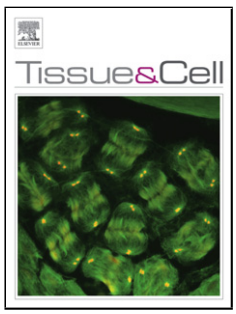

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The importance of insect sperm: Sperm ultrastructure of Hermetia illucens (black soldier fly)

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Running title: The importance of insect sperm: Sperm ultrastructure of *Hermetia illucens* (black soldier fly)

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Highlights

- General sperm structure represents typical insect sperm structural characteristics
- Electron-dense centriolar adjunct material separates mitochondrial derivatives and axoneme
- Accessory bodies flanks axoneme in flagellar region
- Large mitochondrial derivatives located along flagellum

Keywords: *Hermetia illucens*, Sperm structure, Sperm ultrastructure, Centriolar adjunct, Accessory bodies, Transmission Electron Microscopy

1. Introduction

Insects are the most diverse and probably the most successful group of animals, owed to their method of reproduction and their survival abilities (O'woma et al., 2016). Moreover, insects are very important in affecting entire ecosystems; for example, a keystone species, such as honey bees (Kratochwil, 2003; O'woma et al., 2016).

Insects mainly reproduce oviparously, whereby the female produces eggs in the ovaries, and sperm, produced by the testes and transferred during copulation to be stored within spermathecae, fertilize the eggs, which is finally released by the ovipositor (O'woma et al., 2016).

Successful fertilization in insects depends on several factors, including sperm structure and function as well as sperm competition and cryptic female choice (Snook, 2005). A typical insect sperm consists of a head, containing an apical acrosome and nucleus; neck or mid-piece, containing the centriole region and surrounding centriolar adjunct, and a tail or flagellum, containing the mitochondrial derivatives, accessory bodies, and axoneme (Dallai, 2014). However, insects, particularly Diptera, exhibit large diversity between and within species, in sperm structure and morphology (Snook, 2005; Werner and Simmons, 2008; Name et al., 2010), for example, sperm polymorphism based on sperm with different lengths, and the production of both fertilizing and non-fertilizing sperm in a species (Gottardo et al., 2016). Evolution of insect sperm structure, *per se*, has greatly been attributed to sperm competition (Holman and Snook, 2006; Werner and Simmons, 2008), while less is known about how the absence of sperm competition affects sperm characteristics, function and subsequently fertilization in animals (van der Horst and Maree, 2014).

The black soldier fly, *Hermetia illucens*, (Diptera: Stratiomyidae) is known for its use in waste-management, studies of forensic entomology, and animal feeds (Tomberlin and Sheppard, 2001; Park, 2016). Interest in this species for its economic and environmental benefits is rapidly growing, along with mass production, supported by their relatively short adult life cycle (5-8 days) and ability to frequently reproduce (Park, 2016). Sexual dimorphism between adult male and female external genitalia has been described (Oliveira et al., 2016), as well as the female's three spermathecae (sperm storage organs) location and structural components (Ururahy-Rodrigues and Pujol-Luz, 2000). However, detailed descriptions of the complete reproductive tracts in this species are lacking.

Mating in the black soldier fly occurs approximately two days after eclosion (adult fly emerging from pupal case) and certain mating strategies or behaviors exist. For example, the males await females at lekking sites and perform territorial lekking behavior where a male resting at the lekking site interacts aggressively with an intruder male arriving within his territory. After these events the intruder leaves and the resting male returns to the lekking site (Tomberlin and Sheppard, 2001; Diclaro and Kaufman, 2009). Females passing the lekking site can be grasped by males, and mating occurs in flight where after the mating pair descends while still copulating (Tomberlin and Sheppard, 2001). In addition to the lekking behavior, preliminary results from a current study, determining paternity, indicates polyandry (one female mating with more than one male) and accordingly the presence of sperm competition in this species (personal communication). Mating strategies are known to play a role in sperm competition, which in turn can influence sperm structure and function (Snook, 2002; Safonkin, 2011; Blengini et al., 2014), with subsequent effects on fertilization. However, to date, neither sperm morphology nor the effect of possible sperm competition has been described in the black soldier fly.

The aim of this study was to, for the first time, determine and describe sperm structure and ultrastructure of sperm in the spermathecae of the economic viable species, *Hermetia illucens*. These are accordingly the sperm deposited by the male in the spermathecae and those that will compete for fertilization.

