular changes (Guraya, 1987; Fawcett, 1994; Hess, 1999). During this process the paired, homologous chromosomes cross over and then proceed with division 1 of meiosis to produce two secondary spermatocytes. A maturation division of each secondary spermatocyte reduces the somatic number of chromosomes by half and produces a cluster of four small nondescript round cells, the spermatids. The final phase of spermatogenesis occurs when the haploid spermatids undergo cytological transformations into mature spermatozoa, which are released into the fluid-filled lumen of the seminiferous tubules (Hess, 1999). This sequence of postmeiotic changes by which spermatids are transformed into spermatozoa is known as spermiogenesis (Fawcett, 1994).

A number of authors have suggested that a study of spermiogenesis can also provide clues for studying systematic relationships (e.g. Justine, 1991; Bunke, 2005). The genetic diversity of spermatozoa is attributed to the meiotic events of gene recombination and chromosome reassortment (Guraya, 1987). In any given species, the characteristic steps of spermatogenesis, which may differ slightly between species, are responsible for shaping sperm architecture (e.g. Friedlander, 1997). Therefore, any morphological variations in sperm structure between and within species may be traced back to the particular steps involved during the complex process of spermatogenesis. Some spermiogenic events e.g. the development of the acrosome, and the reshaping of the sperm head by elongation coupled with the condensation of chromatin in the nucleus,

appear to be generally similar, especially in those species that are closely related (Fawcett *et al.*, 1971). Differences occur only in the extent of the nuclear elongation, which to an extent determines the shape of the sperm head, and the position and extent of the acrosome (Phillips, 1970; Fawcett *et al.*, 1971). The development of polymorphic sperm, an unusual phenomenon that is prevalent in some insect groups, including cicadas, can be studied by tracing the characteristic steps involved in spermiogenesis of the given insect that displays sperm polymorphism. Different sperm morphs would be expected to show polymorphic variation in size when germ cells undergoing spermiogenesis are examined microscopically.

In the majority of insects that live a very short time as adults, e.g. mayflies and caddis flies, spermatogenesis has ceased in adult testis, which contains mature spermatozoa only (Phillips, 1971). Myers (1929) suggested that this would be expected in cicadas because they have a short adult life. Whilst spermatogenesis has been well studied in insects (e.g. Phillips, 1970; 1974; Friedländer and Meisel, 1977; Fuller, 1993; Friedländer, 1997; Hess, 1999), there is little information on cicadas. Two studies have shown that in cicadas spermatogenesis commences in the early nymphal stages (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003). Unfortunately, studies of such early spermatogenesis are a challenge because early cicada nymphs are very small and extremely difficult to locate in their subterranean habitats. Consequently, in their studies of spermiogenesis Folliot and Maillet (1970) and Kubo-Irie *et al.* (2003) examined the last larval instars and some adults. They found that unlike the prediction of Myers (1929), spermiogenesis was still underway in adult male cicadas. The species they examined (*Cicada orni*, *Lyristes*

*plebejus* and *Graptosaltria nigrofuscata*) all belong to the subfamily Cicadinae. Some minor variations in the sperm morphology of cicadine and cicadettine cicadas have since been noted (Chapters 2 and 3) and therefore there might be variations during spermatogenesis in these cicada subfamilies.

The present study describes and compares some stages of spermiogenesis in the subfamilies Cicadinae and Cicadettinae, with an emphasis on the formation of the centriolar adjunct and the formation of sperm morphs. Results from Chapters 2 and 3 indicate that a large centriolar adjunct with a characteristic lamella-like substructure could be considered to be a synapomorphic character that separates the cicadettine from the cicadine cicadas. One objective of this study was to examine the early centriolar adjunct or surrounding structures to determine whether there is a direct link to the centriole. This is because in Chapters 2 and 3 it was suggested that there is no certainty on whether the purported centriolar adjunct in cicadas is indeed such a structure. A further aim was to examine the nature and origin of the homogenous matrix into which sperm heads are embedded. For this reason histochemical investigations were done. The ultrastructure of the seminal vesicles, which play a role in sperm storage prior to ejaculation and possibly in the formation of the homogenous matrix, was also examined.

## **4.2. MATERIALS AND METHODS**

### **4.2.1. MATERIALS**

Male cicadas from five species, representing three tribes, were collected in the Eastern Cape, South Africa, southeastern Zimbabwe and Florida, United States of America

(Table 4.1). *Pycna semiclara* (Germar, 1834) was collected in the Thomas Baines Nature Reserve (33° 23' S 26° 29'E) in December 2004. *Kongota punctigera* (Walker, 1850) was collected in the vicinity of East London (33° 1' S 27° 55' E) in December 2002. *Monomatapa matoposa* (Boulard, 1980) was collected in Malilangwe Private Game Reserve, Zimbabwe (20° 58' S 31° 47' E) in December 2003. *Diceroprocta biconica* (Walker, 1850) was collected by Prof. Allen Sanborn (Barry University, Florida) and Dr Polly Phillips (Florida International University, Miami) in Florida, United States.

Table 4.1. Cicada species used for the study.

Species	Subfamily	Tribe	Location
<i>Platypleura capensis</i>	Cicadinae	Platypleurini	Kasouga, South Africa
<i>Kongota punctigera</i>	Cicadinae	Platypleurini	Kasouga, South Africa
<i>Pycna semiclara</i>	Cicadinae	Platypleurini	Thomas Baines Nature Reserve, South Africa
<i>Monomatapa matoposa</i>	Cicadettinae	Taphurini	Malilangwe Game Reserve, Zimbabwe
<i>Diceroprocta biconica</i>	Cicadinae	Cryptotympanini	Florida, United States

#### 4.2.2. MICROSCOPY

Testes and seminal vesicles from *K. punctigera*, *M. matoposa* and *D. biconica* were dissected in 2 % saline solution before fixation overnight at 4 °C in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After washing in 0.1 M phosphate buffer, tissues were postfixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 90 minutes at room temperature, dehydrated in ethanol and embedded in Epon 812 resin. Some semi-thin sections were stained with Toluidine Blue and examined and photographed with an Olympus BX-60 compound microscope. Ultrathin sections (silver-gold) were cut using a diamond knife and collected on 300 mesh copper grids before staining with uranyl

acetate and lead citrate. Sections were examined and photographed with a JEOL 1210 transmission electron microscope at 120 kV.

### **4.2.3. HISTOCHEMISTRY**

Testes and seminal vesicles from adult *K. punctigera*, *P. semiclara* and *P. capensis* were dissected in 2 % saline solution before they were placed in aqueous Davidson's fixative for 24 hours (Humason, 1972). Davidson's fixative (sometimes called Hartmann's fixative) has a rapid action and gives good nuclear detail with minimal formalin pigment (Latendresses *et al.*, 2002). Fixation was followed by rinsing in distilled water before dehydration in absolute ethanol and embedding in Paraplast. Serial sagittal sections (approximately 8 µm thick) were cut using a Leica RM 2035 microtome, de-waxed in two changes of 100 % xylene, rehydrated in a graded ethanol series before being treated with the following histochemical reactions: PAS (periodic acid- Schiff reaction) to identify 1-2- glycols (Humason, 1972), Alcian Blue (pH 2.5) followed by counterstaining in nuclear fast red for the detection of acidic mucopolysaccharides (Humason, 1972), Bromophenol Blue for detection of proteins and peptides (Humason, 1972) and Toluidine Blue which produces different colours in various histological or cytological structures. All the tissues were then photographed and examined with an Olympus BX-60 compound microscope.

### **4.3. RESULTS**

#### **4.3.1. Light Microscopy**

Each testis is comprised of a series of follicles which are connected to the vas deferens. Within each follicle are several diverse cysts within which numerous spermatids are visible; these are aligned in a parallel fashion (Fig. 4.1A, B, C). A number of large spherical electron-dense granules are closely associated with spermatids but it is not clear whether they lie within or between them. The interstitial tissue is dominated by large irregularly-shaped cells (Fig. 4.1C). A few granules are found within this region. In all species no early stages (spermatogonia or spermatocytes) were observed. Spermiogenesis is synchronous within the cyst but not within the follicle.

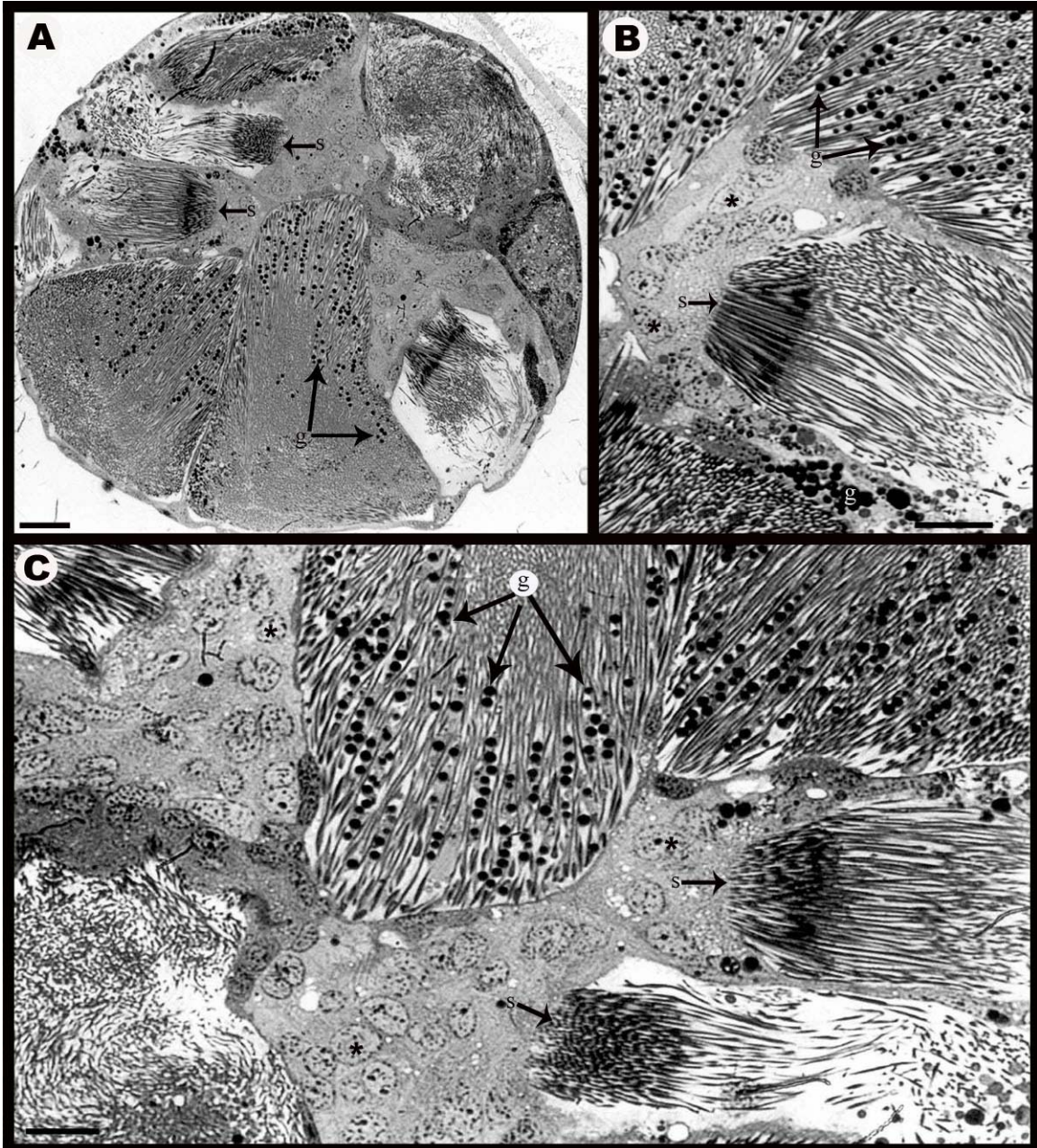


Figure 4.1. A: A light micrograph of a follicle in the testis of *Kongota punctigera* each one showing synchronously developing cells. Numerous spermatids (s) and granules (g) can be seen in each cyst within the follicle. Bar = 10  $\mu$ m. B: A higher magnification light micrograph of some cysts in the follicle of *K. punctigera*. Spermatids (arrows) are already aligned in a parallel fashion within each cyst and are associated with the large spherical granular bodies (g). Several large epithelial cells can be seen within the interstitial tissue. Bar = 10  $\mu$ m. C: A light micrograph of some cysts in the testis of *Pycna semiclara*. Large epithelial cells (\*) line the interstitial spaces. Note the elongated profiles of the spermatids (s) and the large spherical granules (g). Bar = 10  $\mu$ m.

### 4.3.2. Electron Microscopy

Early stages of spermiogenesis were observed in the testis of *Diceroprocta biconica*, but not in *Kongota punctigera* and *Monomotapa matoposa*. Nevertheless, spermiogenesis was occurring in the adults of all species. Spermatids, which are linked by cytoplasmic bridges (Fig. 4.5A), have a close association with cyst cells whose cytoplasmic projections enclose the anterior regions of spermatids (Figs. 4.2F and 4.3D). In addition, the cytoplasm of cyst cells contain large spherical (0.8 – 3  $\mu\text{m}$  diameter) and electron-dense granules (Fig. 4.3F).

#### 4.3.2.1. Development of the acrosome

Early spermatids possess a spherical proacrosomal granule (about 500 nm diameter) that is located between the concave face of the Golgi complex and the spermatid nucleus (Fig. 4.2.A, B). In mid spermatids the developing acrosome becomes positioned at the presumptive anterior of the elongating nucleus and microtubule-like elements start appearing in the developing acrosome (Fig. 4.2C). Both the anterior nucleus and acrosome are embedded in the cytoplasm of cyst cells (Fig. 4.2F). The acrosome elongates, invaginates and develops a posterior subacrosomal space as the spermatid matures (Figs. 4.2F and 4.3A to E). In mid to late spermatids there is a small region of cytoplasm anterior to the acrosome (Figs. 4.2F, 4.3A to E), hereafter referred to as the ante-acrosomal region. This region is circular in cross section and has a thickened plasma membrane (Fig. 4.2D). It is absent in mature sperm. The developing acrosome is surrounded by a ring of microtubules that continue into the ante-acrosomal region (Fig. 4.2D, E). Elongation of the acrosome is concomitantly accompanied by a transformation

of the acrosomal contents which gives rise to an electron-dense peripheral region, the acrosome proper, and an electron-lucent interior, the subacrosomal space (not illustrated).

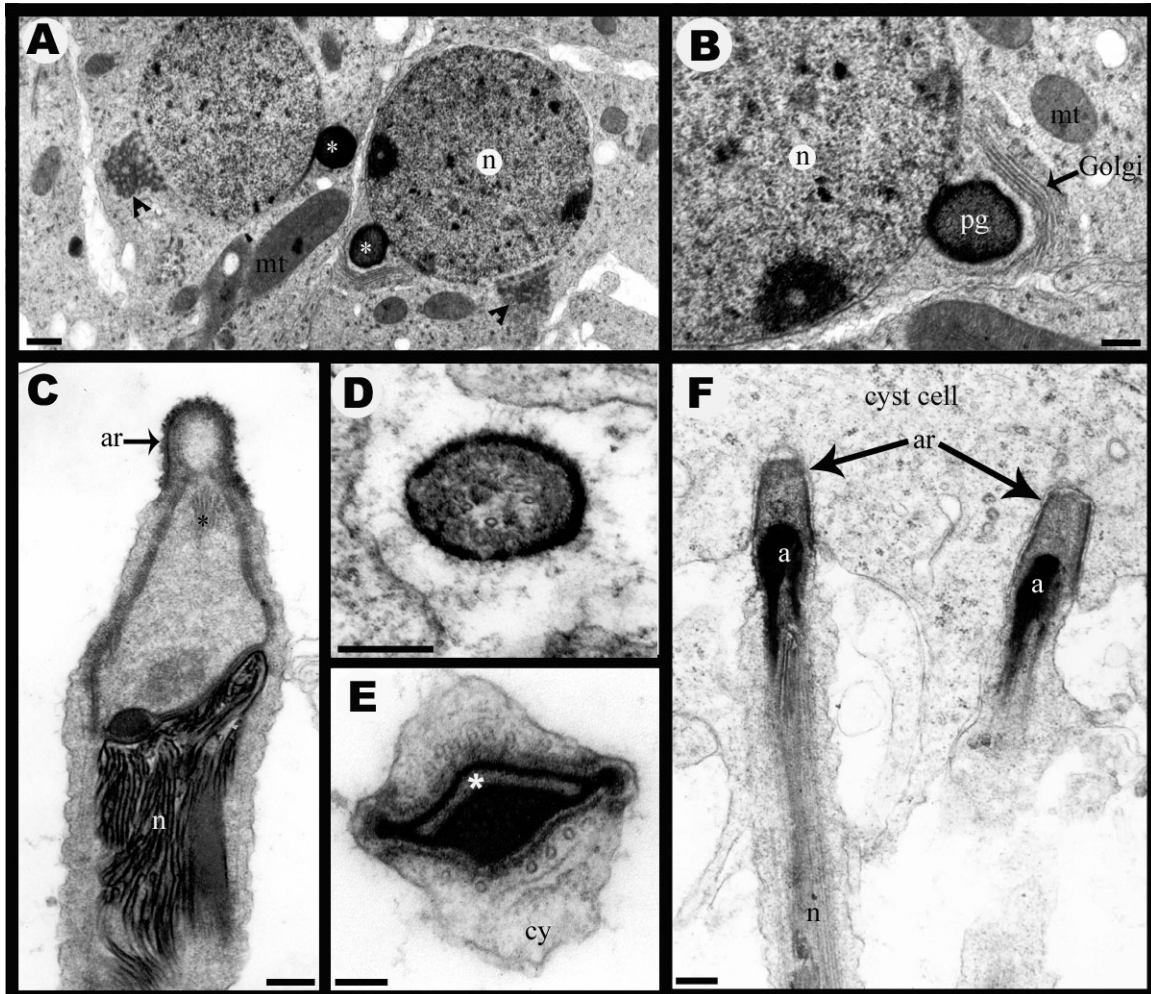


Figure 4.2. A: *Diceroprocta biconica*. Sections of early spermatids. During the early stages some coarse granular material (arrowheads) accumulates in a region adjacent to the nucleus (n) of each spermatid. (\*), proacrosomal granule; mt, mitochondrion. Bar = 500 nm. B: Acrosome formation in a spermatid of *D. biconica*. The proacrosomal granule (pg), which lies adjacent to the nucleus (n), is located below the concave side of the Golgi complex. mt, mitochondrion. Bar = 200 nm. C: Longitudinal section of a developing sperm head of *Kongota punctigera*. At this stage the largely undifferentiated acrosome has an ante-acrosomal region (ar) and microtubule-like elements (\*) start appearing in the developing acrosome. Bar = 200 nm. D: *K. punctigera* spermatid. Transverse section through the ante-acrosomal region. Note the microtubules within the interior. Bar = 200 nm. E: *Monomatapa matoposa*. Transverse section through the developing acrosome. Note the subacrosomal space (asterisk) and the microtubules. Bar = 100 nm. F: Mid-spermatids, *M. matoposa*. Longitudinal sections of the head. At this stage the ante-acrosomal region (ar) has extended along the acrosome. a, acrosome; n, nucleus. Bar = 200 nm.

