UNIVERSIDADE FEDERAL DE VIÇOSA

EXPLORING TAXONOMY AND FUNCTIONAL MORPHOLOGY IN CAMPSURINAE MAYFLIES (EPHEMEROPTERA: POLYMITARCYIDAE)

Gabriel Martins Pantoja
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2024

GABRIEL MARTINS PANTOJA

EXPLORING TAXONOMY AND FUNCTIONAL MORPHOLOGY IN CAMPSURINAE MAYFLIES (EPHEMEROPTERA: POLYMITARCYIDAE)

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Frederico Falcao Salles

Co-advisers: Jose Eduardo Serrao
Carlos Molineri

VIÇOSA - MINAS GERAIS
2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
P198e
2024

Pantoja, Gabriel Martins, 1991-
Exploring taxonomy and functional morphology of Campsurinae mayflies (Ephemeroptera: Polymitarcyidae) / Gabriel Martins Pantoja. – Viçosa, MG, 2024.
1 tese eletrônica (67 f.): il. (algumas color.).

Texto em inglês.
Orientador: Frederico Falcão Salles.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Entomologia, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2024.726
Modo de acesso: World Wide Web.

1. Ephemeroptera. 2. Polymitarcyidae. 3. Campsurinae. 4. Insetos - Classificação. I. Salles, Frederico Falcão, 1975-. II. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. III. Título.

CDD 22. ed. 595.734

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

GABRIEL MARTINS PANTOJA

EXPLORING TAXONOMY AND FUNCTIONAL MORPHOLOGY IN CAMPSURINAE MAYFLIES (EPHEMEROPTERA: POLYMITARCYIDAE)

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: July 10, 2024.

Assent:

_____

Gabriel Martins Pantoja
Author

_____

Frederico Falcao Salles
Adviser

ACKNOWLEDGMENTS

My parents, Sandra Helena and Hodmar, for being my inspiration. To my sister, Ana Clara, for caring about me. To my grandparents, Augusto (in memoriam), Deolinda, Avanete, and Mário (in memoriam), for all the love and affection. To the family I found in Viçosa. To my partner Carolina Arroyo, for all the love, care, and attention, especially during the most difficult times. To Ceniza, for happy moments.To my advisor, Frederico Falcão Salles, for all the support during fieldwork, for sharing his vast knowledge about Ephemeroptera and for introducing me to a group of insects I fell in love with.To my co-advisor, José Eduardo Serrão, for his patience and for the valuable lessons on insect anatomy and histology. My sincere thanks to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for granting the doctoral scholarship (process 140911/2020-0).This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

ABSTRACT

PANTOJA, Gabriel Martins, D.Sc., Universidade Federal de Viçosa, July, 2024. **Exploring taxonomy and functional morphology in Campsurinae mayflies (Ephemeroptera: Polymitarcyidae)**. Adviser: Frederico Falcao Salles. Co-advisers: Jose Eduardo Serrao and Carlos Molineri.

Ephemeroidea is a superfamily of Ephemeroptera that encompasses the only species ofmayflies in which nymphs are adapted to live in buried shelters. Polymitarcyidae is the most diverse family within this superfamily. Recently, the systematic classification of neotropical representatives of Polymitarcyidae has been revised in works based on conventional external morphological characteristics. Polymitarycidae exhibits some unique biological aspects in Ephemeroptera, such as adults having the shortest lifespan among mayflies and nymphs capable of silk production. Campsurinae is the most species-rich family of Polymitarcyidae, with a Pan-American distribution, showing greater diversity in the Neotropical region. Campsurinae nymphs construct U-shaped shelters in various aquatic substrates, and some species have been observed producing silk and manipulating silk fibers on shelter walls. In this study, as a consequence of our investigations on internal morphology, we describe the nymph of Tortopsis canum (Chapter I) and a new species of Campsurus (Chapter II). In the main study of this work, the objective was to analyze the histology and histochemistry of Malpighian tubules of Campsurinae species and species not belonging to Polymitarcyidae but still within Ephemeroidea. The studied Campsurinae species were Campsurus truncatus Ulmer, 1920, Campsurus sp., and Tortopsis canum Gonçalves, Da-Silva & Nessimian, 2011, which build shelters in different sediments (Chapter III). The studied species that do not belong to Polymitarcyidae were Campylocia burmeisteri (Hagen, 1888) (Ephemeroptera: Euthyplociidae) and Hexagenia albivitta (Walker, 1853) (Ephemeroptera: Ephemeridae) (Chapter III). Additionally, we analyzed the ultrastructure of the sperm of Campsurus violaceus Needham & Murphy, 1924 (IV). The Malpighian tubules of the studied Campsurinae species exhibit anatomy similar to the Malpighian tubules of C. burmeisteri and H. albivitta. However, the cell wall of the Malpighian tubules of the Campsurinae species contains secretory columnar cells, which are not found in the tubules of C. burmeisteri and H. albivitta. The secretory

columnar cells of the Campsurinae species display cytoplasm containing granules that react positively to Periodic Acid-Schiff (PAS) and Bromophenol Blue tests, confirming the secretion of glycoprotein material by the Malpighian tubules. The morphology found in the sperm of C. violaceus shows variations that may serve as important phylogenetic signals in evolutionary studies of mayflies, such as the pattern of protofilaments in the accessory tubules, the arrangement of accessory bodies, and the number of mitochondria.

Keywords: ephemeroidea, south america, mayflies, silk production, sperm, ultrastructure

RESUMO

PANTOJA, Gabriel Martins, D.Sc., Universidade Federal de Viçosa, julho de 2024. **Explorando a taxonomia e a morfologia funcional em Campsurinae (Ephemeroptera: Polymitarcyidae)**. Orientador: Frederico Falcao Salles. Coorientadores: Jose Eduardo Serrao e Carlos Molineri.

Ephemeroidea é uma superfamília de Ephemeroptera que engloba as únicas espécies cujas ninfas são adaptadas a viver em túneis escavados. Polymitarcyidae é a família mais diversa desta superfamília. Recentemente, a classificação sistemática de representantes neotropicais de Polymitarcyidae tem sido revisada em estudos baseados em características convencionais da morfologia externa. Polymitarcyidae apresenta aspectos biológicos únicos em Ephemeroptera, como os adultos que possuem o menor tempo de vida entre as efemerópteras e as ninfas que são capazes de produzir seda. Campsurinae é a família mais especiosa de Polymitarcyidae, possuindo distribuição panamericana, com maior diversidade na região neotropical. As ninfas de Campsurinae constroem abrigos em formato de U em diferentes substratos e algumas espécies foram observadas produzindo seda e manipulando as fibras nas paredes dos abrigos. Neste trabalho, como consequência da investigação sobre morfologia interna, nós descrevemos a ninfa de Tortopsis canum Gonçalves, Da-Silva & Nessimian, 2011 (Capítulo I) e uma nova espécie de Campsurus (Capítulo II). No principal estudo deste trabalho, o objetivo foi analisar a histologia e a histoquímica de túbulos de Malpighi de espécies de Campsurinae e de espécies não pertencentes à Polymitarcyidae, mas ainda dentro de Ephemeroidea. As espécies de Campsurinae estudadas foram Campsurus truncatus Ulmer, 1920, Campsurus sp. e T. canum, (Capítulo III). As espécies estudadas que não pertencem a Polymitarcyidae foram Campylocia burmeisteri (Hagen, 1888) (Ephemeroptera: Euthyplociidae) e Hexagenia albivitta (Walker, 1853) (Ephemeroptera: Ephemeridae) (Capítulo III). Além disso, nós analisamos a ultraestrutura do espermatozoide de Campsurus violaceus Needham & Murphy, 1924 (IV). Os túbulos de Malpighi das espécies de Campsurinae estudadas apresentam anatomia similar aos túbulos de Malpighi de C. burmeisteri e de H. albivitta. No entanto, a parede celular dos túbulos de Malpighi das espécies de Campsurinae apresentam células colunares secretoras, não encontradas nos túbulos de C. burmeisteri e de H. albivitta. As células

colunares secretoras das espécies de Campsurinae apreentam citoplasma contendo grânulos que reagem positivamente aos testes de Ácido Periódico de Schiff (PAS) e Bromofenol Azul, confirmando a secreção de material glicoproteico pelos túbulos de Malpighi. A morfologia encontrada no espermatozoide de C. violaceus exibe variações que podem servir como importantes sinais filogenéticos em estudos evolutivos de efemerópteros, como o padrão de protofilamentos nos túbulos acessórios, o arranjo de corpos acessórios e o número de mitocôndrias.

Palavras-chave: ephemeroidea, américa do sul, efemerópteras, produção de seda, espermatozoide, ultraestrutura

LIST OF ILLUSTRATIONS

LIST OF TABLES

SUMMARY

INTRODUCTION

Order Ephemeroptera

Ephemeroptera, or mayflies, are the oldest lineage of winged insects, with PanEphemeroptera dating from the Carboniferous period (Kukalová-Peck, 1985; Brittain & Sartori, 2003). Mayflies are the only one among extant pterygotes to retain the primitive characteristics: caudal median filament and subimaginal molt (Grimaldi & Engel, 2005). In addition to these characteristics, adults of mayflies exhibit the following autapomorphies: vestigial mouthparts, enlarged compound eyes and an extra joint between the tibia and the first segment of the tarsus in males, palmén body, midgut filled with air, hindwing reduced, anal regions of wings reduced. The nymphs exhibiting tracheal gills on the lateral of the abdomen as an autapomorphy (Beutel et al., 2013). The nymphs of mayflies are entirely aquatic and get oxygen using tracheal gills (Brittain, 1982). The adults exhibit a swarming behaviour, often named “nuptial dance”, that is initiated by the emergence of males and soon numerous females mixed with the swarms (Brinck, 1957).

Ephemeroidea

Ephemeroidea, commonly known as burrowing mayflies, is a superfamily of Ephemeroptera with worldwide distribution (McCafferty, 1975). The colloquial name "burrowing mayflies" is misleading for this superfamily because, although most Ephemeroidea species build and live in tunnels in various aquatic substrates, the nymphs of some families are found under rocks, on leaf litter, and on gravel (Nguyen & Bae, 2004; Gonçalves et al., 2017). Currently, Ephemeroidea comprises the families Ephemeridae, Euthyplociidae, Ichthybotidae, Palingeniidae, Potamanthidae, Behningiidae, and Polymitarcyidae (Miller et al., 2018). In the most recent phylogenomics of Ephemeroidea (Miller et al., 2018), Polymitarcyidae is recovered as the sister group of the clade (Behningiidae + Euthyplociidae) + (Ephemeridae + Ichthybotidae + Palingeniidae).

Family Polymitarcyidae

Polymitarcyidae is the most diverse family within Ephemeroidea, with 10 genera and 84 species, exhibiting the greatest diversity in the Neotropical Region (Sartori & Brittain, 2015; Salles et al., 2024; Jacobus, 2024). The adults of Polymitarcyidae exhibit one of the shortest life among winged insects, emerging synchronously en masse and having an aerial stage as short as two hours (McCafferty & Bloodgood, 1989; Molineri, 2010). Other interesting

biological aspects of Polymitarcyidae is that the subimaginal molt occurs during the flight, with the cuticle peeling off like sun-burned skin (Domínguez et al., 2023). Furthermore, the legs of the adults are vestigial or reduced except for the forelegs of males wich are used to grasp the female during the copula (Domínguez et al., 2006).

This family is divided into three subfamilies: Polymitarcyinae, Asthenopodinae, and Campsurinae (Molineri et al., 2015). Campsurinae is the most diverse subfamily, comprising 63 species in 3 genera, *Campsurus* Eaton, 1868, *Tortopus* Needham & Murphy, 1924, and *Tortopsis* Molineri, 2010 (Jacobus, 2024; Salles et al., 2024).

