**UNIVERSIDADE FEDERAL DE VIÇOSA**

**PAULO HENRIQUE REZENDE**

# MORPHOLOGY OF THE MALE REPRODUCTIVE SYSTEM AND SPERM IN TINGIDAE (HEMIPTERA)

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# MORPHOLOGY OF THE MALE REPRODUCTIVE SYSTEM AND SPERM IN TINGIDAE (HEMIPTERA)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

Orientador: José Lino Neto

Coorientadores: Glenda Dias
Pietro Lupetti
Romano Dallai

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ASSENTIMENTO:

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Autor

José Lino Neto

Orientador

*Em memória de Laerte Mulinacci,*

*Imperador que ergueu monumentos eternos em meu coração.*

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## RESUMO

REZENDE, Paulo Henrique, D.Sc, Universidade Federal de Viçosa, março de 2024. Morfologia espermática e reprodutiva de Tingidae (Hemiptera). Orientador: José Lino Neto. Coorientadores: Glenda Dias, Pietro Lupetti e Romano Dallai.

Tingidae é uma família de Heteroptera que apresenta padrões rendados no pronoto e hemiélitros, o que lhes dá o nome popular de percevejos de renda. Eles são exclusivamente fitófagos, e muitas espécies são pragas agrícolas. Apesar de sua importância, o grupo tem sido pouco estudado nos últimos anos. Esta tese focou nesta família para abordar duas questões principais: Quão confiável é uma abordagem morfométrica espermatica como uma ferramenta taxonômica ao analisar 16 espécies de uma só vez? E, o que os aspectos ultraestruturais de seus espermatozoides podem nos dizer sobre as relações entre Miroidea e Heteroptera? Dados morfológicos sobre o sistema reprodutivo também foram descritos. A análise morfométrica provou ser útil na distinção de espécies e mostrou ser uma excelente ferramenta taxonômica complementar. Além disso, por meio da análise ultraestrutural de espermatozoides, os Tingidae se assemelharam ao padrão geral encontrado em Heteroptera, corroborando as sinapomorfias do grupo. Entretanto, algumas peculiaridades foram observadas quanto à formação do adjunto de centríolo, que se estende no eixo anteroposterior das espermátides, flanqueando o núcleo. Também confirmou achados anteriores para sua família irmã, Miridae. No geral, esses resutados contribuem para uma melhor compreensão das características reprodutivas de Tingidae, expandem os dados da família e ajudam a delinear cenários evolutivos para tais características e implicações filogenéticas dentro da subordem Heteroptera.



Palavras-chave: Cimicomorpha. Miroidea. Espermatologia. Espemiotaxonomia.

**ABSTRACT**

REZENDE, Paulo Henrique, D.Sc, Universidade Federal de Viçosa, March of 2024. Sperm and reproductive morphology of Tingidae (Hemiptera). Advisor: José Lino Neto. Co-Advisors: Glenda Dias, Pietro Lupetti and Romano Dallai.

Tingidae is a family of Heteroptera that presents lace patterns on the pronotum and hemielytra, which gives them the popular name Lace Bugs. They are exclusively phytophagous, and many species are agricultural pests. Despite their importance, the group has been left underappreciated in recent years. This thesis focused on this family to address two main questions: How reliable is a sperm morphometrical approach as a taxonomical tool when analyzing 16 species at once? What can the ultrastructural aspects of their sperm tell us about the relationships among the Miroidea and Heteroptera? Morphological data on the reproductive system was also described. The morphometric analysis proved helpful in distinguishing species and was shown to be an excellent complementary taxonomic tool. Furthermore, through ultrastructural sperm analysis, the Tingidae resembled the general pattern found in Heteroptera, corroborating the group's synapomorphies. However, some peculiarities were observed regarding the formation of the centriole adjunct, which extends in the anteroposterior axis of the spermatids, flanking the nucleus. It also confirmed previous findings for their sister family, Miridae. Overall, these findings contribute to a better understanding of Tingidae's reproductive characteristics, expand the family's data, and help outline evolutionary scenarios for such characteristics and phylogenetic implications within the suborder Heteroptera.

Keywords: Cimicomorpha. Miroidea. Spermatology. Spemiotaxonomy.

**SUMMARY**

# 1. GENERAL INTRODUCTION

## 1.1. Tingidae (Heteroptera)

Tingidae, or lace bugs, are a family of insects characterized by areoles that adorn their hemihelytra, giving them a lace-like appearance (Schuh & Slater, 1995). The family consists of approximately 2,600 species, subdivided into three subfamilies: Tinginae (the largest), Cantacaderinae, and Vianaidinae, the latter exclusive to the Neotropical region (Guidoti, Montemayor, et al., 2015; Schuh et al., 2006). Tingidae, Miridae, and Thaumastocoridae form the superfamily Miroidea within the infraorder Cimicomorpha (Schuh & Štys, 1991). However, Thaumastocoridae as a member of Cimicomorpha, is not supported in most analyses, indicating that this family is closer to Pentatomomorpha (Schuh et al., 2009).

Lace bugs are exclusively phytophagous and have specific hosts, but they can feed on plants from different families (Drake & Ruhoff, 1965; Guidoti, Montemayor, et al., 2015). They complete their life cycle on the same plant species, so this association is important for ecological and behavioral studies and their identification. Many species feed on cultivated plants, such as cassava, passion fruit, castor oil plant, and cotton (Bellon et al., 2012; Bellotti et al., 1999; Coelho & Da-Silva, 2015; Guidoti, Montemayor, et al., 2015; Varón et al., 2010), thus having an economical impact.

They exhibit gregarious behavior, preferring the abaxial surface of leaves, where they cause chlorosis by feeding on the sap, but they can also feed on the upper part of roots (Guidoti, Montemayor, et al., 2015). Females of some species exhibit parental care (a rare behavior among Cimicomorpha), remaining with the offspring for some time after the emergence of the nymphs (Guidoti, Tallamy et al., 2015). Another unusual behavior is the formation of galls by species of *Copium* and *Paracopium* (Drake & Ruhoff, 1965).

## 1.2. Morphology of the male reproductive system in Heteroptera

Despite their diversity and importance to humans, information related to the biology of numerous groups of Heteroptera has not yet been described,

especially regarding the morphology of reproductive organs and sperm biology. Among the Cimicomorpha, only a few species of Reduviidae, Cimicidae, Anthocoridae, Thaumastocoridae, Tingidae and Miridae have publications regarding the morphology of their reproductive organs (Eguagie, 1976; Horton & Lewis, 2011; Javahery, 2019; Mróz & Wojciechowski, 2011; Pendergrast, 1957; Rezende et al., 2021; Takeda et al., 2019).

Pendergrast (1957) was the first to compile information on the morphology of the reproductive apparatus of Heteroptera families in order to identify morphological patterns and evolutionary relationships among taxa. More recently, Grozeva et al. (2022) updated the list, including data published since then. In general, males of Cimicomorpha, like other Heteroptera, have a pair of testes connected to vas deferens, zero to two pairs of accessory glands, an ejaculatory duct, and, in some species, an ejaculatory bulb (Grozeva et al., 2022; Pendergrast, 1957). The number of testicular follicles also varies from one to nine, with seven being the most frequent. Furthermore, this characteristic tends to be constant in the subfamily or genus (Gomes et al., 2013; Gonçalves et al., 1987; Grozeva et al., 2022; Leston, 1961; Mróz & Wojciechowski, 2011; Munhoz et al., 2021; Rezende et al., 2021; Vélez et al., 2020). Such data have taxonomic and phylogenetic value, in addition to reflecting the reproductive dynamics of a given taxon.

### 1.3. Sperm morphology in Cimicomorpha (Heteroptera)

Sperm are highly specialized and diverse cells among insects, varying in size, shape, number of structures, and ultrastructural organization (Jamieson et al., 1999; Phillips, 1970). In Heteroptera, existing studies describe the ultrastructure of sperm by observing well-conserved characters and support the monophyly of the group (Araújo et al., 2011, 2012; Dallai & Afzelius, 1980, 1982; Dias et al., 2016; Itaya et al., 1980; Jamieson et al., 1999; Lee, 1985; Lee & Lee, 1992). These cells have a head containing an acrosome and a nucleus, a centriole adjunct that connects the head to the flagellum elements. The latter is composed of a long axoneme partially surrounded by two generally symmetrical mitochondrial derivatives anchored to the axoneme microtubules by two bridges, which help coordinate the beating of the flagellum during sperm movement

(Mercati et al., 2009). Furthermore, each mitochondrial derivative contains two to three paracrystalline inclusions (Dallai & Afzelius, 1982; Dolder, 1988; Jamieson et al., 1999). Likewise, sperm commonly exhibit specific morphometric characteristics, aiding the taxonomic identification and association of highly dimorphic conspecifics in different groups of animals (Barcellos et al., 2015; Baffa et al., 2017; Cursino & Duarte, 2016; Pereira et al., 2008; Phillips, 1970), although, sperm morphometry as a taxonomical tool needs to be further cohobored and explored.

Among the Cimicomorpha, publications on sperm morphology comprehend the Reduviidae (Baffa et al., 2017; Báo & De Souza, 1994), Cimicidae (Dallai & Afzelius, 1980), Anthocoridae (Santos & Lino-Neto, 2018), Miridae (Rezende et al., 2023), four out of sixteen families. Thus, considering the lack of data regarding the anatomy of the male reproductive system and sperm morphology of several groups of Cimicomorpha and their usefulness for taxonomy and phylogenetics, the objective of this thesis was to describe the morphology of the male reproductive system and the sperm of Tingidae. This particular family has been underrepresented in recent years and, from the data obtained here, would help us infer phylogenetic relationships and from sperm morphometry, help in the identification and discrimination of Tingidae species with an integrative taxonomic approach, especially considering that there are few morphological descriptions of the genitalia for the family, as in the past it was said to have little taxonomic value (Guidoti, Montemayor, et al., 2015), as for the bibliography with identification keys for the species is dispersed and very old in some cases (Bisson et al., 2003; Drake & Ruhoff, 1965; Gibson, 1919; Guidoti, Montemayor, et al., 2015; Knudson, 2018; Krishnankutty & Scher, 2020), making the results presented here even more helpful.

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## 2. CHAPTER I

# MORPHOLOGY OF THE SPERM AND MALE REPRODUCTIVE SYSTEM OF LACE BUGS (TINGIDAE: HETEROPTERA)

Paulo Henrique Rezende, Dayvson Ayala Costa, José Lino-Neto.

**Male reproductive morphology and sperm morphometric analysis of lace bugs (Tingidae: Heteroptera) and applications to taxonomy**

Paulo Henrique Rezende, Dayvson Ayala Costa, José Lino-Neto.

## ABSTRACT

In this paper, we described the male reproductive apparatus and provided sperm measurements for 16 species of nine genera of Tingidae. We have conducted a sperm morphometry analysis to determine if this approach could be used as a valid taxonomical tool to distinguish species. While the spermatozoa of Tingidae showed common morphology among insects, being elongated and slender, some species had unique features, such as a long acrosome in the *Gargaphia* species (up to 30 µm). The morphometric analysis could distinguish all species, especially those in the same genus, and can be used as a complementary taxonomic tool. The tingid male apparatus exhibited one or two testicular follicles, a pair of seminal vesicles, and one pair of long tubular accessory glands. A second pair of glands was present in one species and was short and round. Also, specific traits in their morphology, such as the number and shape of follicles, length of the glands, their shape, and how they fold, made very clear differences among species, especially when combined with sperm morphometry.

Keywords: Internal morphology. Spermatozoa. Hemiptera. Cimicomorpha. Integrative Taxonomy.

## 2.1. INTRODUCTION

Spermatozoa are an inherent part of sexual reproduction. They show an enormous morphological diversity as a response to sexual selection, especially sperm competition and cryptic female choice (Kahrl et al., 2021; Pitnick et al., 2009). This variation lies in many aspects, such as ultrastructural organization, the formation of sperm conjugations, polymorphism, atypical sperm types, and variations in sperm size, among other features (Dallai, 2014). Arthropods present the largest variation in sperm size, ranging up to six orders of magnitude (Fitzpatrick et al., 2022; Pitnick et al., 2009). Insects, the largest and most diverse clade, are responsible for much of this variation, with species showing extremely short sperm and also the longest sperm known to date - less than 7 µm in the wasp *Cotesia congregate*, and nearly 6 cm in the fruit fly *Drosophila bifurca* (Pitnick et al., 1995; Uzbekov et al., 2017).

Considering the conjunct of traits and high specificity, we are led to agree with Jamieson (Jamieson, 1987 in Pitnick et al., 2009), who said that "it is, at least in principle, possible to determine from the examination of a single sperm cell the phylum, order, family, genus, and species of the male from which the cell has come." However, such a thing is not as simple and must be looked at carefully, and "in practice, we suspect such statements will prove more true of some taxa than of others" Pitnick (2009). Several works have demonstrated the applicability of sperm ultrastructural traits for phylogenetic inference (e.g.: Dallai, 2014; Dallai et al., 2014; Dallai & Afzelius, 1980; Gottardo et al., 2016; Jamieson et al., 1999). Moreover, some authors have also applied the sperm morphometric specificity for taxonomical purposes, aiding the identification and association of sexes in highly dimorphic conspecifics (Baffa et al., 2017; Barcellos et al., 2015; Cursino & Duarte, 2016; Pereira et al., 2008; Phillips, 1970), although, these studies dealt with a limited number of species in the same test, thus requiring more support. So, in order to analyze this approach as a viable tool for integrative taxonomy, we have chosen to study the lace bugs (Tingidae), an underemphasized group in recent works.

Tingidae (Heteroptera) is a family of small insects common in the Neotropics, comprising around 2,600 species, out of which nearly 2,500 species belong to the subfamily Tinginae (Guidoti, Montemayor et al., 2015; *ITIS*, 2023).

They are phytophagous, and many species have agricultural importance for being pests of cassava, eggplant, passion fruit, castor bean, cotton, and rubber trees (Bellon et al., 2012; Bellotti et al., 1999; Coelho & Da-Silva, 2015; Guidoti, Montemayor, et al., 2015; Varón et al., 2010). We have found several species during field incursions and noted only a few works on their reproductive morphology (Javahery, 2019; Pendergrast, 1957) and one publication concerning their sperm ultrastructure by the present author (Rezende et al., 2024). So, here we describe the anatomy of the male reproductive apparatus of several species alongside the sperm morphometry analysis.

## 2.2. MATERIALS AND METHODS

This work was conducted at the Departamento de Biologia Geral at the Universidade Federal (UFV) de Viçosa, MG - Brazil. The lace bugs were collected between November 2019 and October 2022 at the UFV campus, Mata do Paraiso, Viçosa - MG, and in Diamantina municipality, MG - Brazil.

We collected and analyzed 16 lace bug species from nine genera, all belonging to the tribe Tingini (Tinginae). Listed below are the species identified to the lowest taxonomic level possible, where they were found, and their host plant (as available). We have used identification keys from the following works: Bisson et al., 2003; Drake & Ruhoff, 1965; Gibson, 1919; Guidoti, Montemayor, et al., 2015; Knudson, 2018; Krishnankutty & Scher, 2020.

- *Gargaphia munda* Drake & Hambleton, 1938 (n=1) (Fig. 6A-C – supplementary material). Viçosa, MG – Brazil (20°45'09.9"S 42°52'15.7"W). Host plant not available.
- *Gargaphia paula* A Drake, 1939 (n=5) (Fig. 6D-F – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant not available.
- *Gargaphia paula* B Drake, 1939 (n=5) (Fig. 6G-I – supplementary material). Viçosa, MG – Brazil (20°45'38.5"S 42°52'03.9"W). Host plants: *Arachis sp* (Fabaceae) and *Passiflora edulis* (Passifloraceae).
- *Gargaphia brunfelsiae* Monte, 1938 (n=4) (Fig. 7A-C – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant not available.

- *Gargaphia* sp1 (n=5) (Fig. 7D-F – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant not available.
- *Gargaphia* sp2 (n=5) (Fig. 7G-I – supplementary material). Viçosa, MG – Brazil (20°45'53.2"S 42°52'13.3"W). Host plant not available.
- *Pliobyrsa* sp1 (n=5) (Fig. 8A-C – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant: Rubiaceae.
- *Pliobyrsa* sp2 (n=3) (Fig. 8D-F – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant: Rubiaceae.
- *Leptodictya* sp1 (n=5) (Fig. 8G-I – supplementary material). Viçosa, MG – Brazil (20°48'05.7"S 42°51'58.7"W). Host plant: Bamboo (Poaceae).
- *Vatiga illudens* Drake, 1922 (n=5) (Fig. 9A-C – supplementary material). Viçosa, MG – Brazil (20°45'40.1"S 42°51'56.4"W). Host plant: *Bauhinia sp.* (Fabaceae).
- *Atheas fuscipes* Champion, 1898 (n=5) (Fig. 9D-F – supplementary material). Viçosa, MG – Brazil (20°45'38.5"S 42°52'03.9"W). Host plant: *Arachis pintoi* (Fabaceae).
- *Corythaica misionera* Ajmat 2000 (n=3) (Fig. 9G-I – supplementary material). Diamantina, MG – Brazil. Host plant: Solanaceae.
- *Corythaica leprosa* Montemayor & Melo 2012 (n=1) (Fig. 10A-C – supplementary material). Viçosa, MG – Brazil (20°45'32.0"S 42°52'13.3"W). Host plant not available.
- *Pleseobyrsa* sp (n=3) (Fig. 10D-F – supplementary material). Diamantina, MG – Brazil. Host plant: Araceae.
- *Teleonemia scrupulosa* Stål, 1873 (n=5) (Fig. 10G-I – supplementary material). Viçosa, MG – Brazil (20°45'35.2"S 42°51'41.4"W). Host plant: *Lantana camara* (Verbenaceae).
- *Leptopharsa* sp (n=4) (Fig. 11A-C – supplementary material). Viçosa, MG – Brazil (20°45'33.7"S 42°51'46.9"W). Host plant: Rubiaceae.

### 2.2.1. Light microscopy of the Spermatozoa

Drops of sperm suspension, extracted from seminal vesicles of adult individuals, were spread on histological slides in 0.1 M sodium phosphate buffer,

pH 7.2, and fixed with 4% paraformaldehyde for 15-20 minutes at room temperature. Then the slides were washed in running water and dried at room temperature. To measure sperm, part of these preparations was stained with Giemsa, washed in running water, and dried at room temperature. Photo documentation was carried out in a photomicroscope with a digital camera. For the nuclei length, some preparations were stained with DAPI (4,6-diamino-2-phenylindole) 0.2 µg/ml in PBS buffer for 20 minutes, washed in running water, dried at room temperature, and mounted with 50% sucrose. These slides were photographed under an epifluorescence microscope equipped with a BP 360-370 nm filter. All measurements were made using the image analysis program *Sperm-Sizer-1.6.6* (McDiarmid et al., 2021).

### 2.2.2. Morphometric analysis

We analysed one to three males from each species and measured at least 20 spermatozoa of each individual. The parameters examined were the lengths of nuclei, whole sperm, and acrosomes. We conducted a multivariate analysis test (MANOVA) to check for differences when analysing all data together. We proceed with a Kruskal-Wallis one-way analysis for each data group and a Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons between species. All analyses were made using the free software jamovi, version 2.3 (Jamovi, 2022).