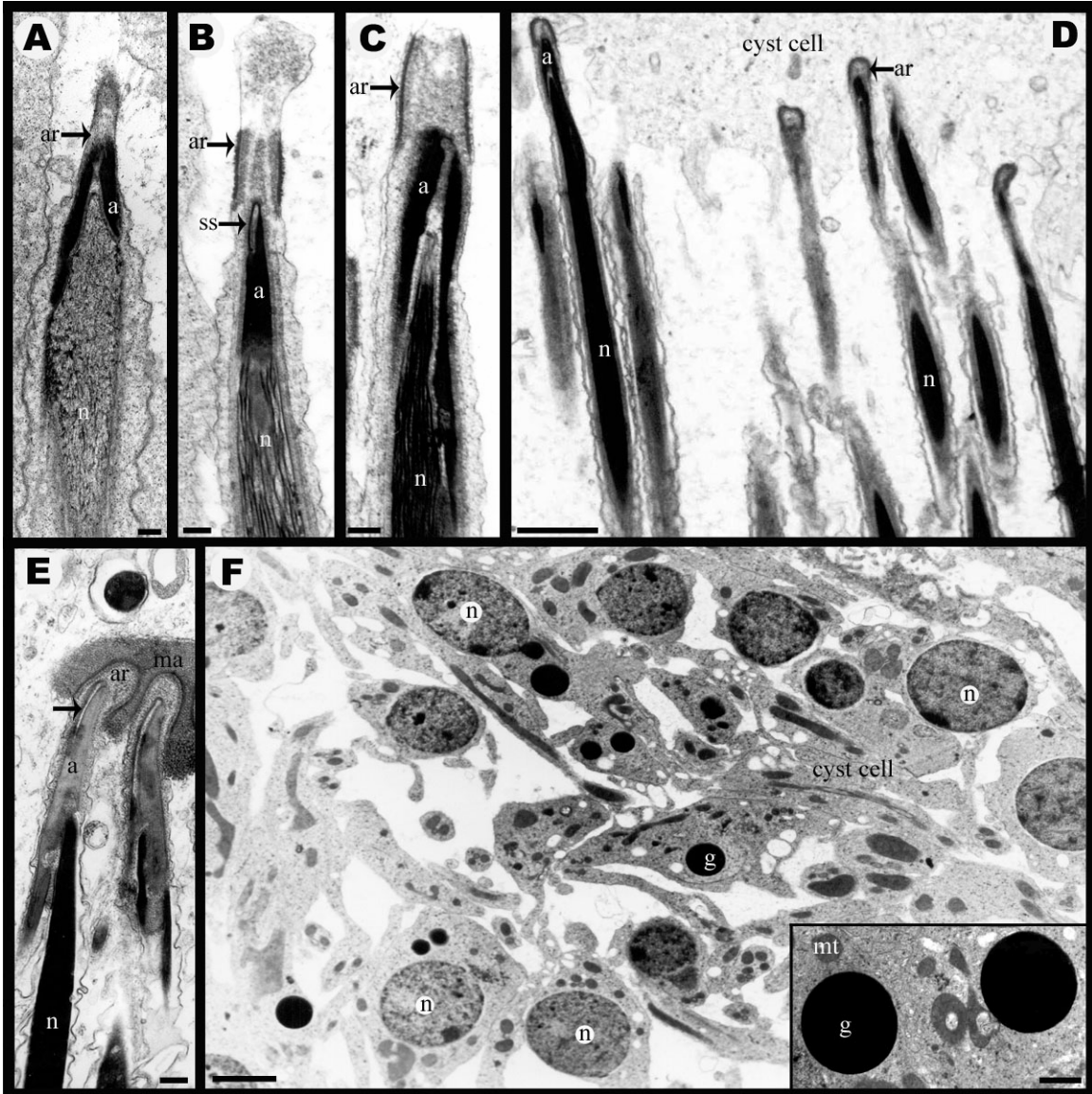


Fig. 4.3. A: Mid spermatid of *Kongota punctigera*. Longitudinal section of the head. The developing acrosome (a) extends as two processes on either side of the condensing nucleus (n). ar, ante-acrosomal region; n, nucleus. Bar = 200 nm. B: *K. punctigera*. Longitudinal section through a spermatid head. The developing acrosome (a) has developed a subacrosomal space (ss). Chromatin condensation in the nucleus (n) has begun. The plasma membrane of the ante-acrosomal region (ar) has ruptured. Bar = 200 nm. C: *K. punctigera*. Longitudinal section of a developing spermatid head. The acrosome (a) now has a well developed subacrosomal space; chromatin condensation in the nucleus (n) has produced thick lamellae. Bar = 200 nm. D: *K. punctigera*. Longitudinal section of late spermatids. The anterior nucleus (n) and acrosome (a) are surrounded by cytoplasmic extensions of cyst cells. ar, ante-acrosomal region. Bar = 1  $\mu$ m. E: *M. matoposa*. Longitudinal sections of late spermatids. a, acrosome; ar, ante-acrosomal region; arrow, subacrosomal space; ma, matrix; n, nucleus. Bar = 200 nm. F: Early spermatids of *D. biconica*. At this stage early spermatids, which are surrounded by cytoplasmic extensions of cyst cells, have a round nucleus (n) with diffuse chromatin. g, granule. Bar = 2  $\mu$ m. Inset: Higher magnification showing the electron-dense granules (g) in *D. biconica*. mt, mitochondrion. Bar = 1  $\mu$ m.

#### 4.3.2.2. Development of the nucleus

The early spermatid nucleus (Figs. 4.3F, 4.4A, 4.5A and 4.7B) is characterized by an ovoid shape and heterogenous chromatin distribution with a granular appearance. The spermatid nucleus elongates and chromatin condensation begins during spermiogenesis (Fig. 4.4B, C). A lateral invagination develops posteriorly as the nucleus elongates (Fig. 4.4E).

Chromatin condensation commences at two lateral regions of the spermatid nucleus in all species (Fig. 4.4D, E). The degree of chromatin condensation is not uniform within an individual spermatid. The spatial arrangement of the individual chromatin strands is variable within different regions of the nucleus, especially around the edges and within the centre (Figs. 4.4D, E, 4.5B, C). The elongation of the spermatid nucleus is accompanied by an arrangement of the chromatin fibers parallel to the elongating axis (Fig. 4.3A to E). The chromatin gradually condenses during the subsequent transformations, increasing in electron density and becoming organized as lamellae before forming an intricate labyrinth (Figs. 4.4D and 4.6A). Further condensation produces a conglomerate of coarse granular subunits (Figs. 4.4E, 4.5B to D). Finally, the chromatin material becomes compact, displays considerable electron opacity and is devoid of visible substructures (Figs. 4.5E and 4.6D). The chromatin condensation stages in *K. punctigera* and *M. matoposa* are generally similar. However, in *D. biconica* the condensing nucleus develops a characteristic freckled appearance (Fig. 4.6B, C). This is caused by isolated nucleoplasm forming numerous small light regions in the dark chromatin.

The nucleus becomes surrounded by longitudinal microtubules that form a manchette during chromatin condensation and nuclear elongation (Figs. 4.4D, E and 4.5C); these appear to be continuous with and around the acrosome (Fig. 4.2D). The microtubules are in turn surrounded by an endomembrane (Figs. 4.4D, E and 4.5C) in all species. Neighbouring microtubules are very close to each other and to the nuclear envelope in *K. punctigera* (Fig. 4.4E). However, the microtubules in *M. matoposa* are arranged sparsely and seem not to be connected to the nuclear envelope (Fig. 4.5C). In *M. matoposa* a single layer of microtubules surrounds all stages of spermatids (Figs. 4.4D and 4.5C), whilst in *K. punctigera* and *D. biconica* one side of the nucleus is flanked by either a single or double layer of microtubules while along the opposite side there may be three to six layers of microtubules clumped together in sheets (Figs. 4.4E and 4.6B, C, D). As many as 220 microtubules are visible in late *D. biconica* spermatids (Fig. 4.6D). The spermatid nucleus becomes laterally invaginated in a region sandwiched between the two zones where chromatin condensation commences (Fig. 4.4E). Within this region granular material becomes closely associated with the nucleus by adhering to it laterally (Fig. 4.6F). Cross-sections of the spermatid nucleus show its intimate association with the layer of microtubules (Fig. 4.4E). However, more posterior cross-sections below the level of the acrosomal processes show a space between the microtubules and the nuclear membrane. This space houses the early so-called centriolar adjunct (Fig. 4.6F).

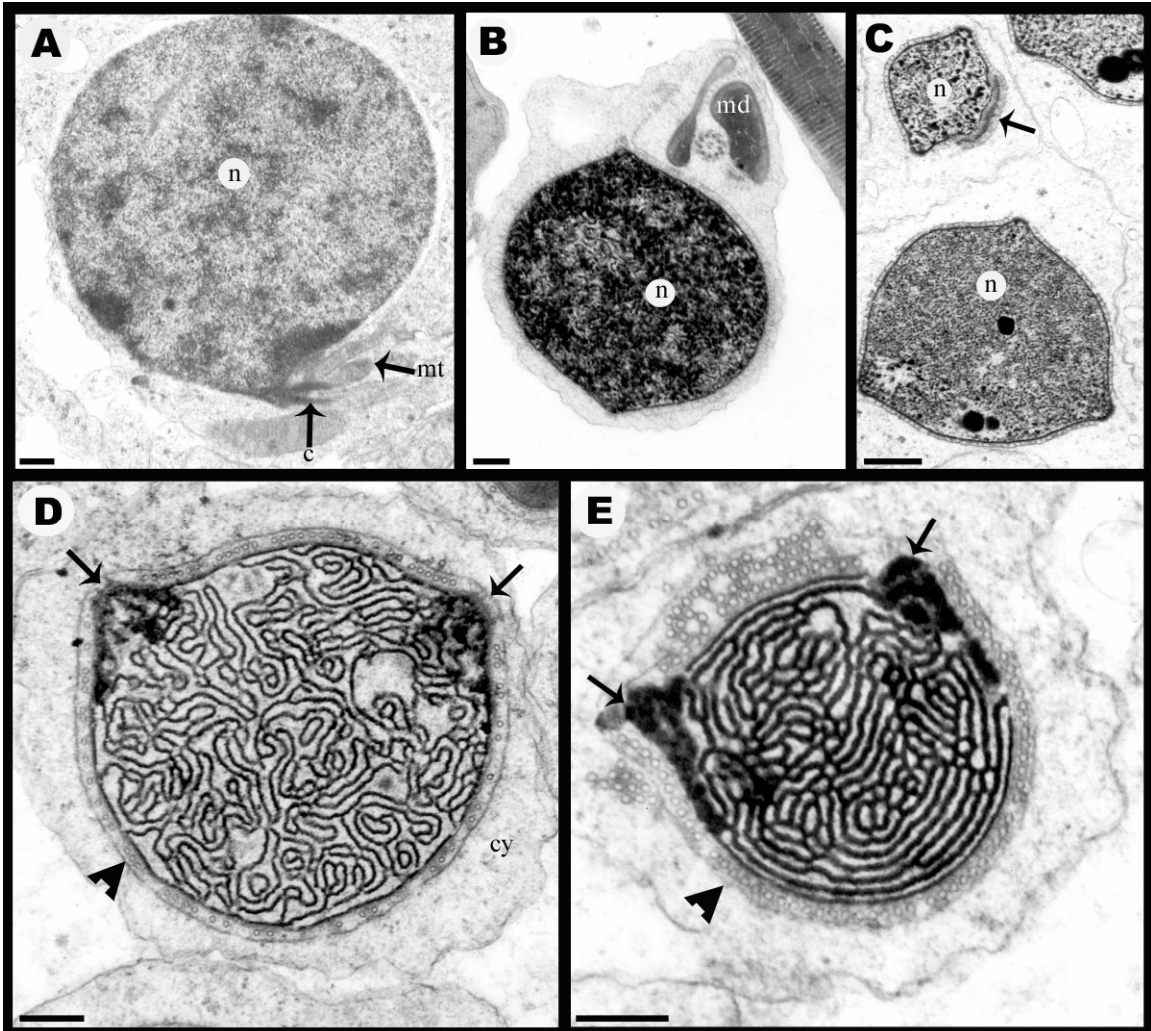


Figure 4.4. A: Early spermatid of *Diceroprocta biconica*. The spherical nucleus (n) has dispersed chromatin. The centriole (c) and mitochondria (mt) have assumed their positions posterior at the presumptive of the nucleus. Bar = 200 nm. B: Maturing spermatid of *M. matoposa*. The nucleus (n) is still spherical but chromatin has condensed into clumps. The two mitochondrial derivatives (md) are extending along the flagellum. Bar = 200 nm. C: *M. matoposa*. Transverse section through developing spermatids to show nuclei of different volumes and shapes. The development of the centriolar adjunct (arrow) has begun. Bar = 500 nm. D: *M. matoposa*. Transverse section of a spermatid nucleus. The nucleus is surrounded by a manchette of microtubules. The microtubules are in turn surrounded by an endomembrane (arrowhead). cy, cytoplasm. The chromatin of the condensing nuclei appears as thin lamellae. Chromatin condensation commences from two lateral regions of the nucleus (arrows). Bar = 200 nm. E: *K. punctigera*. Transverse section of a spermatid nucleus. The condensing chromatin now appears as thick dense strands. However, in the region where condensation commences (arrows), the chromatin has formed thick granules. arrowhead, endomembrane. Bar = 200 nm.

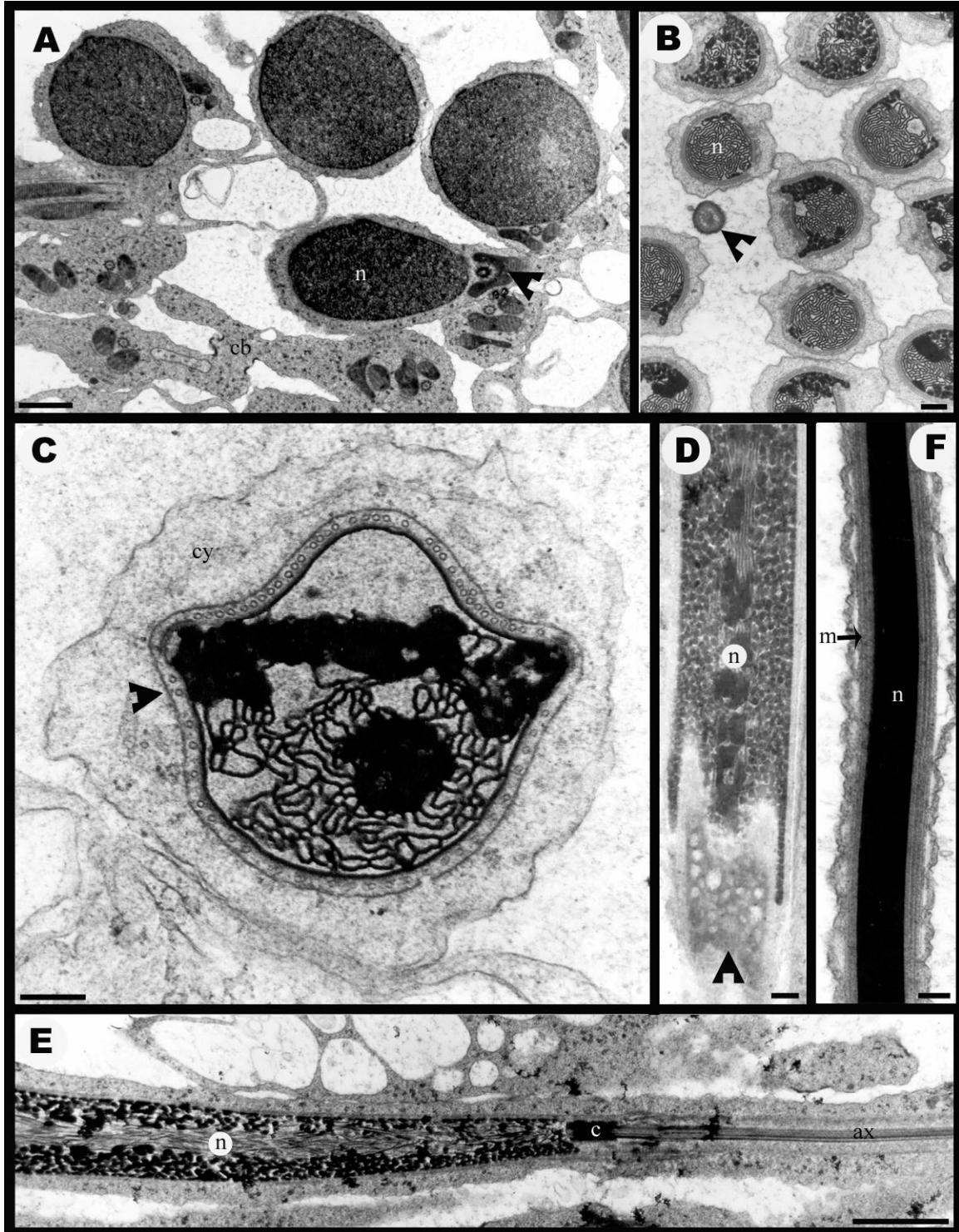


Figure 4.5. A: Early spermatids of *D. biconica*. Within the nucleus (n), chromatin condensation has begun and mitochondrial derivatives have formed and are extending along each flagellum. arrowhead, spermatid tail; cb, cytoplasmic bridge. Bar = 1 $\mu$ m. B: Transverse sections of *K. punctigera* spermatids. Chromatin condensation is not uniform both within and between nuclei. arrowhead, ante-acrosomal region; n, nucleus. Bar = 200 nm. C: *M. matoposa* spermatid. Transverse section of the nucleus. At this stage chromatin condensation has produced thick

strands and large clumps. Note the manchette, endomembrane (arrowhead) and the cytoplasm (cy) surrounding the spermatid. Bar = 200 nm. D: *D. biconica* spermatid. Longitudinal section of a region in the mid-piece where the developing nucleus is invaginated. Centriolar adjunct material (arrowhead) is housed in the invagination. Bar = 200 nm. E: *D. biconica* spermatid. Longitudinal section of the mid-piece. ax, axoneme; c, centriole; n, nucleus. Bar = 1  $\mu$ m. F: Late spermatid of *K. punctigera*. Longitudinal section of the elongated nucleus. Here the nucleus has completely condensed. Note the microtubules (m) aligned parallel to the long axis of the nucleus (n). Bar = 200 nm.

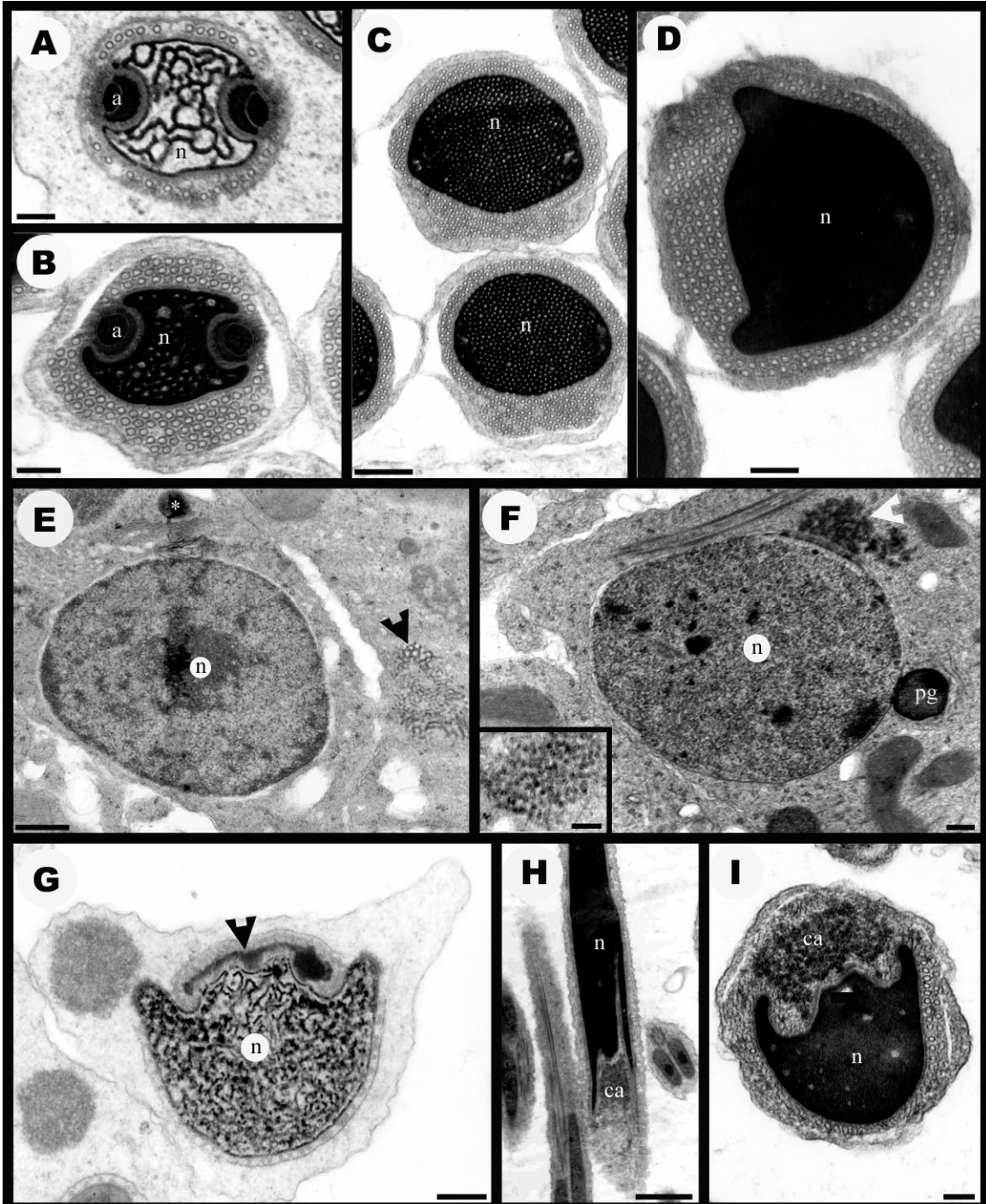


Figure 4.6. A: Mid-spermatid of *K punctigera*. Transverse section through the anterior nuclear region (n), showing the two acrosomal processes (a). Note the ring of microtubules surrounding the spermatid. Bar = 100 nm. B: Late spermatid of *D. biconica* cross-sectioned at the same level as in A. Condensation of the nucleus (n) is almost complete. The freckled appearance of the nucleus is caused by isolated nucleoplasm forming numerous small light regions in the dark chromatin. a, acrosome. Bar = 100 nm. C: Late spermatids of *D. biconica*. Transverse sections to show two condensing nuclei (n). Note the manchette surrounding each spermatid. Bar = 200 nm. D: Late spermatid of *D. biconica*. Here the nucleus (n) is no longer speckled and chromatin condensation has been completed. However, the manchette is still present. Bar = 100 nm. E:

Transverse section of an early *D. biconica* spermatid. Some material (arrowhead) lies close to the developing nucleus (n). At this stage the proacrosomal granule (\*) has not yet attached to the nucleus. Bar = 500 nm. F: *D. biconica*. Transverse section of an early spermatid. A high magnification of the nucleus (n) to show the granular material (arrowhead) that presumably forms the centriolar adjunct. pg, proacrosomal granule. Bar = 200 nm. Inset: A higher magnification to show the granular material. Bar = 100 nm. G: Mid spermatid of *M. matoposa*. Transverse section through the level of the nuclear invagination. arrowhead, centriolar adjunct material; n, nucleus. Bar = 200 nm. H: Late-spermatid of *K. punctigera*. A longitudinal section at a level where the nucleus (n) is invaginated. Here the material of the centriolar adjunct (ca) is accumulating within the nuclear invagination. Bar = 1  $\mu\text{m}$ . I: Late spermatid of *M. matoposa*. Transverse section of the nucleus (n) showing the lateral invagination and the material of the centriolar adjunct (ca). Note that the number of microtubules in the manchette has decreased. Bar = 100 nm.

