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Research article

Insights into abdominal structures and spermatozoal ultrastructure of coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae)

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Abstract

Importance of the work: The coffee berry borer, *Hypothenemus hampei* is an insect pest of coffee worldwide; however, its abdominal ultrastructure remains undescribed, despite its importance for informing targeted strategies to reduce its population and economic impact.
Objectives: To describe, using light and transmission electron microscopy (TEM), the structure of the abdominal region of *H. hampei* with a focus on the ultrastructure of the spermatozoa.
Materials and Methods: A sample of 20 mature male *H. hampei*, each with a total length of 0.5–1.2 mm, was collected and processed using TEM.
Results: The epithelial ultrastructure of the midgut comprised enterocytes and regenerative cells. Numerous mitochondria and lysosomes were observed together with well-developed rough endoplasmic reticulum in the enterocytes. Abundant lipid droplets and glycogen molecules were scattered in the cytoplasm of lipid cells, which were mostly found close to the integument. Testicular maturation was associated with the appearance of spermatids and spermatozoa. The elongated spermatozoon was composed of a head that had two main layers (an acrosomal layer and a central nucleus), a midpiece of elongated and twisted mitochondria and a tail with a typical 9 + 9 + 2 flagellar axoneme.
Main finding: The presence of uniflagellate and biflagellate forms not only sheds light on insect evolution and phylogeny but also raises questions about their functional role in the reproductive success of *H. hampei*.

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Introduction

The coffee berry borer, *Hypothenemus hampei* (Ferrari, 1867), is a small, cylindrical beetle that belongs to the subfamily Scolytinae of the family Curculionidae (Vega et al., 2015). The species is a pest of coffee plantations that causes substantial economic losses in coffee production globally (Vega et al., 2015; Damon, 2000). The females bore into coffee berries to lay eggs and the larvae feed on the coffee seeds, causing extensive damage (Damon, 2000). This pest is a major challenge for coffee growers, especially in regions where coffee is a key agricultural product. Often, integrated pest management strategies, including biological control and cultural practices, are used to manage infestations (Aristizábal et al., 2023; MorenoRamirez et al., 2024).

Studies on *H. hampei* have focused mainly on its ecology, life cycle and methods of control (Damon, 2000; Infante et al., 2014; García-Méndez et al., 2024; Moreno-Ramirez et al., 2024). However, there is a noticeable gap in understanding the detailed structure of its reproductive system, which could be a vital advance in managing its population and developing effective control methods (Jaramillo et al., 2006). Examining the ultrastructure of the abdomen, including the arrangement of internal organs and tissues, can provide valuable insights into the physiology and reproductive potential of this insect (Chapman, 2013). Furthermore, studying the morphology and ultrastructure of the spermatozoa can enhance understanding of sperm competition, fertilization mechanisms and evolutionary relationships within the Scolytinae subfamily (Dallai et al., 2016). Such insights are particularly valuable as they can inform the development of targeted control measures aimed at reducing the reproductive success of this pest (Damon, 2000; Jaramillo et al., 2006).

The current study used transmission electron microscopy (TEM) to analyze some organs in the abdominal ultrastructure of *H. hampei*, focusing specifically on the spermatozoa. The aims of this study were: 1) to describe the overall organization and ultrastructure of the *H. hampei* abdomen; 2) to examine the detailed structure of the male reproductive organs; and 3) to analyze the morphology and ultrastructure of *H. hampei* spermatozoa.

Materials and Methods

Sampled collection and tissue techniques

During January–March 2022, 20 mature male *H. hampei*, each with a total length of 0.5–1.2 mm, were collected by hand from infested coffee plants in Yala province, Thailand (6°51′20.0″ N; 100°21′41.2″ E). The sample size of 20 individuals from a single geographic area was selected as appropriate for ultrastructural analysis because TEM is a detailed and time-consuming technique, requiring high technical precision and costly reagents. Other similar studies have used comparable or even smaller sample sizes to investigate cellular and subcellular features (Wandall, 1986; Jamieson et al., 1999). Additionally, collecting individuals from one area minimized environmental variability that could affect the sperm ultrastructure, ensuring consistency and reliability in the data.