## 2.3. RESULTS

### 2.3.1. Sperm Morphology

The lace bugs spermatozoa are elongated and filiform (Fig. 1-3). Under conventional light microscopy, it was not possible to differentiate the head and tail portions. The nucleus and acrosome were only distinguished through fluorescent staining (DAPI). The longest sperm was observed in *C. leprosa* (around 360 µm; Fig. 3A), while *Leptopharsa* sp. had the shortest (around 220 µm; Fig. 3G) (see Table 1 for complete information). The *Gargaphia* species had an extremely long acrosome, close to or longer than the nucleus, and ranging

from 14 µm in *G. munda* (Fig. 1A), to 29 µm in *G. paula* (Fig. 1D-F). It was also long in *Atheas fuscipes* (Fig. 2J), close to 21 µm (65% of the nuclear length). For the remaining species it was about half the nucleus length and ranged between 6 to 10 µm.

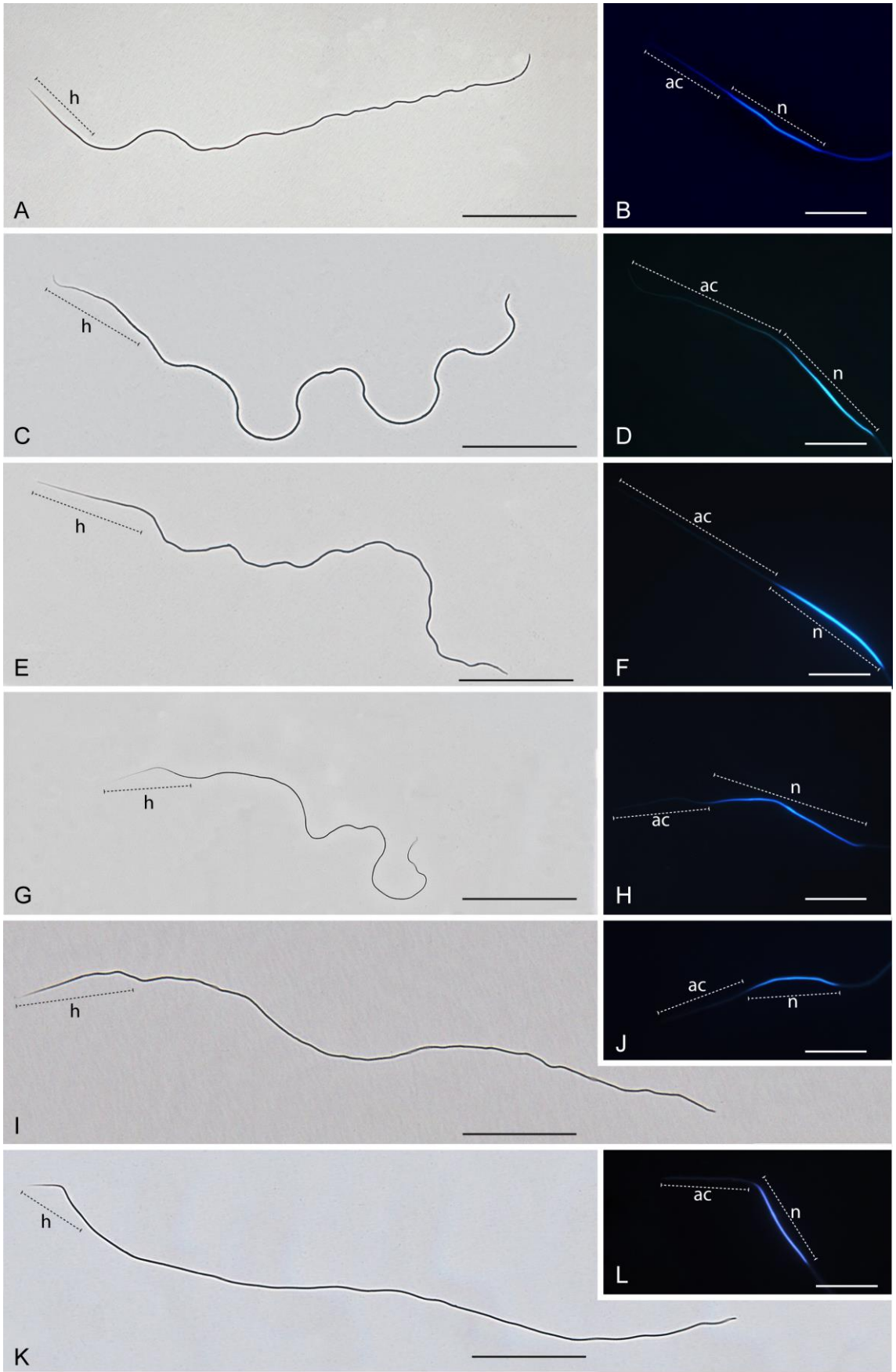



**Figure 1**. Tingidae sperm under light microscopy. A-B: *Gargaphia munda*; C-D: *G. paula* A; E-F: *G. paula* B; G-H: *G. brunfelsiae*; I-J: *Gargaphia* sp1; K-L: *Gargaphia* sp2. **h**: sperm head, **ac**: acrosome, **n**: nucleus. Scale bars: black= 50 µm, white= 10 µm.

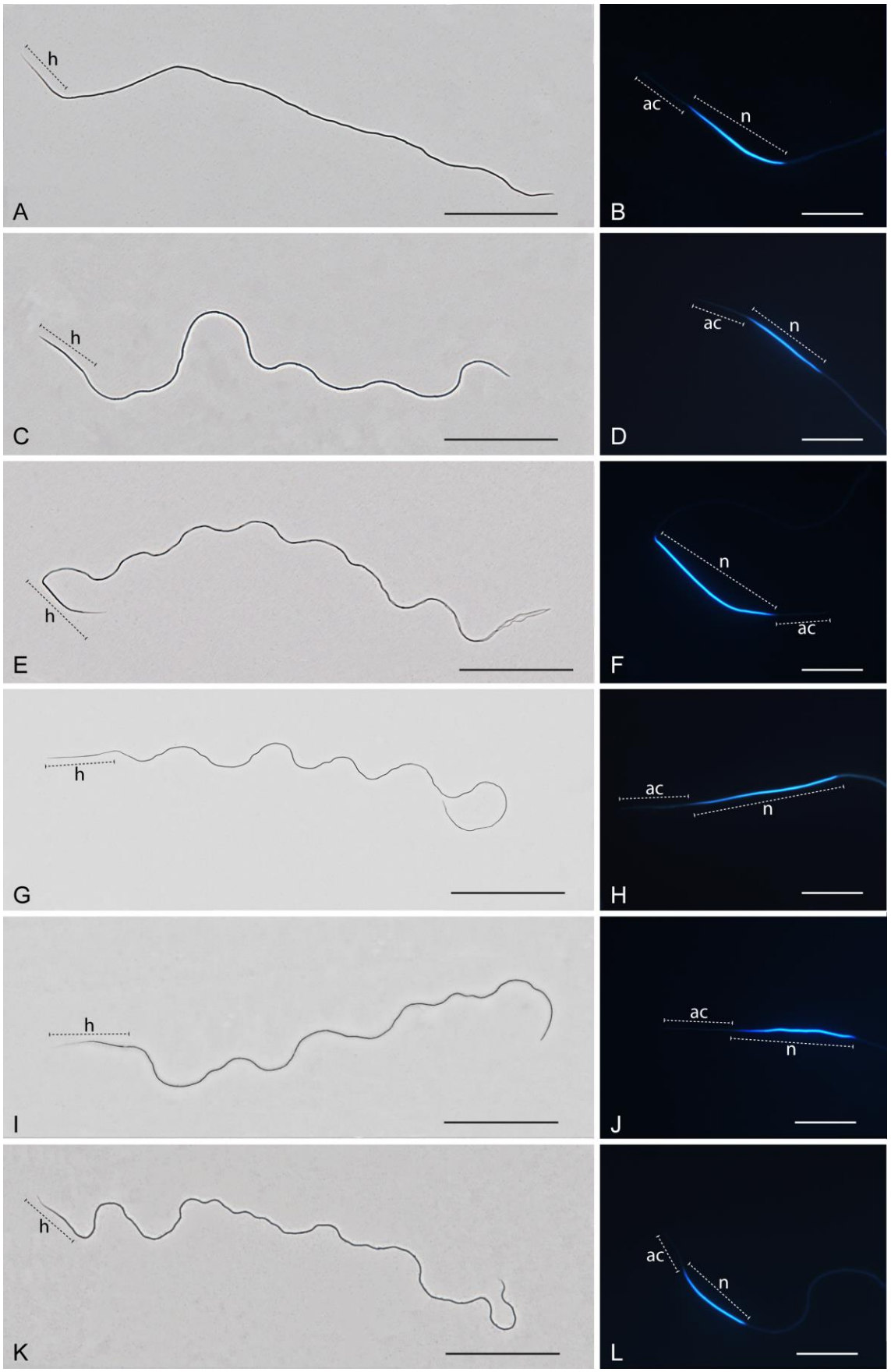



**Figure 2**. Tingidae sperm under light microscopy. A-B: • *Pliobyrsa* sp1; C-D: • *Pliobyrsa* sp2; E-F: *Leptodictya* sp1; G-H: *Vatiga illudens*; I-J: *Atheas fuscipes*; K-L: *Corythaica misionera.* **h**: sperm head, **ac**: acrosome, **n**: nucleus. Scale bars: black= 50 µm, white= 10 µm.

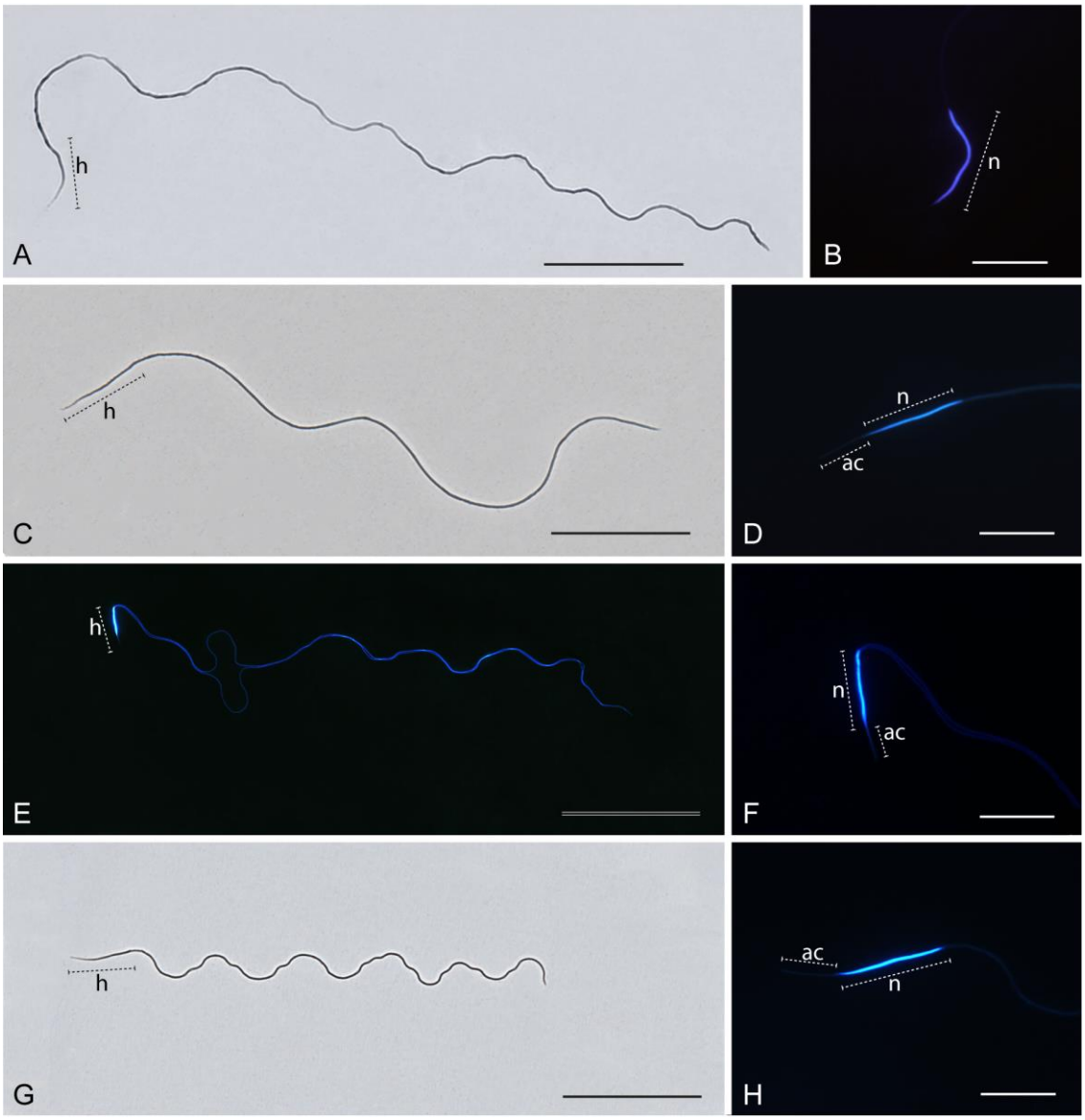



**Figure 3.** Tingidae sperm under light microscopy. A-B: *Corythaica leprosa*; C-D: *Pleseobyrsa* sp; E-F: *Teleonemia scrupulosa*; G-H: *Leptopharsa sp.* **h**: sperm head, **ac**: acrosome, **n**: nucleus. Scale bars: black= 50 µm, white= 10 µm.

### 2.3.2. Morphometric analyses

The MANOVA test considering the three variables together showed significant differences among species ($F=451$; $p<0.001$). The Kruskal-Wallis tests also showed differences for each measurement alone: Total length ($F=351$; $p<0.001$), nucleus ($F=821$; $p<0.001$) and acrosome ($F=653$; $p<0.001$). The pairwise comparisons for total length showed it was possible to distinguish most species, except the following: *G. munda* from *Pliobyrsa* sp1 ($W=1.12$; $p=1$), *G. munda* from *Pleseobyrsa* sp ($W=-0.516$; $p=1$), *G. paula* A from *C. misionera*

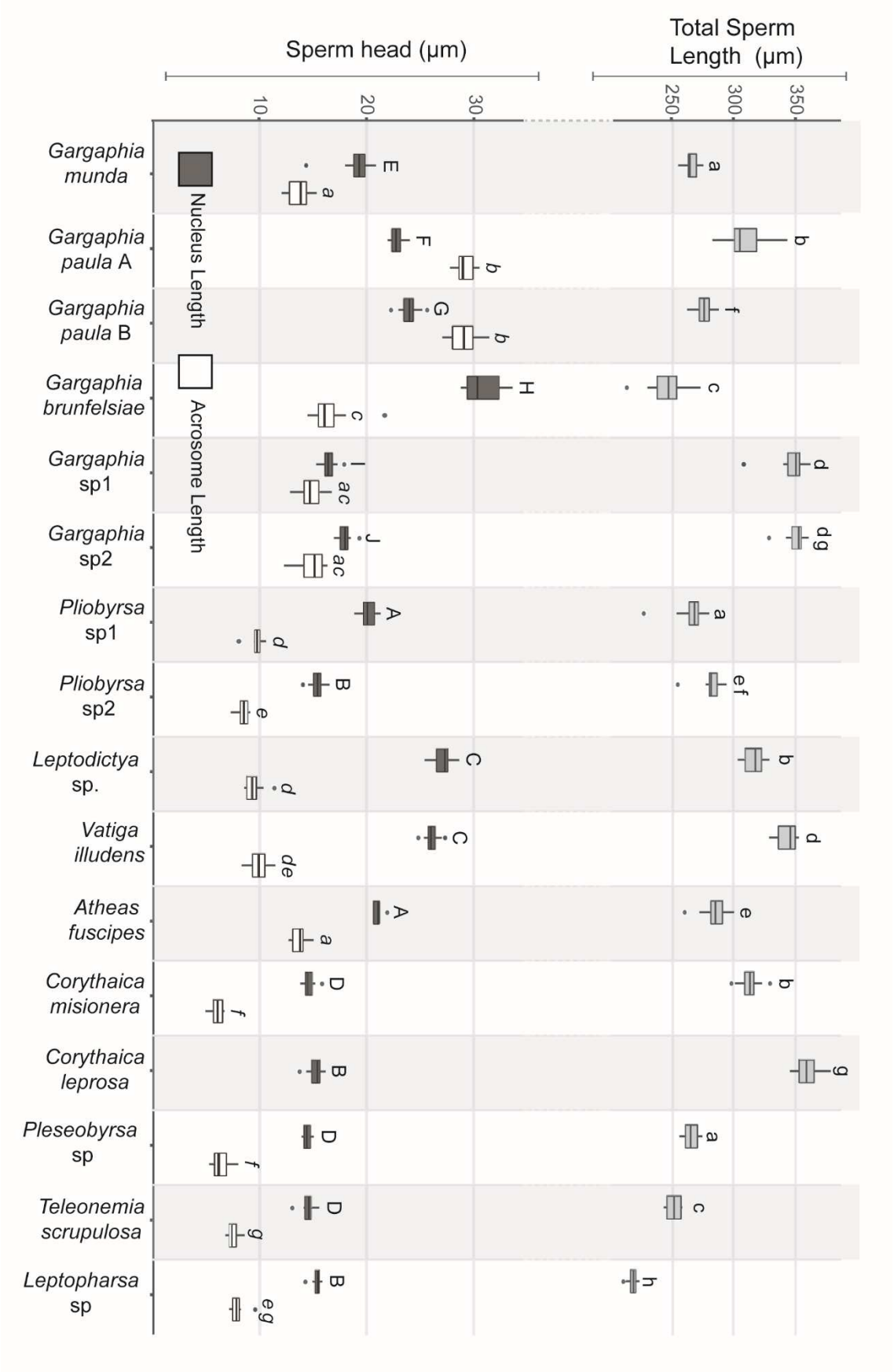
(W=1.886; p=0.995), *G. paula* A from *Leptodictya* sp. (W=3.476; p=0.504), *G. brunfelsiae* from *T. scrupulosa* (W=2.174; p=0.978), *Pliobyrsa* sp1 from *Pleseobyrsa* sp (W=-1.665; p=0.999), *Gargaphia sp1* from *V. illudens* (W=-2.858; p=0.814), *Gargaphia* sp1 from *Gargaphia* sp2 (W=1.523; p=1), *Pliobyrsa* sp2 from *A. fuscipes* (W=1.98; p=0.991), *Pliobyrsa* sp2 from *G. paula* B (W=-4.563; p=0.092), *V. illudens* from *Gargaphia* sp2 (W=3.857; p=0.313), *C. misionera* from *Pliobyrsa* sp1 (W=2.003; p=0.99), and *C. leprosa* from *Gargaphia* sp2 (W=-4.964; p=0.07) (Graph 1; See Table 2 in the Supplementary Materials for complete comparisons).