#### 4.3.2.3. Development of the sperm mid-piece, mitochondrial derivatives and axoneme

Early spermatids possess a single centriole that is positioned in a shallow nuclear fossa distal to the nucleus (Fig. 4.7B, C). The centriole consists of doublets rather than triplets of microtubules. It is in turn flanked by a number of small mitochondria (Figs. 4.5A and 4.7C). The early stages in the development of the mitochondrial derivatives presumably occur in the early nymphal stages. Only the mid and late stages were found in the testes of the three species examined. The early derivatives have an almost circular cross-sectional profile (Fig. 4.8A) and the matrix of each is fairly homogenous. They are surrounded by cytoplasm and very few microtubules. The latter is not initially surrounded by an endomembrane, but after further transformations, an endomembrane develops between the space enclosed by the microtubules and the peripheral spermatid membrane (Fig. 4.8B, C). Electron-dense crystallized regions start to form in the core of each derivative until they almost fill up the spaces within the mitochondrial matrix. The peripheral cristae become regularly aligned and perpendicular to the longitudinal axis of each derivative to form striations of consistent periodicity (Fig. 4.7E).

There is a change in the cross-sectional profiles concurrent with all these transformations. The gradual elongation and narrowing of each derivative (Fig. 4.7D, E) is accompanied by an alteration in the cross-sectional profile during the course of spermiogenesis. The circular outline of the early derivatives eventually gives way to a transitory semi-elliptic profile before becoming a somewhat flattened cylinder in mature sperm (Fig. 4.8A to D). The spermatid tail is surrounded by a manchette of longitudinal microtubules during all these stages but their numbers are reduced compared to those found surrounding the spermatid nucleus. Between 30 and 60 microtubules surround the tail; some lie between the mitochondrial derivatives. Cross-sectional profiles of the spermatid tail reveal two membranes that surround the two derivatives and the axoneme (Fig. 4.8B, C). The inner membrane delimits the region where the microtubules are whilst the outer membrane delimits the extent of the cytoplasm. Further transformations result in a complete elimination of the cytoplasm and an elongation of each derivative until it flanks the axoneme alongside most of the sperm flagellum (not illustrated).

The axoneme emerges from the centriole and is surrounded by longitudinal microtubules and cytoplasm (Fig.4.7B); these are lost at the end of spermiogenesis. In both *D. biconica* and *K. punctigera* the regions with several layers of microtubules are associated with the early so-called centriolar adjunct. The material that forms this structure appears in early stages as a conglomerate of small diffuse moderately electron dense granules adjacent to the nucleus (Figs. 4.2A and 4.6E, F). Cross-sections of the spermatid nucleus in later stages show the same material within the lateral invagination of the nucleus (Fig. 4.6F to I). It has a granular appearance and is surrounded, together with the nucleus, by

the microtubular manchette. Vesicle-like elements are associated with this material (Fig. 4.7A). In later stages, the material becomes more electron-dense, increases in size by elongation and spreads along one surface of the nucleus until termination in the neck region (not illustrated).

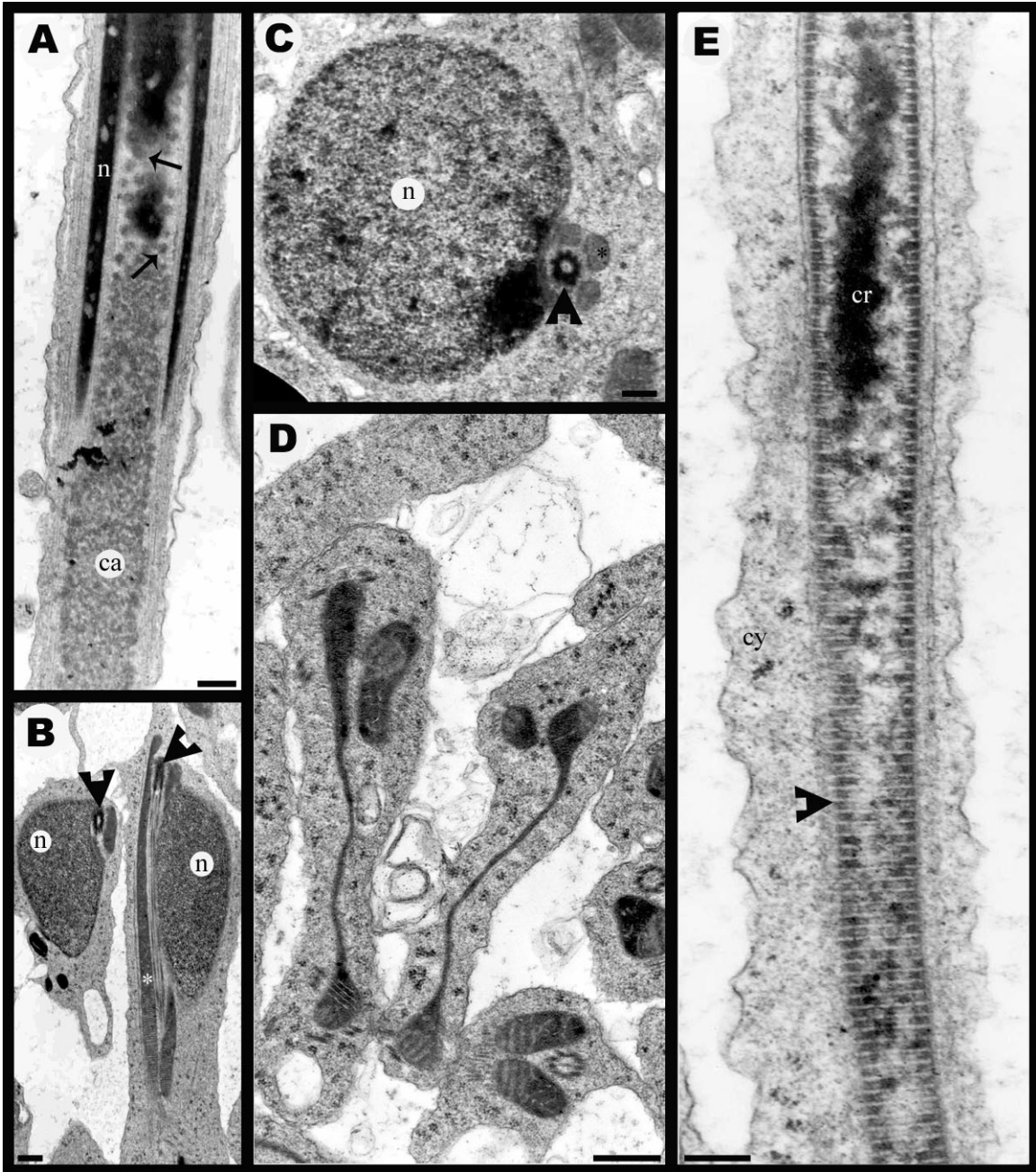


Figure 4.7. A: Late spermatid of *M. matoposa*. Longitudinal section along the mid-piece region to show the nucleus (n), centriolar adjunct (ca) and the vesicle-like elements (arrows). Bar = 200 nm. B: Longitudinal section of a *D. biconica* spermatid to show the centriole (arrowhead),

developing axoneme and mitochondrial derivatives (\*) which lie lateral to the nucleus (n). Bar = 500 nm. C: Transverse section of a *D. biconica* spermatid. Here the nucleus (n) is spherical and a centriole (c) is surrounded by mitochondria (mt). Bar = 200 nm. D: Mid-spermatids of *D. biconica*. Two longitudinal sections show the mitochondrial derivatives undergoing elongation. Bar = 500 nm. E: Longitudinal section through a mitochondrial derivative of a *K. punctigera* spermatid. Here the mitochondrial cristae (arrow) are arranged into regularly spaced lamellae, which only extend only part way across the mitochondrion, leaving most of the space for crystalline material (cr). cy, cytoplasm. Bar = 200 nm.

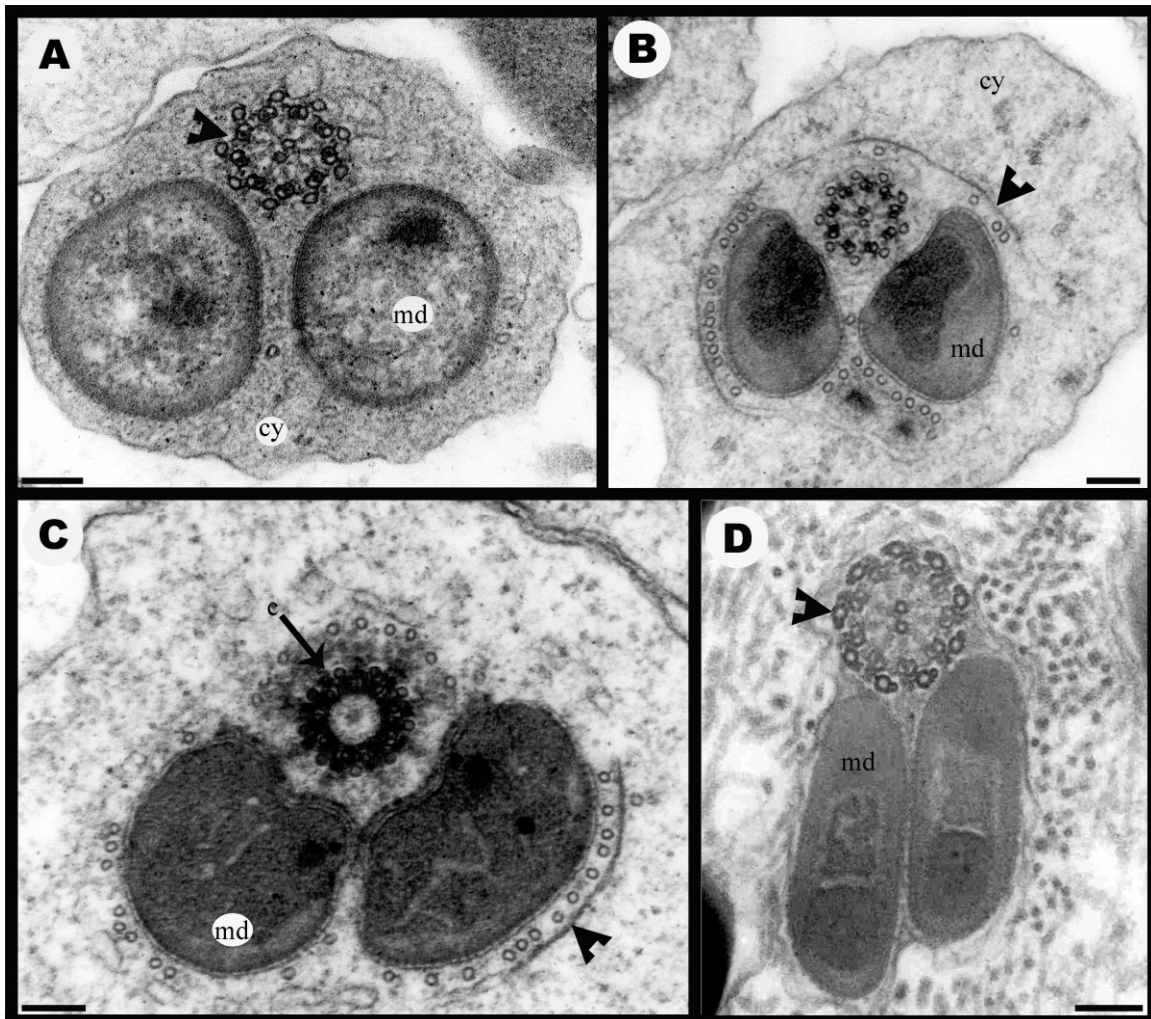


Figure 4.8. A: Mid spermatid of *M. matoposa*. Transverse section through the tail region. Crystalline structures have started forming in each mitochondrial derivative (md). arrowhead, axoneme. Bar = 100 nm. B: Late spermatid of *M. matoposa*. Transverse section through the tail region. Most of the space in each mitochondrial derivative is occupied by crystalline material. The manchette microtubules, arranged in an intermittent pattern, surround a centriole and both derivatives. arrowhead, endomembrane; cy, cytoplasm. Bar = 100 nm. C: Late spermatid of *K. punctigera* cross-sectioned at the level of the centriole (c). The single tubules at the centriolar level, outside the centriole, are accessory tubules. arrowhead, endomembrane; md, mitochondrial derivative. Bar = 200 nm. D: Transverse section of the mature sperm tail of *K. punctigera*. The manchette microtubules and cytoplasm have been lost. arrowhead, axoneme; md, mitochondrial derivative. Bar = 100 nm.

#### 4.3.2.4. Ultrastructure of seminal vesicles

The thick seminal vesicle wall (about 6  $\mu\text{m}$  width) is double layered with an inner epithelium and a peripheral muscular sheath (Fig. 4.10A, B). The wall of the vas deferens has a similar arrangement. The epithelium is pseudostratified and consists of a number of irregularly-shaped cells that are interconnected to each other by septate junctions (Fig. 4.10A). Some of the cells in the seminal vesicle wall have large nuclei containing large chromatin clumps that often are distributed peripherally (Figs. 4.9B, C and 4.10A, B). Extensive polygonal infoldings are prominent within the cytoplasm; they partly surround each of these large nuclei. The cells of the seminal vesicle wall possess a number of granules containing material of high electron density and numerous mitochondria and vesicles of varying sizes (Figs. 4.9B, C and 4.10B). Generally mitochondria are distributed throughout the cells of the seminal vesicle wall (Figs. 4.9B, and 4.10B). Some material of varying electron density is contained in some of the vesicles. However, some vesicles appear to be empty (Fig. 4.9C, D). The vesicles are distributed throughout the cells of the seminal vesicle wall. On the luminal side of the epithelium the vesicles appear to be connected to microvilli which are found on the surface of the epithelium (Fig. 4.9C, D). The microvilli appear as either uniformly cylindrical, short pointed projections or as long bulbous structures containing some material. In other regions of the seminal vesicle microvilli are not present on the apical surface of the epithelium that borders the lumen. The lumen of the seminal vesicles contains spermatodesmata and a number of free sperm (Figs. 4.9A, B, C and 4.10A).

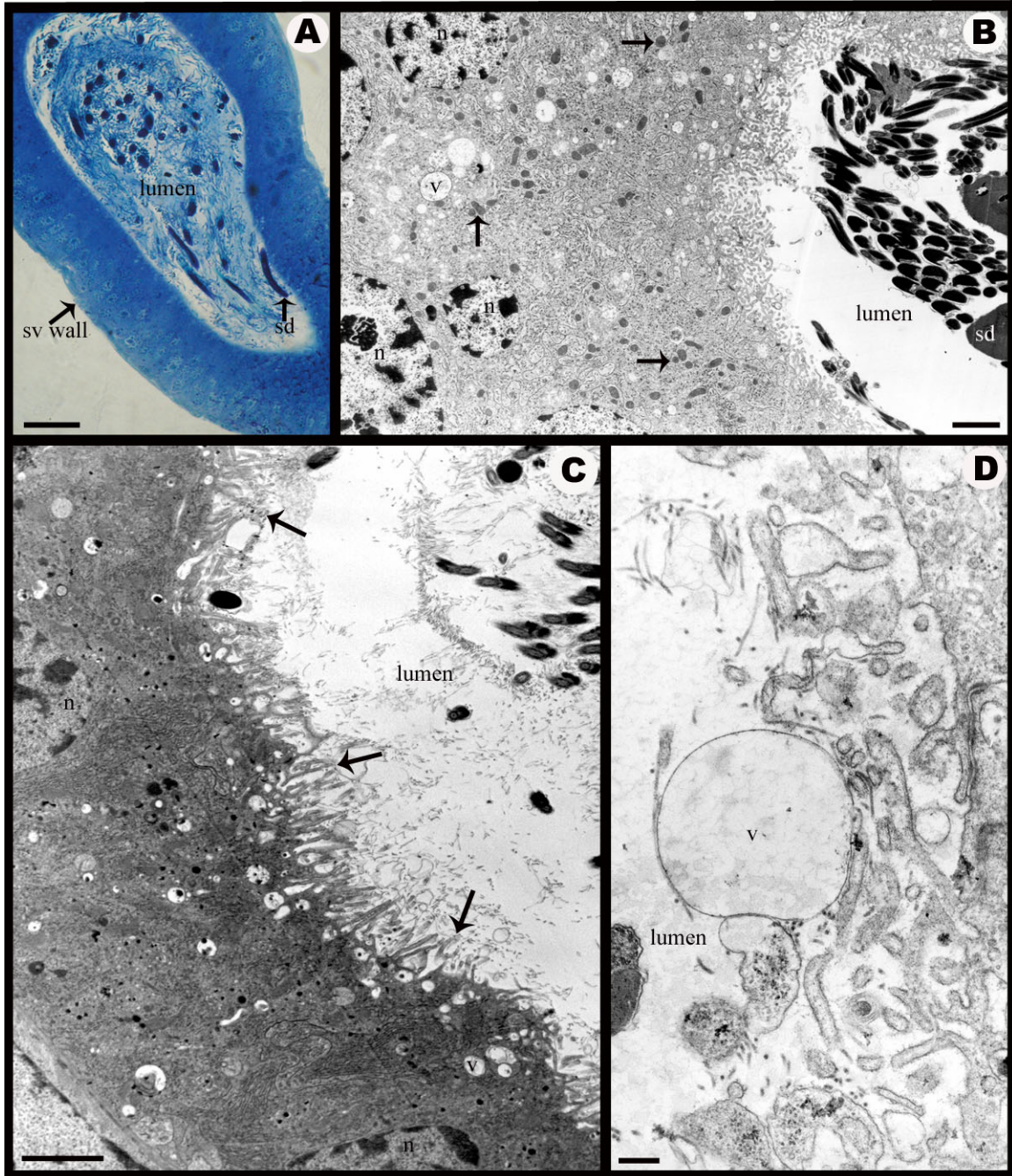


Figure 4.9. A: Transverse section through the seminal vesicle of *K. punctigera* stained with Toluidine Blue. The thick wall of the seminal vesicle contains a number of large cells. Several spermatodesms (sd) are visible within the lumen. Bar = 100  $\mu$ m. B: Transverse section of the seminal vesicle of *K. punctigera*. Numerous mitochondria (arrows), vesicles (v) and dense granular structures are visible within the cytoplasm. Note the large nuclei (n) and the mature sperm in the lumen (lm). sd, spermatodesm. Bar = 2  $\mu$ m. C: Transverse section of the seminal vesicle in *P. semiclara*. Numerous microvilli (arrows) line the luminal side of the epithelium. The microvilli appear as either uniformly cylindrical, short pointed projections or as long bulbous structures containing some material. n, nucleus; v, vesicle. Bar = 2  $\mu$ m. D: Transverse section of the seminal vesicle of *P. semiclara* to show microvilli lining the epithelium on the lumen side. v, vesicle. Bar = 200 nm.