2. Materials and methods

2.1. Collection and husbandry

Adult flies of *Hermetia illucens* were collected from a colony reared in the insectary of Agriprotein Technologies (Philippi, Cape Town, South Africa). Flies were bred and reared at Agriprotein under controlled conditions, temperature 28^0C, 80% relative humidity and

standard 5000 Kelvin fluorescent tubes are run as ambient light. Adult black soldier flies do not feed and is provided with water only.

The methodology and ethics of this study was approved by the Animal Research Ethics Committee of the University of the Western Cape, South Africa (AR17/5/3; AREC-130416-019).

2.2. Light microscopy

Three females of *Hermetia illucens* were collected from the colony and exposed to cold-induced dormancy (Name et al., 2010) for five minutes, after copulation was completed. Following cold-induced dormancy the female was decapitated and dissected under a stereo microscope using 50 uL of phosphate buffered saline. Dissected spermathecae were transferred to physiological media (Hams F10®, Sigma) and kept at 4^0C. In order to recover sperm from spermathecae, without damaging the sperm, a precision drill (Dremel ® 3000, Mexico) was used to drill the spermathecae and release sperm. A sharpened 1.5 mm drill point was inserted into an Eppendorf tube containing the spermathecae covered by a small amount of Hams F10® medium. Sperm smears were made on a microscope slide and covered with a coverslip. Sperm was viewed using a Nikon Eclipse 50i light microscope (60 and 100 x objectives) and imaged using the Morphology module of Sperm Class Analyzer (SCA) ® (Microptic, Spain) and Nikon ACA1300-200uc digital camera. Micrographs were obtained by application of positive phase microscopy.

Additionally, intact spermathecal capsules and capsular ducts were fixed and processed using routine transmission electron microscopy (TEM) techniques with the purpose of making one micrometer sections for light microscopy. Semi-thin sections (approximately 1 µm thick) of the resin-embedded material were mounted on microscope glass slides, stained with 1% toluidine blue for 30 seconds and viewed with an Olympus BX63 light microscope using a 100x oil immersion objective and equipped with an Olympus DP72 camera.

2.3. Transmission electron microscopy

Dissected spermathecae (including capsule and capsular ducts) from six females were removed and fixed by using different fixatives. Additionally a pair of testes from one male was removed and fixed. Samples were fixed in either 2.5% glutaraldehyde or were first fixed in 5% formaldehyde, for 12 hours, and then in 2.5% glutaraldehyde at 4^0C. The samples were washed twice for 30 minutes each in 0.075 M phosphate buffer pH 7.4, before post-fixation in 1% osmium tetroxide for 1 hour. Routine procedures of washing, dehydration and

embedding were used. Thin sections (70-90 nm) were then cut of the appropriate blocks using a Leica EM UC7 ultra-microtome and a Diatome diamond knife. These sections were stained with lead citrate and uranyl acetate before viewing in either a Philips CM10 or a Joel Jem 1011 transmission electron microscope operated at 80 kV.

3. Results

3.1 Sperm structure (bright field microscopy)

Female adult flies possess three spermathecae, consisting of a capsule (Fig. 1A) attached to a capsular duct, and additional ducts attached to the latter, but which were not dissected nor investigated in this study. Spermathecal capsules have an oval shape in longitudinal view and receive sperm from the capsular duct, which opens into its base. Sperm is visible inside the lumen of the spermathecal capsule (Fig. 1B).

Sperm recovered from the spermathecae had thin, elongated heads, and a long flagellum (Fig. 1C). The nucleus is visible as the chromatin condensed part of the head. From the head region, a less darkened region, the flagellum extends. Flagellae of most sperm appear to have a helical or sinusoidal shape. We have not been able to measure sufficient numbers of sperm for statistical purposes. However, it appears that the range for sperm head length seems to vary from 9 to 21 µm.

Transmission electron microscopy was performed on intact spermathecae (spermathecal capsule and capsular duct), containing bundles of sperm following copulation. Multiple transverse and longitudinal sections have been obtained of different sperm components, allowing the identification of representative sperm structures. It is important to note that these sperm are the ones that will ultimately compete for the oocyte(s).

3.2. Sperm ultrastructure

3.2.1. Head region

The head region consists of a nucleus (Fig. 2A), but no acrosome was present. In longitudinal and transverse sections, the elongated nucleus is characterized by highly condensed chromatin. Just posterior to the base of the nucleur tip region, transverse section reveals an almost circular shaped nucleus (Fig. 3A).