All sampled insects were euthanized using a rapid cooling shock (Wilson et al., 2009) and their abdomens were dissected in 0.1 M phosphate buffer (PB) at pH 7.2 under stereo and light microscopes (Leica 750-U; Germany). Samples were fixed overnight at 4°C in 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4), before being transferred and kept in PB for TEM study. Samples were post-fixed in 1% OsO_4 for 2 hr, dehydrated in a graded series of ethanol and embedded in a mixture of Epon-Araldite resins (Sigma Company; Darmstadt). Semi-thin sections (100 nm thick) were stained with 1% toluidine blue and then observed under the light microscope. Thin sections (80 nm) were cut and routinely stained with uranyl acetate and lead citrate, following the standard method from Rowden and Lewis (1974). Finally, cell organization and sperm ultrastructure were examined using TEM (Philips/FEI Tecnai G2 F20; FEI Co.; Eindhoven, the Netherlands).

Ethics statement

The experimental protocol was approved by the Animal Care and Use Committee, Prince of Songkla University (protocol number 2023-NAT13-08).

Results

General observation

The current observations of the abdominal region of *H. hampei* identified the large midgut, hindgut and testis among muscular and adipose tissues (Fig. 1A), all of which were surrounded by the integument (Fig. 1A). Malpighian tubules were present between the midgut wall and adipose tissue (Fig. 1B). However, the TEM observations of representative organs (midgut, adipose tissue and testis) were limited. This study described for the first time the clearly observed ultrastructures of the midgut, adipose tissue and male reproductive tissue in *H. hampei*.

Midgut

The midgut comprised three layers—the single-layered epithelium, the muscularis with prominent muscular cells and the serosa barrier (Figs. 1C and 2). The epithelium was composed of regenerative cells and enterocytes (digestive cells), as shown in Fig. 1C. The regenerative cells with microvilli were grouped between enterocytes (Fig. 1C). The nucleus of the regenerative cells was irregular with electron-dense cytoplasm containing vacuoles (Fig. 1D). Limited organelles were identified, with accumulations of mitochondria being observed in the regenerative cells (Fig. 1C). The predominant cell type on the basement membrane was low columnar enterocyte, which presented a clear organelle distribution (Figs. 1C and 2). Its oval and large nucleus contained several electron-dense nucleoli and was surrounded by the nuclear membrane (Fig. 1C). In the basal region of the columnar enterocyte, accumulations of rough endoplasmic reticulum (RER) were observed close to the oval mitochondria in the light electron-dense cytoplasm (Figs. 1C and 2). The RER was surrounded and penetrated by electron-transparent vesicles (Fig. 1E).

Adipose tissue

Several adipocytes of *H. hampei* were found along the abdominal body (Fig. 3A). Each cell contained large, slightly irregular nuclei (data not shown). The cytoplasm contained only the glycogen rosette with its light electron-dense membrane and lipid droplets among the electron-dense angular granules of the regular inner structures (Fig. 3B).