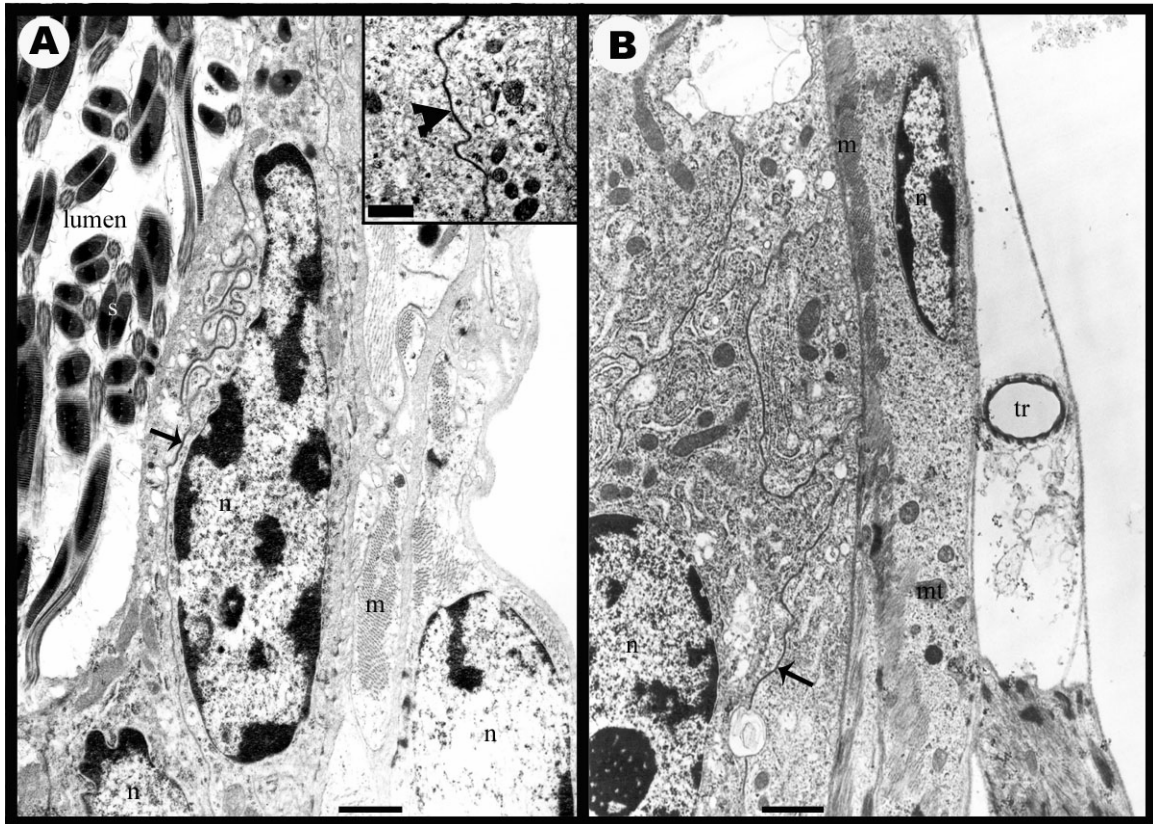


Figure 4.10. A: Transverse section through the wall of the seminal vesicle of *K. punctigera* showing its internal morphology. Note the large nuclei (n) containing large chromatin clumps and the muscle layer (m). arrow, septate junction; mt, mitochondrion. Bar = 1  $\mu$ m. Inset: Higher magnification of the septate junction (arrowhead) in the seminal vesicle wall of *K. punctigera*. Bar = 500 nm. B: Transverse section of the outer seminal wall of *K. punctigera*. arrow, septate junction; m, muscle layer; mt, mitochondrion; n, nucleus; tr, tracheole. Bar = 1  $\mu$ m.

### 4.3.3. Histochemistry

There is no evidence to suggest that the homogenous matrix originates from within the testis. Instead, it appears to originate within the vas deferens and part of the seminal vesicle. Acidic carbohydrates, specifically 1-2- glycols or closely related structures, such as one in which an amino group replaces one of the hydroxyl groups (Kiernan, 1999) are present in the matrix (Table 4.2). Neutral carbohydrates (mucopolysaccharides) and basic proteins are also part of the matrix (Table 4.2). Spermatid nuclei stain a dark blue colouration with PAS and a reddish pink with Alcian Blue. All tissues are metachromatic with Toluidine Blue.

Table 4.2. Histochemical investigation of the homogenous matrix.

Test	Homogenous matrix of spermatodesm	Epithelium surrounding seminal vesicle
PAS	+ (bright magenta)	+
Alcian Blue	+ (bluish green)	+
Bromophenol Blue	+ (blue)	+
Toluidine Blue	+ (metachromatic)	+

#### 4.4. DISCUSSION

Spermiogenesis in *Diceroprocta biconica*, *Kongota punctigera* and *Monomotapa matoposa* is similar to that described in other cicadas (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003) as well as hemipterans (Lee, 1985; Fernandes *et al.*, 2001) and other insects (Baccetti, 1998; Jamieson *et al.*, 1999; Dallai *et al.*, 2001c; Ndiaye and Mattei, 2005). In early spermatids a proacrosomal granule is in close proximity to the Golgi complex suggesting that it is involved in the formation of the acrosome. The role of the Golgi complex in the formation of the proacrosomal granule and its differentiation into a proper acrosome has been reported in several insects e.g. spitbugs (Folliot and Maillet, 1970), butterflies (Phillips, 1970; Mancini and Dolder, 2004), beetles (Baccetti, 1975), honeybees (Peng *et al.*, 1993) and silverfish (Dallai *et al.*, 2001c). As the acrosome matures an ante-acrosomal region develops. This region persists in late spermatids (Fig. 4.3E) but is absent in mature sperm (Chapters 2 and 3). In insects like lacewings, antlions and beetles an ante-acrosomal layer still persists in mature sperm (Baccetti *et al.*, 1973a; 1973b). According to Baccetti (1998) this layer in which granular cytoplasmic material is aggregated, originates from the spermatid proacrosome granule. The gradual transformation of the acrosomal shape is universal and in most cases this generally involves a complex modification from a spherical profile at the apex of the spermatid nucleus to a more streamlined conical profile with each step determined by the characteristics of the species involved. The antecrosomal bleb described from the cicadas, *Lyristes plebejus*, *Cicada orni* (Folliot and Maillet, 1970) and *Graptosaltria nigrofuscata* (Kubo-Irie *et al.*, 2003) could in fact be an ante-acrosomal region because it is so similar to the ante-acrosomal region observed in the present study. In addition, a

layer of cytoplasm which surrounds the spermatid membrane is evident in some electron micrographs used by Folliot and Maillet (1970). Evidence from the present study shows that mature spermatozoa, unlike spermatids, contain a much reduced amount of cytoplasm. In light of this it is possible that the aforementioned authors examined late spermatids instead of mature spermatozoa.

The maturation of the nucleus, characterized by elongation and a reduction in diameter with a simultaneous condensation of chromatin, is typical of most insects (Phillips, 1970, 1974; Baccetti, 1998; Jamieson *et al.*, 1999) as well as many other animals including mammals (Fawcett, 1994), birds (Soley, 1997), reptiles (Ferreira and Dolder, 2003; Al-Dokhi, 2004) and invertebrates (Hodgson and Heller, 2000; Cable and Tinsley, 2001; Dallai *et al.*, 2004). Chromatin condensation involved four phases (granular, fibrillar, lamellar and homogenous, which is typical of sperm with elongate nuclei (Baccetti, 1998). Chromatin condensation commenced at two lateral regions of the spermatid nucleus in *D. biconica*, *K. punctigera* and *M. matoposa*. Studies from other animals have shown that during spermiogenesis, the arrangement of chromatin within the nucleus is mediated by two specific areas, each of which is known as the polar nuclear matrix (Ribes *et al.*, 2004). Such a polar nuclear matrix has been described from spermatids of mammals, fish and a cephalopod (Ribes *et al.*, 2004). In *Octopus vulgaris* (Cephalopoda: Octopodidae), the areas constituting the polar nuclear matrix have the following characteristics: (a) DNA is found anchored to the nuclear membrane in these areas only, (b) chromatin condensation commences from these regions; and (c) mechanical forces (initiated by nuclear elongation) which influence the arrangement of chromatin fibres into

a parallel lamellar disposition, appear to originate from these areas (Ribes *et al.*, 2004). From the present study the zones from which chromatin condensation commenced were not at the poles but the fact that these zones were in similar positions in all three cicadas examined suggests a role in the orientation of chromatin fibres during spermiogenesis.

The packaging of DNA and the architecture of the genome in mature sperm nuclei are the result of a series of structural and chemical modifications including an organized substitution of structural proteins (mainly histones) in the heads of early spermatids by sperm nuclear basic proteins (SNBPs / protamines) in mature sperm nuclei, that are rich in arginine and/or lysine residues (Friedländer and Hauschteck-Jungen, 1982; Hauschteck-Jungen and Rutz, 1982; Ausio, 1995; Hammadeh *et al.*, 1999; Gimenez-Bonafe *et al.*, 2002; Ribes *et al.*, 2004). Nuclear sperm-specific proteins are of three basic types: H-type (histone), P-type (protamine) and PL-type (protamine like) consisting of proteins with an intermediate composition between histones and protamines (Ausio, 1995).

During spermiogenesis, the type of condensation patterns shown by chromatin is influenced by the interaction of nuclear sperm-specific proteins with DNA (Ribes *et al.*, 2004). For example, interaction of DNA with proteins of the histone type (H-type) promotes a granular condensation, while other basic proteins condense chromatin in fibres or lamellae (Ausio, 1995; Saperas *et al.*, 1993; Càceres *et al.*, 1999; Ribes *et al.*, 2004). The evolution of SNBPs appears to be saltatory rather than continuous for these proteins can differ quite markedly between related taxa (Kasinsky, 1995). For example,

different genera of frogs can have either the H-type proteins (histones) or arginine-rich protamine-like SNBPs (Kasinsky, 1995). The variation in chromatin condensation patterns in *D. biconica*, *K. punctigera* and *M. matoposa* can be attributed to the nature of the protamines, which are probably different, and the DNA of each species. This is so because protamines show very little evidence of conservation, and have an elevated degree of heterogeneity, both at the protein and gene levels (Kasinsky, 1989; Ausio, 1995; Lewis *et al.*, 2003). Condensation by these basic amino acids can thus be considered as a first level of genome organization of the sperm nucleus (Ribes *et al.*, 2004).

In *D. biconica*, *K. punctigera* and *M. matoposa*, the sperm nucleus of mature sperm is compact, with no evidence of any chromatin strands. This compaction of DNA is common in the spermatozoa of most animals. According to Ward and Coffey, (1991), the DNA of spermatozoa is sixfold more compact than mitochondrial DNA. The extensive molecular reorganization and condensation of chromatin possibly renders it metabolically inert and ensures the protection of the genome against physical or chemical mutagenesis during its transport to the site of fertilization (Fawcett *et al.*, 1971). The change in shape from a spherical to an elongated outline coupled with a reduction in volume of the sperm nucleus is also an advantage in terms of locomotion to the sites of fertilization (Fawcett *et al.* 1971; Ribes *et al.*, 2004).

A characteristic feature of spermiogenesis in the cicadas studied is the presence of a transient microtubular organelle, the manchette. This organelle that surrounds the

acrosome, nucleus and mitochondria, has been observed in a wide range of invertebrates including insects (Phillips, 1970; Afzelius, 1988; Ndiaye *et al.*, 1997; Baccetti, 1998; Jamieson *et al.*, 1999), spiders (Michalik and Uhl, 2005), annelids (Jamieson and Daddow, 1979; Bunke, 2005) and in some vertebrates including reptiles (Vieira *et al.*, 2001; Al-Dokhi, 2004), mammals (Russell *et al.*, 1994) and birds (Soley, 1997). Some authors (e.g. Kessel, 1966; Tandler and Moriber, 1966; Lee, 1985) are of the opinion that the manchette may be involved in the establishment and maintenance of cell shape, mechanical support and in intracellular transport, in addition to inducing elongation of the nucleus and the mitochondrial derivatives. The presence of both nucleocytoplasmic transport elements and microtubule motor elements such as kinesins and dyneins has been demonstrated in the manchette in mice, suggesting its role in protein or organelle transport in a process termed intramanchette transport (Tovich *et al.*, 2004). The manchette microtubules probably have similar functions in cicada spermatids. The microtubules may also be essential for the redistribution of cytoplasm that takes place during spermatid elongation.

The importance of the manchette during morphogenesis of the spermatid nucleus in cicadas and all other animals is a matter of conjecture. This is so because in some animals its absence causes defects in sperm morphology leading to infertility (e.g. Baccetti *et al.*, 1991) whilst in others it appears not to be important during morphogenesis of the nucleus (e.g. Asa and Phillips, 2005). Absence of the manchette in human spermatids leads to the miniacrosome sperm defect where the acrosome is underdeveloped (Baccetti *et al.*, 1991). In addition a unilateral manchette in the human spermatid nucleus produces

macrocephalic spermatozoa characterized by gargantuan sperm heads with an irregular sperm nucleus (Escalier, 2002). More recently spermatid nuclear morphogenesis in the Thai leaf frog, *Megophrys montana*, has been found to occur in the absence of a microtubular manchette (Asa and Phillips, 2005). This revelation raises questions about its role during spermiogenesis in animals. It might be important in some but not all species; this paradox will be answered by future research. The disappearance of the manchette during the later stages of spermiogenesis in animals gives credence to the assumption that it becomes functionally redundant in the mature sperm (Kessel, 1966). The variation in the number and arrangement of the manchette microtubules that encircle the spermatid nucleus in all three cicada species might have some phylogenetic value. The main difference is in the number of microtubules that are located in the centriolar region of the developing spermatid nucleus. The number of microtubules in the manchette of several insects tends to vary between different species but is consistent within the same species (Jamieson *et al.*, 1999). For example, the microtubules in true bugs (Hemiptera) cluster together in groups of three and five (Danilova *et al.*, 1984), while in stick insects they fill up the entire cytoplasm (Afzelius, 1988).

The morphogenetic events involved in the amalgamation of individual small, spherical mitochondria to form the two mitochondrial derivatives are unique to all higher insects. During this transformation, the two mitochondrial derivatives are encircled by the manchette, suggesting that this transient organelle is also involved during this remarkable process.

The granular material that forms the centriolar adjunct appears in early stages of spermiogenesis. In these early stages it has no direct link to the centriole. In later stages it becomes housed within the lateral invagination of the nucleus where it persists in mature sperm. This arrangement is different from many insect species where this material surrounds the centriole, even to the extent of obscuring it as is the case in Mantophasmatodea, Orthoptera, Blattodea, Mantodea, Phasmatodea, Grylloblattodea and Plecoptera (Fausto *et al.*, 2001). In light of these differences the putative centriolar adjunct in cicadas might not be a true adjunct. Further studies are required to shed more light on its origin and its functional significance.

During spermiogenesis numerous large electron-dense granules were observed. They are not present in mature sperm. Whilst the composition of the granules was not investigated in this study there is a high probability that they could be glycogen granules. This is because of their dense appearance in electron micrographs which is typical of glycogen granules (Stryer, 1988). The presence of glycogen granules scattered throughout the cytoplasm of Sertoli cells during spermiogenesis has been demonstrated in several invertebrates including insects (Cruz-Landim, 2001; Dallai *et al.*, 2001a), platyhelminthes (Ndiaye *et al.*, 2003) and bivalves (Popham and Dickson, 1975). In many animal cells glycogen is a very efficient storage form of glucose. Most of the energy in glycogen is harvested through the process of glycolysis when glucose is converted into pyruvate with the concomitant production of ATP (Stryer, 1988). The granules observed during spermiogenesis, provided they are glycogen-rich, could be important energy sources during the intricate metabolic process of spermatogenesis.

Varying stages of spermiogenesis were observed in the testes of three cicada species. Thus spermiogenesis probably continues after cicada adults eclose. This probably means that cicada males are capable of mating more than once and can replace the mature sperm stored in the seminal vesicle. Whether this applies to all cicadas is not known but after mating for seventy-two minutes one *Oxypleura quadraticollis* male promptly started stridulating again (personal observation). This indicated a readiness to mate again, i.e. another load of sperm was available for insemination. However, because spermatogonia and spermatocytes were not apparent in the adults examined, this suggests that these species will have finite ability to produce sperm and fertilize eggs during the six or so weeks of their short adult lives.

The internal morphology of the seminal vesicle is similar to that in other insects including stingless bees (Dallacqua and Cruz-Landim, 2003) and long-horn grasshoppers, (Viscuso *et al.*, 1999). The numerous mitochondria present in the seminal vesicle wall demonstrate that it is a site of high energy utilization. Mitochondria are the sites of most cellular oxidations, producing ATP required for the cellular biosynthetic and motor activities of each cell. Extending from the apical surface of the seminal vesicle wall into the lumen were numerous microvilli. Their main function is to increase the surface area of epithelial cells. Typically, they are found in epithelia active in secretory activities. From the present study the numerous vesicles present in the seminal vesicle wall appear to release their contents into the lumen. Secretions from the vesicles likely constitute the homogenous matrix. From this study, the secretory activity within the seminal vesicle wall did not appear to be intense. Probably, this indicates that by the time a cicada

emerges it has enough homogenous material. Secretory activity would be expected to be intense induring the last moulting stages and might be influenced by the sexual maturity of individual cicadas. The moderate secretory activity within the seminal vesicles might also be important in the production of material used to preserve the spermatodesms that accumulate in the lumen and so ensure that the spermatodesms are intact as they await transfer to the female reproductive tracts. The structural features of the seminal vesicle wall are characteristic of cells involved in intense synthetic activity. Ultrastructural features of the seminal vesicle in a Brazilian stingless bee, *Melipona bicolor bicolor* (Dallacqua and Cruz-Landim, 2003) and in several species of long-horn grasshoppers (Viscuso *et al.*, 1999) revealed similar morphological signs of secretory activity.

Histochemical reactions indicate that the homogenous matrix surrounding the mature sperm has a glycoproteic nature since it stains strongly positively to both PAS and Bromophenol Blue. The carbohydrates involved are not only 1-2-glycols or closely related structures, but also include mucopolysaccharides which were detected by the Alcian Blue technique. Whilst the transfer of sperm in bundles possibly ensures that a large number of sperm are present in the female reproductive tract, the adhesive glycoprotein which is used for attachment of spermatozoa probably confers other advantages. For example, it might protect the sperm against adverse changes in the environment of the female reproductive tract. The glycoproteins might also be a form of nuptial gift to the female and might be broken down to provide essential nutrients.

In conclusion, spermiogenesis in cicadas is characteristic of insects, and produces spermatozoa typical of pterygote insects. Condensation of chromatin commences from similar regions of nuclei in developing cicada spermatids suggesting a functional role for these regions. The number of microtubules in the manchette surrounding the spermatid nucleus and the chromatin condensation patterns in *D. biconica*, *K. punctigera* and *M. matoposa* also appear to be of paramount significance. Further studies of more cicadine and cicadettine cicadas are required to elucidate the architecture of the chromatin condensation patterns, its functional significance and the significance of the number of manchette microtubules.

## **CHAPTER FIVE**

# **MORPHOLOGY OF THE MALE AND FEMALE REPRODUCTIVE TRACT IN CICADAS**

### 5.1. INTRODUCTION:

Reproduction is an obvious necessity if a species is to survive, because individuals are not immortal (Beck *et al.*, 1991). Most insects are dioecious and reproduction requires copulation between mature adults of opposite sexes (Richards and Davies, 1977). Reproductive organs are very variable in insects, but the arrangement of organs in most insects conforms to a basic pattern (Davey, 1985; Davies, 1988). Individual organs can vary in shape (e.g. of gonads and accessory glands), position (e.g. the site of attachment of the accessory glands) and number (e.g. of the ovarian or testicular tubes or sperm storage organs) between different insect groups (Gullan and Cranston, 2005).

The internal female reproductive system of insects comprises a pair of ovaries connected to a pair of tubular lateral oviducts. These unite into a single median oviduct opening posteriorly into a wider genital chamber. In some insects, the genital chamber forms a tube, the vagina, and this is often developed to form a bursa copulatrix for reception of the male intromittent organ, the aedeagus (Chapman, 1998). A pair of accessory glands (colleterial glands) usually opens into the genital chamber or vagina as well. They often secrete an adhesive substance for cementing the eggs to each other or to the substratum on which they are laid (Davey, 1985; Davies, 1988; Chapman, 1998; Gullan and Cranston, 2005). The spermatheca is usually a sac-like organ which opens into the genital chamber or vagina by a more or less elongate spermathecal duct.