The nucleus length comparisons were also able to differentiate among species, except for the following: *Pliobyrsa* sp2 from *C. leprosa* (W=-1.02; p=1), *Pliobyrsa* sp2 from *Leptopharsa* sp (W=-0.057; p=1), *V. illudens* from *Leptodictya* sp1 (W=4.449; p=0.115), *C. misionera* from *Pleseobyrsa* sp (W=-1.439; p=1), *C. misionera* from *T. scrupulosa* (W=0.111; p=1), *Pleseobyrsa* sp from *T. scrupulosa* (W=1.612; p=0.999), and *C. leprosa* from *Leptopharsa* sp (W=0.539; p=1). The same way was the acrosome measurements, where the species that did not show significant differences were: *G. munda* from *Gargaphia* sp1 (W=3.499; p=0.491), *G. munda* from *A. fuscipes* (W=0.91; p=1), *G. munda* from *Gargaphia* sp2 (W=3.76; p=0.357), *G. paula* A from *G. paula* B (W=-3.21; p=1), *G. brunfelsiae* from *Gargaphia* sp1 (W=-4.189; p=0.185), *G. brunfelsiae* from *Gargaphia* sp2 (W=-3.653; p=0.411), *Pliobyrsa* sp1 from *V. illudens* (W=0.745; p=1), *Pliobyrsa* sp1 from *Leptodictya* sp1 (W=-2.613; p=0.899), *Gargaphia* sp1 from *A. fuscipes* (W=-3.278; p=0.611), *Gargaphia* sp1 from *Gargaphia* sp2 (W=0.549; p=1), *Pliobyrsa* sp2 from *Leptopharsa* sp (W=-4.079; p=0.223), *V. illudens* from *Leptodictya* sp1 (W=-2.721; p=0.865), *A. fuscipes* from *Gargaphia* sp2 (W=3.329; p=0.583), *C. misionera* from *Pleseobyrsa* sp (W=1.652; p=0.999), *T. scrupulosa* from *Leptopharsa* sp (W=3.291; p=0.604). We did not compare the *C. leprosa* acrosome because it was not possible to collect the data.

Overall, it was not possible to separate all species through a single measurement type alone. However, when considering the total and nucleus length, we could distinguish all of them, for no two species matched the same values — the same applies to the acrosome data with a single exception between *Gargaphia* sp1 and sp2 (Table 2, Supplementary materials).

**Table 1**. Tingidae sperm measurements. Species followed by their respective total, nucleus and acrosome length expressed in median, their percentiles (µm) and coefficients of variation (CV).

| Species | Total length | | | | Nucleus length | | | | Acrosome length | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | percentile | | CV | Median | percentile | | CV | Median | percentile | | CV |
| | | 25th | 75th | | | 25th | 75th | | | 25th | 75th | |
| ***Gargaphia munda*** | 265 | 264 | 270 | 2.2% | 19.3 | 18.8 | 19.9 | 4.7% | 13.8 | 12.8 | 14.3 | 22.3% |
| ***Gargaphia paula* A** | 305 | 301 | 318 | 9.8% | 22.7 | 22.4 | 23.1 | 2.2% | 29 | 28.7 | 29.9 | 3.1% |
| ***Gargaphia paula* B** | 276 | 273 | 280 | 1.9% | 24 | 23.5 | 24.3 | 3.1% | 29.1 | 28.1 | 29.9 | 4.6% |
| ***Gargaphia brunfelsiae*** | 248 | 239 | 254 | 4.9% | 30.3 | 29.4 | 32.3 | 5.1% | 16.1 | 15.5 | 16.9 | 10.3% |
| ***Gargaphia* sp1** | 350 | 344 | 353 | 3.8% | 16.4 | 16.2 | 16.8 | 8.2% | 14.7 | 14.2 | 15.5 | 7.9% |
| ***Gargaphia* sp2** | 353 | 347 | 355 | 2.1% | 18 | 17.6 | 18.2 | 3.5% | 15.1 | 14.2 | 15.8 | 8.9% |
| ***Pliobyrsa* sp1** | 269 | 265 | 272 | 3.4% | 20.1 | 19.7 | 20.7 | 3.3% | 9.77 | 9.55 | 10 | 7.0% |
| ***Pliobyrsa* sp2** | 282 | 281 | 287 | 3.0% | 15.5 | 15.1 | 15.7 | 3.9% | 8.52 | 8.22 | 8.86 | 6.2% |
| ***Leptodictya* sp** | 318 | 310 | 323 | 2.5% | 27.3 | 26.5 | 27.5 | 3.0% | 9.3 | 8.84 | 9.66 | 7.3% |
| ***Vatiga illudens*** | 346 | 336 | 350 | 2.4% | 26 | 25.8 | 26.4 | 2.6% | 9.91 | 9.35 | 10.5 | 9.4% |
| ***Atheas fuscipes*** | 285 | 282 | 291 | 3.0% | 21.1 | 20.7 | 21.1 | 1.9% | 13.8 | 13.1 | 14 | 5.3% |
| ***Corythaica misionera*** | 313 | 309 | 316 | 2.2% | 14.6 | 14.4 | 14.9 | 3.3% | 6.07 | 5.69 | 6.5 | 8.8% |
| ***Corythaica leprosa*** | 359 | 353 | 365 | 2.1% | 15.4 | 14.9 | 15.6 | 3.8% | NA | NA | NA | NA |
| ***Pleseobyrsa* sp** | 266 | 262 | 271 | 2.0% | 14.4 | 14.2 | 14.8 | 2.5% | 6.16 | 5.77 | 6.88 | 11.5% |
| ***Teleonemia scrupulosa*** | 252 | 247 | 257 | 2.1% | 14.7 | 14.3 | 14.8 | 3.5% | 7.36 | 7.2 | 7.78 | 6.6% |
| ***Leptopharsa* sp** | 219 | 217 | 221 | 1.6% | 15.5 | 15.2 | 15.6 | 2.3% | 7.86 | 7.5 | 8.07 | 7.9% |




**Graph 1**. Comparison of Tingidae sperm measurements for each species. Total sperm length is shown in the upper part of the graph in light grey. The nucleus (dark grey) and acrosome (white) values are shown side by side.

### 2.3.3. Male Reproductive System

The lace bugs reproductive systems were pretty similar in all species, consisting of one pair of testes with one or two ovoid follicles. The deferent ducts connect the testes to a pair of seminal vesicles (SV). Usually one pair of tubular accessory glands (AG) was present, opening to a medial-posterior ejaculatory duct together with the SV, and in most cases, longer and thicker than the SV.

Eleven of the collected species are described here. *Gargaphia munda*: it presented one follicle per testis and a pair of long accessory glands which folded twice around the middle (Fig. 4A). *Gargaphia paula* A: two follicles per testis were present, the SV were long and narrow, and it had one pair of long accessory glands folding twice around the middle (Fig. 4B). *Gargaphia paula* B: it showed two follicles per testis that appear to be partially fused. The SV were long and filled with a considerable volume of sperm. The pair of AG was long folded twice around the middle (Fig. 4C). *Gargaphia brunfelsiae*: two follicles per testis that appear to be partially fused. The seminal vesicles were long, and the pair of AG was long and folded twice (Fig. 4D). *Gargaphia* sp1: it had one follicle per testis relatively big. The SV and the pair of AG were long, the latter folding twice around the middle (Fig. 4E). *Gargaphia* sp2: it had one follicle per testis, and the SV were long. The pair of accessory glands folding twice around the middle (Fig. 4F). *Pliobyrsa* sp1: It presented asymmetrical testes, with two follicles on one side and one on the other. The seminal vesicles were not very long and thick, and the pair of accessory glands was shorter than the SV and thick, and it did not show foldings (Fig. 4G). *Leptodictya* sp: It displayed two follicles per testis, which seem to be partially fused. The SV were long, and the pair of glands was branched (Fig. 4H). *Vatiga illudens*: one follicle per testis, long and narrow seminal vesicles, and two pairs of accessory glands were observed. One pair was long and lateral to the SV, and the other was very short, rounded and flowing into the ejaculatory duct (Fig. 4I). *Atheas fuscipes*: two follicles per testicle. The seminal vesicles are long and narrow, but their upper portion was slightly wider. The pair accessory glands were short and broad, with no folding (Fig. 5A). *Teleonemia scrupulosa*: it had two follicles per testis and long SV. The pair of AG was long and showed one or two folds (Fig. 5B). *Leptopharsa* sp had two follicles per testis and long SV.

The pair of accessory glands was also long and folding to a lesser degree on its distal portion (Fig. 5C).

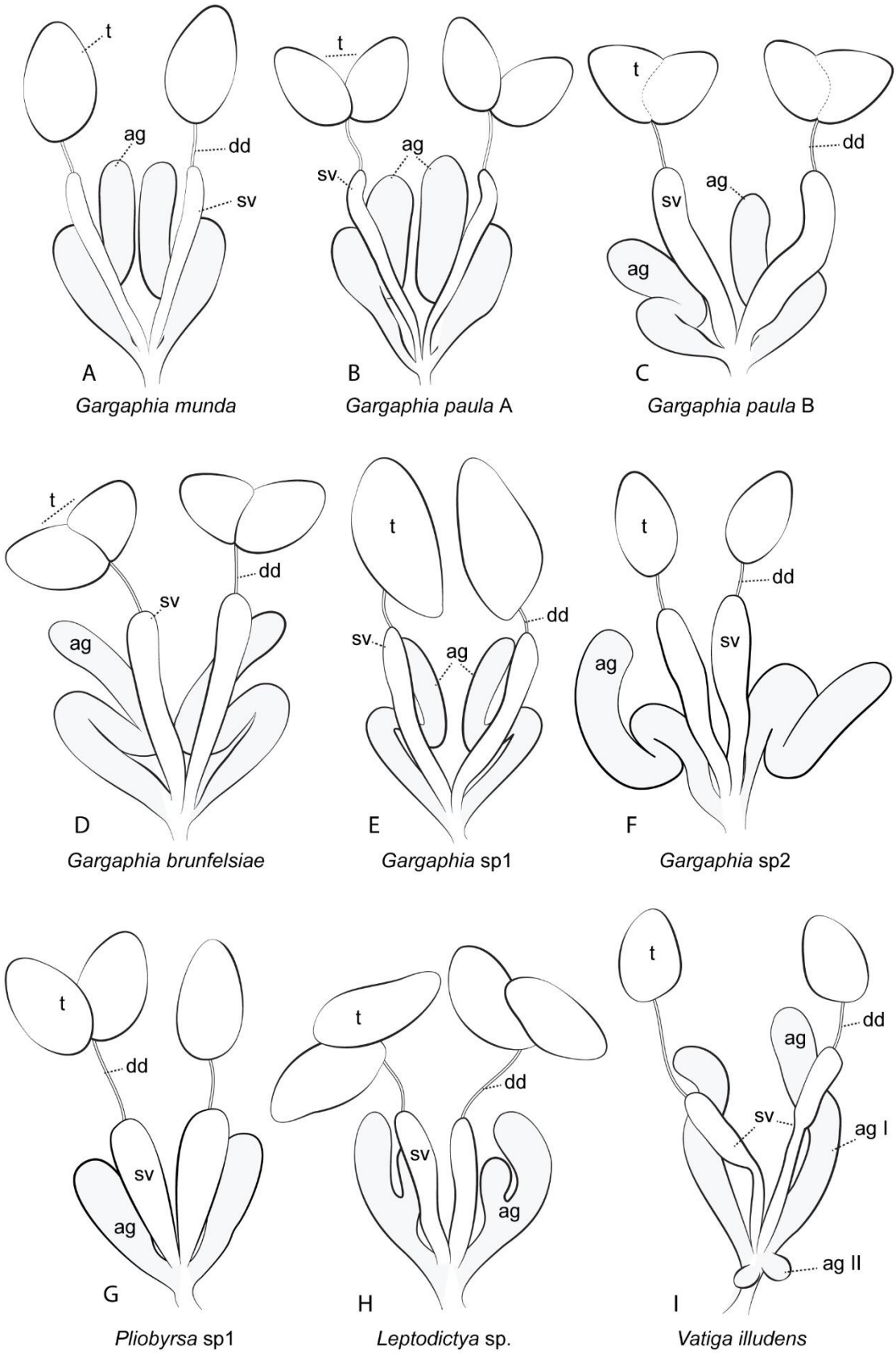



**Figure 4**. Tingidae male reproductive system. **t**: testis, **dd**: deferent duct, **ag**: accessory gland. Schemes not up to scale.

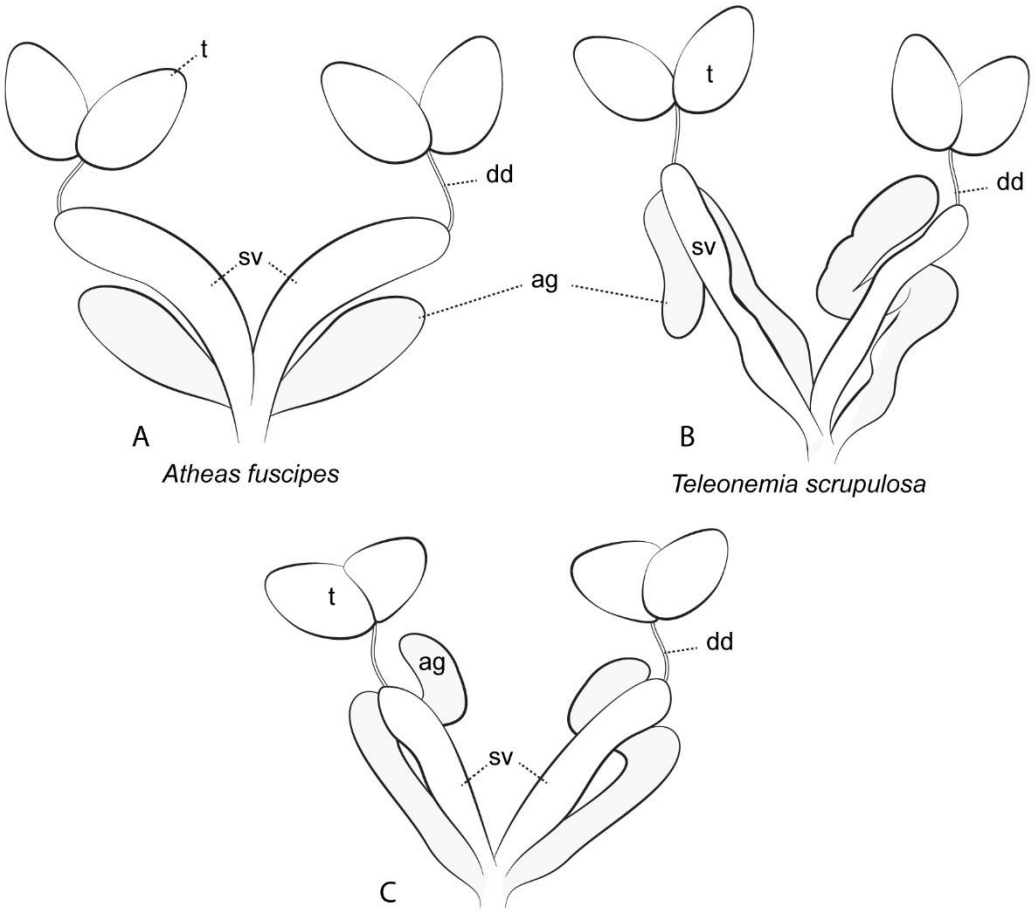



**Figure 5**. Tingidae male reproductive system. **t**: testis, **dd**: deferent duct, **ag**: accessory gland. Schemes not up to scale.

## 2.4. DISCUSSION

The spermatozoa of Tingidae showed the typical morphology among insects, being elongated and slender (Dallai, 2014; Jamieson et al., 1999). However, its parts were not discernible using conventional staining techniques, requiring a fluorescent marker to evidence the sperm head and nucleus. Also, we could not identify a clear centriolar adjunct piece, as observed in their sister group, Miridae (Barcellos, 2018; Rezende et al., 2021). Such observations may indicate that the centriolar adjunct material in Tingidae sperm overlaps with other elements, e.g., flagellar, which is worth further investigation. Another interesting feature was the long acrosome, especially in the *Gargaphia* species. In Miridae, it ranges between 5 to 9 µm long, close to the observed to most lace bugs analyzed (Rezende et al., 2021). In *Ornithocoris palli* (Cimicidae), it measures at approximately 4 µm (Novais, 2017), while in other Heteroptera, it is usually shorter and not so noticeable (Araújo et al., 2011, 2012). It is also short in other insects, such as Cicadidae and Coleoptera (Burrini et al., 1988; Chawanji et al., 2005, 2006; Dallai, 2014), with just a few exceptions (e.g., 300 µm long in *Martarega bentoi*: Notonectidae, (Novais et al., 2017). Concerning the *Gargaphia* acrosome length, we could deduce it is a characteristic of this genus, potentially corroborating this taxon, but more species need to be studied in order to confirm this.

We could distinguish all species through the morphometric analysis, which allowed us to do so, especially those in the same genus. Although, in some cases, it did not show differences when using the total length alone, the species belonged to different genera (except for *Garagaphia* sp1 and sp2), and in four cases, the combination of the nucleus and acrosome metrics could not resolve the species. Considering this, to differentiate among species, we recommend analyzing the total and nuclear length when studying insect sperm, and adding other metrics, such as acrosome length, which can improve the precision. Similar efforts effectively distinguished species closely related and associating males and females in highly dimorphic taxa (Baffa et al., 2017; Barcellos et al., 2015; Cursino & Duarte, 2016; Pereira et al., 2008). These findings are in line with the assertion by Jamieson (Jamieson, 1987 in Pitnick et al., 2009) that sperm cells have particular features to a given taxon, thus being a valuable taxonomic tool.

The male reproductive system anatomy also provided helpful information. It supports that, as in a few published works, the tingids usually present one or two testicular follicles and one pair of long tubular accessory glands. A second pair may be present, but it is very short and round (Javahery, 2019; Pendergrast, 1957). It showed a clear distinction from Miridae, where a bigger number of follicles and two pairs of long glands are usually observed (Mróz, 2012; Rezende et al., 2021; Vélez et al., 2020). Other heteropterans also have more follicles per testis, usually seven and display a vast variation in testes and accessory glands morphology (Horton & Lewis, 2011; Novais et al., 2017; Pendergrast, 1957). Furthermore, each species showed specific traits in their combination of the number and shape of follicles, length of the glands, shape and how they fold.

Another noteworth finding was that, while identifying the specimens, two groups were classified as *Gargaphia paula* (A and B). We kept them as separate groups because they were collected in different plants and locations and they showed significant differences in their sperm morphometry. Also, the follicles in *G. paula* B seemed to be partially fused while in the A*,* they were free and connected independently to the deferent duct. This indicates they are distinct species, although we identified them as the same taxon following the key. This could mean they are a cryptic species complex, and a further investigation is needed to clarify this question.

Based on these results and the current knowledge of sperm morphology, we can conclude that it is a useful taxonomic tool to differentiate among species. Together with male reproductive morphology, it can provide reliable data. However, we highlight that the sperm morphology analysis is a complementary methodology with the advantaged of being very cheap and quick way to obtain data, and it should always be considered together with the other type of data. External characteristics are essential to taxonomic praxis, as molecular, and internal morphology.