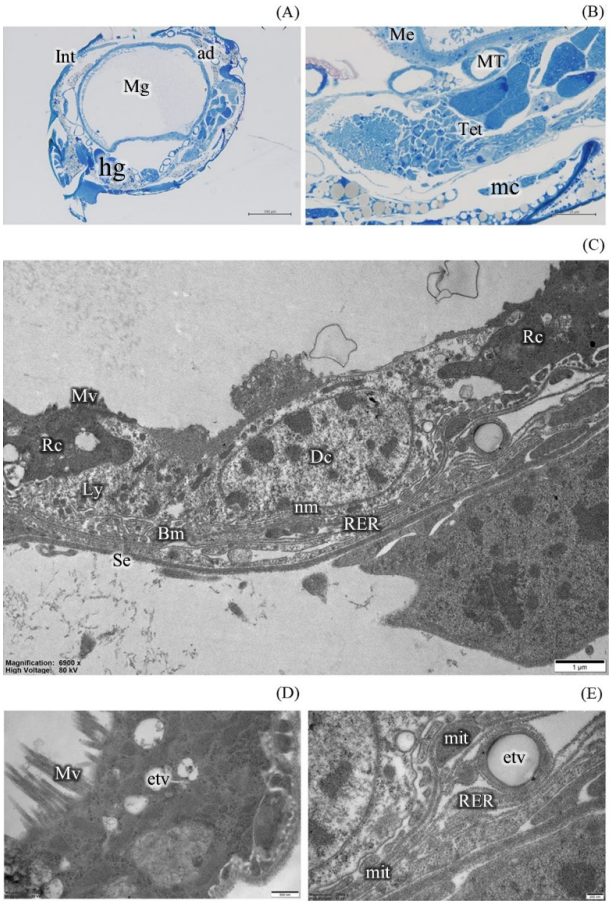

Fig. 1 Light microscopic and ultrastructure imagery of abdominal region of *Hypothenemus hampei*: (A–B) semi-thin section showing longitudinal section of large area of midgut (Mg), hindgut (hg), testicular tubule (Tet), Malpighian tubule (MT), muscular component (Mc) and adipose tissues (ad); (C) digestive cell (Dc) and regenerative cell (Rc), where ultrastructure of midgut wall has three layers (single-layered epithelium on basement membrane (Bm), muscularis with prominent muscular cell (mc) and serosa barrier (Se)); (D) prominent occurrence of electron-transparent vesicles (etv) in degenerative cell; (E) high magnification (×25,500) in enterocyte showing well-developed mitochondria (mit), rough endoplasmic reticulum (RER) and electron-transparent vesicle (etv) throughout lysosome (Ly). Int = integument; Mv = microvilli; nm = nuclear membrane.

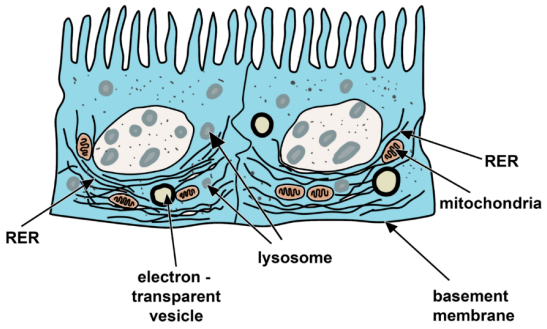

Fig. 2 Ultrastructural diagram of digestive cell in *Hypothenemus hampei*, where RER = rough endoplasmic reticulum.

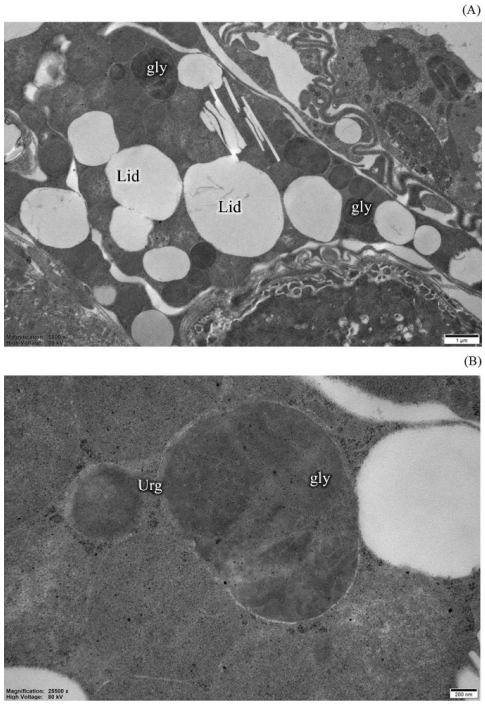

Fig. 3 Ultrastructure of adipose tissue of *Hypothenemus hampei*: (A) adipose cell with lipid droplets (Lid) and glycogen (gly); (B) high magnification revealing electron dense angular granules or ural granules (Urg) between glycogens (gly) for clearly structured, membrane-like structure of glycogen (gly).

Male reproductive tissue

The current study identified several follicles (or spermatocysts) in each testicular lobe, from which it was concluded that the maturation and the production of spermatozoa occur within these structures (Fig. 4A). The follicles were separated from each other by thin connective tissue (Fig. 4B).

The observations of the thin sections revealed that spermiogenesis was characterized by changes in the properties of the nucleus and cytoplasm. Different types of spermatids were identified in the follicle during meiotic division (Fig. 4B). Three distinct stages of spermatid development were identified: early (Est), middle (Mst) and late (Lst), as shown in Figs. 4-5. The Est spermatids were interconnected by cytoplasmic bridges and contained a large amount of cytoplasm (Fig. 4C). There was observed formation of acrosomes and flagellar regions close to the mitochondrial derivative terminates, the nebenkern (Fig. 4D).

Several aspects of the Mst spermatids were observed (Fig. 5A). In the apical region, the Mst spermatids had a compact dense area, corresponding to complex organelle composition. The nucleus was oval with a dense chromatin filament and was surrounded by a row of microtubules (Fig. 5B). The cytoplasm was reduced (Fig. 5B). In the middle area of the Mst spermatids, the mitochondrial derivatives were visible in cross-section as an elliptical shape that reached the adjacent accessory body (Figs. 5C-5D). The short axonemal regions are shown fully developed in Fig. 5D.