Towards the base of the nucleus lateral grooves or indentations are notable in the cross-sectional view (Fig. 2B and C). Close to the posterior part of the nucleus, at the zone of overlap, the diameter is reduced, and centriolar adjunct material becomes evident (Fig. 2A). Furthermore, a “wrinkled” plasma membrane surrounds the head region (Fig. 2A).

Sperm head lengths in this species varied from long to shorter; shorter sperm heads appeared to have shorter nuclear regions. The head and neck region is straight aligned compared to the helical flagellar region.

3.2.2. Zone of overlap

The region of overlap between the head and flagellum contains the mitochondrial derivatives, axoneme and centriolar adjunct. At its basal end, the nucleus has an H or X-shape in cross-sectional view and a larger indentation is present, facing the axoneme (Fig. 3A). In images not shown due to less contrast, it is evident that this indentation is much deeper and hosts the axoneme, and eventually the nucleus embraces the developing mitochondrial derivatives (Fig. 3B). More posteriorly in this region, the nucleus is gradually being replaced by enlarging mitochondrial derivatives and centriolar adjunct material (Figs. 3 C-G). The axoneme is located on the lateral side of the nucleus and has a microtubule formula of 9 + 9 + 2. The axoneme is further, with the developing mitochondrial derivatives, flanked by a small amount of centriolar adjunct material (Fig. 3C). However, distally in this region, when the mitochondrial derivatives become more equally sized, a larger volume of centriolar adjunct material seems to merge into the area between the axoneme and mitochondrial derivatives (Figs. 3E-G). At different levels of the overlap zone, the granular material varies in electron-density (Figs. 3E-G). In Fig. 3F the centriolar adjunct material has conformed into a half-moon shaped electron-dense ring with its two ends adhering to the axoneme, whilst the rest is flanking the mitochondrial derivatives. Mitochondrial derivatives are of more or less equal in size in the zone of overlap and anterior flagellar regions, and tend to increase in diameter reaching the flagellum.

Sperm recovered from the male testes revealed mitochondrial derivatives containing paracrystalline material (supplementary material Fig. B).

3.2.3. Flagellum

The zone of overlap is followed by a helical or wave-shaped flagellum (Fig. 1C). The flagellar region contains the axoneme, two mitochondrial derivatives, and two accessory bodies. In the initial segments of the flagellum, the centriolar adjunct material, previously located between the axoneme and mitochondrial derivatives, is no longer present. Instead, the centriolar adjunct material gives rise to two accessory bodies, partly surrounding the mitochondrial derivatives like “wings” (Fig. 4A). Towards the mid flagellar region, while the mitochondrial derivatives increase in size, the accessory bodies decrease in size (Fig. 4B), until they are no longer present along large mitochondrial derivatives (Fig. 4C). The

axoneme, with a typical 9 + 9 + 2 microtubule arrangement (Fig. 4D), extends along the length of the flagellum and is accompanied by mitochondrial derivatives. The mitochondrial derivatives eventually further decrease in size as it reaches the last part of the flagellum (Fig. 4D). Although not shown, the end-piece of the flagellum only consists of an axoneme with incomplete, disrupted microtubular structure, without mitochondria. Mitochondrial derivative diameter may vary in sperm of different sizes.

4. Discussion

This study describes, for the first time, the detailed sperm structure and sperm ultrastructure of *Hermetia illucens* (Diptera:Stradiomyidae). We investigated sperm structure and ultrastructure from sperm recovered from female storage organs, the spermathecae (capsule and capsular duct), and describes the structure of post-testicular, mature sperm that will take part in fertilization. Contrary, majority of studies reporting on sperm structure and ultrastructure, in particularly brachyceran subspecies, report on sperm recovered from the male reproductive system, such as the testes and deferent ducts (Name et al., 2010; Name et al., 2012; Rego et al., 2016). Although sperm maturation in Diptera is likely to be completed in the male reproductive system before eclosion or shortly thereafter (Spiegel et al., 2013), there are further structural changes that sperm can undergo within the spermathecae in preparation for successful fertilization (Degrugillier, 1985).