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## 2.6. SUPPLEMENTARY MATERIAL

**Table 2.** Sperm morphometry pairwise comparisons between Tingidae species (DSCF). Not sigficant values are marked in dark grey (p>0.05)

| Species | | Total Length | | Nucleus Length | | Acrosome Length | |
|---|---|---|---|---|---|---|---|
| | | W | p | W | p | W | p |
| *G. munda* | *G. paula* A | 6.626 | < .001 | 9.226 | < .001 | 6.164 | 0.001 |
| *G. munda* | *G. paula* B | 5.009 | 0.034 | 8.819 | < .001 | 6.164 | 0.001 |
| *G. munda* | *G. brunfelsiae* | -5.777 | 0.005 | 8.458 | < .001 | 6.833 | < .001 |
| *G. munda* | *Gargaphia* sp1 | 6.414 | < .001 | -6.711 | < .001 | 3.500 | 0.491 |
| *G. munda* | *Gargaphia* sp2 | 5.963 | 0.003 | -5.882 | 0.003 | 3.761 | 0.357 |
| *G. munda* | *Pliobyrsa* sp1 | 1.120 | 1 | 4.927 | 0.042 | -6.164 | 0.001 |
| *G. munda* | *Pliobyrsa* sp2 | 5.099 | 0.028 | -8.477 | < .001 | -7.452 | < .001 |
| *G. munda* | *Leptodictya* sp1 | 6.036 | 0.002 | 8.324 | < .001 | -7.550 | < .001 |
| *G. munda* | *V. illudens* | 5.883 | 0.003 | 7.874 | < .001 | -6.851 | < .001 |
| *G. munda* | *A. fuscipes* | 5.537 | 0.009 | 6.252 | 0.001 | 0.091 | 1 |
| *G. munda* | *C. misionera* | 6.325 | < .001 | -7.694 | < .001 | -6.851 | < .001 |
| *G. munda* | *C. leprosa* | 6.860 | < .001 | -8.502 | < .001 | -2.330 | NA |
| *G. munda* | *Pleseobyrsa* sp | -0.516 | 1 | -8.005 | < .001 | -7.728 | < .001 |
| *G. munda* | *T. scrupulosa* | -5.350 | 0.015 | -7.846 | < .001 | -7.641 | < .001 |
| *G. munda* | *Leptopharsa* sp | -5.963 | 0.003 | -7.186 | < .001 | -7.641 | < .001 |
| *G. paula* A | *G. paula* B | -8.893 | < .001 | 7.254 | < .001 | -0.321 | 1 |
| *G. paula* A | *G. brunfelsiae* | -9.327 | < .001 | 8.216 | < .001 | -6.103 | 0.002 |
| *G. paula* A | *Gargaphia* sp1 | 8.149 | < .001 | -7.517 | < .001 | -5.701 | 0.006 |
| *G. paula* A | *Gargaphia* sp2 | 7.763 | < .001 | -7.517 | < .001 | -5.797 | 0.004 |
| *G. paula* A | *Pliobyrsa* sp1 | -9.770 | < .001 | -7.962 | < .001 | -5.345 | 0.015 |
| *G. paula* A | *Pliobyrsa* sp2 | -7.457 | < .001 | -9.295 | < .001 | -6.164 | 0.001 |
| *G. paula* A | *Leptodictya* sp1 | 3.476 | 0.504 | 8.093 | < .001 | -6.222 | 0.001 |
| *G. paula* A | *V. illudens* | 7.423 | < .001 | 7.675 | < .001 | -5.797 | 0.004 |
| *G. paula* A | *A. fuscipes* | -7.898 | < .001 | -6.277 | < .001 | -5.477 | 0.01 |
| *G. paula* A | *C. misionera* | 1.886 | 0.995 | -8.216 | < .001 | -5.797 | 0.004 |
| *G. paula* A | *C. leprosa* | 10.070 | < .001 | -9.362 | < .001 | -2.236 | NA |
| *G. paula* A | *Pleseobyrsa* sp | -8.964 | < .001 | -8.332 | < .001 | -6.325 | < .001 |
| *G. paula* A | *T. scrupulosa* | -7.293 | < .001 | -8.332 | < .001 | -6.275 | < .001 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *G. paula* A | *Leptopharsa* sp | -7.828 | < .001 | -7.962 | < .001 | -6.275 | < .001 |
| *G. paula* B | *G. brunfelsiae* | -8.648 | < .001 | 7.920 | < .001 | -6.103 | 0.002 |
| *G. paula* B | *Gargaphia* sp1 | 8.485 | < .001 | -7.285 | < .001 | -5.701 | 0.006 |
| *G. paula* B | *Gargaphia* sp2 | 7.559 | < .001 | -7.285 | < .001 | -5.797 | 0.004 |
| *G. paula* B | *Pliobyrsa* sp1 | -6.711 | < .001 | -7.690 | < .001 | -5.345 | 0.015 |
| *G. paula* B | *Pliobyrsa* sp2 | 4.563 | 0.092 | -8.880 | < .001 | -6.164 | 0.001 |
| *G. paula* B | *Leptodictya* sp1 | 7.701 | < .001 | 7.737 | < .001 | -6.222 | 0.001 |
| *G. paula* B | *V. illudens* | 7.408 | < .001 | 7.187 | < .001 | -5.797 | 0.004 |
| *G. paula* B | *A. fuscipes* | 5.459 | 0.011 | -6.135 | 0.002 | -5.477 | 0.01 |
| *G. paula* B | *C. misionera* | 8.292 | < .001 | -7.920 | < .001 | -5.797 | 0.004 |
| *G. paula* B | *C. leprosa* | 9.535 | < .001 | -8.939 | < .001 | -2.236 | NA |
| *G. paula* B | *Pleseobyrsa* sp | -7.395 | < .001 | -8.025 | < .001 | -6.325 | < .001 |
| *G. paula* B | *T. scrupulosa* | -7.071 | < .001 | -8.025 | < .001 | -6.275 | < .001 |
| *G. paula* B | *Leptopharsa* sp | -7.559 | < .001 | -7.690 | < .001 | -6.275 | < .001 |
| *G. brunfelsiae* | *Gargaphia* sp1 | 8.794 | < .001 | -7.071 | < .001 | -4.189 | 0.185 |
| *G. brunfelsiae* | *Gargaphia* sp2 | 7.780 | < .001 | -7.071 | < .001 | -3.653 | 0.411 |
| *G. brunfelsiae* | *Pliobyrsa* sp1 | 8.020 | < .001 | -7.442 | < .001 | -6.103 | 0.002 |
| *G. brunfelsiae* | *Pliobyrsa* sp2 | 7.406 | < .001 | -8.512 | < .001 | -7.349 | < .001 |
| *G. brunfelsiae* | *Leptodictya* sp1 | 7.933 | < .001 | -7.550 | < .001 | -7.442 | < .001 |
| *G. brunfelsiae* | *V. illudens* | 7.616 | < .001 | -7.204 | < .001 | -6.769 | < .001 |
| *G. brunfelsiae* | *A. fuscipes* | 8.199 | < .001 | -6.000 | 0.002 | -5.975 | 0.003 |
| *G. brunfelsiae* | *C. misionera* | 8.580 | < .001 | -7.651 | < .001 | -6.769 | < .001 |
| *G. brunfelsiae* | *C. leprosa* | 9.971 | < .001 | -8.564 | < .001 | -2.324 | NA |
| *G. brunfelsiae* | *Pleseobyrsa* sp | 7.446 | < .001 | -7.746 | < .001 | -7.613 | < .001 |
| *G. brunfelsiae* | *T. scrupulosa* | 2.174 | 0.978 | -7.746 | < .001 | -7.530 | < .001 |
| *G. brunfelsiae* | *Leptopharsa* sp | -7.310 | < .001 | -7.442 | < .001 | -7.530 | < .001 |
| *Gargaphia* sp1 | *Gargaphia* sp2 | 1.523 | 1 | 6.013 | 0.002 | 0.549 | 1 |
| *Gargaphia* sp1 | *Pliobyrsa* sp1 | -9.165 | < .001 | 6.903 | < .001 | -5.701 | 0.006 |
| *Gargaphia* sp1 | *Pliobyrsa* sp2 | -7.348 | < .001 | -6.035 | 0.002 | -6.701 | < .001 |
| *Gargaphia* sp1 | *Leptodictya* sp1 | -6.587 | < .001 | 6.990 | < .001 | -6.774 | < .001 |
| *Gargaphia* sp1 | *V. illudens* | -2.858 | 0.814 | 6.708 | < .001 | -6.245 | 0.001 |
| *Gargaphia* sp1 | *A. fuscipes* | -8.000 | < .001 | 5.692 | 0.006 | -3.278 | 0.611 |
| *Gargaphia* sp1 | *C. misionera* | -7.153 | < .001 | -6.930 | < .001 | -6.245 | 0.001 |
| *Gargaphia* sp1 | *C. leprosa* | 6.178 | 0.001 | -6.496 | < .001 | -2.280 | NA |
| *Gargaphia* sp1 | *Pleseobyrsa* sp | -8.485 | < .001 | -7.147 | < .001 | -6.905 | < .001 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Gargaphia* sp1 | *T. scrupulosa* | -7.019 | < .001 | -7.056 | < .001 | -6.841 | < .001 |
| *Gargaphia* sp1 | *Leptopharsa* sp | -7.496 | < .001 | -5.829 | 0.004 | -6.841 | < .001 |
| *Gargaphia* sp2 | *Pliobyrsa* sp1 | -8.038 | < .001 | 6.749 | < .001 | -5.797 | 0.004 |
| *Gargaphia* sp2 | *Pliobyrsa* sp2 | -6.708 | < .001 | -7.746 | < .001 | -6.851 | < .001 |
| *Gargaphia* sp2 | *Leptodictya* sp1 | -6.877 | < .001 | 6.990 | < .001 | -6.928 | < .001 |
| *Gargaphia* sp2 | *V. illudens* | -3.857 | 0.313 | 6.708 | < .001 | -6.368 | < .001 |
| *Gargaphia* sp2 | *A. fuscipes* | -7.204 | < .001 | 5.692 | 0.006 | -3.329 | 0.583 |
| *Gargaphia* sp2 | *C. misionera* | -7.317 | < .001 | -7.071 | < .001 | -6.368 | < .001 |
| *Gargaphia* sp2 | *C. leprosa* | 4.694 | 0.07 | -7.786 | < .001 | -2.291 | NA |
| *Gargaphia* sp2 | *Pleseobyrsa* sp | -7.559 | < .001 | -7.147 | < .001 | -7.068 | < .001 |
| *Gargaphia* sp2 | *T. scrupulosa* | -6.450 | < .001 | -7.147 | < .001 | -7.000 | < .001 |
| *Gargaphia* sp2 | *Leptopharsa* sp | -6.822 | < .001 | -6.903 | < .001 | -7.000 | < .001 |
| *Pliobyrsa* sp1 | *Pliobyrsa* sp2 | 6.691 | < .001 | -8.232 | < .001 | -5.191 | 0.022 |
| *Pliobyrsa* sp1 | *Leptodictya* sp1 | 8.207 | < .001 | 7.349 | < .001 | -2.613 | 0.899 |
| *Pliobyrsa* sp1 | *V. illudens* | 7.859 | < .001 | 7.027 | < .001 | 0.745 | 1 |
| *Pliobyrsa* sp1 | *A. fuscipes* | 7.695 | < .001 | 4.146 | 0.199 | 5.477 | 0.01 |
| *Pliobyrsa* sp1 | *C. misionera* | 8.925 | < .001 | -7.442 | < .001 | -5.797 | 0.004 |
| *Pliobyrsa* sp1 | *C. leprosa* | 10.513 | < .001 | -8.279 | < .001 | -2.236 | NA |
| *Pliobyrsa* sp1 | *Pleseobyrsa* sp | -1.665 | 0.999 | -7.530 | < .001 | -6.325 | < .001 |
| *Pliobyrsa* sp1 | *T. scrupulosa* | -6.677 | < .001 | -7.530 | < .001 | -6.036 | 0.002 |
| *Pliobyrsa* sp1 | *Leptopharsa* sp | -8.038 | < .001 | -7.249 | < .001 | -5.618 | 0.007 |
| *Pliobyrsa* sp2 | *Leptodictya* sp1 | 6.809 | < .001 | 8.376 | < .001 | 5.802 | 0.004 |
| *Pliobyrsa* sp2 | *V. illudens* | 6.599 | < .001 | 7.918 | < .001 | 5.693 | 0.006 |
| *Pliobyrsa* sp2 | *A. fuscipes* | 1.980 | 0.991 | 6.414 | < .001 | 6.360 | < .001 |
| *Pliobyrsa* sp2 | *C. misionera* | 7.218 | < .001 | -5.799 | 0.004 | -6.851 | < .001 |
| *Pliobyrsa* sp2 | *C. leprosa* | 8.018 | < .001 | -1.021 | 1 | -2.330 | NA |
| *Pliobyrsa* sp2 | *Pleseobyrsa* sp | -6.420 | < .001 | -6.712 | < .001 | -7.506 | < .001 |
| *Pliobyrsa* sp2 | *T. scrupulosa* | -6.091 | 0.002 | -5.889 | 0.003 | -5.956 | 0.003 |
| *Pliobyrsa* sp2 | *Leptopharsa* sp | -6.708 | < .001 | -0.057 | 1 | -4.079 | 0.223 |
| *Leptodictya* sp1 | *V. illudens* | 6.756 | < .001 | -4.449 | 0.115 | 2.722 | 0.865 |
| *Leptodictya* sp1 | *A. fuscipes* | -7.327 | < .001 | -5.948 | 0.003 | 6.423 | < .001 |
| *Leptodictya* sp1 | *C. misionera* | -2.003 | 0.99 | -7.550 | < .001 | -6.928 | < .001 |
| *Leptodictya* sp1 | *C. leprosa* | 8.387 | < .001 | -8.425 | < .001 | -2.336 | NA |
| *Leptodictya* sp1 | *Pleseobyrsa* sp | -7.701 | < .001 | -7.641 | < .001 | -7.836 | < .001 |
| *Leptodictya* sp1 | *T. scrupulosa* | -6.540 | < .001 | -7.641 | < .001 | -7.709 | < .001 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Leptodictya* sp1 | *Leptopharsa* sp | -6.928 | < .001 | -7.349 | < .001 | -6.787 | < .001 |
| *V. illudens* | *A. fuscipes* | -7.071 | < .001 | -5.765 | 0.005 | 5.961 | 0.003 |
| *V. illudens* | *C. misionera* | -7.131 | < .001 | -7.204 | < .001 | -6.368 | < .001 |
| *V. illudens* | *C. leprosa* | 6.949 | < .001 | -7.960 | < .001 | -2.291 | NA |
| *V. illudens* | *Pleseobyrsa* sp | -7.408 | < .001 | -7.284 | < .001 | -7.068 | < .001 |
| *V. illudens* | *T. scrupulosa* | -6.352 | < .001 | -7.284 | < .001 | -6.905 | < .001 |
| *V. illudens* | *Leptopharsa* sp | -6.708 | < .001 | -7.027 | < .001 | -6.571 | < .001 |
| *A. fuscipes* | *C. misionera* | 7.800 | < .001 | -6.000 | 0.002 | -5.961 | 0.003 |
| *A. fuscipes* | *C. leprosa* | 8.871 | < .001 | -6.438 | < .001 | -2.253 | NA |
| *A. fuscipes* | *Pleseobyrsa* sp | -7.430 | < .001 | -6.048 | 0.002 | -6.536 | < .001 |
| *A. fuscipes* | *T. scrupulosa* | -6.774 | < .001 | -6.048 | 0.002 | -6.481 | < .001 |
| *A. fuscipes* | *Leptopharsa* sp | -7.204 | < .001 | -5.892 | 0.003 | -6.481 | < .001 |
| *C. misionera* | *C. leprosa* | 9.155 | < .001 | 5.242 | 0.019 | -2.291 | NA |
| *C. misionera* | *Pleseobyrsa* sp | -8.292 | < .001 | -1.439 | 1 | 1.652 | 0.999 |
| *C. misionera* | *T. scrupulosa* | -6.904 | < .001 | 0.111 | 1 | 7.000 | < .001 |
| *C. misionera* | *Leptopharsa* sp | -7.359 | < .001 | 5.871 | 0.003 | 7.000 | < .001 |
| *C. leprosa* | *Pleseobyrsa* sp | -9.535 | < .001 | -6.311 | < .001 | 2.346 | NA |
| *C. leprosa* | *T. scrupulosa* | -7.601 | < .001 | -5.358 | 0.014 | 2.341 | NA |
| *C. leprosa* | *Leptopharsa* sp | -8.208 | < .001 | 0.530 | 1 | 2.341 | NA |
| *Pleseobyrsa* sp | *T. scrupulosa* | -6.766 | < .001 | 1.619 | 0.999 | 6.426 | < .001 |
| *Pleseobyrsa* sp | *Leptopharsa* sp | -7.559 | < .001 | 6.893 | < .001 | 7.319 | < .001 |
| *T. scrupulosa* | *Leptopharsa* sp | -6.450 | < .001 | 6.215 | 0.001 | 3.291 | 0.604 |