Campsurus is the most diverse genus of Campsurinae, comprising 44 species, with 40 of them found in South America (Jacobus, 2024; Salles et al., 2024). The main diagnostic feature of the genus is the reduction of legs in the adult stage, with tibiae and tarsi absent, except for the male forelegs (Molineri & Salles, 2017). Other characteristics found in adults include forceps 1 segmented and an additional penis lobe, named thumb (Molineri et al., 2015; Molineri & Salles, 2017). *Campsurus* nymphs can be diagnosed by the following characteristics: mandibular tusks with prominent basal or sub-basal tubercule on the inner margin, outer margin of mandibular tusks with numerous setae, and bilamellate abdominal gill I (Molineri, 2010; Molineri et al., 2015).

Tortopsis Molineri, 2010 is the most recently established genus of Polymitarcyidae, with a pan-American distribution, comprising 12 species, 9 of which are exclusive to South America (Molineri, 2010; Jacobus & McCafferty, 2024; Salles et al., 2024). The recent erection of *Tortopsis* from certain *Tortopus* species is justified by several morphological differences, such as in adults: male ninth abdominal sternum entire; parastyli more than 5 times the length of parastyli base, penes completely divided, and female forewing without intercalary veins between R2+3 and IR veins (Molineri, 2010). The diagnostic characteristics of the nymphs are: mandibular tusks with a single subapical tubercle on the median margin, and distal projection of fore-tibia tarsus ⅔ the length of the claw (Molineri, 2010).

Silk production in Polymitarcyidae

Polymitarcyidae is one of the families within Ephemeroidea whose nymphs build U-shaped tunnels in various aquatic substrates (McCafferty, 1975). Nymphs of Asthenopodinae build tunnels in aquatic vegetation, whereas nymphs of Campsurinae construct tunnels in both soft and hard clay sediments (Hartland-Rowe, 1958). An interesting aspect from an evolutionary perspective in certain Polymitarcyidae is the silk production by nymphs (Hartland-Rowe, 1958; Satler, 1967; Molineri & Emmerich, 2010). Nymphs of *Povilla adusta* Navas (Asthenopodinae) were observed secreting silk through the anus and moving the fibers

through the mouthparts and the front legs in a controlled environment (Hartland-Rowe, 1958). While for genera of Campsurinae, there is only field observation of silk-based structures built by species of *Campsurus* living in external shelters on rocks (Satler, 1967; Molineri & Emmerich, 2010; Salles, Boldrini & Pantoja, personal observation). Besides that, the organ responsible for silk production in Polymitarcyidae nymphs is still uncertain (Sutherland et al., 2010). Satler (1967) analyzed the lumen of the Malpighian tubules of *Asthenopus* species and observed granules that test positive for the presence of amino acids. However, the histological characteristics of the Malpighian tubules of Polymitarcyidae has never been investigated.

The silk production is an interesting biological aspect of mayflies. Compared with other insects, the physiology of silk-production is poorly known, with just observational and non-histological chemical studies (Hartland-Rowe, 1953; Hartland-Rowe, 1958; Satler, 1967), without a confirmation of the silk-producing organ.

Sperm morphology

Mayfly imagos copulate immediately after the emergence, showing rapid one-time mating and high fecundity values (Brittain & Sartori; Araújo et al., 2021). The short mating period in mayflies must have exerted selective pressure for adaptations in the reproductive system of the imagos, particularly in males, which have testes that are partially or entirely disintegrated and a swollen seminal duct, full of sperm, that opens directly onto the penis (Soldán, 1979a; Araújo et al., 2021).

Despite these unique characteristics, the stage does not show great interspecific variation in the anatomy of components, aside from variations in the intrinsic musculature associated with the seminal ducts (Landa & Soldán, 1985; Britto et al., 2015). The best model for a comparative anatomy of the reproductive system in Ephemeroptera is the mature nymph, which is the last stage of mayflies in which the testes are complete (Sóldan, 1979a, Araújo et al., 2021). The mature nymphs of mayflies exhibit a pair of testes consisting of testicular follicles that connect directly to the seminal duct, not forming vasa efferentia and lacking accessory glands (Soldán, 1979a). The variation in the anatomical pattern of the male reproductive system in mature nymphs can be found in the shape and arrangement of the testes in relation to the body; in the shape, arrangement, and positioning of the testicular follicles in relation to the seminal duct; and in the shape of the seminal duct (Landa & Soldán, 1985).

The variation in the morphological pattern of the anatomy of the reproductive system must be related to differences in characteristics of sperm in Ephemeroptera (Gaino & Mazzini, 1991). The external morphology of mayflies' sperm exhibit a great variation, with very remarkable difference in shape and size of the head and in the length of the flagellum (Soldán,

1979b). The flagellum is so variable in mayflies' sperm that in some families it is 3 or 5 times the head length, and in one family, Leptophlebiidae, the sperms are aflagellate (Soldán, 1979b; Gaino & Mazzini, 1991; Brito et al., 2011).

The ultrastructure of mayflies' sperm is well studied in terms of representing each family (Bacceti et al., 1969; Gaino & Mazzini, 1991; Brito, 2012; Brito et al., 2015). Among the mayflies occurring in Brazil, for example, out of 10 families, only one lacks a representative species with known spermatic ultrastructure (Brito, 2012). There is a great variation in sperms from different families, but there is a lack of information about variation among species within the same family, either to confirm a family autapomorphy or to identify variations in sperm among species. The most studied species are from Leptophlebiidae, with 6 species described, whereas Polymitarcyidae only has 2 species with known spermatic ultrastructure (Bacceti et al., 1969; Grimm, 1985; Gaino & Mazzini, 1991; Brito, 2012; Brito et al., 2015).

Polymitarcyidae is one of the most diverse families of mayflies, and the sperm ultrastructure for this family is known for only two species (Brito, 2012). The study of sperm morphology can contribute to species identification and to outlining the phylogenetic relationships in Polymitarcyidae based on the knowledge of variations in characteristics at the ultrastructural level among sperm of different species.

OBJECTIVES

Main Objective: The objective of this study is to expand the knowledge on the taxonomy, physiology and reproductive biology of the Ephemerotpera, with a focus on the Campsurinae (Polymitarcyidae).

Specific Objectives:

- To contribute to the systematics of *Tortopsis*, with the description of the nymph of *Tortopsis canum;*
- To provide a key to the nymphs of all South American species of *Tortopsis*;
- To describe a new species of *Campsurus* Eaton, 1868*;*
- To describe the anatomy of the Malpighian tubules of *Campsurus truncatus* Ulmer, 1920, a silk-producing species, and *Campylocia burmeisteri* (Hagen, 1888) a non-silk-producing species;
- To analyze the histology and histochemistry of epithelial cells in the Malpighiantubules of *Campsurus truncatus*; *Campsurus* sp.; *Tortopsis canum*; *Campylociaburmeisteri* and *Hexagenia albivitta* (Walker, 1853);

- To understand the ultrastructural characteristics of sperm from *Campsurus* and to generate potential informative characters for new phylogenetic studies of Polymitarcyidae.

REFERENCES

ARAÚJO, V. A.; DIAS, L. G.; SERRÃO, J. E. Rapid and efficient mating in mayflies (Ephemeroptera): morphological and reproductive strategies in primitive winged insects. **The Science of Nature**, v. 108, p. 1-5, 2021.

BACCETTI, B.; DALLAI, R.; GIUSTI F. The Spermatozoon of Arthropoda VI. Ephemeroptera. **Journal Ultrastructure Research**, v. 29, p. 343-349, 1969.

BEUTEL, R. G.; Friedrich, F; GE, S. K.; Yang, X. **Insect morphology and phylogeny: a textbook for students of entomology**. Berlin: Walter de Gruyter, 2013. 516 p.

BRITO, P.; MANCINI, K.; SALLES, F.F., RIZZI, E.A.; Dolder, H. The sperm of *Hexagenia (Pseudeatonica) albivitta* Walker (Ephemeroptera: F ossoriae: E phemeridae). ***Acta Zoologica***, v. 96, n. 2, p. 160-166, 2015.

BRITO, P.; SALLES, F. F.; DOLDER, H. Characteristics of the male reproductive system and spermatozoa of Leptophlebiidae (Ephemeroptera). **Neotropical entomology**, v. 40, p. 103-107, 2011.

BRITO, P. **Morfologia do sistema reprodutivo masculino e dos espermatozoides de Ephemeroptera (Insecta) e análise do seu potencial filogenético**. Doutorado. Campinas: Unicamp. 2012

BRITTAIN, J. E. Biology of mayflies. **Annual review of entomology**, v. 27, n. 1, p. 119-147, 1982.

BRITTAIN J. E.; SARTORI M. Ephemeroptera (Mayflies). In: RESH, V. H.; CARDÉ, R. T. (eds). **Encylopedia of Insects**. Amsterdam: Academic Press, 2003. pp. 373-380

BRINCK, P. Reproductive system and mating in Ephemeroptera. **Opuscula Entomol**, v. 22, p. 1-37, 1957.

DOMÍNGUEZ, E.; MOLINERI, C.; PESCADOR; M. L., HUBBARD; M. D. & NIETO, C. Ephemeroptera of South America. In: ADIS, J. R.; ARIAS, G.; WANTZEN K. M. (Eds). **Aquatic Biodiversity of Latin America. Volume 2**. Moscow-Sofia: Pensoft, 2006. 646 p.

DOMÍNGUEZ, E., VAN DE KAMP, T., MIKÓ, I., CUEZZO, M. G., & STANICZEK, A. H. The function of wing bullae in mayflies (Insecta: Ephemeroptera) reveals new insights into theearly evolution of Pterygota. **BMC biology**, v. 21, n. 1, 268, 2023.

EATON, A. E. An outline of a re-arrangement of the genera of Ephemeridae. **Entomologist's Monthly Magazine**, v. 6, p. 131–132, 1868

ESBEN-PETERSEN, T. New and little-known species of Ephemerida from Argentine. (Neuropt.). **Deutsche Entomologische Zeitschrift**, 333–342, 1912.

GAINO, E.; MAZZINI, M. Aflagellate sperm in three species of Leptophlebiidae (Ephemeroptera). **International Journal of Insect Morphology and Embryology**, v. 20, n. 3, p. 119-125, 1991.

GRIMALDI, D.; ENGEL, M. S. **Evolution of the Insects**. Cambridge: University Press, 2005. 755 p.

GRIMM, V. R.Vergleichend-anatomische Untersuchung der abdominalen Muskulatur und der Gonoducte männlicher Ephemeroptera-Imagines (Insecta). **Stuttgarter Beiträge zur Naturkunde**

(Serie A) v. 59, p. 1-59,1985.

GONCALVES, I. C.; DA-SILVA, E. R.; NESSIMIAN, J. L. First record and new species of *Tortopsis Molineri*, 2010 (Ephemeroptera, Polymitarcyidae) from Brazil. **Zootaxa**, v. 2866, n. 1, p. 50–54, 2011.

GONÇALVES, I. C., TAKIYA, D. M., SALLES, F. F., PETERS, J. G., & NESSIMIAN, J. L. (2017). Integrative taxonomic revision of Campylocia (mayflies: Ephemeroptera, Euthyplociidae). **Systematics and Biodiversity**, v. 15, n. 6, p. 564-581, 2017.

HAGEN, H. Unsere gegenw€ artige Kenntniss der Ephemeren. **Stettiner Entomologische Zeitung**, 49, 221–232, 1888.

HARTLAND-ROWE, R. Feeding mechanism of an ephemeropteran nymph. **Nature**, v. 172, p. 1109-1110, 1953.

HARTLAND-ROWE, R. The biology of a tropical mayfly Povilla adusta Navas (Ephemeroptera, Polymitarcidae) with special reference to the lunar rhythm of emergence. **Revue de Zoologie et de Botanique Africaines**, v. 58, n. 3-4, p. 185-202, 1958.

JACOBUS, L. M. (2024) **Mayfly Central**. Availabe from: https://www.entm.purdue.edu/mayfly/index.php (access 31 Jan 2024)

KUKALOVÁ-PECK, J. Ephemeroid wing venation based upon new gigantic Carboniferous mayfl ies and basic morphology, phylogeny, and metamorphosis of pterygote insects (Insecta, Ephemerida). **Canadian Journal of Zoology**, v. 63, p. 933-955, 1985.