The internal reproductive system in male insects comprises a pair of testes connecting with paired seminal vesicles and a main ejaculatory duct. A number of accessory glands

are present; these open into the ejaculatory duct (Davey, 1985; Chapman, 1998). Each testis is a more or less ovoid body, partly or completely divided into a variable number of tubules, or follicles, which open by narrow passages into the vas deferens (Richards and Davies, 1977; Davies, 1988). Part of the vas deferens is usually expanded to form a seminal vesicle in which mature spermatozoa accumulate.

In insects the testes contain a number of testicular follicles in which a number of cysts are present. Spermatogenesis occurs within the hollow cysts (Jamieson *et al.*, 1999). Among animals, there is a high diversity in the structure and size of testes. The relative testis size is generally greater in promiscuous than in monogamous species (Harcourt *et al.*, 1981; Jennions and Passmore, 1993; Gage, 1994). In some animals, both the volume of the ejaculate and the number of sperm per ejaculate can be predicted from testis mass (Møller, 1988; Jennions and Passmore, 1993; Gage, 1994; Pitnick, 1996; Rising, 1996). Individuals with relatively larger testes potentially deliver more sperm to the females than those individuals with smaller ones (Møller, 1988; Gage, 1995; Pitnick, 1996; Birkhead and Møller, 1998; Simmons *et al.*, 1999b; Pitnick and Miller, 2000). Intensity of sperm competition has been found to be positively correlated with relative investment in spermatogenesis across a wide range of animals including insects (Svard and Wilkund, 1989; Gage, 1996; Pitnick, 1996), birds (Møller, 1988; Pitcher *et al.*, 2005), equids, (Ginsberg and Rubenstein, 1990); fish (Stockley *et al.*, 1997), frogs (Jennions and Passmore, 1993) and mammals (Harcourt *et al.*, 1981).

In sexually promiscuous species, female choice continues after insemination through cryptic female choice (Tregenza and Wedell, 2000). The complexity of the female reproductive tract in most insects suggests it plays a central role in post-copulatory sexual selection, possibly a function in providing a challenging arena for sperm competition, and in controlling or at least influencing offspring paternity (Eberhard, 1985; 1996; Birkhead *et al.*, 1993; Hellgriegl and Ward, 1998; Miller and Pitnick, 2003).

A great deal of information has been published on the reproductive system of cicadas. Whilst it has been easy to identify all the organs in males, several authors (e.g. Myers, 1928; 1929; Snodgrass, 1933; Torres, 1963; Dugdale, 1972; Matsuda, 1976) have failed to identify all the organs involved in the female reproductive system. By contrast Boulard (1965) and Moulds (2005) managed to completely describe the female reproductive tract in *Cicada orni* and 23 Australian cicada species respectively. Furthermore, they confirmed the taxonomic value of the reproductive system in cicadas, an idea initially suggested by Torres (1963). According to Moulds (2005) the male reproductive system in cicadas, in which length of accessory glands often differs between species, is generally much simpler than that of the female. One aspect of the reproductive system in male cicadas that has received less attention is the relative mass of the testes with respect to body mass. This aspect has important implications because many studies have demonstrated a link between testis size and breeding system, with males of species in which females are mated by more than one male within a reproductive period having larger testes for their body size than males of monogamous species (Pitnick, 1996; Kappeler, 1997; Stockley *et al.*, 1997).

Moulds (2005) found a variation in the number of genital apertures and the length of accessory glands of the female reproductive tract of Australian cicadas. If the reproductive tract in cicadas is to be of any value in exploring a role in post-copulatory sexual selection, and in exploring systematic and phylogenetic relationships, comparative information from a number of taxa is required. This preliminary study describes the reproductive system in some male and female African cicadas. For female cicadas, the emphasis was on the external morphology of the spermatheca, which may influence fertilization success of males. A further aim of this study was to identify the sperm morph (s) involved in the fertilization of eggs. The seven species examined, representing two subfamilies and three tribes, were chosen on the basis of availability and the intactness of the dissected reproductive systems.

## **5.2. MATERIALS and METHODS**

### **5.2.1. Materials**

Seven species of mostly male and a few female cicadas were either collected in the Eastern Cape, South Africa or southeastern Zimbabwe (Table 5.1). *Azanicada zuluensis* (Villet, 1989), *Kongota punctigera* (Distant, 1904), *Platypleura hirtipennis* (Germar, 1834), *Pycna semiclara* (Germar, 1834) and *Quintilia carinata* (Thunberg, 1822) were collected in the Grahamstown (33° 18' S 26° 32'E), Kasouga (33° 38' S 26° 44'E), Thomas Baines Nature Reserve (33° 23' S 26° 29'E) and East London (33° 15' S 27° 36'E) areas of the Eastern Cape in December and January 2004. *Oxypleura quadraticollis* (Butler, 1874) was collected in the Malilangwe Private Game Reserve (20° 58' S 31° 47'E), Zimbabwe in December 2003. *Diceroprocta biconica* (Walker, 1850) was

collected by Prof. Allen Sanborn (Barry University, Florida) and Dr Polly Phillips (Florida International University, Miami) in Florida, United States.

Table 5.1 Cicada species examined for the study.

Species	Subfamily	Tribe	Location
<i>Azanicada zuluensis</i>	Cicadinae	Platypleurini	Kasouga, South Africa
<i>Kongota punctigera</i>	Cicadinae	Platypleurini	East London, South Africa
<i>Pycna semiclara</i>	Cicadinae	Platypleurini	Thomas Baines Nature Reserve, South Africa
<i>Platypleura hirtipennis</i>	Cicadinae	Platypleurini	Grahamstown, South Africa
<i>Quintilia carinata</i>	Cicadettinae	Parnisini	Grahamstown, South Africa
<i>Oxypleura quadraticollis</i>	Cicadinae	Platypleurini	Malilangwe Game Reserve, Zimbabwe
<i>Diceroprocta biconica</i>	Cicadinae	Cryptotympanini	Florida, United States

### 5.2.2. Morphology of reproductive tracts

Reproductive tracts from *A. zuluensis*, *D. biconica*, *K. punctigera*, *P. semiclara*, *P. hirtipennis*, *Q. carinata* and *O. quadraticollis* were dissected under a dissecting microscope in 2 % saline solution. Using fine forceps, the intact reproductive tracts were isolated into a drop of 2 % saline solution in a petri dish. The male reproductive tract in *D. biconica* and *P. hirtipennis* and the female tract in *A. zuluensis* and *O. quadraticollis* were photographed using an Olympus BX-61 microscope and digital camera. The bursa copulatrix and the spermatheca were also pared free from the remainder of the female reproductive tract. Each organ was ruptured with fine probes and examined under a light microscope for the presence of spermatozoa. The testes in *K. punctigera*, *P. semiclara*, *P. hirtipennis* and *Q. carinata* were pared free from the remainder of the male

reproductive tract and the average testis mass was calculated to the nearest 0.01mg on an electronic balance. For each species average testis mass was calculated as the mean for at least five males.

### **5.2.3. Examination of eggs**

To identify the sperm morph (s) involved in fertilization, *A. zuluensis* females were caught using a net and kept in a small cage. Some females were caught in the process of oviposition whilst others were caught prior to mating. Pieces of fresh plant material were then placed in the cage. After about ten hours the plants were checked for the presence of egg nests and eggs. Twenty five cicada eggs were collected from the slits in plants and squashed on a microscope slide coated with gelatin and chrome alum. A drop of glycerol was added before drying in an oven overnight at 60 °C. The slides were prepared for epifluorescence microscopy as described in Chapters 1 and 2 before examination with an Olympus BX-61 epifluorescence microscope at a wavelength of 343 nm. Digital images of sperm inside eggs were captured and the length of each sperm nucleus was measured using the analysis Soft Imaging System programme ([www.softimaging.net](http://www.softimaging.net)).

## **5.3. RESULTS**

### **5.3.1. Morphology of the Male Reproductive Tract**

The male genital system of both *Platypleura hirtipennis* and *Diceroprocta biconica* comprises a single pair of almost spherical testes and a long (about 120 mm) and coiled vas deferens (Fig. 5.1A, B). The testes (Fig. 5.2A, B) are composed of a series of follicles (more than fifty in most individuals). Each slender vas deferens is expanded

along its posterior section to form a seminal vesicle where mature sperm are stored (Fig. 5.1A, B). Each seminal vesicle opens into the common ejaculatory duct in which the aedeagus is housed. The ejaculatory duct opens to the outside as the gonopore. A pair of accessory glands opens into the seminal vesicle at a point just above the base of the ejaculatory duct. In both species, the accessory glands are differentiated into two regions. In *D. biconica*, each accessory gland is distally slender and tubular, especially at its confluence with the seminal vesicle, but becomes slightly expanded proximally (Fig. 5.1A.) The slender portion is much shorter than the expanded portion. In *P. hirtipennis*, the gland is expanded distally, assuming a more slender outline proximally (Fig. 5.1B). The thickened region is much shorter than the slender region and has a golden yellowish appearance. Spermatozoa were found in part of the vas deferens and the seminal vesicles.

Preliminary data on body and testis mass in four cicada species are shown in Table 5.2. Testis mass in the four cicadas varied from 0.63 mg to 5.85 mg and the values of testis mass as a percentage of body mass ranged from 0.68 to 3.83 %. In all species (Fig. 5.3) testes mass was positively related to body mass (r values ranged from 0.83 to 0.99, n = 10 individuals for each species except *Pycna semiclara*,  $P = < 0.0005$ ). The testis mass in *Kongota punctigera* was almost equivalent to that in *P. semiclara* yet there is a distinct difference in body size between these two species. *P. semiclara* is a much larger cicada, while *K. punctigera* is more the size of *P. hirtipennis*. Of the four species, *Quintilia carinata* had the smallest body mass; the testes were also much smaller.

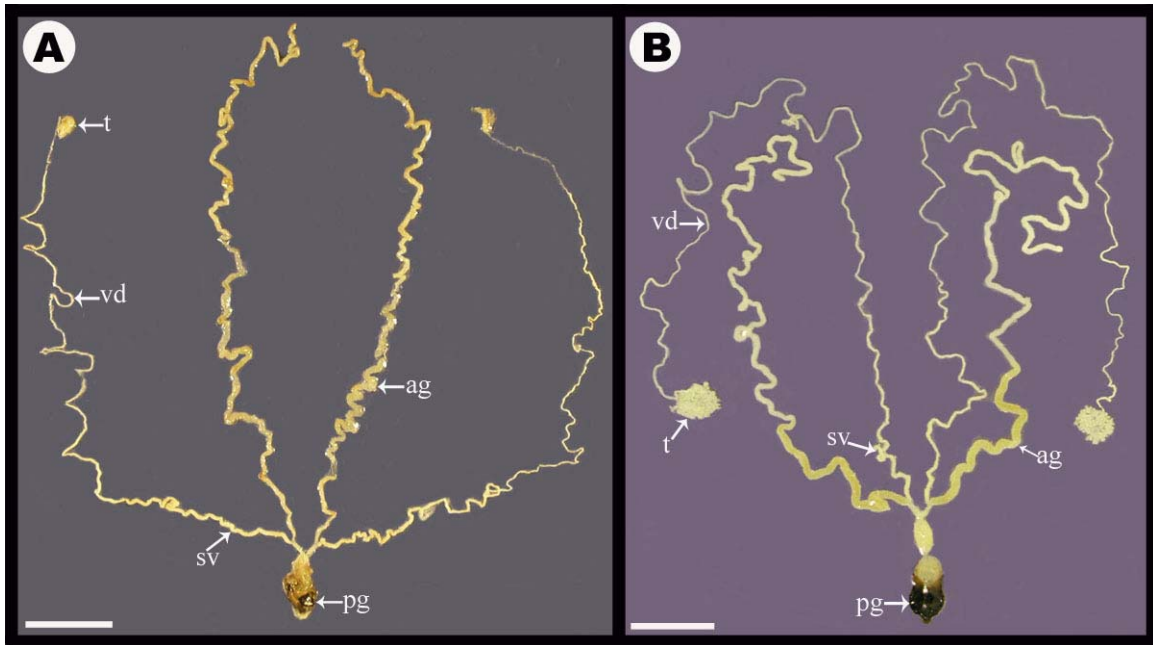


Figure 5.1. Male reproductive system in A: *Diceroprocta biconica*; B: *Platyleura hirtipennis*. The paired testes (t) are connected to the pygopher (pg) through the convoluted vas deferens (vd) and the seminal vesicle (sv). ag, accessory gland. Bar = 10 mm.

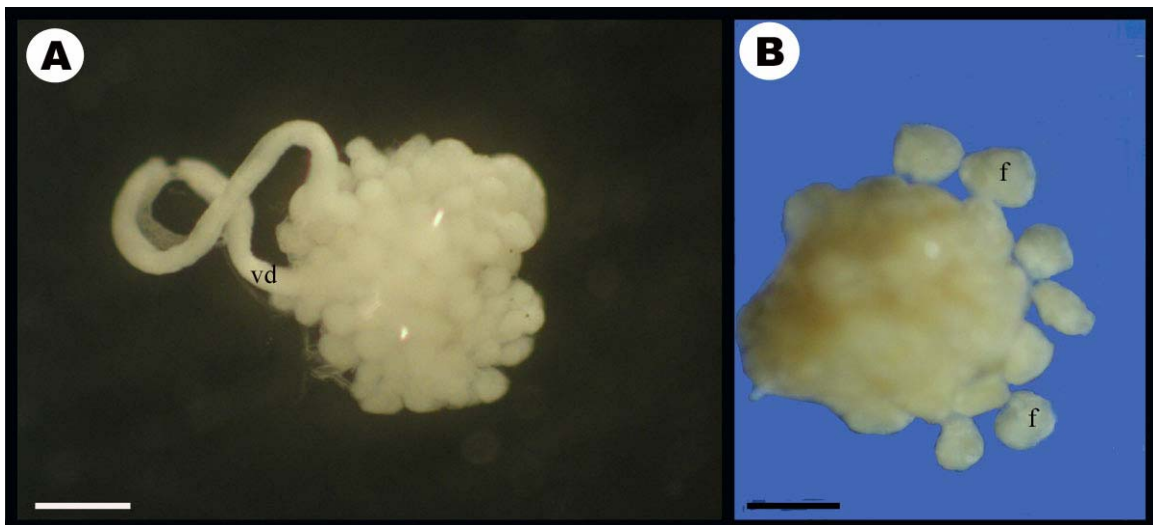


Figure 5.2. Cicada testes as seen under a dissecting microscope. Each testis comprises a series of follicles which are connected to the vas deferens. A: *P. hirtipennis*. vd, vas deferens. Bar = 200  $\mu$ m. B: *A. zuluensis*. f, follicle. Bar = 300  $\mu$ m.

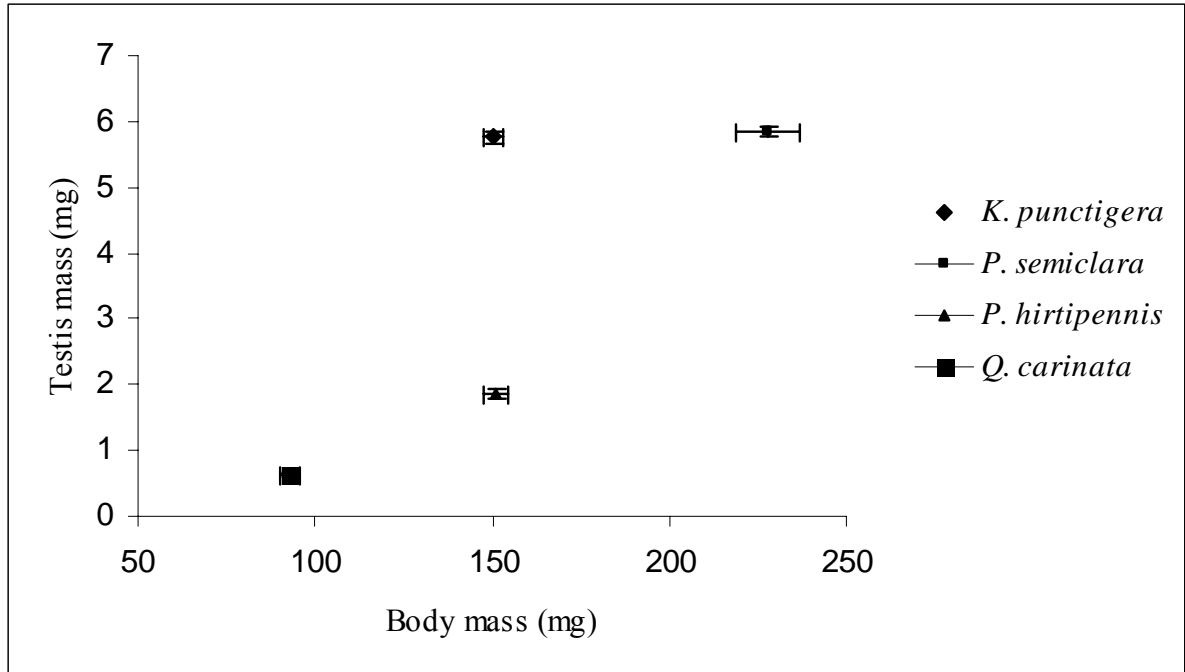


Figure 5.3. Relationship between mean testis mass and mean body mass in four cicada species. Bars indicate the standard deviation.

Table 5.2. Body mass, testis mass (mean  $\pm$  SE) and percentage of testis mass/body mass for four species of African cicadas. n, number of males.

Species	n	Body mass (mg)	Testis mass (mg)	Testis/Body (%)	r	Reproductive behaviour
<i>Q. carinata</i>	10	92.77 $\pm$ 0.92	0.63 $\pm$ 0.01	0.68	0.83	No choruses
<i>P. hirtipennis</i>	10	150.99 $\pm$ 1.06	1.85 $\pm$ 0.02	1.23	0.89	Forms choruses
<i>K. punctigera</i>	10	150.49 $\pm$ 0.88	5.76 $\pm$ 0.03	3.83	0.85	No choruses
<i>P. semiclara</i>	15	227.88 $\pm$ 2.35	5.85 $\pm$ 0.10	2.57	0.99	Forms choruses

### 5.3.2. Morphology of the Female Reproductive Tract

The female reproductive tract in *Azanicada zuluensis* and *Oxypleura quadraticollis* consists of a pair of large ovoid ovaries, which contain numerous ovarioles (Fig. 5.4A, B). Each ovary is connected to a pair of lateral oviducts of approximately 1.5 mm in diameter. These join to form a median oviduct, which opens, posteriorly, into a genital chamber. The genital chamber forms a short, stout pear-shaped tube, the bursa copulatrix, approximately 1.8 mm in diameter. Opening from the bursa copulatrix is a large sac-like structure, with a transparent outer membrane and which is differentiated into a soft-walled apical pouch and a very stout proximal portion. This structure, which Boulard (1965) termed a “dorso-vaginal pocket”, was once believed to be the spermatheca (Myers, 1929; Snodgrass, 1933). A pair of winding accessory glands (often termed colleterial or glue glands) of approximately 20 mm in length is also connected to opposite sides of the bursa copulatrix near the external opening. A long, extremely coiled, tapered, thread-like white blind-ending gland (spermathecal gland) is inserted dorsally into the reproductive tract, in a region in close proximity to the anterior of the ovipositor. Boulard (1965) suggested that this gland is the original spermatheca that has been modified and subsequently lost its previous function of storing sperm.

Numerous sperm were observed in the bursa copulatrix of those female insects that were ready to oviposit. In contrast, no sperm were observed in the spermathecal gland and only a few sperm were observed in the sac-like dorso-vaginal pocket. The terminal segment of the reproductive tract forms the blade-like ovipositor, used to cut slits in twigs and branches where eggs are deposited. Eggs are released from each oviduct and pass

down the median duct and through the bursa copulatrix before being transported through the ovipositor to be laid in slits in plants (Fig. 5.4C, D). The diameter of each oviduct, which is slightly greater than the diameter of a single egg, means that only one egg can pass down the oviduct tract and subsequently, through the bursa copulatrix at any one time.

### **5.3.3. Sperm morph (s) involved in fertilization**

Under the fluorescence microscope, the brightly stained sperm nuclei were visible within the cytoplasm of newly laid *A. zuluensis* eggs (Fig. 5.4E, F). This was observed in 48 % of the eggs examined (n = 25). All the sperm nuclei observed were of the long type (> 30  $\mu\text{m}$ ); no fertilized egg was observed to contain short nuclei. Sperm tails were barely visible within the cytoplasm of fertilized eggs making it impossible to measure their lengths.