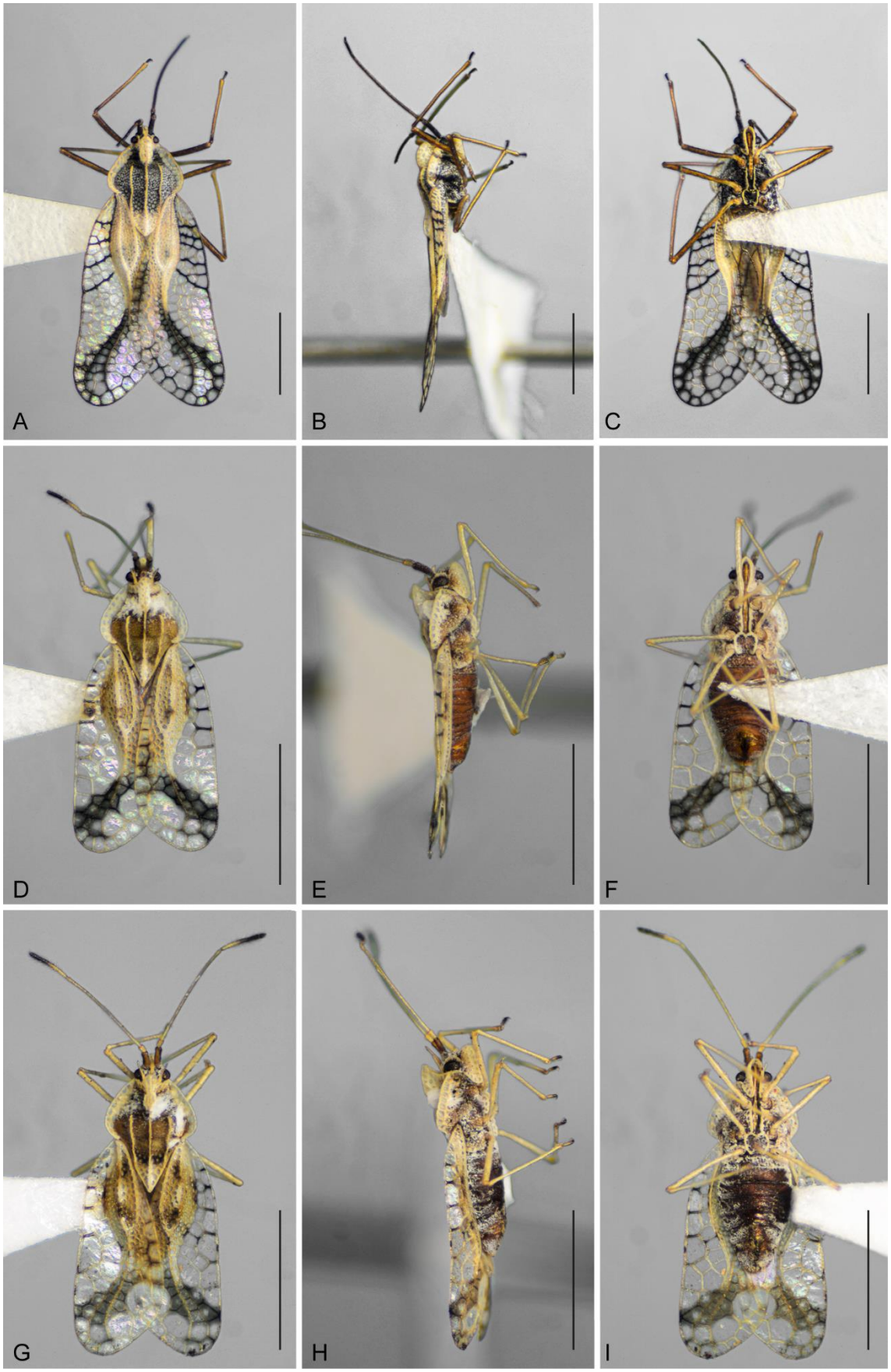


**Figure 6.** *Gargaphia* species. A-C: *Gargaphia munda*. D-F: *G. paula* A. G-I: *G. paula* B. Scale bars= 1.5 mm.

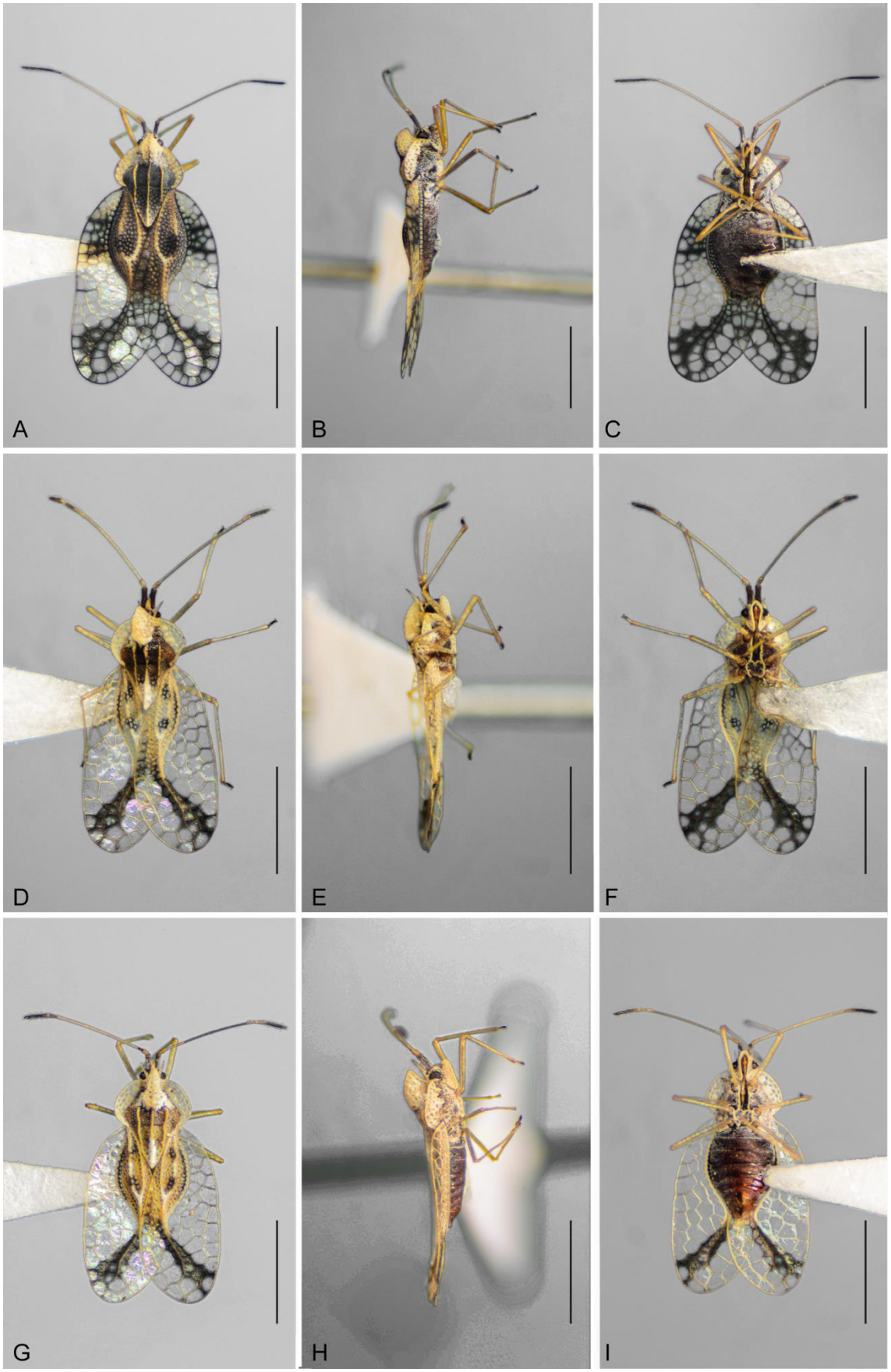


**Figure 7.** *Gargaphia* species. A-C: *G. brunfelsiae*. D-F: *Gargaphia* sp1. G-I: *Gargaphia* sp2. Scale bars= 1.5 mm

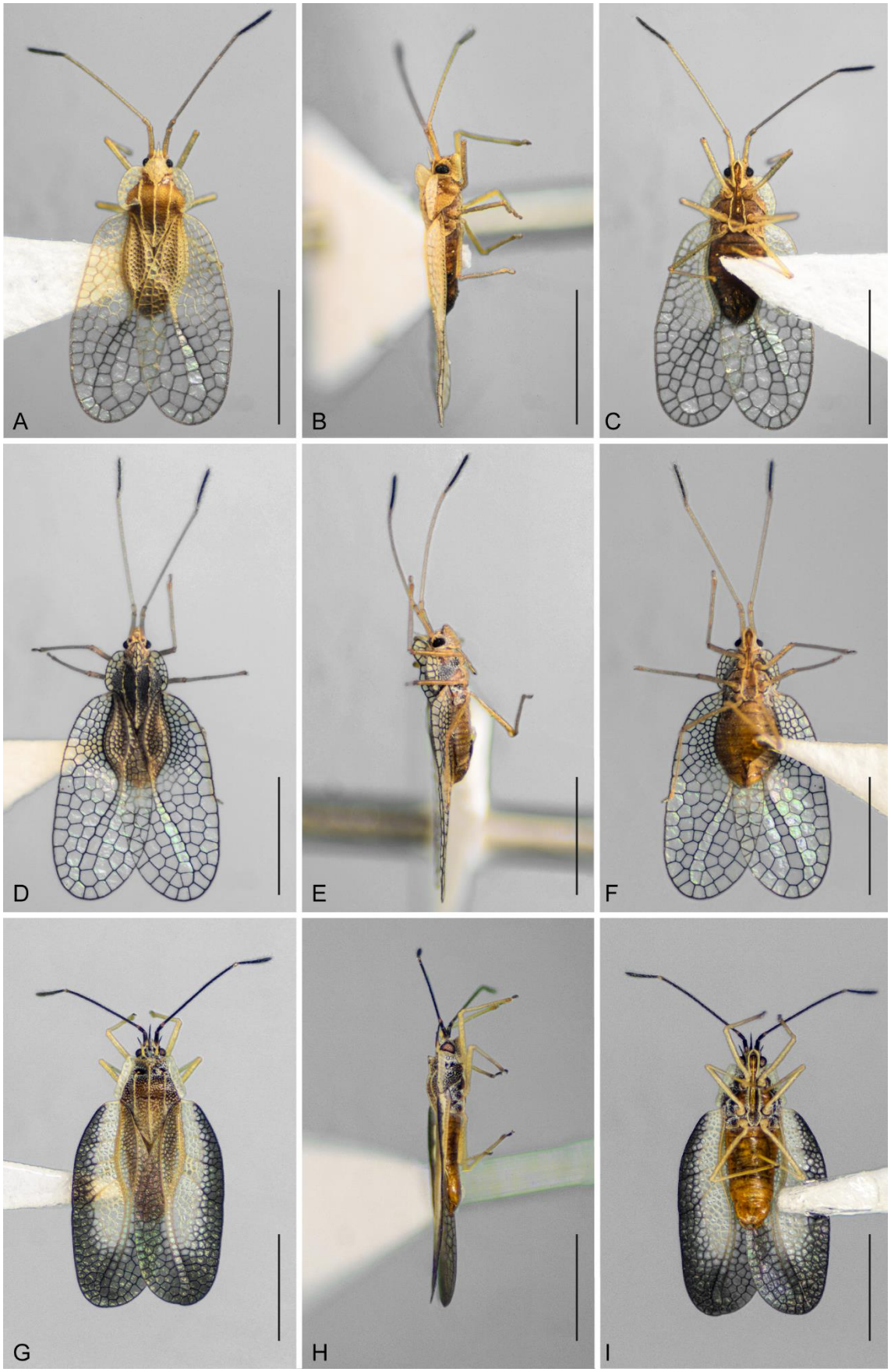


**Figure 8**. Tingidae species. A-C: *Pliobyrsa* sp1. D-F: *Pliobyrsa* sp2. G-I: *Leptodictya* sp1. Scales bars= 1.5 mm.

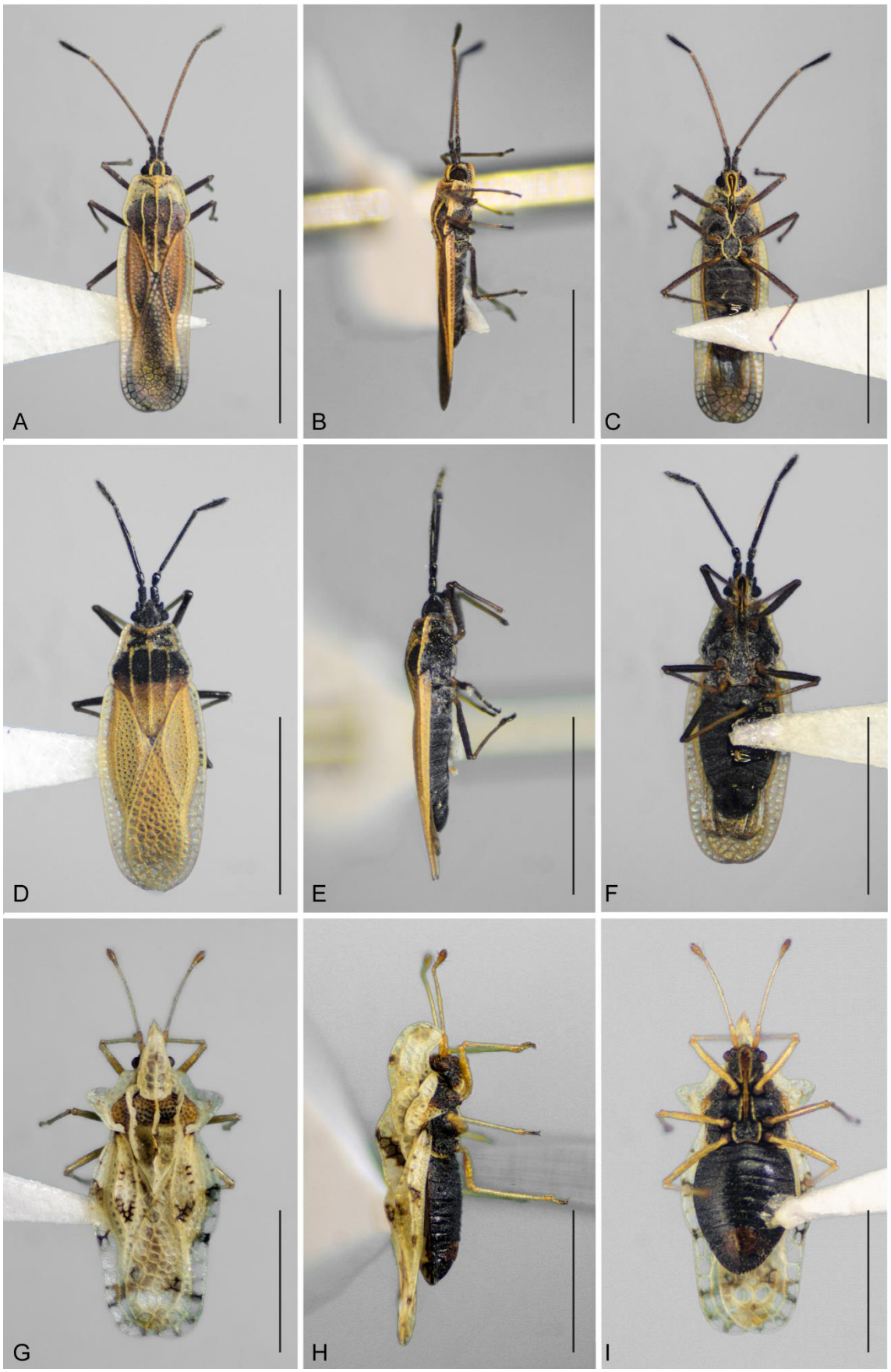


**Figure 9**. Tingidae species. A-C: *Vatiga illudens*. D-F: *Atheas fuscipes*. G-I: *Corythaica misionera*. Scales bars= 1.5 mm.

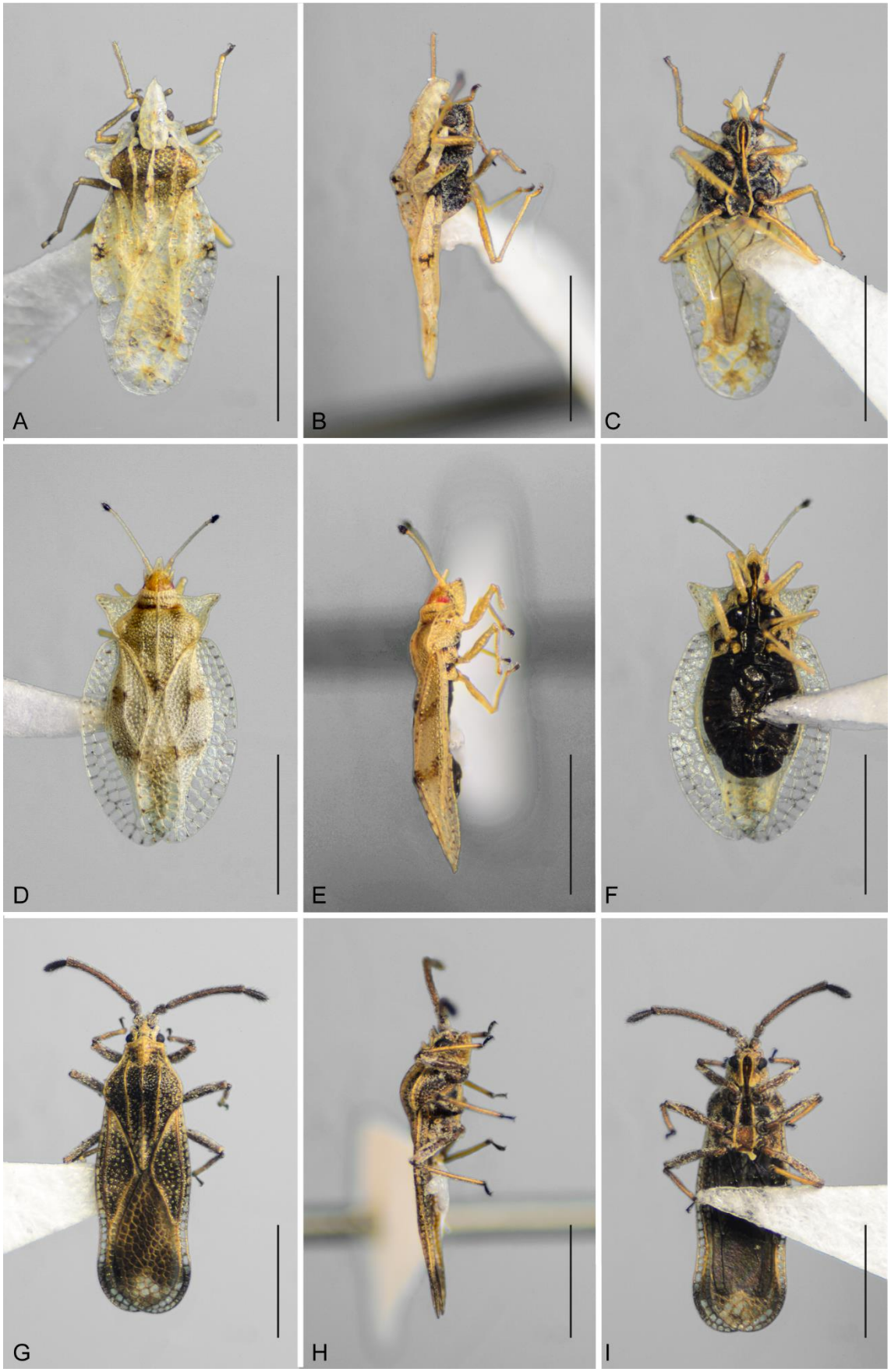


**Figure 10**. Tingidae species. A-C: *Corythaica leprosa*. D-F: *Pleseobyrsa* sp2. G-I: *Teleonemia scrupulosa*. Scales bars= 1.5 mm.


**Figure 11**. Tingidae species. A-C: *Leptopharsa* sp. Scales bars= 1.5 mm.

## 3. CHAPTER II

**SPERM MORPHOLOGY OF TINGIDAE Laporte, 1833 (MIROIDEA: CIMICOMORPHA)**

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# Sperm morphology of Tingidae Laporte, 1833 (Miroidea: *Cimicomorpha*)

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## Abstract

Here, we describe for the first time the sperm morphology of Tingidae (Heteroptera). They are small insects presenting lacy patterns on their pronotum and hemielytra and are exclusively phytophagous, with many economically important species. We studied five species of the tribe Tingini (Tinginae): *Teleonemia scrupulosa*, *Vatiga illudens*, *Gargaphia lunulata*, *Leptopharsa* sp., and *Corythucha arcuata*. Their spermiogenesis process is similar to other Heteroptera, with some differences in the formation of the centriole adjunct. This structure extends in the anteroposterior spermatid axis, flanking the nucleus, possibly contributing to nucleus remodeling and sperm elongation. The mature sperm of Tingidae is also similar to that of other Heteroptera, with features that corroborate the group's monophyly. Our data support previous results for their sister family, Miridae, which exhibits some characteristics exclusive to this taxon, not present in Tingidae or other Heteroptera. They also support the sister relationship of the genera *Gargaphia* and *Leptopharsa* and suggest closer relationship between *Vatiga* and *Corythucha*. Overall, this study sheds light on the sperm ultrastructure of Tingidae and provides information for understanding the evolution and diversity of Heteroptera.