LANDA, V.; SOLDÁN, T. Phylogeny and higher classification of the order Ephemeroptera: a discussion from the comparative anatomical point of view. Praga: Ceskolovenske Akademie, 1985. 128 p

MCCAFFERTY W. P.; BLOODGOOD D. W. The female and male coupling apparatus in *Tortopus* mayflies. **Aquatic Insects**, v. 11, p. 141–146, 1989.

MCCAFFERTY, W. P. The burrowing mayflies (Ephemeroptera: Ephemeroidea) of the UnitedStates. **Transactions of the American Entomological Society**, v. 101, p. 447-504, 1975.

MILLER, D. B.; BARTLETT, S.; SARTORI, M.; BREINHOLT, J. W.; OGDEN, T. H. Anchored phylogenomics of burrowing mayflies (Ephemeroptera) and the evolution of tusks. **Systematic Entomology**, v. 43, p. 692-701, 2018.

MOLINERI, C. A cladistic revision of Tortopus Needham & Murphy with description of the new genus Tortopsis (Ephemeroptera: Polymitarcyidae). **Zootaxa**, v. 2481, n. 1, p. 1-36, 2010.

MOLINERI, C.; EMMERICH, D. New species and new stage descriptions of Campsurus major species group (Polymitarcyidae: Campsurinae), with first report of silk-case construction in mayfly nymphs. **Aquatic Insects**, v. 32, n. 4, p. 265-280, 2010.

MOLINERI, C.; SALLES, F. F. Review of selected species of *Campsurus* Eaton 1868 (Ephemeroptera: Polymitarcyidae), with description of eleven new species and a key to male imagos of the genus. **Zootaxa**, v. 4300, n. 3, p. 301-354, 2017.

MOLINERI, C.; SALLES, F. F.; PETERS, J. G. Phylogeny and biogeography of Asthenopodinae with a revision of Asthenopus, reinstatement of Asthenopodes, and the description of the new genera Hubbardipes and Priasthenopus (Ephemeroptera,

Polymitarcyidae). **Zookeys**, n. 478, p. 45, 2015.

NGUYEN, V. V.; BAE, Y. J. Descriptions of Rhoenanthus sapa, new species, and larval stage of R. magnificus Ulmer (Ephemeroptera: Potamanthidae) from Vietnam. **Aquatic Insects**, v. 26, n. 1, p. 9-17, 2004.

SALLES, F. F., MOLINERI, C., NIETO, C., LIMA, L. R. C., DIAS, L. C., BOLDRINI, R., MARIANO, R. & DOMÍNGUEZ, E. **Ephemeroptera da América do Sul**. 2024. Available from: http://ephemeroptera.com.br/ (access 31 Jan 2024).

SATTLER, W. Über die Lebensweise, insbesondere das Bauverhalten, neotropischer Eintagsfliegen-Larven (Ephemeroptera, Polymitarcidae) Mit 14 abbildungen. **Studies on Neotropical Fauna and Environment**, v. 5, n. 2, p. 89-110, 1967.

SÓLDAN, T. The structure and development of the male internal reproductive organs in six European species of Ephemeroptera. **Acta Etnomologica Bohemolovasca**, v. 76, 9. 22-33, 1979a.

SOLDÁN, T. A comparative study of spermatozoa of some Central European Ephemeroptera. **Acta entomologica bohemoslovaca**, v. 76, p. 223-230, 1979b.

SUTHERLAND, T. D.; YOUNG, J. H.; WEISMAN, S.; HAYASHI, C. Y.; MERRITT, D. J. Insect silk: one name, many materials. **Annual review of entomology**, v. 55, p. 171- 188, 2010.

ULMER, G. Neue Ephemeropteren. **Archiv fu¨r Naturgeschichte**, v. 85(A), p. 1–80, 1920.

WALKER, F. **Catalogue of the specimens of neurpterous insects in the collection of the British Museum**. Edward Newman: London, 1853.

CHAPTER I - THE NYMPH OF *Tortopsis canum* Gonçalves, Da-Silva & Nessimian, 2011 (Ephemeroptera: Polymitarcyidae)

Zootaxa 5447 (2): 289–294
https://www.mapress.com/zt/

Correspondence

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5447.2.10
http://zoobank.org/urn:lsid:zoobank.org:pub:3ADCA725-B6BF-4A3C-BFE0-B3254DDA43FD

The nymph of *Tortopsis canum* Gonçalves, Da-Silva & Nessimian (Ephemeroptera: Polymitarcyidae)

GABRIEL M. PANTOJA[1,2,3] & FREDERICO F. SALLES[1,*]
[1]*Museu de Entomologia, Depto. de Entomologia, Universidade Federal de Viçosa, CEP 36.570-900, Viçosa, MG, Brazil*
[2]*Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa, CEP 36.570-900, Viçosa, MG, Brazil*
[3] *gabriel.pantoja@ufv.br; https://orcid.org/0000-0002-1769-6070*
**Corresponding author: frederico.salles@ufv.br; https://orcid.org/0000-0002-3016-9902*

The nymph of *Tortopsis canum* Gonçalves, Da-Silva & Nessimian, 2011 is described based on material from the Rio Doce Basin. It can be distinguished from other species of the genus by the following combination of characteristics: mandibles with around 10 spines on inner margin of tusk, basal to subdistal tubercle; outer margin of tusk with row of around 30 stout spines; occiput with gray anastomosed lines distributed throughout the area; wing pads shaded with gray on costal margin and some longitudinal veins. A key to the nymphs of all South American species known in this stage is presented.

Key words: mayfly, Ephemeroidea, Campsurinae, Taxonomy, Neotropics, South America

The genus *Tortopsis* Molineri, 2010 (Ephemeroptera: Polymitarcyidae) was described to include some species previously assigned to *Tortopus* Needham & Murphy, 1924. It can be differentiated among other characteristics, based on mandibular tusks with one large subapical denticle on inner margin; fore tibia-tarsus with a distal projection, with ⅔ the length of the claw in nymphs; male gonopore associated with a claw-like structure and penes separated from the base in adults (Molineri, 2010).

Currently, *Tortopsis* is represented by 12 species distributed along the American continent, but only for five of them the nymphal stage has been described: *Tortopsis toro* Molineri, Dias & Zuñiga, 2021, *T. andaki* Molineri, Dias & Zuñiga, 2021, *T. sarae* (Domínguez, 1985), *T. puella* (Pictet, 1843), and *T. obscuripennis* (Domínguez, 1985). The lack of knowledge on the nymphal stage not only precludes the identification of the immatures at the specific level, but also hinders additional efforts to understand the evolutionary history of *Tortopsis* and its sister group, *Tortopus*. Of the 30 characters selected by Molineri *et al.* (2021) in the most recent cladistic analysis of the group, none include the nymphal stage.

Tortopsis canum Gonçalves, Da-Silva & Nessimian, 2011 was the first species of the genus reported from Brazil and its description was based on males and females collected at Macaé river, Rio de Janeiro state (Gonçalves, Da-Silva & Nessimian, 2011). Its known distribution includes two localities in Southeastern Brazil, Rio de Janeiro state and Espírito Santo state (Gonçalves, Da-Silva & Nessimian, 2011; Molineri, Salles & Boldrini, 2012). In order to diminish this shortfall, the aim of the present study is to describe the unknown nymphal stage of *T. canum* based on material from the Rio Doce Basin (Fig. 1A–C). Additionally, a key to the nymphs of all South American species known in this stage is presented.

Material and methods

The nymphs were collected by removing hard clay substrate of the river margins. All material studied herein was fixed and preserved in 80% alcohol and is deposited at the Museu de Entomologia, Universidade Federal de Viçosa (UFVB). Pictures were taken using a Leica DM-205A stereomicroscope and then stacked with Leica Application Suite V4.11 software. Final photographs were improved on Adobe Photoshop® and drawings were prepared with Adobe Illustrator CC® software. QGis ver. 3.22.8 free software was used to create the distributional map (Figs. 1A and 1B).

Accepted by C. Molineri: 9 Apr. 2024; published: 6 May 2024

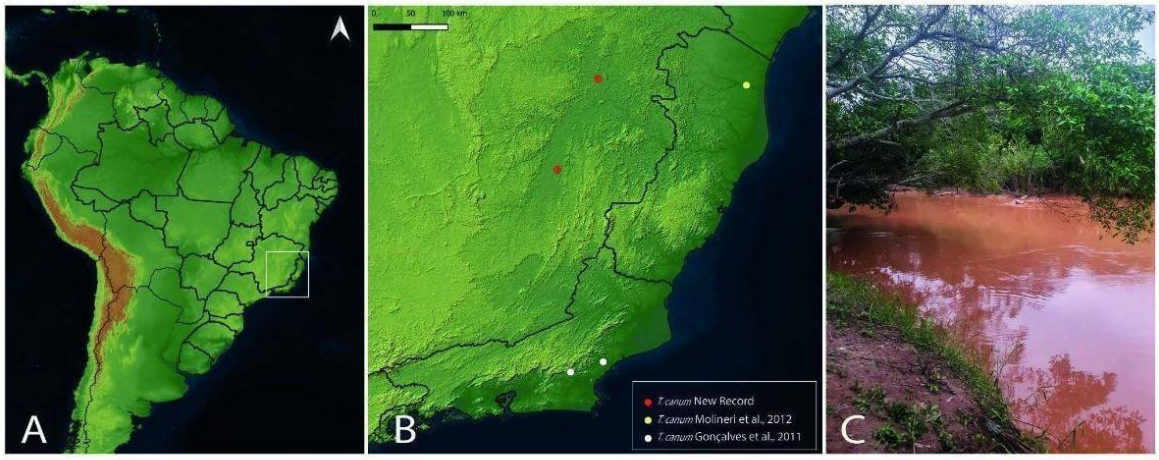

FIGURE 1. Distribution of *Tortopsis canum*. A, Map of South America with white rectangle highlighting the States of Rio de Janeiro, Espírito Santo and part of Minas Gerais (thick lines delimiting Brazil and Brazilian states, narrow lines delimiting other South American countries); B, Map of the states of Rio de Janeiro, Espírito Santo and southeast of Minas Gerais with circles showing the known distribution of *Tortopsis canum*; C, Collection site of *Tortopsis canum* nymphs in the Suaçuí Grande River, Governador Valadares, Minas Gerais, Brasil.

Tortopsis canum Gonçalves, Da-Silva & Nessimian

Tortopsis canum Gonçalves, Da-Silva & Nessimian, 2011: 51; Molineri, Salles & Boldrini, 2012: 466.

Examined material: 2 nymphs (UFVB) from Brazil, Minas Gerais, Pingo-dÁgua, Doce river basin, 19°46'03.3"S, 42°28'35.7"W, drag, 20-IX-2022, Pantoja, G. M., Rippel, M. L. S., & Orlando, T. Y. S., col.; 2 nymphs (UFVB) from Brazil, Minas Gerais, Governador Valadares, Mathias Lobato, Suaçuí Grande river, 18°34'23.5"S, 41°56'52.3"W, drag, 11-IV-2023. Pantoja, G. M., Jabeen, F., Orlando, T. Y. S., cols. 61 adults (UFVB) from Brazil, Minas Gerais, Pingo-dÁgua, Doce river basin, 19°46'03.3"S, 42°28'35.7"W, light trap, 20-IX-2022, Pantoja, G. M., Rippel, M. L. S., & Orlando, T. Y. S., col.; and 135 adults (UFVB) from Brazil, Minas Gerais, Governador Valadares, Mathias Lobato, Suaçuí Grande river, 18°34'23.5"S, 41°56'52.3"W, light trap, 11-IV-2023. Pantoja, G. M., Jabeen, F., Orlando, T. Y. S., cols.

Diagnosis. The nymphs of *T. canum* can be distinguished from all other species of the genus by: 1) mandibles with around 10 spines on inner margin of tusk, basal to subdistal tubercle (Fig. 3C); 2) outer margin of tusk with row of around 30 stout spines (Fig. 3A–C); 3) occiput with gray anastomosed lines distributed throughout the area (Fig. 2B); 4) wing pads shaded with gray on costal margin and some longitudinal veins (Fig. 2F).