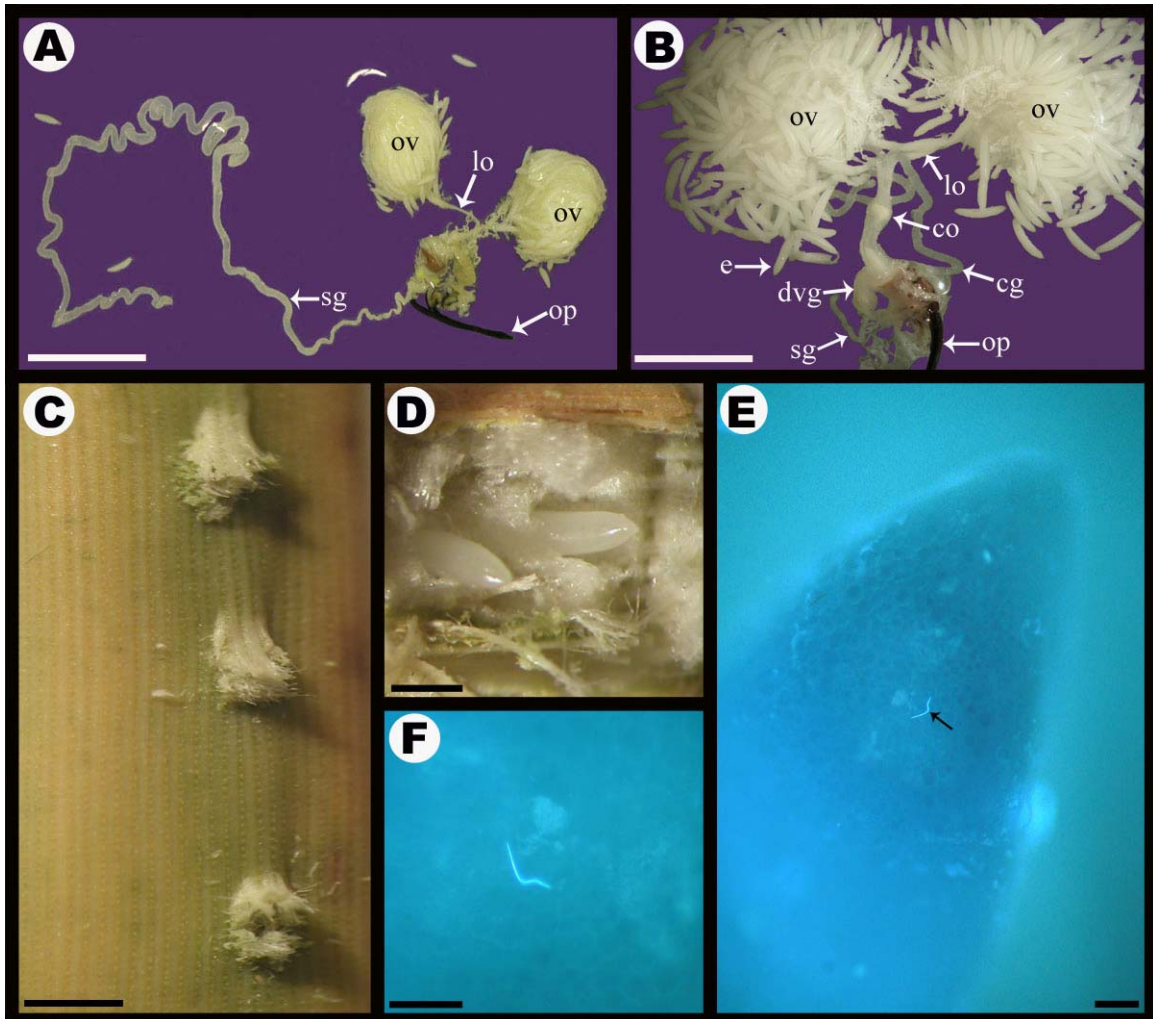


Figure 5.4. A: The female reproductive system in *Oxypleura quadraticollis* consists of a pair of large ovaries (ov), each connected to a lateral oviduct (lo) and uniting to form a common oviduct or genital chamber. op, ovipositor; sg, spermathecal gland. Bar = 10 mm. B: Female reproductive system in *Azanicada zuluensis*. Eggs (e) are released from each ovary and pass down the common oviduct (co) to the outside through the ovipositor (op). cg, colleterial gland; dvg, dorso-vaginal pocket; lo, lateral oviduct; sg, spermathecal gland. Bar = 20 mm. C: Egg nests of *A. zuluensis*. The blade-like ovipositor is used to create slits in plants where eggs are laid. Bar = 2 mm. D: *A. zuluensis* eggs laid in plant slits. Bar = 2mm. E: Fertilized *A. zuluensis* egg to show a spermatozoon with a long nucleus, stained with Hoechst 33258 and examined with a fluorescence microscope at 343 nm. Bar = 50  $\mu$ m. F: Fertilized *A. zuluensis* egg. Higher magnification of a spermatozoon with a long nucleus. Bar = 40  $\mu$ m.

## 5.4. DISCUSSION

### 5.4.1. Male Reproductive Tract

The gross morphology of the male reproductive system in both *Diceroprocta biconica* and *Platypleura hirtipennis* is similar. Typical features of the reproductive system in both cicada species are: (a) two almost spherical testes of equal size; (b) a slender considerably long and convoluted vas deferens connected to each testis; (c) a seminal vesicle formed by dilation of the vas deferens along part of its length; (d) a pair of long accessory glands that are regionally differentiated; and (e) a pygopher that houses the male intromittent organ, the aedeagus. A similar organization of the reproductive system has been reported in the cicada *Carineta formosa* (Myers, 1929), *Graptosaltria nigrofuscata* (Kato, 1956) and in several other species of insects from different orders (Richards and Davies, 1977; Davey, 1985; Davies, 1988; Chapman, 1998; Gullan and Cranston, 2005). However, there is a slight variation in the reproductive system of the two cicadas with respect to the morphology of the accessory glands and the relative thickness of the seminal vesicles.

The accessory glands in both *D. biconica* and *P. hirtipennis* are very long and regionally differentiated. An elongated accessory gland most likely results in an increase in the production of accessory gland secretions and might play a significant role in the determination of male reproductive success. For example, the length of the accessory gland in the stalk-eyed fly, *Cryptodiopsis dalmanni*, was found to be both phenotypically and genetically correlated with male mating frequency (Rogers *et al.*, 2005). Regional differentiation of the accessory glands has also been reported in bees (Ferreira *et al.*

2004). In their study of 51 bee species representing six families these authors found two portions constituting the accessory gland: a long tubular duct and a dilated loop, with the latter usually presenting anatomical variation among the species. They suggested that these differences in the structure of the accessory glands might be of phylogenetic importance.

In a study of 23 Australian cicada species representing 19 genera Moulds (2005) found two discrete groupings based on the length of the accessory glands; those with short and those with long accessory glands. There were no species with glands of intermediate length. Like other members of the tribe Cryptotympanini (Moulds, 2005), *Diceroprocta biconica* males possesses long accessory glands (Table 5.3). The three species of platypleurine cicadas examined in this study also possessed long accessory glands suggesting that all members of this tribe will have this feature. This is not surprising because the platypleurines and cryptotympanines are phylogenetically closely related (Fig. 1.1).

Table 5.3. Comparison of male and female accessory gland length and number of testicular follicles in cicadas. \*, Data from Moulds (2005);? Missing data as published by Moulds (2005).

Species	Tribe	Length of ♂ accessory glands	Length of ♀ accessory glands
<i>Aleeta curvicosta</i> *	Taphurini	long	short
<i>Azanicada zuluensis</i>	Platypleurini	long	long
<i>Cicada orni</i> *	Cicadini	long	?
<i>Diceroprocta biconica</i>	Cryptotampanini	long	long
<i>Henicopsaltria eydouxiis</i> *	Cryptotampanini	long	short
<i>Macrotristria angularis</i> *	Cryptotympanini	long	short
<i>Magiccicada septendecim</i> *	Taphurini	long	?
<i>Oxypleura quadraticollis</i>	Platypleurini	long	long
<i>Pauropsalta mneme</i> *	Cicadettini	short	long
<i>Platypleura hirtipennis</i>	Platypleurini	long	long

Secretions of the accessory glands of insects are often used to form a kind of capsule or spermatophore, in which spermatozoa are enclosed, to “lower” the female’s receptivity to mating again or to stimulate oviposition (Davies, 1988). Cicadas do not produce spermatophores; instead, sperm are aggregated in bundles with their heads held in a matrix of glycoproteic material (Chapters 2, 3 and 4). These bundles are transferred to the females, where free sperm dissociate and the homogenous matrix degenerates. The homogenous matrix does not include secretions from the accessory gland because it is formed somewhere within the vas deferens and part of the seminal vesicle (Chapter 4). Secretions of the accessory glands must join the spermatozoa at the confluence of the glands and the seminal vesicles during the process of insemination and could be used to form mating plugs which are inserted into the female reproductive tract to prevent female remating. Mating plugs have been demonstrated in bumblebees (Sauter *et al.*, 2001) and in periodical cicadas (Cooley, 1999). According to Cooley (1999), mating plugs in *Magicicada septendecim* are a result of antagonistic coevolution between time-limited females and males that seek to guarantee their paternity by endeavouring to devalue females’ remating incentives. It is reasonable to assume that similar mating plugs are present in other cicadas, especially in those species where huge numbers forming fairly dense aggregations emerge after long subterranean sojourns, because there would be a struggle for mating opportunities in such situations.

In most insects both the vas deferens and seminal vesicles vary greatly in length (Richards and Davies, 1977; Chapman, 1998). These structures are especially long in both *D. biconica* and *P. hirtipennis* and this might be linked to the synthesis of the

homogenous matrix in which sperm heads assemble, and formation of spermatodesmata. Part of the vas deferens might also be important as a sperm storage organ. To date there is no evidence in literature to support this idea. The length of the vas deferens varies considerably within different groups of insects but there is no information linking the length of the vas deferens to the synthesis of spermatodesmata (Chapman, 1998). More importantly, the spermatodesmata of some Orthoptera and Odonata, unlike those in cicadas, are formed within the testis cysts from where they are released into the vas deferens (Szöllösi, 1974; Abro, 1998). Hence an elongated vas deferens might not predict the formation of a spermatodesm.

#### **5.4.2. Female Reproductive Tract**

The morphology of the female reproductive system in *Azanicada zuluensis* and *Oxypleura quadraticollis* is similar to that of other cicadas described to date (Myers, 1929; Snodgrass, 1933; Kato, 1956; Torres, 1963; Boulard, 1965; Moulds, 2005). According to Boulard (1965, 1996), the organization of the genital system in most cicadas is generally similar. However, there are variations at generic and even specific levels with respect to (a) the degree of isolation of the copulatory and egg-laying paths; (b) the position of the gonopore on the genital junction; and (c) the shape of the common oviduct. The distinction between the common oviduct and the bursa copulatrix is clear because the latter is distinctly swollen and pear-shaped. This swelling of the common oviduct is brought about by accumulation of sperm. Boulard (1965) examined the common oviduct in both virgin and inseminated *Cicada orni* females. The swelling was less pronounced in virgin females, while in inseminated females it was large and the

oviduct was filled with spermatozoa. In light of these studies it could be assumed that the swollen and differentiated portion of the oviduct in *A. zuluensis* and *O. quadraticollis* females is the site of sperm storage. In many insects the spermatheca is the site for sperm storage (Chapman, 1998, Gullan and Cranston, 2005). From this study no sperm were found in the uneven blind-ending structure, which Boulard (1965) and Moulds (2005) refer to as the original spermatheca. This blind-ending structure seems not to be a site for sperm storage. Boulard (1965) found that the dorso-vaginal pocket is the site for sperm destruction and not for sperm storage. Therefore the function of this blind-ending tube is obscure and is a question that needs to be addressed in future.

In the vast majority of oviparous insects there is no obvious distinction between the common oviduct (bursa copulatrix) and the vagina, especially in those insects where the bursa copulatrix receives the aedeagus (Richards and Davies, 1977; Davies, 1988; Chapman, 1998; Gullan and Cranston, 2005). In these insects, eggs from the ovaries do not develop in the bursa copulatrix once they have been fertilized; instead they pass through the sharp ovipositor to the outside where they will hatch into larvae. In general, fertilization in insects occurs in the genital chamber when sperm move from the spermatheca into the chamber via a duct. The opening of the spermatheca into the genital chamber is independent of the oviduct (Chapman, 1998). The swollen and differentiated nature of the common oviduct in *A. zuluensis* and *O. quadraticollis* appears to be a functional adaptation for the storage of spermatozoa since the original spermatheca has lost its function. Modification of the common oviduct or vagina to suit a particular function is not uncommon in insects. For example in the dipteran family Glossinidae

(tsetse flies), the vagina is greatly enlarged to form a uterus for the reception of the developing larva. The larva develops within the uterus of the mother's reproductive system and is nourished by secretions from modified accessory glands. Once deposited, the fully grown larva pupariates promptly. This form of reproduction is termed adenotrophic viviparity (Tobe and Langley, 1978).

The dual role of the common oviduct, i.e. to receive oocytes from the ovaries and to store sperm, introduces remarkable scenarios that may be important in the determination of the sperm morph (s) that is/are involved in fertilization. Once mature oocytes are released from each ovary, they are moved by peristalsis down each lateral oviduct and into the common oviduct where they are fertilized before passing through the sharp ovipositor to be laid (Chapman, 1998; Gullan and Cranston, 2005). From this study the diameter of each oviduct, which is slightly greater than the diameter of each egg, means that only one egg can pass down each oviduct at any one time. The confluence of the two oviducts is also narrow and can only allow passage of one egg. Consequently, only a single egg at a time passes through the common oviduct where fertilization occurs. Since any sperm morph is potentially capable of fertilizing eggs (they all have a nucleus with DNA and a flagellum), the position within the common oviduct of an individual sperm, and possibly its ability to resist displacement from a perfect vantage position, may be critical if it is to succeed in fertilization. If the common oviduct is the site of sperm storage then there is a situation where the first sperm in get precedence for fertilization. This is because these sperm are positioned closer to the confluence of the two oviducts.

### 5.4.3. Reproductive Competition

Only long nuclei were observed in the fertilized eggs of *Azanicada zuluensis*. Kubo-Irie *et al.* (2003) obtained similar results after examining *Graptosaltria nigrofuscata* eggs. The small sample sizes used for both investigations make it difficult to come up with a conclusive statement concerning the identity of sperm morph (s) involved in the fertilization of all eggs. The numerical superiority of spermatozoa with long nuclei in the seminal vesicles (Chapter 2) should give them a higher probability of fertilizing eggs especially since eggs are fertilized singly. But this does not necessarily mean that spermatozoa with short nuclei are not functional in egg penetration and karyogamy, since they all have DNA in the nucleus and a flagellum. They might have a lower probability of fertilizing eggs on the basis of their numerical inferiority but their potential in fertilizing eggs cannot be categorically dismissed. In addition, the sperm nucleus in other eggs may have fused with the DNA of the oocyte before the eggs were prepared for fluorescence microscopy thereby rendering visualization of the nuclei impossible. There is a need to examine a large number of fertilized eggs immediately after oviposition in order to successfully identify the sperm morph (s) involved in fertilization.

Absolute variations in cicada testes size have never been documented before. However, Moulds (2005) highlighted some variations in the number of testicular follicles between cicada species. By contrast, variations in absolute testis size have been documented in a number of insects, including the vinegar fly, *Drosophila hydei* (Pitnick and Miller, 2000) and the dung-fly, *Scathophaga stercoraria* (Hosken and Ward, 2001). *S. stercoraria* males with larger testes had elevated levels of polyandry compared to those with smaller

testes (Hosken and Ward, 2001; Hosken *et al.*, 2001). The variations in testis size between species recorded in this study may in part be due to the age of the males sampled. Cicada testes appear to shrink in size as the season progresses and males get older (pers. obs). Nevertheless, this probably does not explain the large difference in relative testis size between species. Intensity of sperm competition has been found to be positively correlated with relative investment in spermatogenesis across a wide range of animals including insects (Svard and Wilkund, 1989; Gage, 1994; Pitnick, 1996), birds (Møller, 1988; Pitcher *et al.*, 2005), equids (Ginsberg and Rubenstein, 1990), fish (Stockley *et al.*, 1997), frogs (Jennions and Passmore, 1993); mammals, Harcourt *et al.*, 1981). In addition, the amount of spermatogenic tissue in reproductively promiscuous species is often linked to fertilization success (Parker, 1970; 1984; 1990a; 1990b). These findings strongly indicate that larger testis size is a predictive indicator of both the volume of the ejaculate and the number of sperm per ejaculate and agree with sperm competition theory which predicts increased spermatogenic investment with an increase in sperm competition risk (Parker, 1970; 1982; 1990a; 1990b). Therefore based on the preliminary findings of this study, *Kongota punctigera*, with its larger testis relative to body size, would be the ideal candidate to show the greatest levels of sperm competition.

In conclusion the reproductive tracts in both male and female cicadas exhibit an organization similar to that in most oviparous insects. The blind-ending spermathecal gland is the only exceptional feature. The common oviduct has been modified into a swollen, differentiated structure with a dual role of receiving oocytes from the paired ovaries and to store spermatozoa.

**CHAPTER SIX**  
**GENERAL DISCUSSION**

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## 6.1. PHYLOGENETIC PATTERNS

Prior to the present study, the structure of the sperm of just three species of cicadas, *Cicada orni*, *Lyristes plebejus* (Folliot and Maillet, 1970) and *Graptosaltria nigrofuscata* (Kubo-Irie *et al.*, 2003) had been investigated at the ultrastructural level. In their study, Folliot and Maillet (1970) did not mention polymorphic sperm, whereas Kubo-Irie *et al.* (2003) noted the occurrence of more than one size of sperm in *G. nigrofuscata*. The paucity of information on cicada sperm thus made it difficult to comment on the value of sperm morphology in systematic and phylogenetic studies. The present study of the morphology of the spermatozoa in the cicadine species *Azanicada zuluensis*, *Albanycada albiger*, *Diceroprocta biconica*, *Kongota punctigera*, *Platypleura capensis*, *P. hirtipennis*, *Pycna semiclara* and the cicadettine species *Melampsalta leucoptera*, *Quintilia walkeri*, *Xosopsaltria thunbergi*, *Stagira simplex* and *Monomatapa matoposa* now allows this to be done.

### 6.1.1. RELATIONSHIPS WITHIN AND BETWEEN CICADA TAXA

The results from the current study suggest that it is not possible to readily distinguish cicadas from the same genus or tribe on the basis of the morphology of individual sperm. However, it is possible to distinguish cicadine species from their cicadettine counterparts on the basis of sperm ultrastructure. In particular, the structure of the centriolar adjunct appears to be an informative feature within the Cicadidae and is thus a potentially useful character when studying phylogeny at a subfamily level. The extent to which this character could be used will only be established after examination of the sperm from representative taxa of other tribes.

The bilaterally symmetrical, anteriorly positioned acrosome, which has a subacrosomal space in cicadas, does not seem to have characters of phylogenetic value because its morphology was found to be similar in all species examined. Folliot and Maillet (1970) reported the presence of an antecrosomal bleb between the tip of the nucleus and the acrosome proper in *C. orni*, *L. plebejus* and representative members of the families Cicadidae, Cercopidae, Cicadellidae, Typhlocybinae and Ulopinae. The presence of an antecrosomal bleb was also reported in the sperm heads of *G. nigrofuscata* (Kubo-Irie *et al.*, 2003). The findings from the present study show no evidence of the presence of the antecrosomal bleb. What is evident is that during spermiogenesis there is a small region of cytoplasm anterior to the acrosome (the ante-acrosomal region) that might be the same feature reported to be the antecrosomal bleb. This region is absent in mature spermatozoa. There is a need for more ultrastructural work to clarify this.

There is a great deal of variation in nuclear and tail lengths both between and within tribes in the cicadas studied making it impossible to use this information to infer systematic relationships between and within taxa. It is difficult to link interspecific variation in these sperm dimensions with reproductive behaviour (e.g. forming choruses or not) in these cicadas with sperm dimensions. Hence in the case of these cicadas, sperm length is probably of little phylogenetic significance. However, it might be important in other situations e.g. where sperm have to compete for fertilization of the ova.