Overall, the general sperm structure of *Hermetia illucens* is similar to that of a typical Dipteran insect sperm (Phillips, 1970; Dallai, 2014; Gottardo et al., 2016). Light microscopy revealed a needle-like head region, followed by an elongated, helical flagellum. Furthermore, variation in sperm length was observed, possibly indicating sperm polymorphism, a phenomenon which is not uncommon in brachycera, where the same testis produces sperm of different sizes (Snook and Karr, 1998; Name et al., 2012). Although the role of variation in sperm length is not fully understood, data from the well-studied *Drosophila* showed that different sperm sizes can exhibit different structural characteristics, including head and tail length (polymegaly), as well as altered functionality and fertilization success (Snook et al., 1994; Snook and Karr, 1998). The production of sperm with different lengths is suggested to be a result of sperm competition and coevolution, involving female reproductive tract morphology (Hosken, 2003; Pitnick et al., 2003). However, it should be noted that the spermathecae, as evaluated in this study, could have contained sperm of more than one male. Furthermore, dissection of male testes in *Hermetia illucens* was difficult, and it is yet to be confirmed whether one male produces sperm with different sizes or not. The interplay between sperm size, sperm abundance, and fertilization success is of interest, and further analysis is needed to determine morphological and functional

differences between different types of sperm produced by *Hermetia illucens*, and the role of sperm competition, is yet to be determined.

Hermetia illucens share many features with other brachyceran subspecies as well as some nematoceran subspecies, confirming their close relationship as indicated (Dallai et al., 2007; Dallai et al., 2008). However, several structural differences were observed pertaining to the head region, zone of overlap, and anterior flagellar region. The head region of *Hermetia illucens* presents with an apical nuclear region consistent with other brachycera (Degrugillier, 1985; Curtis et al., 1989; Name et al., 2010; Name et al., 2012) and some nematocera (Dallai et al., 1984; Dallai et al., 2007), but despite several sections viewed, the presence and structure of an acrosome could not be determined. Additionally, as observed in sperm derived from the spermathecae of *Musca domestica* (Degrugillier and Leopold, 1976; Degrugillier, 1985), a loosening or "wrinkled" plasma membrane surrounding the anterior head region was observed. Loosening or shedding of the plasma membrane from the sperm head had been associated with the acrosome reaction in this species, and has further been suggested to occur within the spermathecal capsule or spermathecal ducts as a result of contact with female accessory gland secretions, and is of importance for egg penetration (Degrugillier and Leopold, 1976). However, a plasma membrane that forms many wrinkles or riffles is unfortunately often associated as a swelling or fixation artefact, and is evident even in other animal species (Soley, 1993; Swan and Alboghobeish, 1997). It should also be noted that in some insect species (Phillips, 1970), including subspecies belonging to the Dipteran suborder nematocera (Dallai, 2014), the acrosome is lacking and may be lacking in *Hermetia illucens*.

In accordance with nematoceran Chironomoidea subspecies (Dallai et al., 2007), the more posterior nuclear region contains lateral cavities or grooves, from which one cavity deepens eventually to accommodate the axoneme. However, such nuclear features seem to not have been previously described for brachyceran subspecies. Furthermore, these nuclear cavities in the Chironomoidea subspecies corresponded to a helicoidal nucleus, which has been suggested to contribute to progressive swimming (Dallai et al., 2007). At the basal segment of the nucleus, centriolar adjunct material appears and is present throughout the region of overlap as in majority of brachyceran sperm (Name et al., 2010; Name et al., 2012), and nematoceran Chironomoidea (Dallai et al., 2007) and Tipulidae subspecies (Dallai et al., 2008). However, in brachyceran Phoridae subspecies, similar shaped structures have not been referred to as centriolar adjunct material, but rather an accessory body (Curtis et al., 1989). There further seems to be some variation regarding the location and arrangement of centriolar adjunct material in the zone of overlap. For example, in brachycera the Calliphoridae subspecies *Chrysomya megacephala* (Name et al., 2010) and