Description. Nearly mature nymph. Length of body (from apex of tusks to apex of abdominal tergum X): 8.3 mm (N=1). General coloration yellowish white with gray markings dorsally (Fig. 2A). Head. Coloration whitish yellow shaded dark gray among ocelli, occiput with gray anastomosed lines, area beneath preocular tuft unpigmented (Fig. 2B), anterolateral spine 1.5 length of scape (Fig. 2E). Antennae and mouthparts whitish yellow, except apex of tusk orangeish, darker towards apex, and spines orange. Finger-like gill present near base of maxillae (Fig. 3H). Mandibular tusks with around 10 stout spines on inner margin, basal to subdistal tubercle; outer margin of tusk with row of around 30 stout spines (Fig. 3C). Thorax. Pronotum, anterior ring shaded black almost completely including the anterolateral pointed projection, posterior ring with medial gray band and lateral light gray band, both with a median pale line (Fig. 2A–B). Pigmentation on meso- and metanotum poorly marked. Wing pads shaded with gray costal margin and some veins (Fig. 2F). Thoracic pleura and sterna whitish, except for gray metasternum (Fig. 2C). Legs yellowish white, with yellowish setae and apex of tarsal claws orange (Figs. 4A–D). Subdistal transversal row of setae on hind femur composed of a single row (Fig. 4E). Inner distal margin of hind tibia with few strong setae (Fig. 4F). Abdomen. Color pattern as in figures 2A and 2C. Gills: vestigial gills I translucent white; gills II–VII well developed, formed by a pair of large whitish lamellae, the outer (dorsal) lamellae of each pair are shaded with black on a medio longitudinal band, wider on basal portion, thinner towards apex on distal portion (Fig. 4G). Caudal filaments broken and missing.

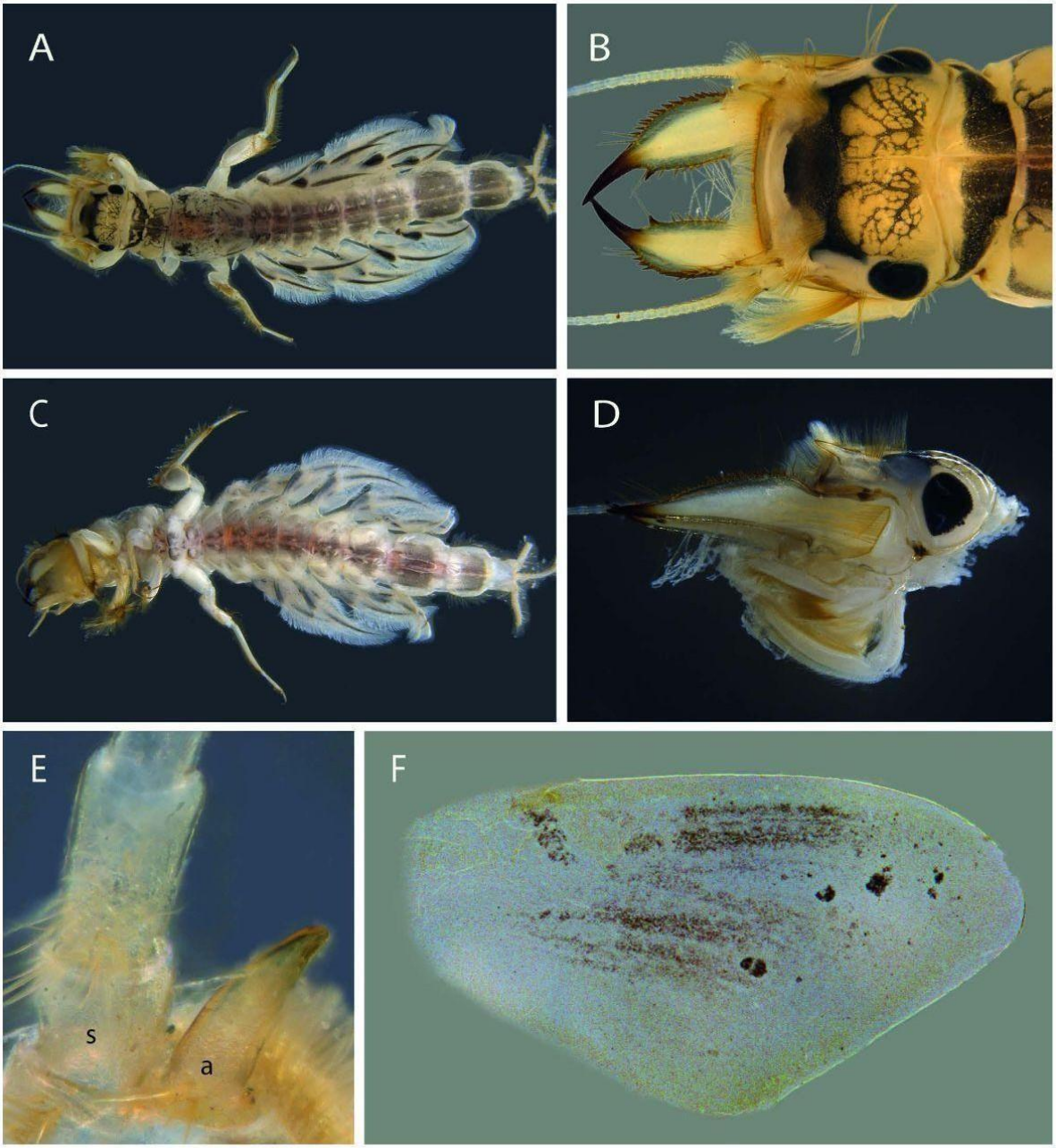

FIGURE 2. *Tortopsis canum*, nymph: A, general view, dorsal; B, head, dorsal view; C, general view, ventral; D, head, lateral view; E, base of antennae and anterolateral projection, dorsal view; F, Fore wing pad, dorsal view. Abbreviations: s=scape, a=anterolateral spine.

Key to the known nymphs of South American species of *Tortopsis*

1	Wing pads completely whitish	*T. andaki*
1'	Wing pads, at least, with costal margin pigmented	2
2(1')	More than 8 stout spines on inner margin of tusk	3
2'	Less than 3 stout spines on inner margin of tusk	4
3(2)	Wing pads with costal margin and some longitudinal veins shaded with gray (Fig. 2F); occiput with gray markings throughout the area (Fig. 2B)	*T. canum*
3'	Wing pads shaded gray only on costal margin (Fig 15 in Molineri, 2010); occiput without marks or with slightly marked sublateral pattern (Fig. 12 in Molineri, 2010)	*T. sarae*

4(2) Wing pads with costal margin and base of longitudinal veins slightly gray (similar to fig. 16 of Molineri 2008); outer margin of tusk with a subdistal indentation (Fig. 25 in Molineri *et al.* 2021); outer margin of tusks with 18 to 26 spines. . . . . *T. toro*

4' Wing pads shaded extensively with gray on costal margin and longitudinal veins (Fig. 14 in Molineri, 2008); outer margin of tusk with smoother outline, without a subdistal indentation (Fig. 96 in Molineri 2010); outer margin of tusks with around 30 spines . . . . . *T. obscuripennis*

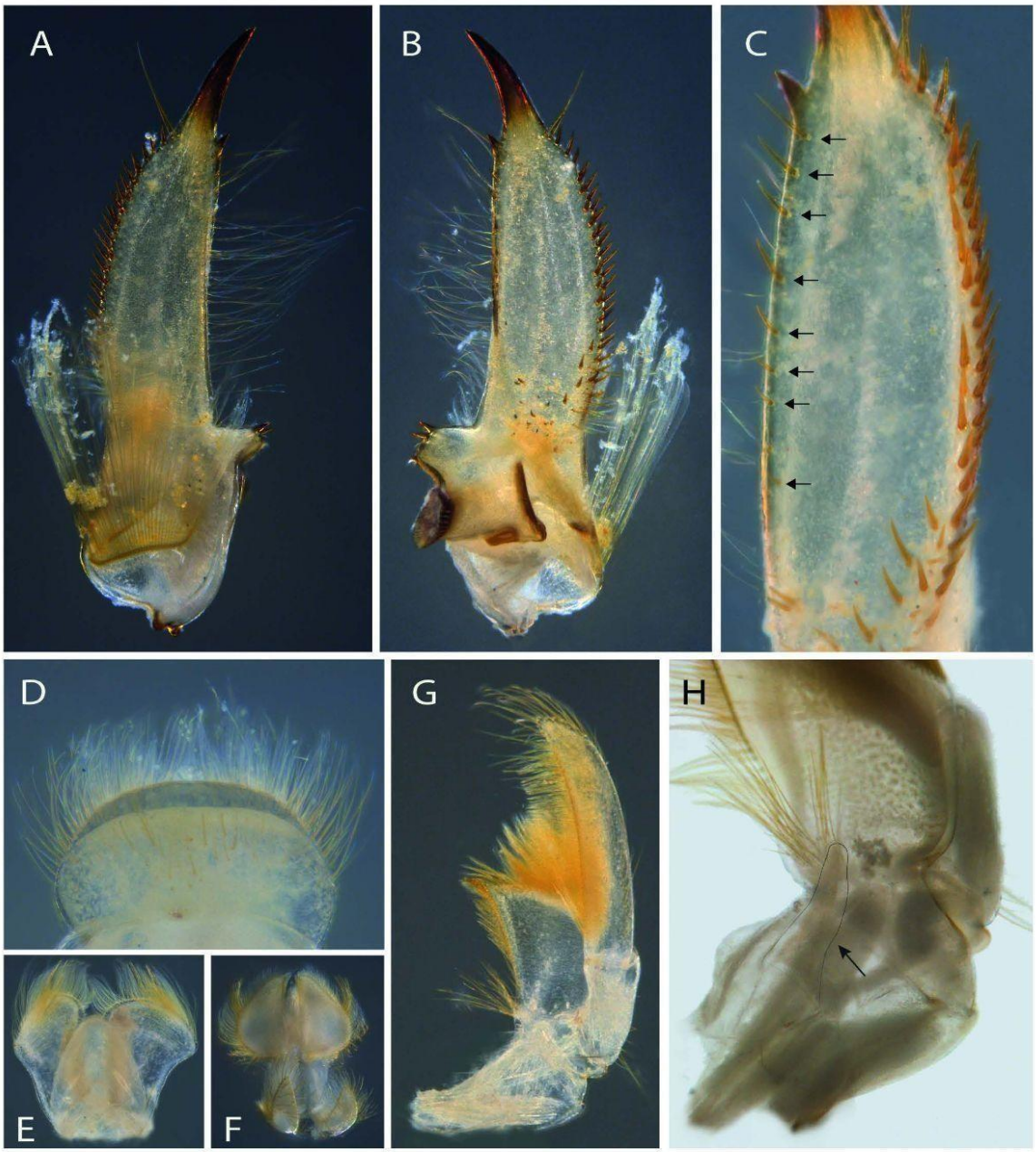

FIGURE 3. *Tortopsis canum*, nymph: A, right mandible, ventral view; B, right mandible, dorsal view; C, detail of right mandible, dorsal view. Arrow: spines on inner margin; D, labrum, dorsal view; E, hypopharynx, ventral view; F, labium, ventral view; G, right maxilla, dorsal view; H, right maxilla, ventral view, detail. Arrow: gill.