### Research Highlights

- The spermiogenesis process and mature sperm are similar to other Heteroptera
- The centriole adjunct is derived from a strip of a pericentriolar material extending from the centriole
- Tingidae and Miridae are distinguishable using sperm morphology.

KEYWORDS
lace bug, spermatogenesis, sperm biology, systematics

## 1 | INTRODUCTION

The Tingidae, or lace bugs, are a family of Heteroptera (Hemiptera) characterized by areolas and expansions adorning their body (Schuh & Slater, 1995). They belong to the superfamily Miroidea (*Cimicomorpha*), comprising nearly 2600 species in about 300 genera, subdivided into three subfamilies: Cantacaderinae, Vianaidinae (exclusive to the Neotropical region), and the speciose Tinginae with more than 2500 species (Guidoti et al., 2015; ITIS, 2023; Schuh et al., 2006). They are critical agricultural pests for being phytophagous with gregarious behavior and fast reproduction. There are reported attacks on crops such as cassava, eggplant, passion fruit, castor bean, cotton, and rubber trees, among others (Bellon et al., 2012; Bellotti et al., 1999; Coelho & Da-Silva, 2015; Guidoti et al., 2015; Varón et al., 2010). Besides their importance, the family has been overlooked, and relationships within Tingidae subfamilies and tribes are yet unclear

  

(Guidoti et al., 2015). So, new information may contribute to their better understanding.

In past decades, several authors have described the sperm morphology of many Heteroptera and shown many characteristics supporting the clade, as well as specific traits of some taxa (Araújo et al., 2011, 2012; Dallai & Afzelius, 1980, 1982; Dias et al., 2016; Dolder, 1988; Itaya et al., 1980; Jamieson et al., 1999; Lee, 1985; Mercati et al., 2009). However, such data are still scarce for some bug groups, such as the superfamily Miroidea (*Cimicomorpha*), which includes Tingidae. A recent work concerning the sperm of Miridae, the sister group Tingidae, found some peculiar sperm features not observed in any other Heteroptera so far (Rezende et al., 2023). These are the twisted acrosome and the long centriole adjunct as a sole structure between the sperm head and the flagellar components. To contribute to the knowledge of the Miroidea, we aim to characterize for the first time the sperm ultrastructure of Tingidae. Then, we propose which sperm features can be considered exclusive to Miridae and compare our findings with those of other heteropterans looking for potential phylogenetic signs.

## 2 | MATERIALS AND METHODS

We analyzed spermatids and spermatozoa of adult male lace bugs of five species: *Teleonemia scrupulosa Stål*, 1873, *Vatiga illudens* Drake, 1922, *Gargaphia lunulata* Mayr, 1865, *Leptopharsa* sp., and *Corythucha arcuata* Say, 1832. The specimens were collected in Viçosa, MG, Brazil, and *C. arcuata* in Siena, Italy. We also measured the mature sperm ($n = 30$) of three individuals of species. The cells were stained with DAPI (4,6-diamino-2-32 phenylindole) 0.2 µg/mL in phosphate buffered saline (PBS) and with Giemsa, and measurements were performed using the software *Sperm-Sizer-1.6.6* (McDiarmid et al., 2021).

### 2.1 | Transmission electronic microscopy

For the ultrastructural description of spermatids and spermatozoa, testes and seminal vesicles of three to five adult males of each species were dissected in 0.1 M sodium phosphate buffer, pH 7.2 added 3% sucrose and fixed for 24 h in 2.5% glutaraldehyde plus 3% sucrose solution. Then, samples were washed with the same buffer, postfixed in 1% osmium tetroxide solution for 2 h, dehydrated in an increasing series of alcohol and acetone, infiltrated and embedded in Epoxy resin (Epon 812). Some samples were fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium phosphate buffer, plus 2% sucrose, for 5 days, at 4°C. After washing in the same buffer, the samples were contrasted in an aqueous 1% uranyl acetate solution for 2 h at room temperature. Then they were dehydrated and embedded in Epoxy resin as the previous samples. The ultrathin sections were made in an automatic Reichert Ultracut ultramicrotome with a diamond knife, collected on copper grids, and contrasted with 3% uranyl acetate and 2% lead citrate. Analyses and photographic records were carried out using a transmission electron microscope CM10 at the Department of Evolutionary Biology at the University of Siena, Italy.

## 3 | RESULTS

The general process of spermiogenesis is similar in the five studied species here and happens as follows. The early spermatids have a large and round nucleus (2–3 µm in diameter), which occupies a significant part of their cytoplasm (Figure 1a). It shows uncondensed chromatin, with some denser areas closer to the nuclear envelope. A proacrosomal vesicle is attached to the nuclear envelope (Figures 1a, 2a, and 3a,b). It first appears as a less dense structure (Figure 3a); later, it shows a compact and electron-dense appearance (Figures 1c, 2a,b, and 3c). It is possible to see the Golgi apparatus near it (Figure 2a).

Adjacent to the nucleus, the centriole is visible and inserted at its base (Figures 1b, 2a, and 4a,b,d). Around it, pericentriolar material (Pm) forms a strip alongside the nuclear membrane, extending to the contact point between the nucleus and the proacrosomal vesicle (Figures 1a,b, 2a, and 3a,b). The strip flanks the nucleus, and both elongate simultaneously (Figure 4a,b). From the medial portion of the Pm strip, a "budding" appears, increasing in size as it shortens from both sides (Figures 2a,b, and 3c). This centriolar material gives rise to the centriole adjunct in the late spermatids (Figures 2d, 4g, and 5a–c) and is present in the mature sperm. Numerous microtubules are observed in the cytoplasm of spermatids (Figures 1f, 4f,g, and 5a–e); they also seem to attach to the Pm strip (Figure 4b). The number of microtubules decreases throughout spermiogenesis, no longer present in the final stage and mature sperm cells.

The acrosomal vesicle also elongates, partially or totally surrounding the anterior tip of the nucleus (Figure 4a,c). It varies in shape and size depending on which point it is observed in the cross-section (Figures 1d, 2c, 4e, and 5a). As chromatin condenses, the nucleus elongates, and its diameter decreases to its final shape (Figures 1d–f, 2c,d, and 4a,c,g). At first, the chromatin is somewhat granular (Figures 1a,b,d, 2, 3a,b, and 4c,e,f), then shows a filamentous aspect (Figure 2d and 4g), and finally becomes compact and uniform (Figures 5a, 6e, 7b, and 8a–d). However, in *V. illudens* and *T. scrupulosa*, it does not compact entirely, with less dense areas in some points of later-stage spermatids that persist in the mature sperm (Figures 5b and 9b,d). *C. arcuata*, *Leptopharsa* sp. and *G. lunulata* all show nuclei with fully compacted chromatin (Figures 6e, 7a, and 8a–d).

The axoneme is formed from the centriole inserted at the nucleus base (Figures 1b, 2a, 3c, and 4d). The axoneme (~240 nm in diameter), reaches its typical form in insects with nine microtubule doublets, nine accessories tubules and a central pair of microtubules (Figures 3f, 5d,e, and 6b–d). It elongates, accompanied by two mitochondrial derivatives. The latter originated by the fusion of several mitochondria into a large Nebenkern (Figure 3d). Then, it divides into two (Figure 3e), disposed on either side of the axoneme (Figures 3c,f and

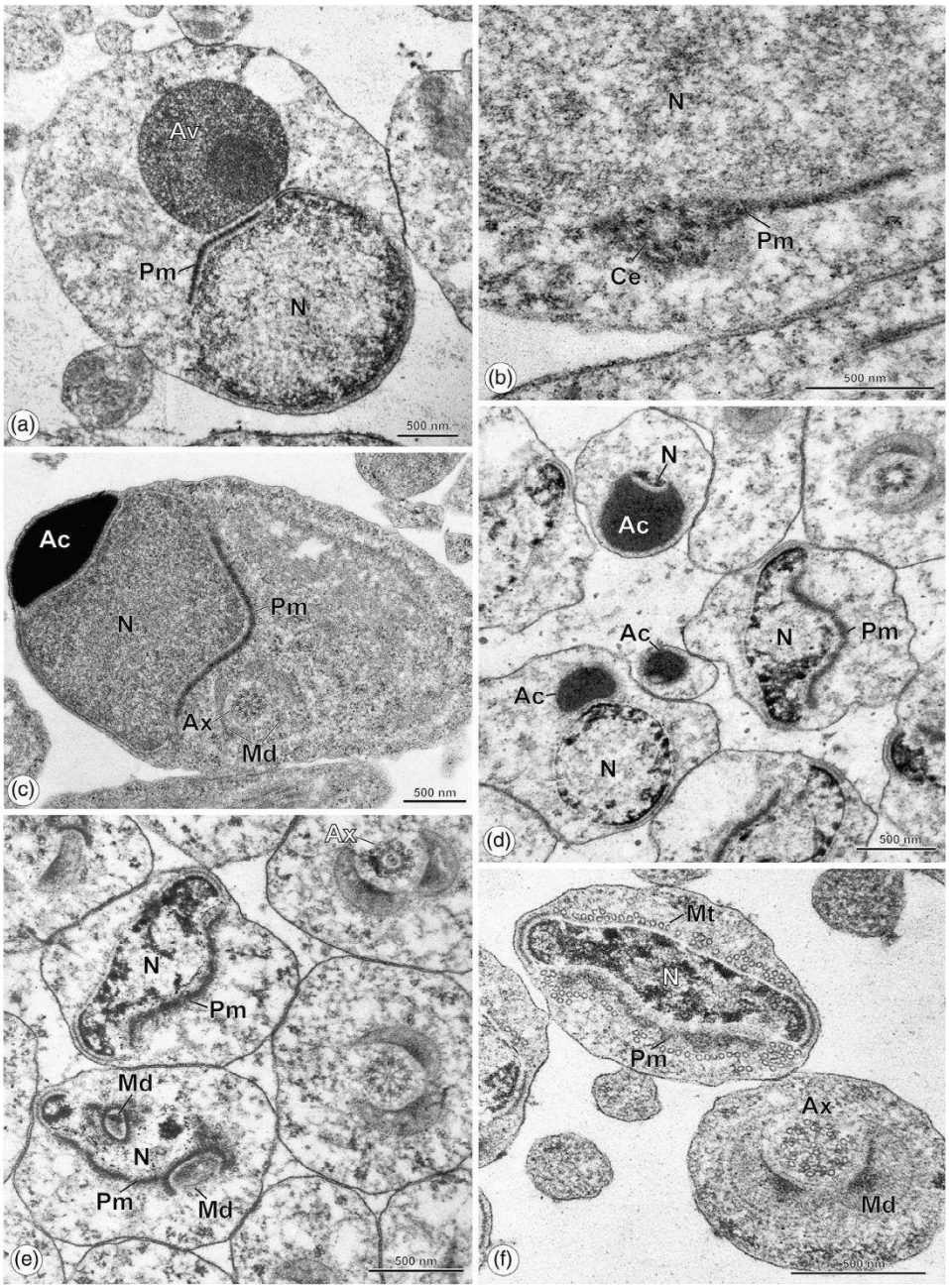


**FIGURE 1** Legend on next page.

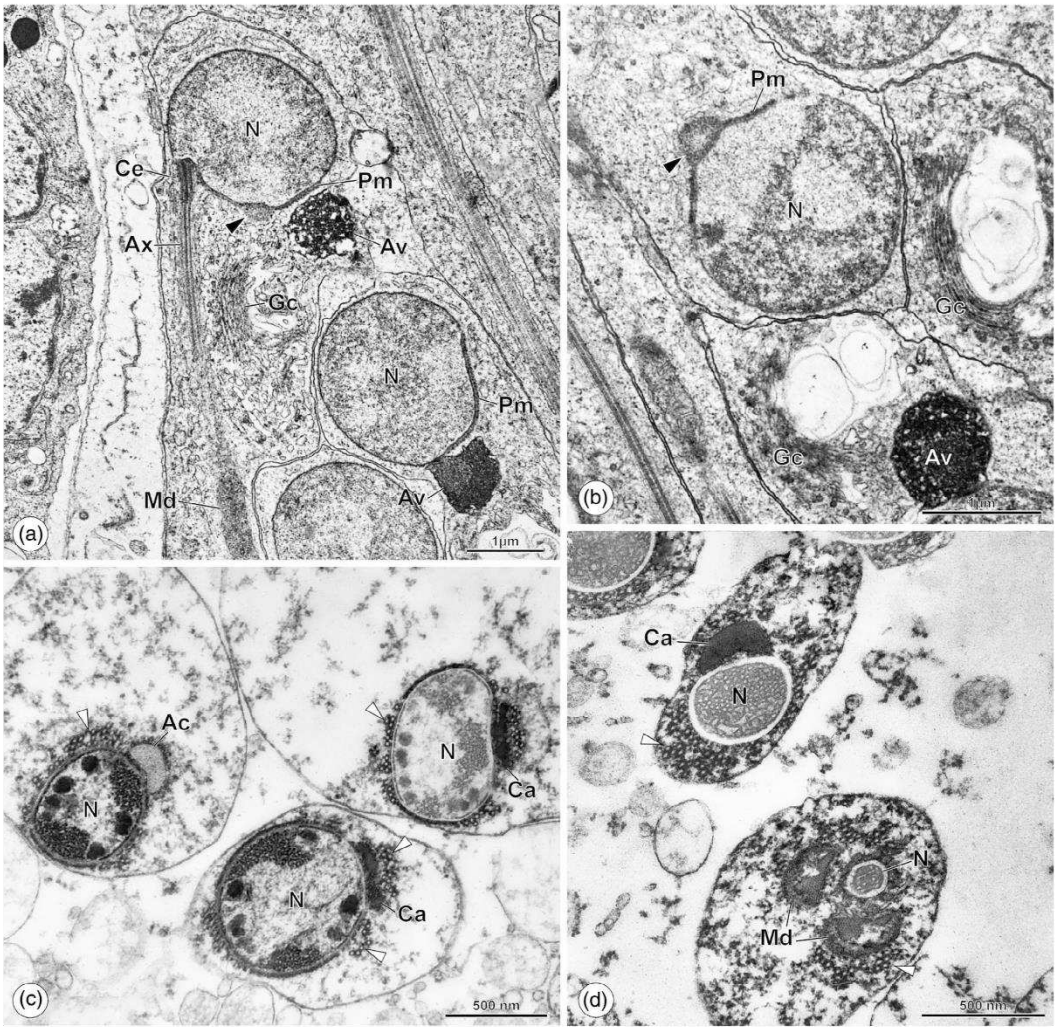


**FIGURE 2** *Corythucha arcuata* spermatids. (a) Early sperm cell with roundish nucleus (N). At its base, the centriole is inserted, from which a strip of pericentriolar material (Pm) extends. A dense proacrosomal vesicle is evident, with a Golgi complex (Gc) near it. (b) Detail of the nucleus with chromatin starting to become denser, also being flanked by the Pm strip. In its medial part, it shows an enlargement or "budding" (arrowhead). (c) The nucleus in more advanced spermatids. The chromatin is more condensed in some points, giving it a granular aspect. The acrosome (Ac) and centriole adjunct material (Ca) flank the nucleus at different points of its length. (d) The chromatin acquires a filamentous aspect before fully condensing in mid to late spermatids. The centriole adjunct flanks the nucleus, and this later has a tapered end inserted between the mitochondrial derivatives (Md). Microtubules (white arrowheads) in the cytoplasm of elongating cells.

**FIGURE 1** *Teleonemia scrupulosa* spermatids. (a) Early spermatid with a large proacrosomal vesicle (Av) is attached to a round nucleus (N) with uncondensed chromatin. Note a piece of a dense strip of pericentriolar material (Pm) in contact with the nuclear envelope. (b) The centriole (Ce) is inserted at the base of the nucleus. Observe the Pm that surrounds it and extends, flanking the nucleus. (c) The proacrosomal vesicle becomes denser and elongates, forming the acrosome (Ac) disposed lateral to the nucleus. (d–f) The nuclei show areas of chromatin start to condense. As it elongates, it diminishes in diameter, as well as the acrosome in the anterior portion of the spermatid. The Pm strips flank the nucleus in the anteroposterior axis, and in (e) we can see the mitochondrial derivatives (Md) tips inserted in the Pm lateral to the posterior nuclear end. They then become symmetrical and partially surround the axoneme (Ax).

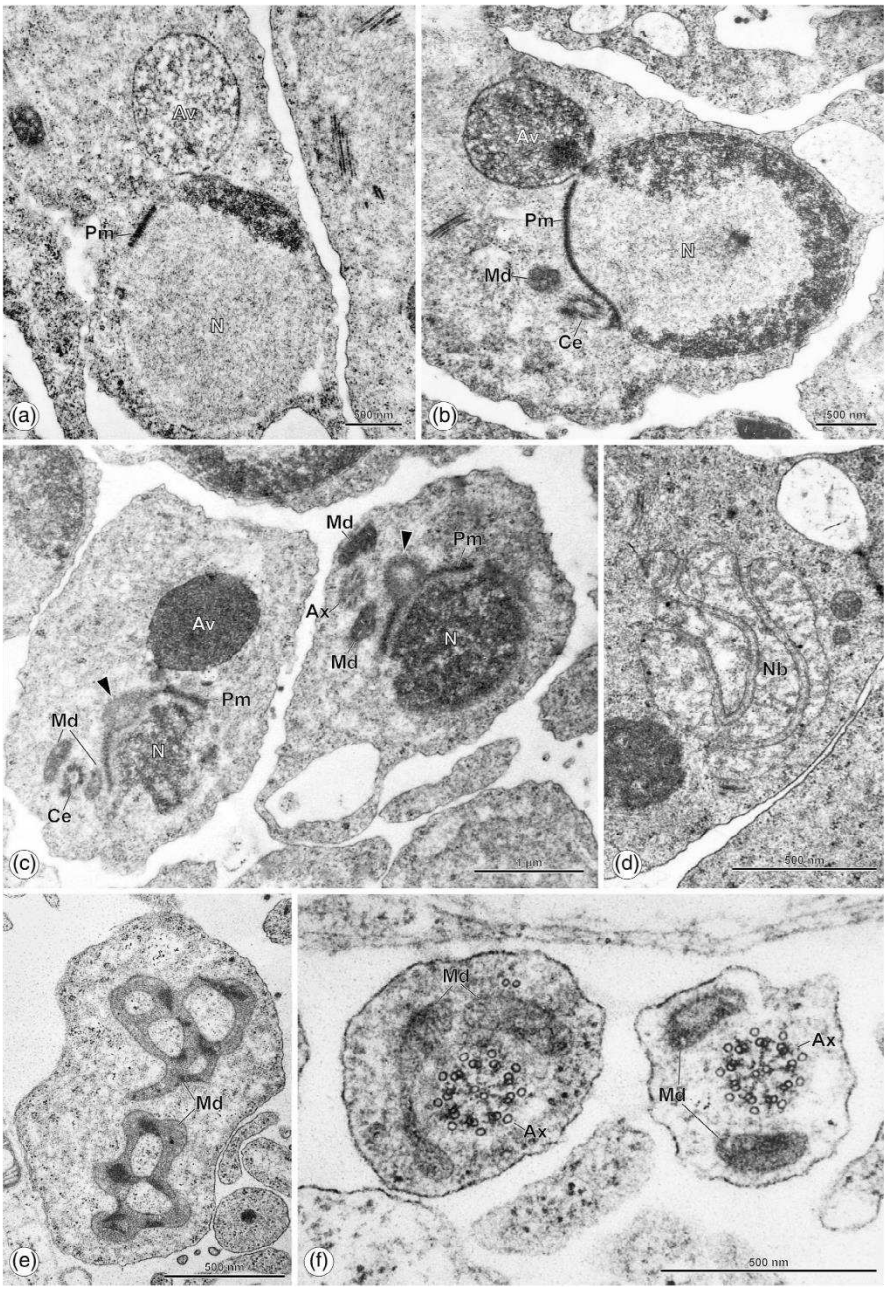


**FIGURE 3** *Vatiga illudens* spermatids. (a, b) Early spermatids with round nuclei, with areas of chromatin condensing at the periphery. The proacrosomal vesicle (Av) is evident, first electron lucent, and then becoming denser. As the strip of pericentriolar material (Pm) that extends from the centriole (Ce). (c) Slightly more advanced spermatids. The chromatin appears more uniform and condensed. The Pm strip shows a "budding" in the medial portion (arrowhead), and two mitochondria (Md) flank the centriole and, below, the axoneme (Ax). (d) Nebenkern (Nb). (e, f) The nebenkern divides into two large mitochondrial bodies (Md) that reshape and elongate following the formation of the flagella.