Lucilia cuprina (Name et al., 2012) showed centriolar adjunct material that is surrounded by the nucleus, mitochondrial derivatives and axoneme, while in *Lucilia peruviana* and *eximia* (Name et al., 2012) it is located laterally on one side only, between the axoneme and nucleus. In *Hermetia illucens*, small amounts of centriolar adjunct material seem to be arranged laterally, as in the latter, but on both sides of the sperm cell. Furthermore, a unique organization of centriolar adjunct material has been noted in the zone of overlap and the anterior flagellar region. Here centriolar adjunct material, separating the mitochondrial derivatives and axoneme, resembles a half moon shape consisting of cylindrical structures, of which form an electron dense half-moon shaped ring peripherally. Similar cylindrical centriolar adjunct material was described by Dallai et al. (2008) for nematoceran Tipulidae subspecies. The suggested function of the centriolar adjunct is to tighten or strengthen the head and flagellum together (Chawanji et al., 2006; Dallai et al., 2016). However, it is not clear what role the centriolar adjunct may play (Dallai, 2014). The presence of the centriolar adjunct mainly in the zone of overlap and anterior part of the flagellum in *Hermetia illucens*, indicates a possible role for stabilizing the elongated, immotile head and supporting its connection with the flagellum, particularly in sperm with long, twisted flagella. One could further postulate that such firm head-tail connection possibly supports the head in successful egg penetration, and preventing a heavy, immotile, twisted flagellum to prematurely detach from the head region before egg penetration has been completed.

In *Hermetia illucens*, the centriolar adjunct material gives rise to two accessory bodies of peculiar shape. Accessory bodies, derived from the centriolar adjunct, which is flanking the axoneme and running along mitochondrial derivatives in the flagellar region, are known structures in most insects (Werner and Simmons, 2008; Dallai, 2014), but a less common phenomenon in brachycerans. The exact levels of transition from centriolar adjunct material to accessory bodies in the flagellar region could not be distinguished. Furthermore, the presence of accessory bodies containing adenosine triphosphatase activity has been associated with the double helical movement pattern of sperm in some insect species (Werner and Simmons, 2008). However, in other insect species with similar swimming patterns, accessory bodies are lacking (Werner and Simmons, 2008).

Flagellar features of *Hermetia illucens* include an axoneme with the most commonly observed Brachyceran axonemal formula of 9 + 9 + 2 (Dallai and Afzelius, 1993). Furthermore, accessory tubules with electron dense intertubular material has been noted, while more detail, such as the number of protofilaments in the accessory tubules could not be determined. Although it seems likely that *Hermetia illucens*, as most Brachyceran species (Afzelius et al., 1993), may also exhibit 13 protofilaments in the accessory tubules, the exact number of protofilaments must be confirmed. Additionally, the axoneme is

accompanied by two mitochondrial derivatives for most of the flagellum. Mitochondrial derivatives are almost equally sized, and vary from small to large structures, possibly owed to the presence of sperm polymorphism (Dallai, 2014). The role of mitochondrial derivatives in sperm functionality is unclear. It has been suggested to rather play a passive role in insect sperm motility (Werner and Simmons, 2008).

Furthermore, the presence of mitochondrial paracrystalline structures of sperm derived from the spermathecae could not be confirmed, while sections through the zone of overlap from sperm derived from the testes revealed mitochondrial derivatives consisting of paracrystalline material. Paracrystalline inclusions (cristae) in mitochondrial derivatives have been shown to exhibit cytochrome-c oxidase activity (Werner et al., 1999), which may assist in energy production for motility. Additionally, it has been suggested that the presence of mitochondrial derivative crystalline inclusions allow for elasticity, which could alter the axoneme beating (Werner and Simmons, 2008). However, despite the presence of cytochrome-c oxidase activity in the mitochondrial derivatives of Staphylinidae, subspecies *Aleochara bilineata* (rove beetle), the addition of glycolysis inhibitor, iodoacetic acid, stopped sperm motility, indicating the glycolytic pathway as energy source for sperm motility in this species and not the mitochondrial derivatives (Werner et al., 1999).

There are certain insect species exhibiting motile sperm devoid of mitochondrial derivatives, only containing accessory bodies (Werner and Simmons, 2008). Large mitochondria in *Drosophila melanogaster* have further been suggested to play a role in sperm elongation, resulting in different sized sperm, subsequently affecting sexual selection in such a manner (Noguchi et al., 2012). It is thus clear that mitochondrial derivatives may be involved in different sperm functional roles in insects.

In conclusion, *Hermetia illucens* sperm share most structural characteristics with other Brachycerans, but with interesting distinguishing features in the zone of overlap and anterior flagellar region. However, further studies in this species are needed to provide more details regarding the sperm structure, particularly differences between long and short sperm in the head and tail regions and how it may affect fertilization success, sperm competition, and whether sperm exhibit any motility along the reproductive tract in males and females and during storage in the spermathecae as well as acrosome status. Furthermore, a lack of sperm ultrastructure studies in particularly Stratiomyomorpha hinders proper comparison of results and therefore, warrants future investigations in these subspecies for phylogenetic conclusion.

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Declarations of interest

None.

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