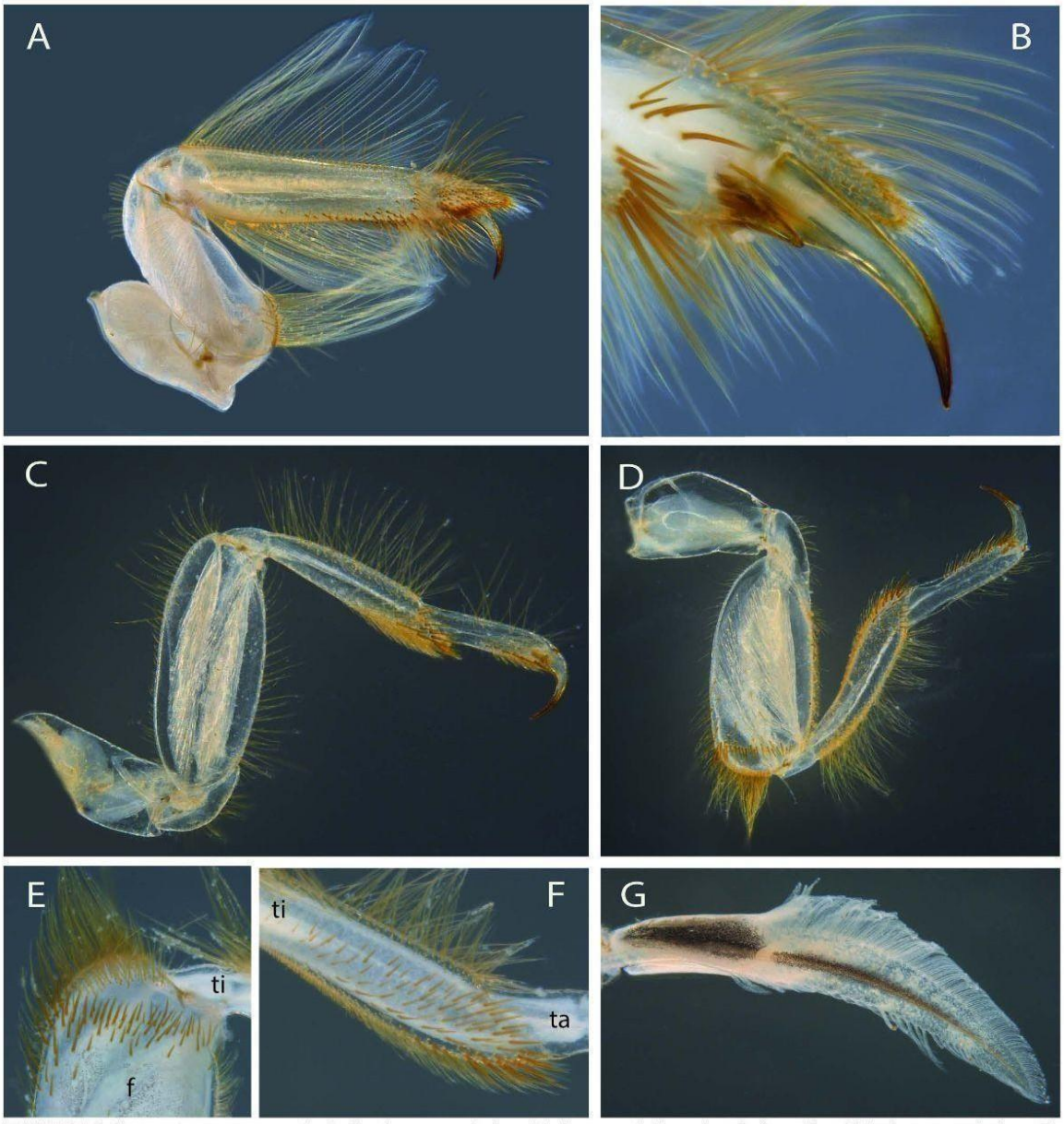

FIGURE 4. *Tortopsis canum*, nymph: A, foreleg, ventral view; B, fore tarsal claw, dorsal view; C, middle leg, ventral view; D, hind leg, ventral view; E, detail of hind leg, ventral view; F, detail of hind tibia, ventral view; G, abdominal gill V, dorsal view. Abbreviations: f=femur, ti=tibia, ta=tarsus.

Discussion

The association between the nymphal and adult stages was made by the site of collection and color pattern. Adults of *T. canum* were collected exactly in the same locations as the nymphs using Pennsylvania and white sheet light traps (see examined material). Besides that, it was the only species of the genus found in these locations. Nymphs and adults also share the same abdominal color pattern (Fig. 2A; Fig. 3 in Gonçalves *et al.*, 2011).

The nymph of *T. canum* exhibits characteristics of generic importance (Molineri, 2010), shared by all species of *Tortopsis* known as nymphs, as the fronto-clipeal region surpassing the madibular tusk ventrally; the presence of a large subapical denticle on the inner margin of tusks (Fig. 3A–C); and the fore tibia-tarsus with a dorsal projection that is 2/3 the length of the claw (Fig. 4A–B).

The most morphologically similar nymph to *T. canum* is *T. puella*, as they share the same occiput and wing pads pigmentation pattern. Concerning the number of spines on the inner margin of the tusk, 8 are present in *T. canum*, while *T. puella* can present from 10 to 20 spines. Unfortunately, due to the limited number of specimens examined, we are unaware at this moment of possible variations in *T. canum*. Nevertheless, *T. puella* has a Nearctic distribution, while *T. canum* is endemic to Brazil. The nymph of *T. canum* can be distinguished from *T. andaki* and *T. sarae* by the pigmentation of wing pads; those of *T. andaki* present whitish wing pads, while in *T. sarae* only the costal margin is shaded with gray. In *T. canum* wing pads show the costal margin and some longitudinal veins shaded with gray. The nymphs of *T.canum* can easily be distinguished from *T. toro* and *T. obsucripennis* by the number of spines on the inner margin of the tusk; *T. canum* present around 8 spines, while *T. toro* and *T. obscuripennis* present a maximum of 2 spines.

Adults of *T. canum* were previously collected in the states of Espírito Santo and Rio de Janeiro, Brazil, and this is the first record of *T. canum* from the state of Minas Gerais, Brazil.

Acknowledgments

We would like to the Conselho Nacional de Desenvolvimento Científico e tecnológico do Brasil (CNPq) for a productivity grant to FFS (process 309666/2019-8) and a scholarship to GMP, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for collection permissions, and Pedro Bonfá (Museu de Entomologia, UFV) for assistance with the map. We would also like to thank all the members of the Museu de Entomologia (UFV) who contributed to the collections and field trips and Inês Gonçalves and Lucas Lima for reviewing the manuscript. Thanks also to prof. Og DeSouza (UFV) for the availability and use of the stereomicroscope with the attached camera.

References

Domínguez, E. (1985) El género Tortopus Needham y Murphy (Ephemeroptera: Polymitarcyidae) en la Argentina. *Physis*, 43, 69–72.

Gonçalves, I.C., Da-Silva, E.R. & Nessimian, J.L. (2011) First record and new species of Tortopsis Molineri, 2010 (Ephemeroptera, Polymitarcyidae) from Brazil. *Zootaxa*, 2866 (1), 50–54.
https://doi.org/10.11646/zootaxa.2866.1.3

Molineri, C. (2010) A cladistic revision of Tortopus Needham & Murphy with description of the new genus Tortopsis (Ephemeroptera: Polymitarcyidae). *Zootaxa*, 2481 (1), 1–36.
https://doi.org/10.11646/zootaxa.2481.1.1

Molineri, C., Dias, L.G. & Zúñiga, M.D.C. (2021) New insights into the phylogeny of Tortopus Needham and Murphy and Tortopsis Molineri (Ephemeroptera, Polymitarcyidae) with description of three new species. *Arthropod Systematics & Phylogeny*, 79, 151–170.
https://doi.org/10.3897/asp.79.e62735

Molineri, C., Salles, F.F. & Boldrini, R. (2012) Tortopus Needham & Murphy and Tortopsis Molineri (Ephemeroptera, Polymitarcyidae) new species and new records from Brazil and Ecuador. *Revista Brasileira de Entomologia*, 56, 463–467.
https://doi.org/10.1590/S0085-56262012000400010

Needham, J.G. & Murphy, H.E. (1924) Neotropical mayflies. *Bulletin of the Lloyd Library*, Number 24, Entomological Series 4, 1–79.

Pictet, F.J. (1843–1845) *Histoire naturelle générale et particulière des insectes névroptères. Famille des Éphémérines*. Baillière édit., Paris (Kessmann et Cherbuliez édit., aussi à Genève), 300 pp.
https://doi.org/10.5962/bhl.title.48625

CHAPTER II – A NEW SPECIES OF Campsurus Eaton, 1868 (Ephemeroptera: Polymitarcyidae) from the Doce River Basin, Brazil

Zootaxa 5352 (1): 145–150
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5352.1.6
http://zoobank.org/urn:lsid:zoobank.org:pub:E1B1ADA5-60C5-4185-B531-6708FD714A1B

A new species of *Campsurus* Eaton, 1868 (Ephemeroptera: Polymitarcyidae) from the Doce River Basin, Brazil

GABRIEL M. PANTOJA[1,2,4], ANA D. L. VIANA[1,3,5] & FREDERICO F. SALLES[1*]
[1]*Museu de Entomologia, Depto. de Entomologia, Universidade Federal de Viçosa, CEP 36.570-900, Viçosa, MG, Brazil*
[2]*Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa, CEP 36.570-900, Viçosa, MG, Brazil*
[3]*Programa de Pós-graduação em Ecologia, Universidade Federal de Viçosa, CEP 36.570-900, Viçosa, MG, Brazil*
[4] *gabriel.pantoja@ufv.br; https://orcid.org/0000-0002-1769-6070*
[5] *ana.leite@ufv.br; https://orcid.org/0000-0001-5374-1257*
**Corresponding author: frederico.salles@ufv.br; https://orcid.org/0000-0001-8331-5929*

Abstract

The male imago of *Campsurus mirim* **sp. nov.**, an integrant of the C. major species group, is described based on material from a lake at the Rio Doce basin, Southeastern Brazil. It can be distinguished from other species of the group by the following combination of characteristics: small to medium size (body 7.3 mm, forewings 6.1 mm); blackish pigments on dorsum of head widely and strongly marked in fresh material; abdominal color pattern with pale transverse dashes on terga III–VII; posterior margin of sternum IX triangular at middle; pedestals flat with inner margin broadly rounded and with both posterolateral corners (inner and outer) approximately of the same length, both acute; penes relatively wide and large, ventrally curved on apical ½ and with apex of main lobe slightly twisted.

Key words: mayfly, Campsurinae, Ephemeroidea, Taxonomy, Neotropics, South America

Introduction

Campsurus Eaton, 1868 is the most diverse genus of the family Polymitarcyidae and one of the most diverse mayflies (Ephemeroptera) in the New World (Jacobus, 2023; Salles *et al.*, 2023). Until now, 43 species have been recognized in *Campsurus*, the vast majority of them in Tropical South America (Molineri *et al.*, 2015; Salles *et al.*, 2023). Given this diversity, several authors attempted to organize the genus in species-group (Domínguez *et al.*, 2006, Molineri & Emmerich, 2010, Molineri & Salles, 2013). Following the most recent papers dealing with *Campsurus*, the genus is divided in four species groups: violaceus (formerly notatus), segnis, albifilum, and major (Domínguez *et al.*, 2006; Molineri & Emmerich, 2010; Molineri & Salles, 2013, 2017).

The *Campsurus major* species group is the less diverse of these groups, comprising the following species: *C. major* Needham & Murphy, 1924; *C. argentinus* Esben-Petersen, 1912; and *C. amapaensis* Molineri & Emmerich, 2010. Besides the apomorphies listed by Molineri and Emmerich (2010), all of them related to the morphology of the male genitalia, the known nymphs of this group (*C. argentinus* and *C. major*) are able to construct external cases with silk. At least in the case of *C. argentinus*, in which a huge number of nymphs were studied, they are able to use this silk for the construction of protection cases attached to the surface of rocks on the streams (Molineri and Emmerich, 2010). Recently, based on light traps within the Rio Doce river basin, Southeastern Brazil (Figs. 1–2), a new species of *Campsurus major* species group was found. The aim of this study, therefore, is to describe this species based on the male imago.

Material and methods

Types are deposited at the Museu de Entomologia, Universidade Federal de Viçosa, Viçosa, Brazil (UFVB) and Museu Nacional do Rio de Janeiro (MNRJ, Rio de Janeiro, Brazil). Pictures were taken using a Leica DM-205A

Accepted by C. Molineri: 19 Sep. 2023; published: 2 Oct. 2023

stereomicroscope and then stacked with Leica Application Suite V4.11 software. Final photographs were improved on Adobe Photoshop® and drawings were prepared with Adobe Illustrator CC® software according to Coleman (2003, 2006). QGis ver. 3.22.8 free software was used to create the distributional map (Fig. 1).