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Sperm characters (Table 6.1) were used to construct a cladogram of the cicada species (Fig. 6.1). The analysis was done with HENNIG86 and all characters were treated as unordered, with the aphid, *Megoura viciae* as the outgroup. The analysis used the “implicit enumeration” routine, which searches exhaustively to find the most parsimonious tree. Fourteen trees were found with lengths of 31 steps. The consistency index was 87 % and retention index was 88 %. A Nelson consensus tree was generated to summarize the congruency of the original trees. It shows cercopids as the sister group to the cicadas and separates the latter, with the exception of *G. nigrofuscata* and *P. semiclara*, into two subfamilies (Fig. 6.1). However, the tree generated does not support a close relationship between *X. thunbergi* and *S. simplex*. This is surprising because the two species belong to the same tribe, Tettigomyiini. The sperm characters used might not be phylogenetically informative and so cannot resolve the relationship between species. There is need to incorporate more variable and phylogenetically informative data to have a better resolution.

Cryan (2005) and Moulds (2005), using different approaches, found a similar relationship between cicadas and cercopids. They also clarified the relationships between cicadomorphan families. Cryan (2005) analyzed representatives of the cicadomorphan families Cicadidae, Tettigarctidae, Cercopidae, Aphrophoridae, Clastopteridae, Machaerotidae, Epipygidae, Cicadellidae, Membracidae, and Aetalionidae based on DNA nucleotide sequence data from multiple genetic markers (18S rDNA, 28s rDNA and histone 3). Moulds (2005) examined the internal and external morphology of nymphs and adult Australian cicadas. He used a total of 117 characters in the cladistic analysis.

Table 6.1 Sperm character matrix of cicadas, cercopids and an aphid used in the analysis. Based on evidence from related species assumptions on presence/absence of characters were made for those species where information on characters was unavailable. Published characters (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003) which seem to be erroneous and have been modified for this analysis are in bold. They have been surmised from other species.

Species	Family	Character A	Character B	Character C	Character D	Character E	Character F	Character G	Character H
<i>M. viciae</i>	Aphididae	1	1	0	0	0	0	0	?
<i>P. spumarius</i>	Cercopidae	1	1	1	1	2	1	0	?
<i>C. sanguinea</i>	Cercopidae	1	1	1	1	2	1	0	?
<i>C. orni</i>	Cicadidae	?	?	<b>0</b>	1	2	1	1	?
<i>L. plebejus</i>	Cicadidae	?	?	<b>0</b>	1	2	1	1	?
<i>G. nigrofuscata</i>	Cicadidae	2	1	<b>0</b>	1	2	1	1	2
<i>A. albigera</i>	Cicadidae	3	3	0	1	2	1	1	2
<i>A. zuluensis</i>	Cicadidae	2	2	0	1	2	1	1	1
<i>P. capensis</i>	Cicadidae	2	3	0	1	2	1	1	1
<i>P. hirtipennis</i>	Cicadidae	2	3	0	1	2	1	1	1
<i>P. semiclara</i>	Cicadidae	2	2	0	1	2	1	1	2
<i>M. leucoptera</i>	Cicadidae	2	2	0	1	2	1	1	2
<i>M. matoposa</i>	Cicadidae	2	2	0	1	2	1	1	4
<i>Q. walkeri</i>	Cicadidae	2	2	0	1	2	1	1	2
<i>S. simplex</i>	Cicadidae	2	2	0	1	2	1	1	2
<i>X. thunbergi</i>	Cicadidae	3	2	0	1	2	1	1	2

Character A: Modes of Nucleus Length	Character B: Modes of Tail Length	Character C: Anteacrosomal bleb	Character D: Spermatodesm	Character E: Part of sperm head in spermatodesm	Character F: Accessory Bodies	Character G: Occasional biflagellarity	Character H: Modal length of long nuclei
1. unimodal	1. unimodal	0. absent	0. absent	0. no spermatodesm	0. present	0. absent	0. ≤ 30 μm
2. bimodal	2. bimodal	1. present	1. present	1. anterior acrosome	1. absent	1. present	1. 30-40 μm
3. trimodal	3. trimodal			2. acros + nucleus			2. 40-50 μm 3. 50-60 μm 4. 60-70 μm

Table 6.1 continued.....

Species	Character I	Character J	Character K	Character L	Character M	Character N	Character O	Character P
<i>M. viciae</i>	?	?	?	?	1	2	1	2
<i>P. spumarius</i>	?	?	?	?	1	2	2	2
<i>C. sanguinea</i>	?	?	?	?	1	2	2	2
<i>C. orni</i>	?	?	?	?	1	1	2	1
<i>L. plebejus</i>	?	?	?	?	1	1	2	1
<i>G. nigrofuscata</i>	3	1	2	3	1	1	2	1
<i>A. albigera</i>	1	4	5	3	1	1	2	1
<i>A. zuluensis</i>	2	4	4	4	1	1	2	1
<i>P. capensis</i>	2	3	4	3	1	1	2	1
<i>P. hirtipennis</i>	2	3	4	3	1	1	2	1
<i>P. semiclara</i>	3	1	2	3	1	1	2	1
<i>M. leucoptera</i>	2	2	3	3	2	1	2	1
<i>M. matoposa</i>	4	2	3	5	2	1	2	1
<i>Q. walkeri</i>	2	1	2	2	2	1	2	1
<i>S. simplex</i>	1	2	3	3	2	1	2	1
<i>X. thunbergi</i>	1	1	2	3	2	1	2	1

Character I: Modal length of shortest nuclei	Character J: Modal length of long tails	Character K: Modal total length of long sperm	Character L: Modal length of shortest tails	Character M: Structure of centriolar adjunct	Character N: Position of centriolar adjunct	Character O: Accessory bodies	Character P: Microtubule-like elements in nucleus
0. $\leq 10 \mu\text{m}$	1. $\leq 100 \mu\text{m}$	1. $\leq 100 \mu\text{m}$	1. 1-25 $\mu\text{m}$	1. non-lamellate	1. Extends out	1. present	1. present
1. 11-15 $\mu\text{m}$	2. 101-150 $\mu\text{m}$	2. 101-150 $\mu\text{m}$	2. 26-50 $\mu\text{m}$	2. lamellate	of lateral nuclear canal	2. absent	2. absent
2. 16-20 $\mu\text{m}$	3. 151-200 $\mu\text{m}$	3. 151-200 $\mu\text{m}$	3. 51-75 $\mu\text{m}$		2. posterior to nucleus		
3. 21-25 $\mu\text{m}$	4. 201-250 $\mu\text{m}$	4. 201-250 $\mu\text{m}$	4. 76-100 $\mu\text{m}$				
4. 26-30 $\mu\text{m}$		5. 251-300 $\mu\text{m}$	5. 101-150 $\mu\text{m}$				

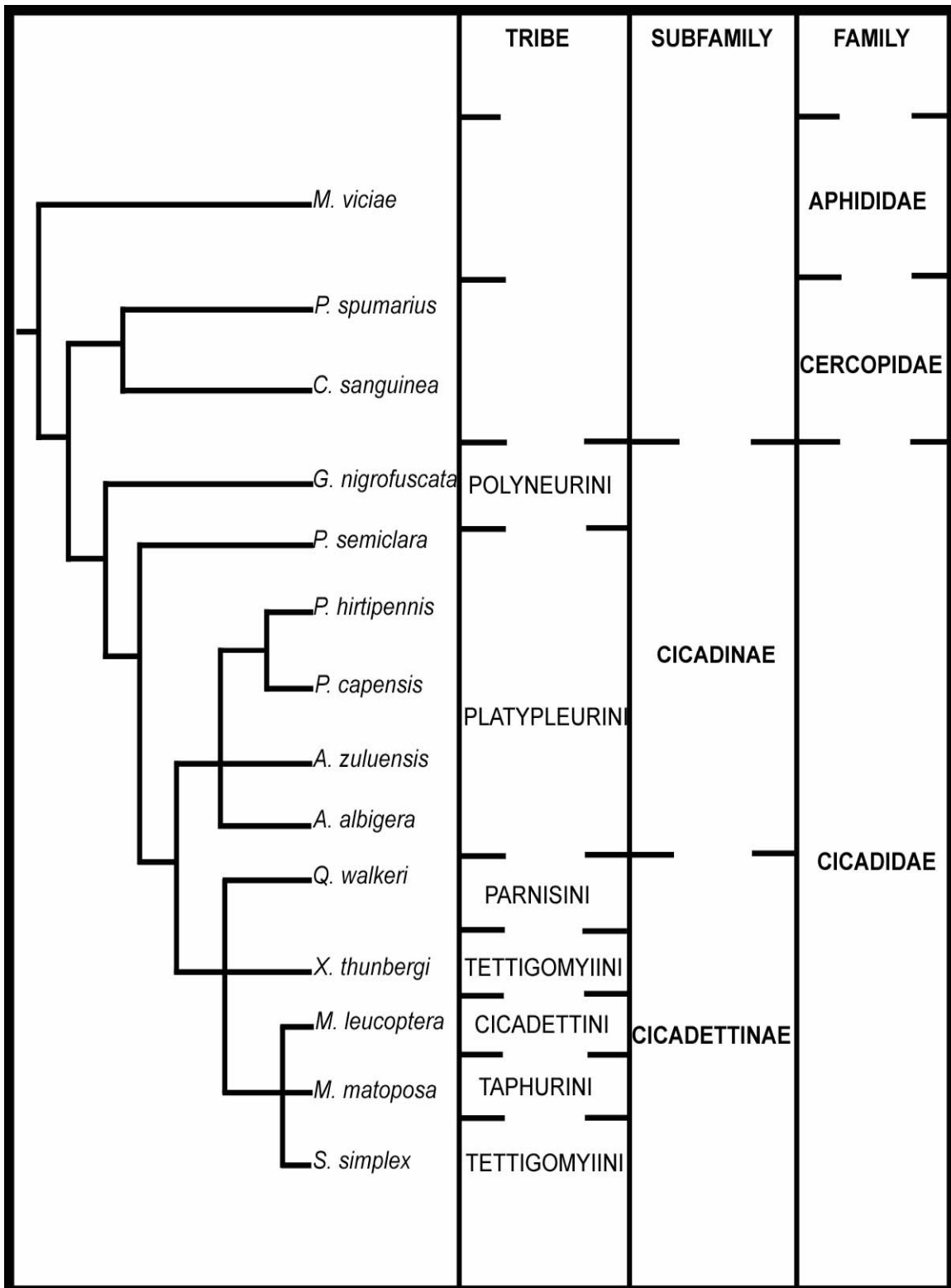


Figure 6.1. Nelson consensus tree of maximum parsimony analysis of sperm characters of cicadas and other auchenorrhynchs. The aphid, *Megoura viciae* was used as the outgroup. Fourteen trees were found with lengths of 31 steps. Consistency index was 87 % and retention index was 88 %.

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### 6.1.2. RELATIONSHIPS BETWEEN CICADAS AND OTHER TAXA

The morphological features exhibited by cicada sperm investigated in this study share some similarities with the sperm of most other hexapods (Phillips, 1970; Quicke *et al.*, 1992; Baccetti, 1998; Jamieson *et al.*, 1999). Nevertheless, some differences in sperm architecture were noted and, collectively, these show that evolution of apomorphies has occurred in cicada sperm.

Features that are plesiomorphic to a hypothetical hexapod sperm are (a) an acrosome with a perforatorium or with some periacrosomal material; (b) an elongate nucleus with condensed chromatin; (c) a centriole with doublets only and surrounded by a centriole adjunct which is posterior to the nucleus; (d) absence of accessory bodies; (e) two elongate, partly cristate, non-crystalline mitochondrial derivatives; (f) a variable number of small mitochondria persistent from spermatids; (g) axonemal arrangement of 9 + 9 + 2 microtubules; and (h) presence of peripheral coarse fibrous material (Jamieson, 1987; Jamieson *et al.*, 1999). Most of these features are present in the sperm of cicadas although some have been modified. Six main features distinguish cicada sperm from the basic hexapod ground plan: (a) presence of an ante-acrosomal region; (b) absence of additional smaller mitochondria which persist from spermatids; (c) loss of the perforatorium; (d) a centriole with a triplet organization of microtubules; (e) a centriolar adjunct not associated with the centriole but which lies within a lateral nuclear canal; and (f) a pair of mitochondrial derivatives with a paracrystalline core, which flank a central axoneme. The loss of the perforatorium in cicadas, reflecting the common ancestry of cicadas and [Anoplura (sucking lice), Psocoptera (booklice), Mallophaga (biting lice),

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Hemiptera (true bugs) and Thysanoptera (thrips)] is a synapomorphic character uniting the rhynchotoid group (Jamieson *et al.*, 1999).

In all species of cicadas examined there was a small number of unusual sperm which had two flagella. Occasional biflagellarity is not unique to the Cicadidae or other auchenorrhynchs; it occurs more extensively and regularly in other hemipteroid orders (Jamieson *et al.*, 1999). Abnormal spermatozoa that are biflagellate are very common in several insect groups; they result from the failure of the second meiotic division (see White, 1973; Paccagnini *et al.*, 2006). Thus, biflagellarity of the cicadas mentioned in the present study might be a result of aberrant meiosis during spermatogenesis. Biflagellarity was considered to be basic to all hemipteroids above the Psocoptera (Baccetti, 1979; Jamieson *et al.*, 1999). In certain species of book lice, some sperm have two axonemes and four mitochondrial derivatives and the percentage of biflagellarity ranges from 2 to 100 % (King and Ahmed, 1989). In some membracid leafhoppers (Cicadellidae) spermatozoa have either two (Folliot, 1970) or four flagella (Phillips, 1974). In cicadellid corn-hoppers the flagellum is divided into five components (Kitajima and Cruz Landim, 1972). In thrips the functional flagellum has an unusual organization; an amalgamated triaxonemal structure (Dallai *et al.*, 1991). Despite the tendency of the axoneme to branch in the rhynchotoids, monoflagellarity has been retained in some members, especially in most hemipterans (Jamieson *et al.*, 1999).

Fig. 6.2 shows a phylogeny of some hemipteroid orders including cicadas, planthoppers (Fulgoromorpha), leafhoppers and treehoppers (Membracoidea) with spermatozoal

apomorphies superimposed onto an existing cladogram. Many of the branches lack support because some descriptions of sperm morphology (e.g. Folliot and Maillet, 1970) were incomplete. In addition, it seems sperm characters may not exclusively be used to decipher evolutionary relationships. The results of the present study show that the sperm characters used are not sufficient on their own to offer considerable phylogenetic information on cicadas since all the taxa sampled cannot be grouped on terminal autapomorphies. It should also be pointed out that spermiocladistics in insects is notoriously refractory. Hence there is a necessity for further sampling which would allow more rigorous testing of relationships. In addition, sperm characters can be incorporated into other phylogenetic analyses.

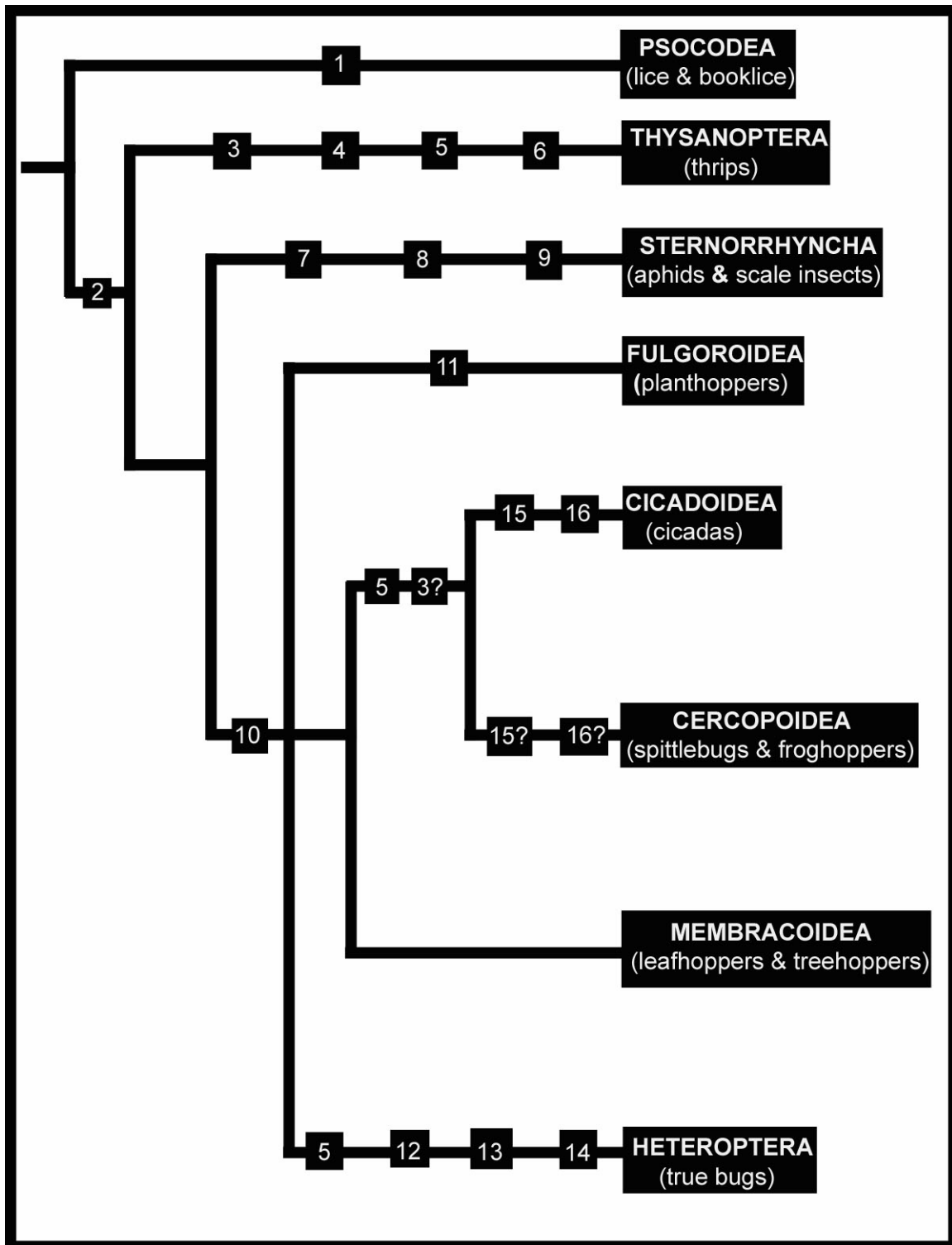


Figure 6.2. Cladogram of postulated relationships between Psocodea, Thysanoptera and Hemiptera (Stenorrhyncha, Fulgoroidea, Cicadoidea, Cercopoidea, Membracoidea, Heteroptera) based on combined morphological and nucleotide sequence data. Spermatozoal apomorphies are superimposed. Spermatozoal characters from this study from Jamieson *et al.* (1999), phylogeny from various sources e.g. Gullan and Cranston (2005). 1: acrosome and axoneme with outer cytoplasmic microtubules (not linked to

doublets; 2: 1 x (9 + 9 + 2) axoneme and two mitochondrial derivatives; 3: centriolar adjunct lost; 4: bizarre flagellum (amalgamated triaxonemal structure); 5: accessory bodies absent; 6: acrosome reduced; 7: feebly motile spermatozoa (Psyloidea); 8: degenerate immotile axoneme (Aleyroidoidea); 9: aflagellate spermatozoa with motile microtubules (Coccoidea); 10: occasional biflagellarity?; 11: two large, scroll-like accessory bodies; 12: more than one crystalline region per mitochondrial derivative; 13: two bridges from mitochondrial derivatives; 14: 'zipper-like' array near one mitochondrial derivative; 15: polymorphic sperm; 16: centriolar adjunct lateral to nucleus and not linked to centriole.

## 6.2. SPERMIOGENESIS

The development of the acrosome, condensation of chromatin in the nucleus, development and elongation of mitochondrial derivatives and elongation of the axoneme were the main events associated with spermiogenesis in the cicada species examined. The pattern and progression of these complex spermiogenic events is generally similar to that reported in other insects (Jamieson *et al.*, 1999; Mancini and Dolder, 2004). The chromatin condensation patterns (granular, fibrillar and lamellar) in *Kongota punctigera*, *Monomatapa matoposa* and *Diceroprocta biconica* were very clear and distinct within each species. These patterns occur during reorganization of DNA when histones are replaced by protamines, a series of small, arginine-rich proteins with high positive charge densities and that become highly  $\alpha$ -helical on binding to DNA (Gimenez-Botafe *et al.*, 2002; Ribes *et al.*, 2004). The  $\alpha$ -helices of protamine lie in the major grooves of DNA, where they neutralize the negatively charged phosphate backbone through phosphorylation and later dephosphorylation to induce DNA duplexes to pack tightly (Stryer, 1988; Càceres *et al.*, 1999; Raukas and Mikelsaar, 1999; Lewis *et al.*, 2003).