The Lst spermatids were observed within spermatocytes (Fig. 5E). The chromatin in the nucleus of the Lst spermatids was transformed, coalescing into dense masses (Fig. 5F); however, some cells were still visible being dispersed to a granular state (Fig. 5G). The acrosomal structure and two mitochondria were identified with dense deposited materials on the side facing the paired axoneme (Figs. 5F and 5H). Accessory bodies were formed close to the axoneme (Fig. 5H). The axoneme exhibited had a 9 + 2 pattern with 9 doublet microtubules and 2 central microtubules (Figs. 5H and 6). A cylindrical arrangement of the microtubular doublets of the flagellar axoneme revealed a backward-oriented projection from the B-subtubule (Figs. 5H and 6).

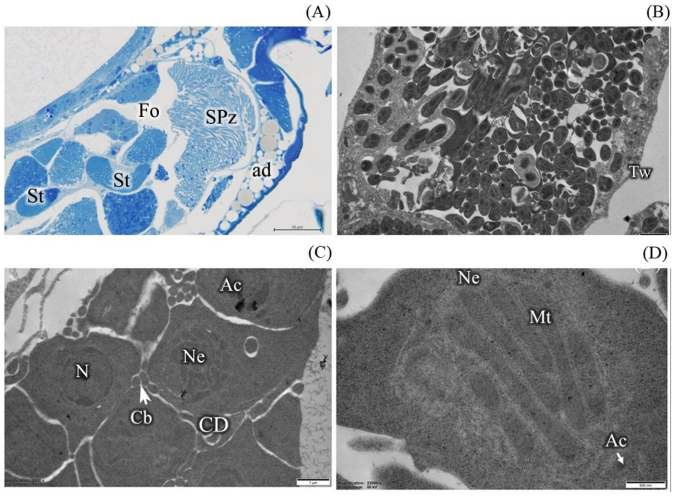

Fig. 4 Ultrastructure of testicular follicle in spermiogenesis of *Hypothenemus hampei*: (A) testicular follicle (Fo) having prominent spermatids (St) and spermatozoa (SPz); (B) several cell types in spermatocytes (or follicle) surround by the testicular wall (Tw); (C–D) initial development of acrosome-like structure (Ac) and nebenkern (Ne) having mitochondrial derivative terminates (Mt) in early spermatid, with each cell linked with cytoplasmic bridges (Cb) and cytoplasmic droplets (CD) also evident, reflecting active cytoplasmic remodeling during early spermiogenesis. ad = adipose tissue, N = nucleus.