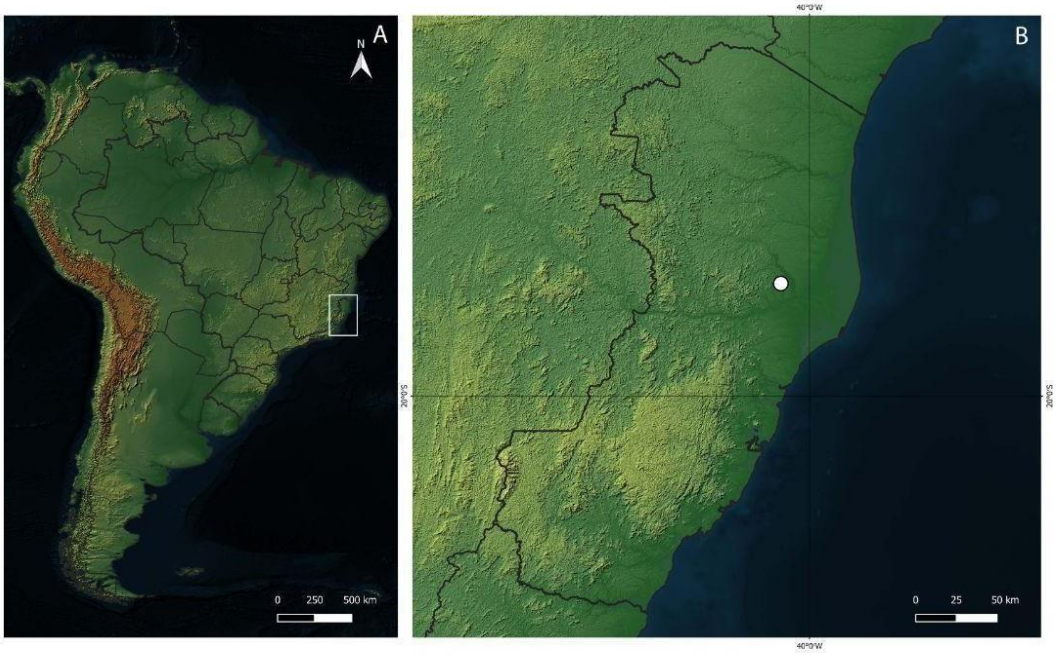

FIGURE 1. Distribution of *Campsurus mirim* **sp. nov.** A, Map of South America with white rectangle highlighting the State of Espírito Santo (thick lines separating Brazil from other countries; narrow lines within Brazil delimiting states; narrow lines outside Brazil delimiting South American countries); B, Map of Espírito Santo with white circle showing the known distribution of *Campsurus mirim* **sp. nov.**

FIGURE 2. Distinct views of the type-locality (Juparanã Mirim Lake, Espírito Santo State, Brazil).

Results

Campsurus mirim sp. nov. (Figs. 3–5)

Type material. Holotype: ♂ imago in alcohol, Brazil, Espírito Santo State, Linhares municipality, Lagoa Juparanã Mirim (Lagoa Nova), S19°19'49.0", W40°10'12.3", 32 m, 13.ix.2022, Viana, A.D.L., Bonfá, P., Ataide, A. col. (UFVB). **Paratypes**: 26 ♂ imagos, same data as holotype (16 at UFVB, 10 at MNRJ).

FIGURE 3. *Campsurus mirim* **sp. nov.** male imago (holotype): A, head and thorax, dorsal view; B, head and thorax, lateral view; C, abdomen, dorsal view; D, abdomen, lateral view.

Diagnosis. The male imago of *C. mirim* **sp. nov.** can be distinguished from other species of the genus by the following combination of characteristics: small to medium size (body 7.3 mm, forewings 6.1 mm); blackish pigments on dorsum of head widely and strongly marked in fresh material (Fig. 3A); abdominal color pattern with pale transverse dashes on terga III–VII (Figs. 3C and 3D); posterior margin of sternum IX triangular at middle (Fig. 5A); pedestals with both posterior margins (inner and outer) approximately of the same length, both acute (Fig. 5A);

penes relatively wide and large, ventrally curved on apical ½ and with apex of main lobe slightly twisted, gonopore not visible (Figs. 5A and B).

Male imago (Figs. 3–5). Length (mm): body, 7.3; forewing, 6.1; hind wing, 2.9; fore leg, 3.06. General coloration yellowish white with gray markings dorsally. Head (Figs. 3A–B) heavily washed with black on dorsum, especially at base of ocelli, scape and pedicel slightly washed with black, flagellum hyaline. Thorax (Figs. 3A–B). Pronotum translucent shaded with black on anterior portion and on lateral and posterior margins of posterior portion; medially with blackish medial line well marked. Meso- and metanota yellowish white diffusely washed with black; black marks more heavily on anteronotal impression, longitudinal median suture, and area between posteroscutal protuberance. Pleura and sterna yellowish white except on prothorax shaded purplish grey. Forelegs shaded with purplish black, lighter on tarsi and even lighter on apex of claws; vestiges of middle and hind legs yellowish white, except for black middle coxa. Wings (Fig. 4). Membrane hyaline, except ¾ of C and Sc areas tinged with purple; veins slightly tinged with purple at base, becoming lighter towards outer margin. Abdomen (Figs. 3C–D) whitish translucent, shaded with black on terga: terga I–III lighter, terga VIII–X darker; thin medial line on terga V–X; pale lateral transversal band present on terga III–VII. Genitalia (Fig. 5) whitish yellow, forceps translucent white, slightly tinged with purple at base, sclerotized apex of penes yellowish brown. Posterior margin of sternum IX triangular at middle. Penis curved ventrally, especially at apical ½; main lobe of penis with apex twisted outwards and with its prominent dorsal (posterior) margin very sclerotized; secondary lobe membranous and cylindrical; gonopore not visible. Pedestal short and somewhat flattened, inner-posterior margin well developed and broadly acute, outer-posterior margin equal in length to outer margin, with angle more acute. Caudal filaments whitish translucent.

Female imago and nymph. Unknown.

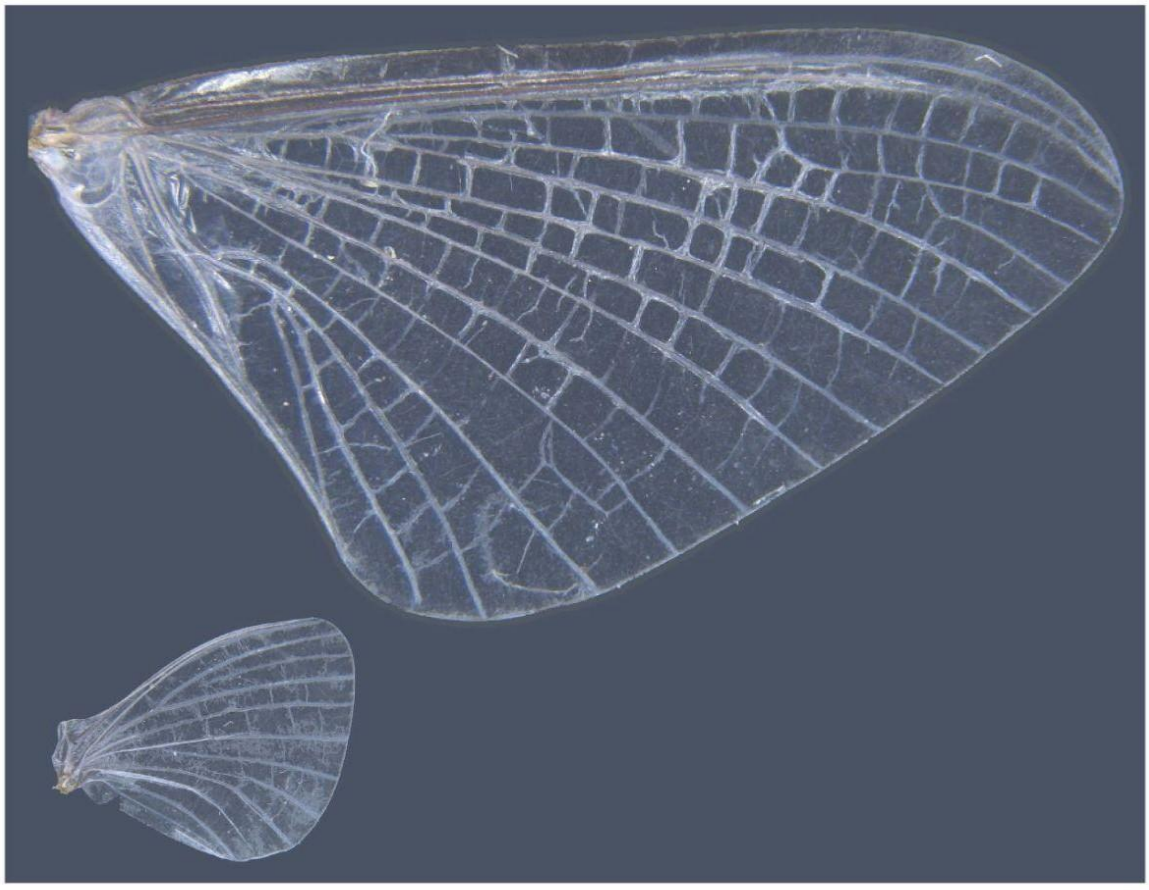

FIGURE 4. *Campsurus mirim* **sp. nov.** male imago (holotype): Fore and hind wing.

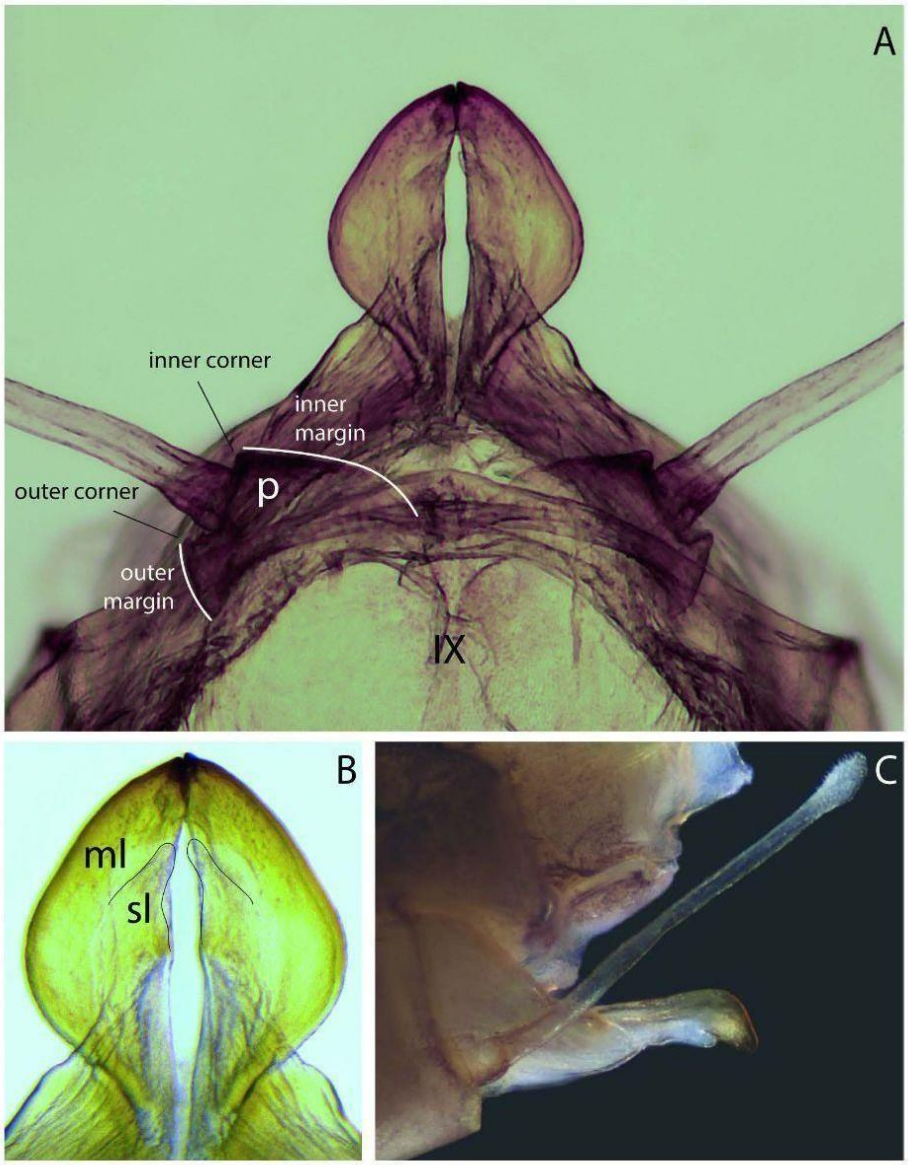

FIGURE 5. *Campsurus mirim* **sp. nov.** genitalia of male imago (holotype): A, genitalia, ventral view; B, penes lobes, ventral view; C, genitalia, lateral view. Abbreviations: IX, sternum IX; ml, main lobe; sl, secondary lobe; p, pedestal.