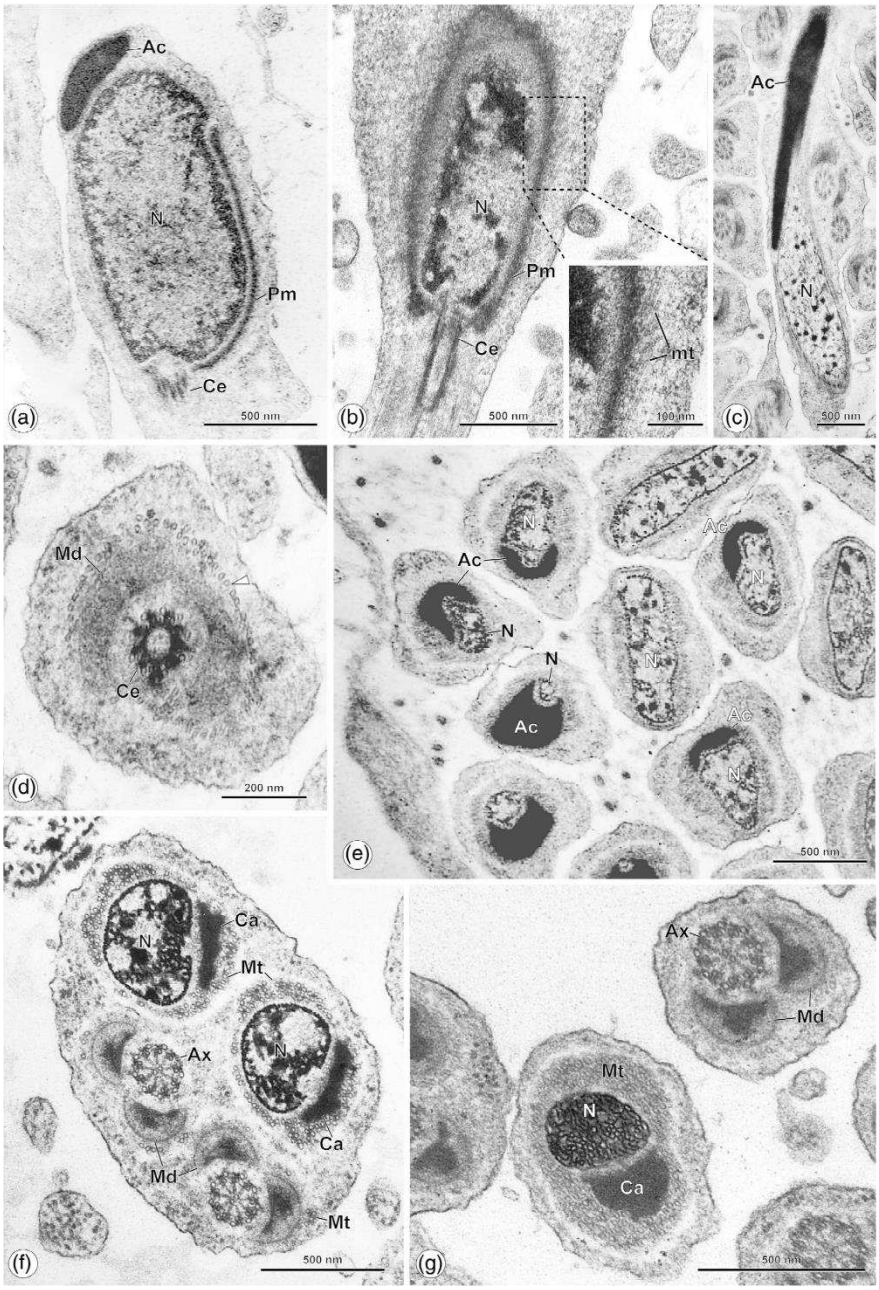


**FIGURE 4** *Vatiga illudens* mid to late spermatids. (a, b) Acrosome (Ac) and nucleus (N) elongating. A centriole (Ce) is inserted in the nuclear base and surrounded by pericentriolar material (Pm) that extends anteriorly, flanking the nucleus. Inset in (b) shows microtubules (Mt) associated with this material. (c) Section of narrow and elongated acrosome and nucleus, which has a somewhat granular chromatin. (d) Detail of the centriole in cross-section. (e) Cross-section of the anterior-medial nuclear portion. The acrosome flanks the tips of the nuclei with granular chromatin. (f) Four cells with fused cytoplasm. The nuclei show a higher chromatin condensation and are flanked by centriole adjunct material (Ca). (g) Late spermatids. The nucleus with filamentous chromatin. In the upper corner, the symmetrical, comma-shaped mitochondrial derivatives (Md) encircle the axoneme (Ax).

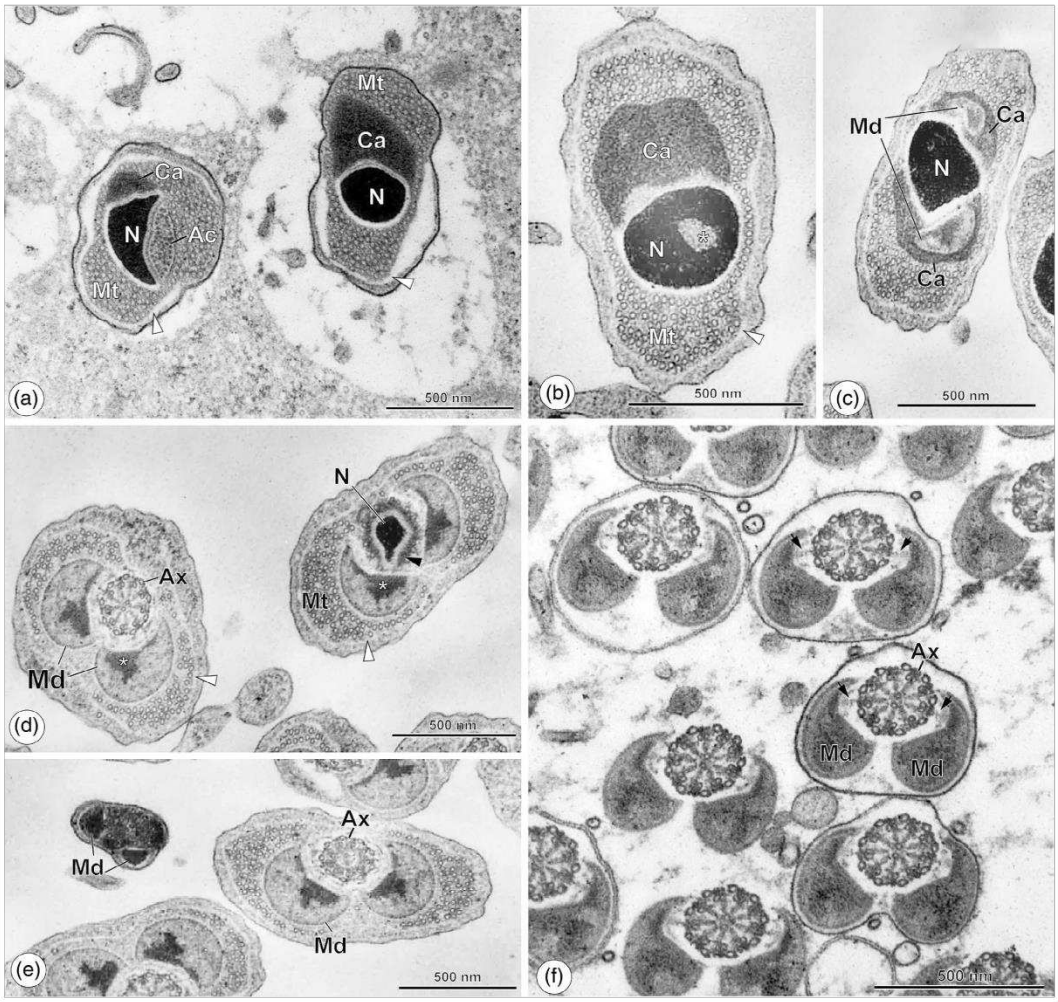


**FIGURE 5** *Teleonemia scrupulosa* late spermatids. (a) In the anterior portion, the nucleus is accompanied by the centriole adjunct (Ca) and the posterior extension of the acrosome (Ac), which show paracrystalline content. In the spermatid medial portion, the centriole adjunct, now wider, flanks the nucleus. (b) The nucleus presents dense chromatin but shows a less condensed area (*). Numerous microtubules (Mt) are present in the cytoplasm around the main structures, which are delimited from the thin periphery cytoplasm by a clear limit (white arrowhead). (c) The centriole adjunct bifurcate in its posterior-most portion, and it houses the anterior tips of the mitochondrial derivatives (Dm) disposed on either side of the nucleus. (d) The nucleus becomes tapered in its posterior end. It is surrounded by microtubules embedded in a dense material (black arrowhead). This portion is replaced by the axoneme (Ax) below this point. Note the presence of paracrystalline formations (*) in the mitochondrial matrix. (e) The end tip of the flagellum can be seen in the left corner. It is much narrower, with a disorganized axoneme and the end tips of the mitochondrial derivatives. (F) Cross-section of the mature sperm flagella. The axoneme is connected to symmetrical derivatives (Md) by bridges (arrows).

6a). They elongate and diminish in diameter, and after undergoing several changes (Figures 3f and 6a,b), they become symmetrical and comma-shaped, with areas of protein crystallization in their matrix (Figures 4g, 5d, 6c, and 7e,f).

During the flagellum formation, the tips of the mitochondrial derivatives are inserted into the centriole adjunct material surrounding the posterior-most part of the nucleus (Figures 1e and 5c). In the later spermatids, the derivatives show a similar arrangement, but the nucleus now has condensed chromatin, with its tapered tip surrounded by some dense material and microtubules not well organized (Figures 2d and 5d). The mitochondrial derivatives are now symmetrical and comma-shaped with paracrystalline areas on their

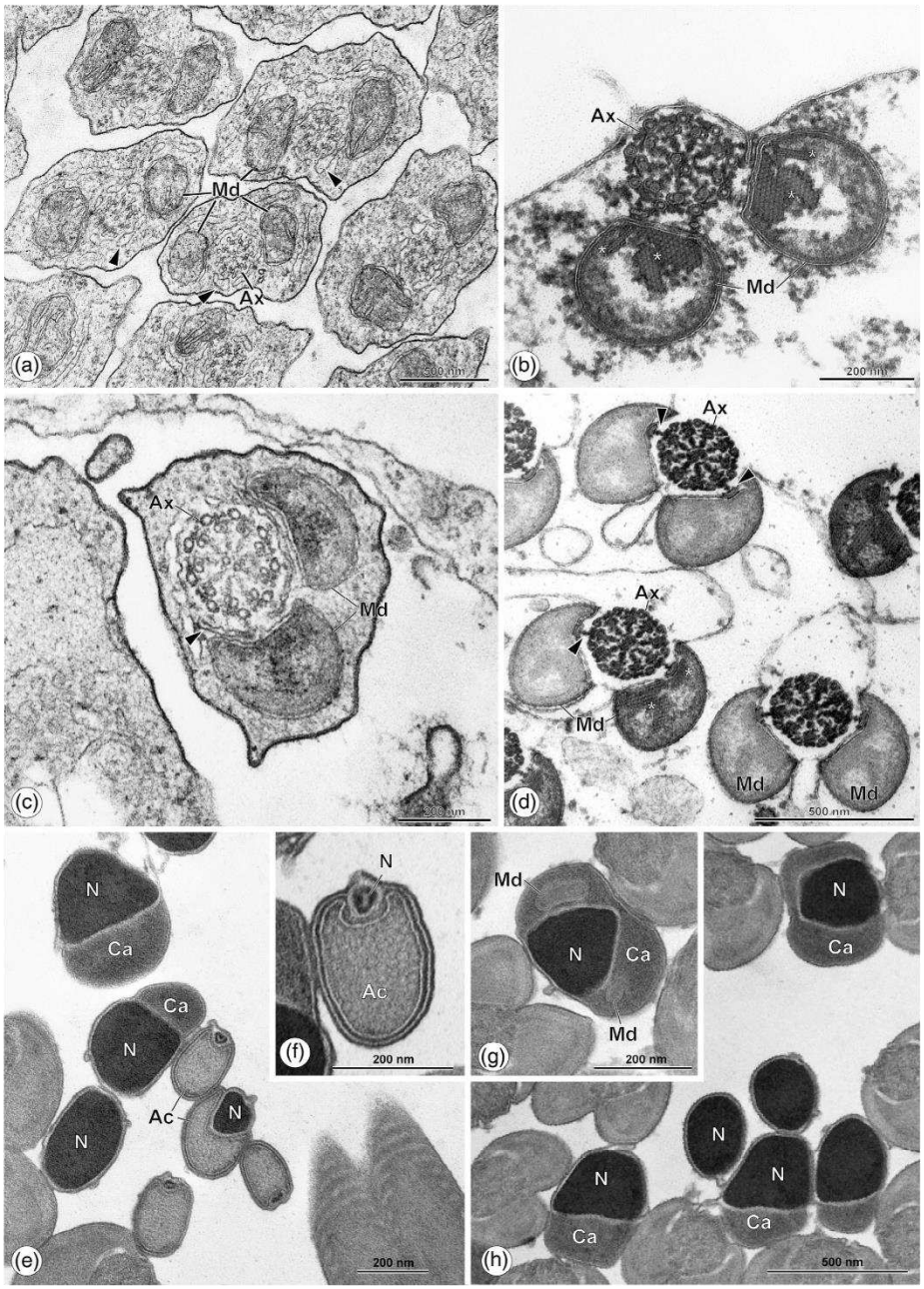


**FIGURE 6** Legend on next page.

matrix (Figures 2d and 5d). Flattened cisterns (periaxonemal cisterns) surround the axoneme (Figure 6a,c). A clear area delimits the region containing the main structures and the layer of microtubules from the thin cytoplasmic periphery (Figure 5a–e), and might be also derived from cisterns.

The mature sperm of all species also show a similar pattern. They are long and filiform cells (*T. scrupulosa*: 252.1 ± 5.3 μm in total length, and nucleus 14.6 ± 0.5 μm long; *V. illudens*: 342.7 ± 8.5 μm long, and nucleus 26.1 ± 0.7 μm; *G. lunulata*: 346.8 ± 13.2 μm long, and nucleus 16.5 ± 0.7 μm; *Leptopharsa* sp.: 218.7 ± 3.5 μm, and nucleus 15.4 ± 0.4 μm; *C. arcuata*: 231 ± 8.7 μm long, and nucleus 16 ± 0.6 μm long), with an acrosome and the centriole adjunct flanking the nucleus. The acrosome is disposed anterolateral to the nucleus and shows low electron density (Figures 6e,f, 8a–d, and 9a), consisting of a monolayer of paracrystalline material (Figures 6e,f and 7a), also seen in late-stage spermatids (Figure 5a). The most notable differences among the species are the degree of condensation of the chromatin and whether the nucleus is flanked along its entire length by other elements (i.e., acrosome and centriole adjunct). In *G. lunulata*, *Leptopharsa* sp., and *T. scrupulosa*, the anterior part of the centriole adjunct and posterior end of the acrosome overlap, disposed lateral to the nucleus (Figure 5a, 7b,c, and 8a,d). In *C. arcuata* and *V. illudens*, the nucleus can be seen "alone" between the acrosome and centriole adjunct regions, with no overlapping portion of the aforementioned structures (Figures 6e,h and 9d).

The flagellum shows the axoneme with a 9 + 9 + 2 microtubular pattern, with the accessory tubules having 16 protofilaments on their wall (Figure 7f). The symmetric mitochondrial derivatives along the axoneme show two paracrystalline inclusions in their matrix, in addition to two bridges in close contact with short cisterns connecting the mitochondrial derivatives to the axoneme (Figures 5f, 6d, 7e, and 9c). In the end portion of the flagellum, the derivatives become more tapered until they are no longer present (Figure 5e); at this level, however, the axoneme still maintains its regular organization. Then, at the flagellum end, only the accessory microtubules are present but still hold a circular organization (Figure 9d).

## 4 | DISCUSSION

Based on our findings, the spermiogenesis process in Tingidae is similar to the overall pattern of Heteroptera. Proacrosomal granules secreted by the Golgi apparatus undergo fusion to form a single large proacrosomal vesicle at the nuclear surface, later becoming the acrosome (Khawar et al., 2019). Changes in the nucleus, with chromatin becoming condensed in mature sperm, as the nebenkern differentiation into the two mitochondrial derivatives lying on either side of the axoneme are similar to other Heteropera, as for insects in general (Baccetti, 1972; Itaya et al., 1980; Lee, 1985). The cisterns delimiting cytoplasmic regions have also been described in several groups, and according to Baccetti (1972), they originate from the Golgi complex. They enclose the main organelles, including the axoneme and mitochondria, whilst unnecessary cytoplasm and other organelles are shed (Dias et al., 2016). The periaxonemal cisterns are also formed by the Golgi complex (Itaya et al., 1980). As Mercati et al. (2009) noted, they give rise to small cisterns that connect the mitochondrial derivatives to the axoneme through bridges, as seen in all heteropterans.