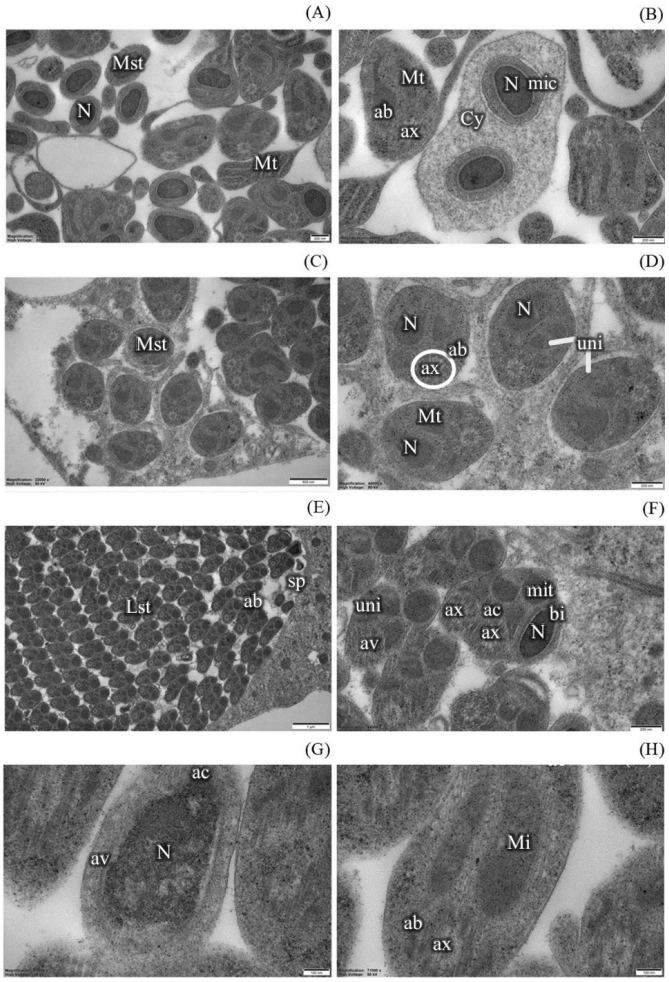

Fig. 5 Ultrastructure of middle and late spermatids of *Hypothenemus hampei*: (A) middle spermatid (Mst) revealing nuclear area (N) and mitochondria (Mt); (B) formation of axoneme (ax), accessory body (ab) and microtubule (mic) in Mst; (C–D) end stage of Est, with development of tail-like structure (asterisks) in parallel to mitochondria (Mt) close to the axoneme (ax); (E) spermatocyte (Spc) with prominent late spermatids (Lst); (F) biflagellate (bi) and uniflagellate (uni) forms; (G–H) high magnification (×71,000) revealing clear observation of acrosome (ac) in acrosomal vesicle (av) close to nucleus (N) in Lst, ax = axoneme, mic = microtubule, Cy = cytoplasm, Spc = spermatocytes, ac = acrosome, mit = mitochondrial accumulation, Mi = mitochondria.

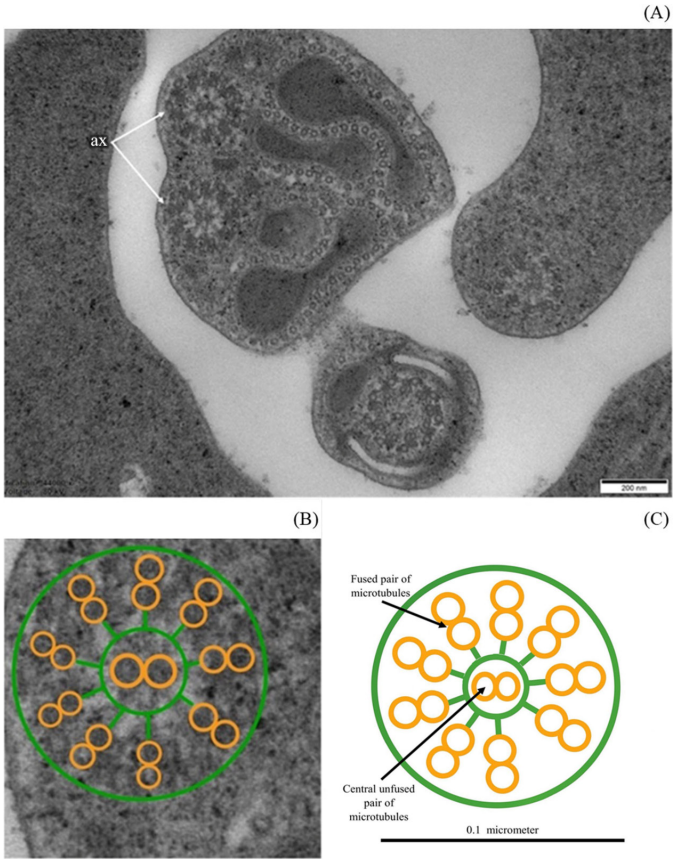

Fig. 6 Representative axoneme with nine doublet microtubules and two central microtubules in flagellar region. (A) Cross-sectional view of the flagellar axoneme of *H. hampei* spermatozoon, showing the typical 9 + 2 microtubule arrangement, consisting of nine outer doublets and two central singlets. (B) High-magnification of the axoneme highlighting detailed microtubule architecture, including outer dynein arms and radial spokes, which contribute to structural integrity and motility. (C) Longitudinal section of the flagellar axoneme illustrating the parallel alignment of microtubules along the tail axis, facilitating coordinated wave-like motion during sperm motility. ax = axoneme.

The mature sperm cysts contained several packed spermatozoa (Fig. 7A). The process of elongation was driven by a nucleus and axonemal manchette that surrounded all the organelles (Figs. 7B-7C). In the mature sperm head, the nucleus was cylindrical, containing condensed chromatin and some light granules (Fig. 5D). There was a thick pericentriolar material, corresponding to the centriole adjunct in the midpiece (Fig. 7C). The centriole, having proximal and distal centrioles, was located at an invagination of the nuclear envelope and developed by elongation to form the axoneme (Fig. 7C). Longitudinal sections of twisted mitochondria with cristae were arranged in a parallel fashion (Fig. 7E). At the tail end, the mitochondrial derivatives were no longer present and only the axoneme was visible. The axoneme consisted of nine microtubular doublets and nine accessory tubules forming the same structure as observed in the Lst spermatid (Fig. 5H).