Etymology. From the tupi-guarani language, meaning small. An allusion to the size of the species, especially when compared to the relative larger *C. major*, and also an allusion to one of the names of the type-locality, the lake Juparanã Mirim.

Distribution. Brazil: Espírito Santo State (Fig. 1).

Campsurus mirim **sp. nov.** would key out in couplet 3(2) of the key proposed by Molineri & Salles (2017). In order to add the new species to that key, and also to include the recently described *C. fortuitus* Cruz, Molineri & Hamada, 2022, which would also key out in the same couplet, we propose the following change:

3(2)	Forewing smaller than 7 mm	4
3'.	Forewing larger than 9 mm	couplet 4 of Molineri & Salles (2017)

4(3). Penes blade-like, not curved; posterior margin of sternum IX slightly concave 5
4'. Penes curved ventrally; posterior margin of sternum IX triangular at middle . . . . . *C. mirim* **sp. nov.**
5(4). Penes with inner distal margin of main lobe curved outwards. . . . . *C. fortuitus*
5'. Penes with distal margin of main lobe rounded, not curved . . . . . *C. povilla*

Acknowledgements

We would like to the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for a productivity grant to FFS (process 309666/2019-8) and a scholarship to GMP, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for collection permissions, and Pedro Bonfá (Museu de Entomologia, UFV) for assistance with the map and some of the pictures. We also would like to thank all the members of the Museu de Entomologia (UFV) who contributed to the collections and field trips, especially those involved in the project to assess the impact of the Rio Doce basin (Projeto FAPEMIG/RENOVA APQ-05461-18.), mainly the project coordinator prof. Carlos Frankl Sperber. Thanks also to prof. Og DeSouza (UFV) for the availability and use of the stereomicroscope with the attached camera.

References

Coleman, C.O. (2003) "Digital inking": How to make perfect line drawings on computers. *Organisms, Diversity & Evolution*, 14, 1–14.
https://doi.org/10.1078/1439-6092-00081

Coleman, C.O. (2006) Substituting time-consuming pencil drawings in arthropod taxonomy using stacks of digital photographs. *Zootaxa*, 1360 (1), 61–68.
https://doi.org/10.11646/zootaxa.1360.1.4

Domínguez, E., Molineri, C., Pescador, M., Hubbard, M.D. & Nieto, C. (2006) *Ephemeroptera of South America. In*: Adis, J., Arias, J.R., Ru-Delgado, G. & Wantzen, K.M. (Eds.), *Aquatic Biodiversity in Latin America. Vol. 2.* Sofia-Moscow, Pensoft, pp. 1–646.

Esben-Petersen, T. (1912) 'New and little-known species of Ephemerida from Argentine (Neuropt.)'. *Deutsche Entomologische Zeitschrift*, 1912, 333–342.
https://doi.org/10.1002/mmnd.48019120310

Jacobus, L.M. (2023) Mayfly Central. Available from: https://www.entm.purdue.edu/mayfly/index.php (access 31 July 2023)

Molineri, C. & Emmerich, D. (2010) New species and new stage descriptions of *Campsurus* major species group (Polymitarcyidae: Campsurinae), with first report of silk-case construction in mayfly nymphs. *Aquatic Insects*, 32 (4), 265–280.
https://doi.org/10.1080/01650424.2010.533131

Molineri, C. & Salles, F.F. (2017) Review of selected species of *Campsurus* Eaton 1868 (Ephemeroptera: Polymitarcyidae), with description of eleven new species and a key to male imagos of the genus. *Zootaxa*, 4300 (3), 301–354.
https://doi.org/10.11646/zootaxa.4300.3.1

Molineri, C., Salles, F.F. & Emmerich, D. (2015) Revision of *Campsurus violaceus* species group (Ephemeroptera: Polymitarcyidae) with new synonymies and nomina dubia in *Campsurus* Eaton, 1868. *Zootaxa*, 3920 (1), 51–68.
https://doi.org/10.11646/zootaxa.3920.1.3

Needham, J.G. & Murphy, H.E. (1924) 'Neotropical mayflies'. *Bulletin of the Lloyd Library*, Entomological Series 4, No. 24, pp. 1–79.

Salles, F.F., Molineri, C., Nieto, C., Lima, L.R.C., Dias, L.C., Boldrini, R., Mariano, R. & Domínguez, E. (2023) Ephemeroptera da América do Sul. Available from: http://ephemeroptera.com.br/ (access 31 July 2023)

Walker, F. (1853) Ephemerinae. *In*: List of the specimens of neuropterous insects in the collection of the British Museum. Part III. Termitidae—Ephemeridae. The Trustees of the British Museum, London, pp. 533–585.

CHAPTER III - EVIDENCE OF SILK PRODUCTION IN MALPIGHIAN TUBULES OF CAMPSURINAE (EPHEMEROPTERA: POLYMITARCYIDAE) NYMPHS

ABSTRACT

PANTOJA, Gabriel Martins, Federal University of Viçosa, July, 2024. **Evidence of silk production in Malpighian tubules of Campsurinae (Ephemeroptera: Polymitarcyidae) nymphs**. Adviser: Frederico Falcão Salles. Co-adviser: José Eduardo Serrão.

Silk glands are present in species of spiders, myriapods, and insects. Among insects, these glands may be present in the labium, epidermis, or as cells derived from Malpighian tubules. Nymphs of Polymitarcyidae (Insecta: Ephemeroptera) use silk to incase tunnels built in plant tissues and sediments. However, silk production in nymphs of this family has only been observed for species of *Asthenopus*, in wich the Malpighian tubules lumen exhibits protein content. Histological studies of silk production in Ephemeroptera nymphs are lacking. Here, we analyzed the histology and histochemistry of Malpighian tubules cells from three Campsurinae species that construct shelters in different sediments: *Campsurus truncatus*, *Campsurus* sp., and *Tortopsis canum*. Besides these three species, we analyzed the Malpighian tubules of species from two other families of Ephemeroidea, *Campylocia burmeisteri* (Hagen, 1888) (Ephemeroptera: Euthyplociidae) and *Hexagenia albivitta* (Walker, 1853) (Ephemeroptera: Ephemeridae), as a control group. The species of Campsurinae present Malpighian tubules with columnar secretory cells, containing glycoprotein granules in the cytoplasm. This kind of cells is absent in malpighian tubules of *C. burmeisteri* and *H. albivitta* The presence of glycoprotein-secreting cells in the Malpighian tubules of the studied species provides evidence that silk production is widespread among Polymitarcyidae with different habitats, whether they build shelters with mineral materials or construct tunnels in clay. This study presents the first histological evidence of protein-secreting cells in the Malpighian tubules of Ephemeroptera.

INTRODUCTION

Silk is utilized by arthropods to build nests, shelters, cocoons, and webs (Maschwitz et al, 1990; Vollrath, 1999; Quicke et al., 2004; Chang et al., 2006). These structures exhibit a certain resistance to adverse environmental conditions, mainly due to physical properties of silk, such as water insolubility, mechanical resistance, and elasticity (Seibt & Wickler, 1990; Vollrath & Knight, 2001). Silk acquires these properties during the synthesis process, which consists of converting an aqueous solution of proteins into fibers (Jin & Kaplan, 2003). Storing silk as an aqueous solution enables the rapid synthesis of fibers in response to specimen development and environmental cues (Sutherland, 2010).

Silk fibers are synthesized exclusively in arachnid, myriapod and insects by organs named silk glands, which have a range of classes with different evolutionary origins (Sehnal & Akkai, 1990; Craig, 1997 Sutherland, 2010; Scott et al., 2018). Whereas the silk glands in myriapod are unknown, in arachnids they are tubular or acinous and specialized for fiber production in the opisthosoma (Kovoor, 1987). In insect species, silk glands are classified as labial and dermal glands, as well as specialized cells in the Malpighian tubules (Sehnal & Akkai, 1990; Sutherland, 2010).

The labial gland is the most common class of silk gland in insects (Snodgrass, 1935; Sehnal & Craig, 2009). Labial silk glands occur in adults of Gryllacrididae and Anostostomatidae (Orthoptera), Psocoptera, Sphecidae (Hymenoptera) and in larvae of Hymenoptera, Siphonaptera, “Nematocera” (Diptera), Lepidoptera and Trichoptera (Morton & Rentz, 1983; Maschwitz et al., 1990; Lawrence & Foil, 2002). This paired gland is tubular with two regions: the posterior, with silk secretory cells, and the anterior region, with a secretory duct that opens between the labium and the hypopharynx (Snodgrass, 1935; Tashiro, 1968). These regions may exhibit anatomical and histological variations among species. Larvae of Trichoptera and Lepidoptera have the anterior region of the gland with a muscle complex that plays a role in the silk extrusion (Engster, 1976; Guo et al., 2019). Additionally, these insects have an extension of the anterior region of the silk gland that reaches the area outside the mouthparts (Sorensen et al., 2006; Spanhoff, 2003; Guo et al., 2019). Meanwhile, in social wasps, Anthophorinae bees, and poneromorph ants, the posterior glandular region is acinar, providing a larger secretory area to the gland (Landolt & Akre, 1979; Cavasin-Oliveira & Cruz-Landim, 1998; Lommelen et al., 2002).

Dermal silk glands in insects belong to class III, according to the classification by Noirot & Quennedey (1974) for epidermal gland cells (Young, & Merrit, 2003; Serrão, 2005). In the silk gland of this class, the secretory units are located below and distant from the cuticle,

connected to it by a secretory canal (Nagashima et al., 1991; Serrão, 2005). The secretory units are composed of numerous secretory cells and a lumen formed by the invagination of the apical plasma membrane of the cells (Kenchington, 1972; Young & Merrit, 2003).The lumen is connected to a secretory canal, which links the secretory units of the gland to specialized setae (Serrão & Campos, 2000; Young & Merrit, 2003). In immature and adult Embioptera, these setae are present on the first and second tarsomeres of the forelegs, while the secretory setae of males of the tribes Hilarini and Empidini (Diptera: Empididae) are found only on the first tarsomere (Nagashima, 1991; Young & Merrit, 2003). However, in adult females of some Pemphredoninae (Hymenoptera: Sphecidae), the secretory setae are located on segments IV and V of the metasoma (Melo, 1997; Serrão, 2005). Another dermal silk glands are the accessory glands of females of Chrysopidae (Neuroptera) and Hydrophilidae (Coleoptera) (La Mun, 1988). However, the anatomy and histology of these glands are unknown.

Silk-secretory cells derived from Malpighian tubules are found in immature stages of some Cercopidae (Hemiptera), Curculionidae (Coleoptera), and Chrysopidae (Neuroptera) (Spiegler, 1962; Marshall, 1973; Kenchington, 1983). These cells possess invaginations by an arboriform complex of channels, where silk exocytosis occurs, reaching the lumen of the tubules (Marshall, 1973). In species of Cercopidae and Curculionidae, silk secretion occurs in the distal region of the Malpighian tubules (Marshall, 1973; Kenchington, 1983). Meanwhile, in species of Chrysopidae, the secretory cells are present in the proximal region, close to the opening into the gut (Spiegler, 1962).