From the present study, no differences in chromatin condensation patterns (granular, fibrillar and lamellar) were found in *K. punctigera* and *M. matoposa*. In their analysis of the condensation patterns in the spermatid nuclei of *Philaenus spumarius* (a cercopid), *Murex brandaris* (a snail) and *Eledone cirrhosa* (an octopus), Harrison *et al.* (2005) proposed the role of phase separation dynamics in changes that occur in chromatin condensation between the initial and final conformations. They located a constant spacing between pattern repeats through changes from granular to fibrillar to lamellar pattern followed by shrinkage of the spacing. Overall, their results show that chromatin distribution patterns during spermiogenesis are characterized by a “patterning stage” followed by a “condensation stage”. During late spermiogenesis in cicadas, chromatin condensation in *D. biconica* resulted in a pattern completely different from that in *K. punctigera* and *M. matoposa*. The condensing chromatin, which became increasingly dark, had a speckled appearance, a feature caused by isolated nucleoplasm forming numerous small light regions in the dark chromatin. Walker and MacGregor (1976) reported a similar pattern of chromatin condensation in the desert locust, *Locusta migratoria*. More recently, Harrison *et al.* (2005) found a similar pattern in the octopus, *Eledone cirrhosa*.

According to Harrison *et al.* (2005), the initial and final chromatin condensation patterns during spermiogenesis often vary across species. In several bony fish and related vertebrates there is no evidence of a universal pattern of chromatin condensation (Càceres *et al.*, 1999). All these reports suggest that spermatid nuclear chromatin condensation in animals might have species-specific differences mediated by the species-specific

protamines and the structural conformations they form with the DNA in spermatid chromatin. The number of species examined in these studies (three) is too few to comment on whether the data supports species-specificity during chromatin condensation. Hopefully further studies will answer this question.

### **6.3. REPRODUCTIVE BEHAVIOUR**

To date the function of polymorphic sperm in insects (including cicadas) remains uncertain. Nevertheless, a number of authors have suggested that production of polymorphic sperm may be linked to sperm competition (e.g. Joly *et al.*, 1989; Cook and Gage, 1995; Snook, 1998, Simmons, 2001; Swallow and Wilkinson, 2002). One important prerequisite for sperm competition is female promiscuity, especially before females have had an opportunity to utilize stored sperm from the first insemination. Evidence from a wide array of taxa indicates that most females have a proclivity to mate with multiple males (Dewsbury, 1984; Ridley, 1988; Eberhard, 1996; Birkhead and Møller, 1998). In most of these taxa, polyandry is often covert and difficult to detect at the behavioural level (e.g. Newcomer *et al.*, 1999). Nevertheless, DNA-fingerprinting has shown evidence of multiple paternity in organisms that are socially monogamous and that have been hitherto considered to be sexually monogamous (e.g. Birkhead and Møller, 1998; Morell, 1998). Sexual fidelity may actually be rare among animals.

Cicadas are not socially monogamous. They also display a spectrum of reproductive behaviours (Myers, 1929; Alexander and Moore, 1958; 1962; Dunning *et al.*, 1979; Doolan, 1981; Williams and Simon, 1995; Villet and van Noort, 1999; Marshall, 2000;

Cooley and Marshall, 2001; Sueur, 2002; 2003; Sueur and Aubin, 2003; Villet *et al.*, 2003). Regrettably, very few of these works have addressed the question of female cicada promiscuity. Nevertheless, this is to be expected because it is extremely difficult to monitor female cicadas in their natural environments. They are not easily detected because they do not stridulate. Furthermore, most of them utilize different reproductive strategies. For example, in periodical cicadas and some cicadettines, the males stridulate and search for mates by chorusing or alternating bouts of calls with brief flights (Alexander and Moore, 1958; 1962; Williams and Smith, 1991; Villet and van Noort, 1999). Sexually receptive periodical cicada females remain relatively stationary within the chorus and respond by making wing flicks timed to conspecific males (Cooley and Marshall, 2001). In contrast to other cicadettine cicadas, *Quintilia carinata* males have two calling songs which seem to serve different purposes (M. Villet, pers. comm.). Other cicadettine males have only one calling song.

In species like *Stagira simplex* and *Xosopsaltria annulata*, receptive females are cryptic and do not move around. Instead they wait for advertising males to locate them before courtship can be initiated. The males in all cicadettine species are more vulnerable to predators compared to females because of their constant movements. All cicadettines have elevated levels of population densities compared to the cicadines. This makes it easier for females to be located. In contrast, male cicadines (e.g. *Pycna semiclara*, *Platyleura hirtipennis* and *Oxypleura quadraticollis*) advertise from fixed positions and wait for amorous females to approach them. The females of these species are more at risk than in the cicadettines. The probability of a successful insemination and female

promiscuity is higher in gregarious species (e.g. *P. semiclara*) unlike in solitary species (e.g. *O. quadraticollis*) because of the reduced costs of finding mates. As a result of these different reproductive strategies which place constraints on making general statements the question of female promiscuity in cicadas has largely remained uncertain.

A number of works have addressed reproductive behaviour in periodical cicadas (Alexander and Moore, 1958; 1962; Alexander, 1968; Dunning *et al.*, 1979; Williams and Simon, 1995; Cooley, 1999; Marshall, 2000; Cooley and Marshall, 2001). Cooley and Marshall (2004) observed that *Magicicada septendecim* females usually mate once after which they become sexually impassive. Further remating opportunities are only pursued as a way to replenish sperm supplies, especially when previous matings are interrupted (Cooley, 1999). These observations showed that these females benefit by actively seeking insemination from more than one male. These benefits are likely to be indirect (those that benefit her progeny). It therefore seems that sperm competition in these cicadas must occur. What remains to be established is whether female promiscuity occurs in female cicadas from other tribes and under what circumstances.

The second prerequisite for sperm competition is the ability to store spermatozoa. In most insects there is a sperm-storage organ, the spermatheca, which forms part of the female reproductive tract. The hostility and complexity of the female reproductive tract in many insects suggests a function in providing a challenging arena for sperm competition, and in controlling or at least influencing offspring paternity (Birkhead *et al.*, 1993; Hellgriegl and Ward, 1998; Birkhead and Pizarri, 2002). In the cicadas examined in the present study

the original spermatheca (a long blind-ending structure) has become vestigial; its function in sperm storage has been taken over by a modified common oviduct (Chapter 5; Boulard, 1965; Moulds, 2005). Why this has happened in these cicadas remains to be established. However, intense postcopulatory sexual selection is known to influence inter-sexual conflict and drive rapid evolutionary changes that generate a startling diversity of morphological, behavioural and physiological adaptations (Birkhead and Pizzari, 2002). Inter-sexual conflicts in cicadas (if they exist) are yet to be demonstrated. Nevertheless, in the majority of species that are not truly monogamous (this includes cicadas), a conflict between males and females is inevitable because of conflicting interests.

In order to ensure or increase paternity, male insects utilize an assortment of strategies (Birkhead and Møller, 1998; Simmons and Siva-Jothy, 1998). The optimal strategy for most male insects (cicadas included) is not necessarily to mate with as many females as possible, but to fertilize as many eggs as possible (Singh *et al.*, 2002). In contrast, females want only the best males to fertilize their eggs, i.e. they tend to prefer quality over quantity. In essence, female cicadas invest more in their progeny. This is especially important because juveniles develop underground for long periods before they become adults. During their underground sojourns, there is no parental care and they are exposed to environmental fluctuations and soil-dwelling predators. As insurance against the inevitable losses, most cicadas oviposit a large number of eggs. Other unknown strategies to ensure the complete development of juveniles into adults might also be used.

The determination and significance of these strategies in cicadas remains a question for the future.

#### **6.4. CONCLUSIONS**

Several features of the sperm in the cicada species examined are shared with most other auchenorrhynchans (Folliot and Maillet, 1970; Kubo-Irie *et al.*, 2003) and other insects (Jamieson *et al.*, 1999). There are other structural characteristics that seem exclusive to the cicadas. These include the unusual centriolar adjunct which can either be homogenous or lamellate, the laterally invaginated nucleus, the presence of two posterior acrosomal processes and the vesicle-like elements that are associated with both the nucleus and centriolar adjunct. The functional role of the putative centriolar adjunct in cicadas, an almost ubiquitous sperm organelle in insects, remains unresolved. Similarly, the significance of polymegaly and reproductive behaviour in most cicadas are not well understood. The early stages of spermiogenesis in cicadas are not usually apparent in the adults. The rest of the stages have a similar morphology to those in many other insects (Phillips, 1970; Bào *et al.*, 1997; Baccetti, 1998; Jamieson *et al.*, 1999; Mancini and Dolder, 2004). The absence of early stages of spermiogenesis probably indicates that males have a finite number of sperm but they can mate more than once.

#### **6.5. FUTURE WORK**

The results of the current study investigation have raised a number of questions regarding the phylogeny, reproductive behaviour, polymegaly, identity of the “centriolar adjunct”, spermiogenesis and genital morphology in cicadas. These questions provide a number of

avenues for future research. The higher classification of the Cicadoidea, especially the number of families and assignment of species to tribes, has remained a contentious issue. From the present study, examination of sperm structure in some cicadas has shown the potential of the external morphology of sperm and its ultrastructure in providing clues for distinguishing taxa and examining phylogeny at a subfamily level. There is a need to further examine the structure of the vesicles in the sperm mid-piece of these insects in order to establish clearly whether they are vesicles or nuclear pores. In addition, it is also desirable to further examine the acrosome, in order to establish whether the cicada acrosome really has no perforatorium. This can be done using the phalloidin-rhodamine fluorescent probe. Absence of the perforatorium would support the common ancestry of the cicadas with Phthiraptera, Psocoptera, Hemiptera and Thysanoptera. Certainly the number of species examined (ten) in the present study is too few considering the number of cicada species worldwide. Clearly, there is need to examine the sperm structure in more representative cicada taxa from other tribes to clearly define sperm synapomorphies. In addition, there is need to utilize additional characters (not necessarily sperm characters) that are variable and phylogenetically informative in order to clarify the phylogenetic relationships of cicadas.

In some species like thrips and sucking lice the meiotic process of spermatogenesis proceeds abnormally; thus the presence of a couple of centrioles results a normal feature in this groups, and consequently the presence of two axonemes or two flagella could be expected. It would be of importance to investigate how spermatogenesis proceeds in cicadas to verify whether meiosis develops normally.

The present study has shown that sperm polymorphism exists in cicadas with different reproductive behaviours. Therefore the reason for sperm polymorphism needs to be addressed. The first step to this approach would be the determination of the DNA content in all sperm morphs (do sperm with short nuclei have the same DNA content as those with long nuclei ?) and examination of a large number of oocytes to determine whether it is always the sperm with long nuclei that participate in fertilization. These steps represent exciting opportunities to extend an understanding of reproductive behaviours in cicadas, which might help in understanding polymegaly. The present study has also shown that the so-called centriolar adjunct in cicadas (Folliot and Maillet, 1970) might not be a true centriolar adjunct. This is because of its formation (which is not in close proximity to the centriole) and its unusual position (in the majority of insects the centriolar adjunct is posterior to the nucleus and is linked to a centriole). Further work should endeavour to investigate the true nature of this structure.

In most animals, especially mammals and birds, larger testes are associated with higher sperm production rates, large sperm reserves and more sperm per ejaculate (Møller, 1988; 1989). Energy is expended during sperm production and the accrued costs of sperm production can be very important over many generations (Dewsbury, 1982; Parker, 1982; Dewsbury and Sawrey, 1984). The variation in testes size of cicadas observed in the present study merits further studies in order to fully understand this phenomenon and how it may influence reproductive behaviour and ejaculate characteristics of male cicadas.

During spermiogenesis the haploid genome of the early spermatid undergoes an important condensation that leads to a reduction of the nuclear volume (Ribes *et al.*, 2004). Future studies should consider characterizing the DNA-interacting proteins responsible for the profound changes in sperm chromatin together with the protamines present in mature sperm. Information on the composition of protamine in spermatids and mature sperm might be of paramount importance in understanding the variations in chromatin condensation patterns across species. In addition, such information might have important evolutionary implications.

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**Appendix One: Sperm dimension frequencies of cicadine cicadas (50 sperm per individual, 5 individuals per species) and data for chi-square tests.**

Nucleus length classes: 5 -15.9 – short  
 16 - 30.9 – intermediate  
 31 - 80 – long

Tail length classes: 5 – 75.9 – short  
 76 – 150.9 – intermediate  
 151 – 300 – long

Sperm dimensions	<i>A. albiger</i>					<i>A. zuluensis</i>					<i>P. capensis</i>					<i>P. hirtipennis</i>					<i>P. semiclara</i>				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Frequency of short nuclei	8	9	7	10	9	6	5	0	2	5	0	6	11	7	8	4	13	7	1	5	0	0	2	2	3
Frequency of interm. nuclei.	17	19	15	13	11	17	16	25	16	27	9	18	17	8	12	7	12	16	16	15	18	16	13	7	15
Frequency of long nuclei	25	22	28	27	30	27	29	25	32	18	41	26	22	35	30	39	25	27	33	30	32	34	35	41	32
Frequency of short tails	10	17	14	14	14	21	14	9	11	5	8	19	14	38	20	17	11	1	8	15	8	14	25	36	18
Frequency of inter. tails	20	21	19	21	21	12	28	35	37	43	41	23	31	12	28	33	35	47	41	32	42	36	25	14	32
Frequency of long tails	20	12	17	15	15	17	8	6	2	2	1	8	5	0	2	0	4	2	1	3	0	0	0	0	0

**Appendix Two: Sperm dimensions. Chi-square test for homogenous frequencies within cicadine species.**

Test statistic = (Observed frequency-Expected frequency)<sup>2</sup> divided by Expected frequency.

Species	Nucleus Length	Test statistic value	Tail Length	Test statistic value	Critical value at 99% significance level
<i>A. albigera</i>	short	<b>0.6*</b>	short	<b>1.79*</b>	13.28
	intermediate	<b>2.68*</b>	intermediate	<b>0.1*</b>	13.28
	long	<b>1.4*</b>	long	<b>2*.2</b>	13.28
					13.28
<i>A. zuluensis</i>	short	<b>6.99*</b>	short	<b>11.98*</b>	13.28
	intermediate	<b>5.68*</b>	intermediate	<b>18.27**</b>	13.28
	long	<b>4.22*</b>	long	<b>21.71**</b>	13.28
					13.28
<i>P. capensis</i>	short	<b>10.2*</b>	short	<b>25.5**</b>	13.28
	intermediate	<b>6.47*</b>	intermediate	<b>16.81**</b>	13.28
	long	<b>7.23*</b>	long	<b>13.37**</b>	13.28
					13.28
<i>P. hirtipennis</i>	short	<b>17**</b>	short	<b>15.3**</b>	13.28
	intermediate	<b>4.45*</b>	intermediate	<b>4.23*</b>	13.28
	long	<b>3.92*</b>	long	<b>5*</b>	13.28
					13.28
<i>P. semiclara</i>	short	<b>6.16*</b>	short	<b>23**</b>	13.28
	intermediate	<b>5.13*</b>	intermediate	<b>15.6**</b>	13.28
	long	<b>1.58*</b>	long	<b>-</b>	13.28

\* Pattern of distribution can be confidently generalized to the larger population from which the sample was randomly drawn

\*\* A statistically significant departure from homogeneity.

**Appendix Three: Test for homogeneity between cicadine species (250 sperm per species).**

Sperm categories	<i>A. albigera</i>	<i>A. zuluensis</i>	<i>P. capensis</i>	<i>P. hirtipennis</i>	<i>P. semiclara</i>
Short nuclei	15	18	32	30	7
Intermediate nuclei	43	101	64	66	69
Long nuclei	132	131	154	154	174
Short tails	69	60	100	52	101
Intermediate tails	102	155	135	188	149
Long tails	79	35	16	10	0

Test statistic = (Observed frequency-Expected frequency)<sup>2</sup> divided by Expected frequency.

Test statistic for short nuclei = **21.63, P < 0.01**, frequencies are not homogenous.  
 Test statistic for intermediate nuclei = **25.26, P < 0.01**, frequencies are not homogenous.  
 Test statistic for long nuclei = **8.64, P > 0.01**, frequencies are homogenous.

Test statistic for short tails = **27.24, P < 0.01**, frequencies are not homogenous.  
 Test statistic for intermediate tails = **26.82, P < 0.01**, frequencies not homogenous.  
 Test statistic for long tails = **111.36, P < 0.01**, frequencies not homogenous.

**Appendix Four: Sperm dimension frequencies of cicadettine cicadas (50 sperm per individual, 5 individuals per species) and data for chi-square tests.**

Nucleus length classes: 5 -15.9 – short

16 - 30.9 – intermediate

31 - 80 – long

Tail length classes: 5 – 75.9 – short

76 – 150.9 – intermediate

151 – 300 – long

Sperm dimensions	<i>M. leucoptera</i>					<i>Q. walkeri</i>					<i>S. simplex</i>					<i>X. thunbergi</i>					<i>M. matoposa</i>				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Frequency of short nuclei	2	4	7	15	11	5	9	1	6	6	17	12	9	13	15	0	5	11	13	3	0	0	0	0	0
Frequency of interm. nuclei.	6	6	15	8	14	10	8	9	10	11	8	10	9	10	10	10	12	19	18	14	5	4	9	18	9
Frequency of long nuclei	42	40	28	27	25	35	33	40	34	33	25	28	32	27	25	40	33	20	19	33	45	46	41	32	41
Frequency of short tails	20	29	17	15	7	42	40	43	43	37	31	26	34	33	27	39	37	31	27	32	0	0	0	0	0
Frequency of inter. tails	30	21	33	35	43	8	10	7	7	13	19	24	16	17	23	11	13	19	23	18	48	48	49	49	49
Frequency of long tails	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	1	1

**Appendix Five: Sperm dimensions. Chi-square test for homogenous frequencies within cicadettine species.**

Test statistic = (Observed frequency-Expected frequency)<sup>2</sup> divided by Expected frequency.

Species	Nucleus Length	Test statistic value	Tail Length	Test statistic value	Critical value at 99% significance level
<i>M. leucoptera</i>	short	<b>14.2**</b>	short	<b>14.49**</b>	13.28
	intermediate	<b>7.83*</b>	intermediate	<b>7.88*</b>	13.28
	long	<b>7.81*</b>	long	-	13.28
<i>Q. walkeri</i>	short	<b>6.16*</b>	short	<b>0.63*</b>	13.28
	intermediate	<b>0.55*</b>	intermediate	<b>2.88</b>	13.28
	long	<b>0.96*</b>	long	-	13.28
<i>S. simplex</i>	short	<b>2.79*</b>	short	<b>1.68*</b>	13.28
	intermediate	<b>0.35*</b>	intermediate	<b>2.57*</b>	13.28
	long	<b>1.21*</b>	long	-	13.28
<i>X. thunbergi</i>	short	<b>18.64**</b>	short	<b>2.79*</b>	13.28
	intermediate	<b>4.05*</b>	intermediate	<b>5.28*</b>	13.28
	long	<b>11.51*</b>	long	-	13.28
<i>M. matoposa</i>	short	-	short	-	13.28
	intermediate	<b>13.56**</b>	intermediate	<b>0.03*</b>	13.28
	long	<b>4.96*</b>	long	<b>0.85*</b>	13.28

\* Pattern of distribution can be confidently generalized to the larger population from which the sample was randomly drawn.

\*\* A statistically significant departure from homogeneity.

**Appendix Six: Sperm dimensions. Test for homogeneity between cicadettine species (250 sperm per species).**

Sperm categories	<i>M. leucoptera</i>	<i>Q. walkeri</i>	<i>S. simplex</i>	<i>X. thunbergi</i>	<i>M. matoposa</i>
Short nuclei	39	27	66	32	0
Intermediate nuclei	49	48	47	73	45
Long nuclei	162	175	137	145	205
Short tails	88	205	151	166	0
Intermediate tails	162	45	99	84	243
Long tails	0	0	0	0	7

Test statistic for short nuclei = **21.63**, **P < 0.01**, frequencies are not homogenous.

Test statistic for intermediate nuclei = **25.26**, **P < 0.01**, frequencies are not homogenous.

Test statistic for long nuclei = **8.64**, **P > 0.01**, frequencies are homogenous.

Test statistic for short tails = **210.71**, **P < 0.01**, frequencies are not homogenous.

Test statistic for intermediate tails = **189.87**, **P < 0.01**, frequencies not homogenous.

Test statistic for long tails = **28**, **P < 0.01**, frequencies not homogenous.