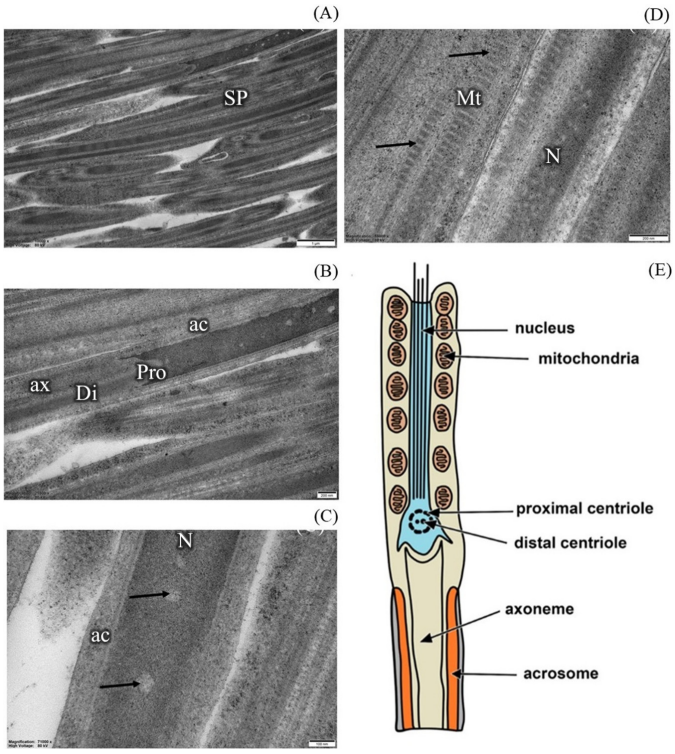

Fig. 7 Ultrastructure of spermatozoa of *Hypothenemus hampei*: (A) prominent spermatozoa (Sp); (B) diagram of spermatozoa features; (C) head regions with nucleus close to acrosome vesicle (ac), with midpiece consisting of two components (proximal (Pro) and distal (Di) centrioles) and axoneme (ax); (D) twisted (arrows) mitochondria (Mt); (E) enlarged illustrated features showing sperm ultrastructure.

Discussion

The abdominal structure of *H. hampei* has been fully observed (Alba-Alejandre et al., 2019: Alba-Tercedor et al., 2019; Rubio et al., 2008)) and the digestive tract and reproductive structure have been reported (Rubio et al., 2008). In this study, the midgut of *H. hampei* is the center of digestion, osmoregulation and immunity (Chapman, 2013). Although several defensive strategies are present in this organ, its organization and function may be disturbed by some insecticidal agents where nutrient absorption and digestion occur efficiently (Napoleão et al., 2019). The cellular composition and specific structures within these cells are tailored to support these functions. An understanding is crucial of the abundant regenerative cells in the midgut, as well as how organelles such as RER, mitochondria and lysosomes contribute to cellular function. The epithelial lining of the midgut undergoes constant renewal due to the mechanical and chemical stresses it faces from ingested food. This high turnover necessitates a substantial population of regenerative cells to continuously replace the lost or damaged cells. Besides routine turnover, the midgut must quickly repair any damage resulting from infections, toxins, or dietary irritants. Regenerative cells are essential for these repair processes, rapidly proliferating to heal injuries and restore normal function (Alba-Alejandre et al., 2019).