The main difference in the development of spermatids of Tingidae is the formation of the centriole adjunct. Usually, in Heteroptera, scattered material is found around the centriole, called pericentriolar material (Pm), restricted to the posterior end of the nucleus (Dallai, 2014). However, Tingidae shows a strip that extends from the centriolar region, posteriorly, to the anterior nuclear portion. Its extension varies among insect orders (Dallai et al., 2016), and such variation within closely related taxa is also significant. This strip also appears to be linked to the several microtubules in the spermatid cytoplasm, thus forming a "manchette," analogous to mammals, though with a distinct origin (Avidor-Reiss et al., 2020; Russell et al., 1991). The Pm contain γ-tubulin and other proteins, suggesting this structure is a nucleation site for the "manchette" of microtubules (Lüders & Stearns, 2007). So, this structure on spermatids has an essential role in shaping the nucleus and sperm elongation. It is important to note that the Pm strip described here is a distinct structure to the arch-like structure described in the Pentatomomorpha *Coptosoma scutellatum* (Plataspidae: Dias et al., 2016), *Pyrrhocoris apterus* (Pyrrhocoridae: Godula, 1979) and *Euschistos heros* (Pentatomidae). It was referred as the Microtubule Organizing Centre (MTOC) by these authors, as it is responsible for the nucleation of many microtubules that are arranged around the main cytoplasmic components of spermatids. This structure extends laterally from the dense material of the centriole adjunct consisting of a ribbon, and is oriented opposite to the nucleus in transversal view and observed below the nuclear base. In contrast, in Tingidae the strip of centriolar material is in close contact with the nuclear envelope, flanking it in the anteroposterior axis. This arch-like structure has not been observed in any member of the *Cimicomorpha* so far, thus being an important distinction between these taxa. Besides their morphological difference, they are functionally similar.

**FIGURE 6** *Corythucha arcuata* late to mature sperm cells. (a) Observe the two mitochondria (Md), each on either side of the axoneme (Ax) in the forming flagellum. Cisterns (arrowheads) can be seen around the axoneme. (b, c) The mitochondria become symmetrical and exhibit two areas of protein crystallization (*) in their matrix. They also become comma-shaped, as typically present in mature sperm. (d–h) Mature sperm cells. (d) Section of the flagella where we can see the axoneme (Ax) connected to the mitochondrial derivatives through a pair of bridges and small cisterns (arrowheads). (e–h) The long nucleus (N) has fully condensed chromatin. Anteriorly, the nuclear tip is involved by the acrosome (Ac), which shows paracrystalline content (f). After the acrosome is no longer present alongside the nucleus, this latter can be seen alone. Below this point, the centriole adjunct (Ca) begins, flanking it until the flagellar elements begin. In (g) we can see this structure surrounding most parts of the nucleus, and with the mitochondrial tips (Md) inserted in it.

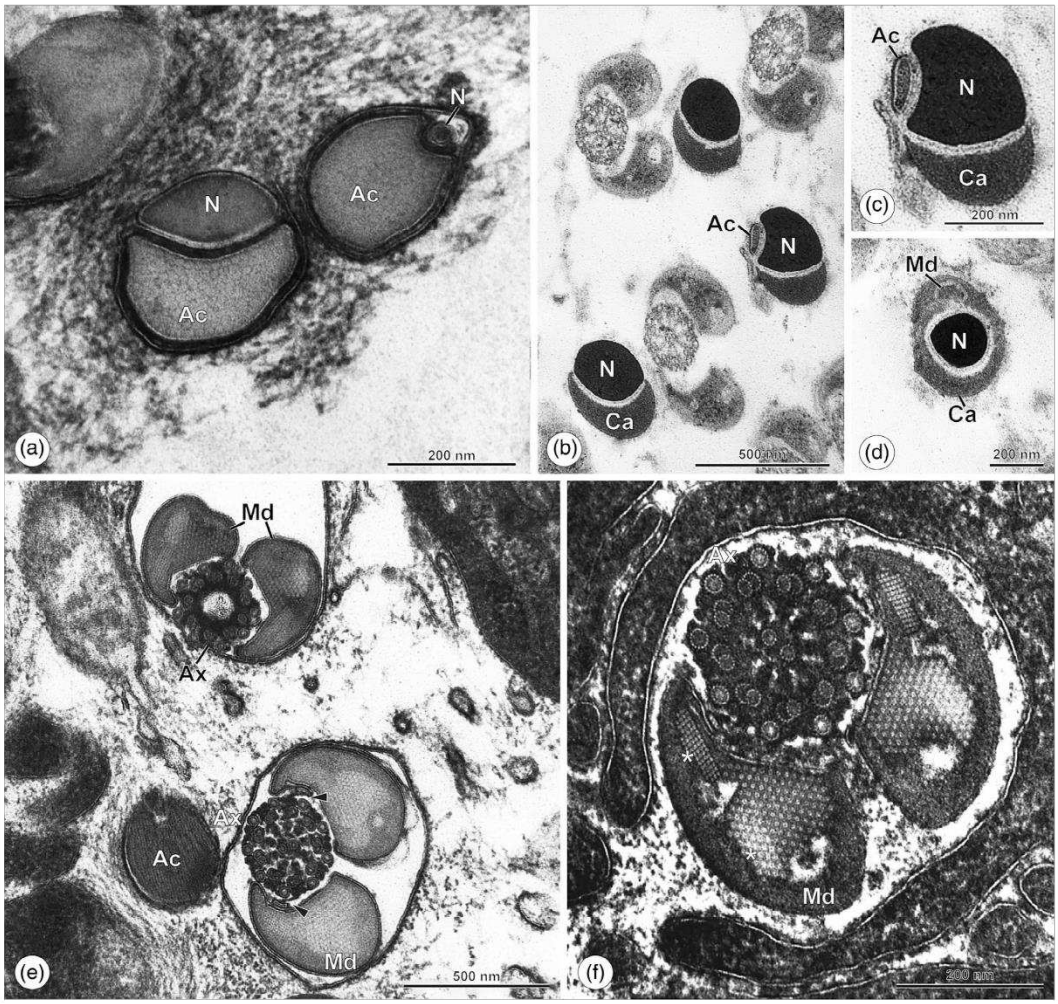


**FIGURE 7** *Leptopharsa* sp. mature sperm. (a) The acrosome (Ac) has paracrystalline content and is disposed alongside the nucleus (N) anteriorly. (b, c) The acrosome becomes narrower before disappearing. Below this point, the nucleus is flanked by the centriole adjunct (Ca). (d) Posteriorly, the centriole adjunct involves the nucleus completely. A tip of mitochondrial derivatives (Md) can be seen inserted in it. (e) In the upper side of the image, an anterior section of a flagellum. It is less wide, and the central pair of microtubules are not yet present in the axoneme (Ax). On the inferior side, a flagellar medial section. The mitochondrial derivatives are symmetrical, and the bridges and cisterns (arrowheads) connecting them to the axoneme can be seen. (f) Detail a flagellum in cross-section. The axoneme exhibits a pair of central microtubules, nine doublets, and nine accessory microtubules made of 16 protofilaments. Two paracrystalline inclusions (*) are evident in the mitochondrial matrix.

The mature sperm of Tingidae has a pretty common morphology compared to other Heteroptera. The sperm cells ranged between 218 and 347 μm, and each species showed specific measurements, allowing us to distinguish them apart when considering total and nucleus length. It contains a head with an acrosome and nucleus, a centriole adjunct that flanks the nucleus for most of it length, and binds the head elements to the flagellum. The acrosome is a monolayer with paracrystalline formations, lacking a perforatorium, and extending alongside the nucleus. The flagellum is made of a long axoneme, partially surrounded by two symmetrical comma-shaped mitochondrial derivatives anchored to the axoneme by two bridges. Furthermore, in each mitochondrial derivative, two paracrystalline inclusions are present. All these features corroborate the group's monophyly, as pointed out by several authors (Araújo et al., 2011,

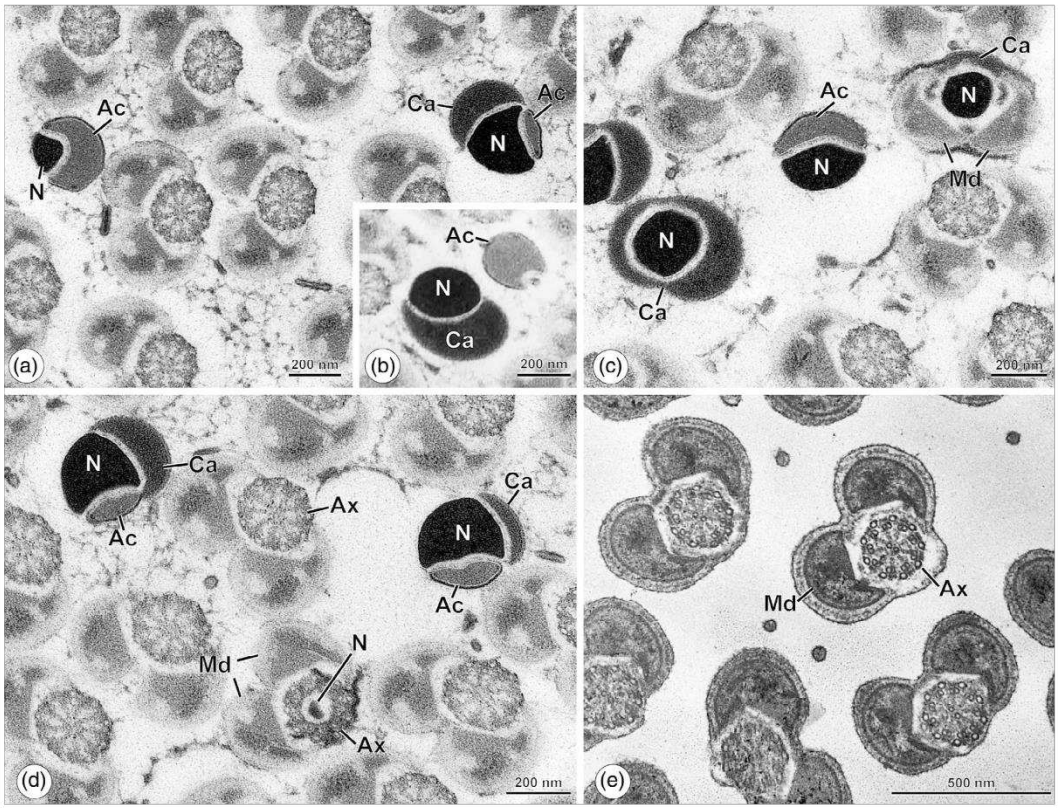


**FIGURE 8** *Gargaphia lunulata* sperm. (a, b) The acrosome (Ac) extends alongside the nucleus (N) anteriorly. It overlaps with the centriole adjunct (Ca) before disappearing towards the medial portion of the nucleus. (c) Below, the centriole adjunct fully encircles the nucleus, and the mitochondrial derivatives (Md) are inserted in this material. (d) The nucleus becomes tapered with its tip inserted in the axoneme (Ax). (e) Flagella in cross-section. The mitochondrial derivatives are symmetrical and surround most of the axoneme (Ax).

2012; Dallai & Afzelius, 1980, 1982; Dias et al., 2016; Dolder, 1988; Itaya et al., 1980; Jamieson et al., 1999; Lee, 1985; Mercati et al., 2009). Thus, these data confirm that the unique characteristics observed in Miridae, as previously hypothesized (Rezende et al., 2023), are exclusive to this taxon. These are the twisted acrosome and the long centriole adjunct as a sole structure between the sperm head and the flagellar components, and not flanking the nucleus, as described here.

The main components of the heteropteran sperm are conserved across the suborder, so any alterations in the basic plan can reveal phylogenetic importance, as we have observed among the species studied here. All the analyzed species belong to the tribe Tingini: Tinginae (ITIS, 2023). *Leptopharsa* and *Gargaphia* are sister genera supported by molecular and morphological data, and their sperm ultrastructure was the most similar, with their long acrosomal vesicle extending alongside the nucleus, overlapping with centriole adjunct anterior extension, thus supporting this relationship (Guilbert et al., 2014). *Teleonemia* and *Corythucha* are more closely related to one another than to the previously mentioned taxa, and the most recent phylogeny did not include the genus *Vatiga* (Guilbert et al., 2014). However, based on the sperm of *V. illudens* being more similar to *C. arcuata*, both showing no overlapping of the acrosome and centriole adjunct extensions, with the nucleus alone in some cross-sections, so we could suggest they are more closely related than *Teleonemia*. Furthermore, both *V. illudens* and *T. scrupulosa* share that their nuclei present areas of uncondensed chromatin, so a further investigation on the position of *Vatiga* within the tribe could reveal if this feature has phylogenetic significance.

In conclusion, the spermiogenesis process in Tingidae shows similarities to the overall pattern of Heteroptera, with some distinct differences in the formation of the centriole adjunct. The mature sperm of Tingidae is similar to other Heteroptera, with features that confirm

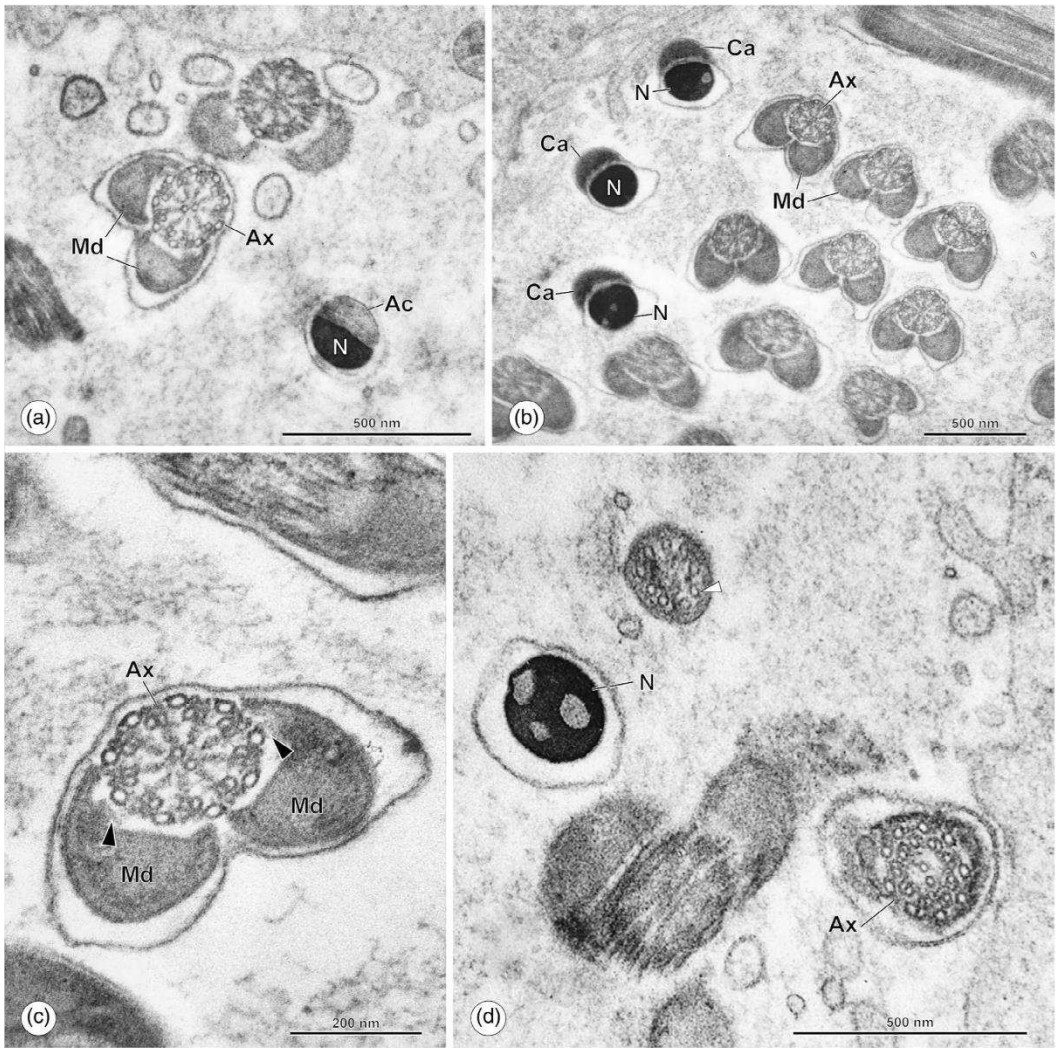


**FIGURE 9** *Vatiga illudens* mature sperm. (a, b) The acrosome (Ac) flanks the anterior tip of the nucleus (N), whilst from the medial to posterior nuclear length, it is accompanied by the centriole adjunct (Ca). Several flagella can be seen. (c) Detail of a flagellum in cross-section. The mitochondrial derivatives (Md) are symmetrical and surround most of the axoneme (Ax). (d) A nucleus not flanked by any other structure can be seen. It exhibits areas of less condensed chromatin. Also, observe the flagellar ends. The axoneme holds its typical organization, but the mitochondria are no longer present, and in its very end, only the accessory microtubules (arrowhead) can be seen.

the group's monophyly. We could also confirm the unique characteristics previously observed in Miridae concerning their twisted acrosome and the long centriole adjunct that do not flank any other structure. Variations in sperm morphology can reveal phylogenetic importance, as observed among the species studied here. Further investigation into the position of the genus *Vatiga* within the tribe could reveal if their sperm features have phylogenetic significance. Overall, these findings contribute to a better understanding of the ultrastructural characteristics of the sperm of Tingidae and their phylogenetic relationships within the suborder.

## AUTHOR CONTRIBUTIONS

**Paulo Henrique Rezende:** Investigation; writing – original draft; writing – review and editing; formal analysis; conceptualization;

methodology; project administration. **Dayvson Ayala Costa:** Visualization; writing – review and editing; investigation; validation. **Mauricio Paulo:** Visualization; writing – review and editing; validation. **Glenda Dias:** Conceptualization; visualization; writing – review and editing; validation; supervision. **Pietro Lupetti:** Validation; visualization; writing – review and editing; supervision; resources. **José Lino-Neto:** Conceptualization; funding acquisition; writing – review and editing; validation; visualization; supervision; formal analysis; resources; project administration. **Romano Dallai:** Formal analysis; conceptualization; funding acquisition; writing – review and editing; visualization; validation; methodology; supervision; resources; investigation.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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## 4. GENERAL CONCLUSION

This thesis explored different aspects of the male reproductive apparatus and sperm morphology of Tingidae. Through sperm morphometric analysis, it was possible to distinguish each species studied by considering at least two parameters that support this approach as a taxonomic tool. The male reproductive system's anatomy was described, showing significant differences among most species. These data sets have taxonomic value and are helpful, especially among closely related species. In the ultrastructural sperm analyses, The Tingidae showed similarities with the general pattern of Heteroptera but with some differences in the formation of the centriole adjunct. Confirming previously described characteristics exclusive to its sister family, Miridae, was also possible. Overall, these findings contribute to a better understanding of Tingidae's reproductive characteristics, expand the family's data, and help outline evolutionary scenarios for such characteristics and phylogenetic implications within the suborder Heteroptera.