The adipose tissue of *H. hampei*, like other insects, contains adipocytes. It can be postulated that the presence of a large number of lipid vacuoles, together with glycogen buildup, is crucial for several functions, namely energy storage, metabolic flexibility, survival and adaptation, development and reproduction, flight and activity and thermoregulation (Arrese and Soulages, 2010; Toprak et al., 2020; Skowronek et al., 2021). In a similar manner, the presence of lipid vacuoles and glycogen in insect adipose cells provides an array of efficient energy storage systems that support various physiological processes while also increasing the ability of the insect to adapt to changing environmental conditions. However, urocytes in the fat body were not clearly observed in this beetle. In one species of termite, *Prorhinotermes simplex*, the fat body is divided into adipocyte and urocytes, which differentiates them from other insects. These specializations are due to their distinct ecological roles, dietary habits and social structures (Sobotník et al., 2006). The coffee berry borer, unlike termites, contains symbiotic microorganisms but different functions. As a result, they may lack distinct urocytes. Termites store uric acid in urocytes, which can be used by symbiotic bacteria to create nitrogenous substances that benefit the host, improving nutrient recycling and availability (Sobotník et al., 2006; Costa-Leonardo et al., 2013; Nozaki et al., 2023). In summary, while direct evidence of urocytes in the coffee berry borer is lacking, the insect likely has analogous mechanisms or cells to manage nitrogen waste and other metabolic functions. Further research is needed to confirm the presence and role of such cells in this species.

During the development of sperm, mitochondria group together to form a structure called the nebenkern or mitochondrial sheath. This grouping is crucial in transforming immature sperm cells into motile sperm capable of successful fertilization (Wu et al., 2017). The main functions of this mitochondrial clustering include energy production, cellular organization and cell stability. This structure allows sperm to use energy efficiently and maintain necessary functions for traveling to and fertilizing the egg effectively. The structure is a pivotal morphological and functional adaptation. This phenomenon, observed across various species,

is particularly evident in insect genera, such as *Drosophila*, where it has been most extensively studied (Vedelek et al., 2024). Typically, in beetles, spermatid development is divided into three distinct stages—early, middle and late spermatids—each characterized by specific morphological transformations (Wu et al., 2017; Özyurt Koçakoğlu et al., 2019). During the early stage, organelles such as mitochondria begin reorganizing, forming the nebenkern. In the middle stage, elongation of the spermatid occurs, along with chromatin condensation and further structural refinement. The late stage involves the final maturation of the sperm, including the formation of the flagellum and the stabilization of mitochondrial architecture (O'Donnell et al., 2012). These staged transformations are critical for producing functionally competent sperm. Sperm motility requires a substantial amount of energy, primarily in the form of ATP. The nebenkern ensures that mitochondria are positioned strategically to meet these high energy demands. By clustering together, mitochondria can manage and supply energy more efficiently where it is most needed, particularly to the flagellum of the sperm, which drives motility. The mitochondrial sheath provides mechanical support to the developing sperm tail, protecting the mitochondrial DNA from potential damage during transit and ensuring longevity and functionality (Köckert et al., 2023). The nebenkern is not just a simple aggregation of mitochondria but a highly organized and dynamic entity that play a pivotal role in sperm structure and function.

The present study of *H. hampei* used TEM to provide a comprehensive analysis of the abdominal ultrastructure and the morphology of spermatozoa. The findings revealed novel insights into the reproductive system of this economically damaging pest species and should contribute to understanding the evolution of reproductive structures in the Coleoptera. By examining the spermatozoa's structural peculiarities and the abdominal glandular tissues, this study has contributed to the broader understanding of reproductive biology in beetles, offering insights into potential pest control measures based on reproductive disruption (Moreno-Ramirez et al., 2024). In addition, the detailed ultrastructural information from this study should serve as a benchmark for future research on pest management strategies and reproductive evolutionary patterns in economically important insect species (Özyurt Koçakoğlu et al., 2021).

The general organization of the *H. hampei* abdomen aligns with the typical coleopteran plan (Chapman, 2013) but with several unique adaptations. The compact arrangement of internal organs, particularly the reproductive system, likely reflects the small size of the species and its adaptation to living within coffee berries. This efficient use of abdominal space may contribute to the reproductive success of this insect in its specific niche (Vega et al., 2015).

The spermatozoa of *H. hampei* have several notable features. The helical shape of the spermatozoa is a common feature among insect species and is thought to facilitate sperm motility and storage (Baccetti, 1972; Alberti and Coons, 1999). The TEM observations revealed a unique morphology characterized by an elongated nucleus, a well-developed and elongated acrosome and a flat acrosomal cap. These features of *H. hampei* spermatozoa are similar to those described in other Coleoptera species, such as *Drusilla canaliculata* (Fabricius, 1787) reported by Werner et al. (2007) and *Rhynchophorus ferrugineus* (Olivier, 1790) mentioned in Alzahrani (2013), indicating a conserved pattern of spermatozoa morphology within this insect order. The morphological characteristics of the spermatozoa have been used as taxonomic and phylogenetic markers in various insect groups (Jamieson et al., 1999). The similarities in spermatozoa ultrastructure between *H. hampei* and other Coleoptera species suggest a close evolutionary relationship within this diverse insect order (Dallai et al., 2016). However, further comparative studies are needed, including a wider range of species from different families and subfamilies, are needed to elucidate the phylogenetic importance of spermatozoa morphology in the Coleoptera.

One of the most striking findings was the presence of both biflagellate and uniflagellate sperm. This dimorphism in sperm morphology is relatively rare in insects and warrants careful consideration. The occurrence of both biflagellate and uniflagellate sperm in *H. hampei* is notable and raises questions about the functional importance of this dimorphism.

The discovery of this dimorphism across a sample of 20 insects from a single location is notable, as it points to a possible adaptive strategy for maximizing reproductive success. One possibility is that the biflagellate sperm may be advantageous for navigating the complex female reproductive tract, while the uniflagellate sperm could offer an energy-efficient alternative, potentially increasing the chances of fertilization under certain conditions (Tsuji and Fukami., 2020). Furthermore, this dual strategy could be related to reproductive behavior and competitive dynamics within the species. The coexistence of both sperm types may reflect a trade-off between speed and energy efficiency, which could be crucial in environments where both fertilization success and reproductive investment are highly variable (Simmon, 2014).

In addition, this dimorphism could be linked to the specific ecological niche occupied by *H. hampei*, as the species is known to thrive within the coffee berry, a confined and resource-limited environment (Sobczyk, et al., 2020). From a pest management perspective, understanding these unique reproductive traits offers valuable insight for developing targeted control strategies. For example, disrupting sperm development or function—particularly one morphotype—could reduce fertility rates. In addition, these findings have potential for informing sterile insect techniques, contributing to sustainable management of this economically important pest.

In *H. hampei*, the uniflagellate sperm coexists with a biflagellate form. This finding aligned with the general pattern observed in the Coleoptera and most other insect orders (Jamieson, 1987; Wolf, 1997; Dallai et al., 2001; Dallai et al., 2016). The presence of biflagellate sperm is more commonly associated with primitive arthropods and some crustaceans (Jamieson et al., 1999). The representation of biflagellate sperm in *H. hampei* could be interpreted as a plesiomorphic character, potentially providing insights into the evolutionary history of Scolytinae within Curculionidae (Dallai et al., 2016). The presence of both sperm types in *H. hampei* may represent a transitional evolutionary state. It could indicate ongoing evolution from a biflagellate ancestral state to a uniflagellate state, which is more common in advanced insects. Alternatively, it might represent a derived condition that has evolved in response to specific selective pressures in the unique ecological niche chosen by this species (Kirkendall et al., 2015).

One possible explanation for the presence of biflagellate sperm is secondary fusion of cells during gonial mitosis. A sophisticated system of cytoplasmic bridges connects the germ cells throughout spermatogenesis (Wolf, 1997). Occasionally, cytoplasmic bridges enlarge, allowing cells to merge. There is also the possibility of supernumerary chromosomes (B chromosomes) which may improve the likelihood of cell fusion during spermatogenesis (Suja et al., 1987; Teruel et al., 2009).

The diversity of sperm morphology in insects, notably the presence of uniflagellate and biflagellate forms, is an intriguing component of insect reproductive biology. This diversity not only sheds light on insect evolution and phylogeny, but it also raises questions concerning the functional role of sperm shape in reproductive success and speciation. Using electron microscopy, the current study has reported for the first time the clearly observed ultrastructures of the midgut, adipose tissue and male reproductive tissue in *H. hampei*. While other research has explored broader aspects of this species' biology, there has been a lack of detailed ultrastructural data for these internal tissues. The current findings should provide a valuable foundation for future comparative and functional studies, which are essential for fully elucidating the evolutionary relevance and practical implications of the structural traits identified.

Understanding the ultrastructural organization of internal tissues in *H. hampei*—particularly those related to digestion, metabolism and reproduction—opens new avenues for targeted pest control strategies. Detailed knowledge of the midgut and adipose tissue architecture may inform the development of insecticides or biological agents that disrupt nutrient absorption or energy storage, while insights into the male reproductive structures could support novel approaches such as sterilization-based methods or sperm-disruption technologies. By linking form to function, this study lays the groundwork for precision-targeted interventions that are more efficient and potentially less harmful to non-target species, contributing to the advancement of sustainable pest management programs for this economically important coffee pest.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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