Some silk-based structures can also be found in aquatic environments, due to the insolubility of the fibers (Case et al., 1994). These structures are built by representatives of Trichoptera, which construct fixed or mobile shelters with gravel and plant parts (Kiel & Roder, 2002), Simuliidae and Chironomidae (Diptera), which anchor themselves to the substrate using silk fibers, facilitating the interception of suspended material (Mackay, 1979; Kiel & Roder, 2002), females of Hydrophilidae (Coleoptera) and Gomphidae (Odonata) that use silk to cover eggs (Anderson, 1976; Trueman, 1990).

In Ephemeroptera, silk production has been exclusively investigated in three genera of Polymitarcyidae (Hartland-Rowe, 1953; Satler, 1967; Molineri & Emmerich, 2010). This family belongs to a group of Ephemeroptera named Ephemeroidea, which includes several families whose nymphs live buried in the substrate (McCafferty, 1975). The first report of silk production in Polymitarcyidae was made by Verrier (1951), who observed tunnels of *Povilla adusta* Navás, 1912 (Asthenopodinae) covered with a film probably produced by nymphs in rivers of Congo, Africa. Satler (1967) observed an adhesive secretion on the walls of tunnels built in plant stem by nymphs of *Asthenopus* sp. Eaton, 1871 (Asthenopodinae:

Polymitarcyidae), and on grains of sand from shelters occupied by *Campsurus* Eaton, 1868 (Campsurinae: Polymitarcyidae) in the Amazon. Decades later, Molineri & Emmerich (2010) reported nymphs of *Campsurus argentinus* Esben-Petersen 1912 (Campsurinae) living in silk structures adhered to the wall of rocks in Uruguay.

The production and release of silk are interesting biological aspects of Polymitarcyidae. Hartland-Rowe (1958) observed nymphs of *P. adusta* secreting silk through the anus and using mouthparts and forelegs to shape and transport the fibers (Hartland-Rowe, 1958). The silk is used to coat the walls of the tunnels built by the nymphs in plant parts and sediments (Hartland-Rowe, 1958; Satler, 1967; Molineri & Emmerich, 2010). It is likely silk provides support to the shelter and enhances the nymphs' adherence to the tunnel, allowing stability during feeding, which occurs through particle filtering (Hartland-Rowe, 1958; Satler; 1967). During feeding, the nymphs rhythmically move their gills, generating the circulation of water and suspended particles into the tunnel (Hartland-Rowe, 1953). Furthermore, the presence of silk may decrease the lumen of the tunnel, resulting in less space between suspended particles and the filtering bristles on the forelegs and mouthparts of the nymphs, thereby facilitating filtration (Hartland-Rowe, 1958).

Despite evidence regarding silk production in Polymitarcyidae nymphs, knowledge about the producing organ is limited to the study by Sattler (1967). The author observed granules positive for amino acids obtained from the lumen of Malpighian tubules of *Asthenopus* (Asthenopodinae). Studies on the biological aspects of silk production in Polymitarcyidae are restricted to two species of Asthenopodinae (Hartland-Rowe, 1953, 1958; Satler, 1967), whereas the family comprises two more subfamilies: Polymitarcyinae and Campsurinae. Campsurinae is the sister group of Asthenopodinae, both the sister group of Polymitarcyinae (Fig.1) (Molineri et al., 2021). In Campsurinae genera, therefore, it has not been verified whether Malpighian tubule cells also produce protein, found in high levels in silk fibers, and whether silk production is restricted only to *Campsurus* species that build external shelters. Additionally, the structure of the protein-secreting cell in Ephemeroptera is still unknown. Therefore, the aim of this study is to search for evidence of silk production by Malpighian tubules in Ephemeroptera and to provide new records of silk-producing species through histological and histochemical analyses of the Malpighian tubules from nymphs of Campsurinae species.

MATERIAL AND METHODS

Selected species

The Campsurinae species studied were *Campsurus truncatus* Ulmer, 1920 (Fig 1A),

Campsurus sp. and *Tortopsis canum* Gonçalves, Da-Silva & Nessimian, 2011 (Fig 1B). In addition, species from two other families of Ephemeroidea were selected: *Campylocia burmeisteri* Hagen, 1888 (Euthyplociidae; Fig 1C) and *Hexagenia albivitta* (Walker, 1853) (Ephemeridae; Fig 1D). Therefore, both clades of Campsurinae [*Campsurus* and (*Tortopsis* + *Tortopus*); Fig 2)] (Molineri et al., 2021) are represented in this study. We could not collect alive nymphs of Polymitarcyinae because this is a holarctic group. As a control group, we selected nymphs of *Campylocia* and *Hexagenia* because these genera are closely related groups to Polymitarcyidae (Fig 2; Miller et al., 2018).

Fig 1. Nymphs of studied species. *Campsurus. truncatus* (A); *Tortopsis canum* (B); *Campylocia burmeisteri* (C); *Hexagenia albivitta*.

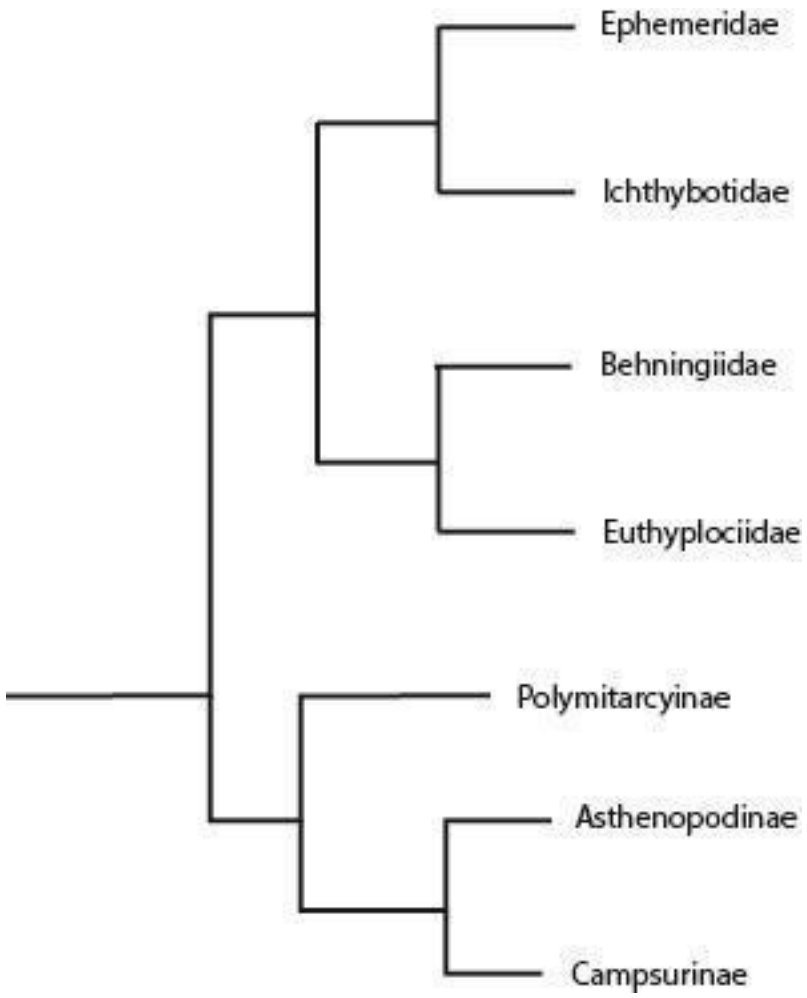

Fig 2. Cladogram of suborder Ephemeroidea (Modified from Miller et al., 2018 and Molineri et al., 2021)

Species habitats

The nymphs of *Campsurus* sp., *T. canum*, *Cy. burmeisteri* and *H. albivitta* were collected in lotic systems, while the nymphs of *Cs. truncatus* were collected in lentic systems (Table 1). The nymphs of *Cy. burmeisteri* were found under rocks in backwater areas, and the nymphs of *H. albivitta* were found buried in silty sediment near the riverbank. Among the Polymitarcyidae nymphs, those of *Campsurus.* sp. inhabited shelters, attached to rocks, built with mineral material and an adhesive substance (Fig 3A). Meanwhile, the nymphs of *T. canum* were found buried in a bank of consolidated clay substrate, and those of *Cs. truncatus* were found buried in soft clay substrate. The Campsurinae species studied here represent the two different types of nymphal behaviour seen in this subfamily: nymphs that build helters on rocks and nymphs that construct U-shaped tunnels in clay sediments (Fig 3B; Molineri & Emmerich, 2010; Molineri et al., 2021; Salles, Pantoja & Boldrini, personal observation), allowing for the comparison of silk production in relation to the different habitats. Nymphs of *H. albivitta*, like

Campsurinae nymphs, construct U-shaped tunnels, but in sediments of larger particle sizes than clay, primarily with a predominant silt fraction (Fremling, 1960; Fremling & Schoening 1973). Nymphs of *Hexagenia* build tunnels without exhibiting silk production activity (Fremling & Schoening 1973). The nymphs of *Cy. burmeisteri* are interstitial dwellers in mixed substrate that do not form burrows (Bae & McCafferty, 1995).

Table 1. Annotated species and locations

Species	Locality	County	State	Coordinates
Cy. burmeisteri	Cruzeiro Waterfall	Ervália	MG	20°46'36.4"S 42°29'50.3"W
H. albivitta	Ribeirão Lajes river	Carolina	MA	7°20'44.9"S 47°26'39.1"W
Cs. sp.	Arraias river	Bonfim	RR	3°21'34.1"N, 59°53'51.6"W
Cs. truncatus	Cond. dos lagos	Viçosa	MG	20°46'11.3"S,42°50'24.8" W
T. canum	Doce river basin	Pingo-d'água	MG	19°46'03.3"S 42°28'35.7"W

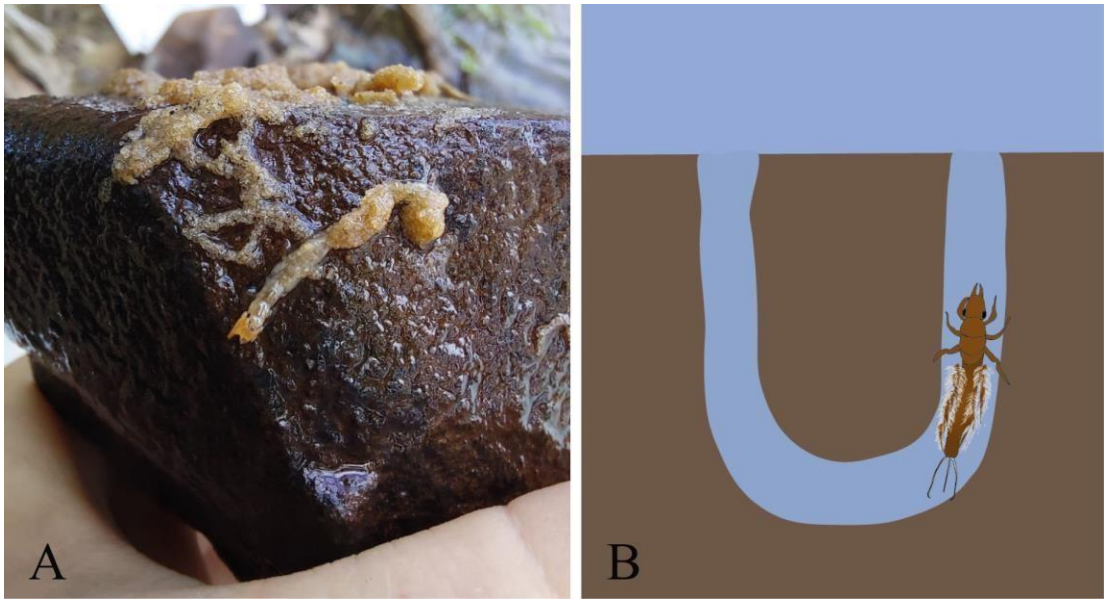

Fig 3. Type of habitats of nymphs in Campsurinae. A) External shelters on rocks made by nymphs of *Campsurus* sp. (Boldrini & Pantoja, 2021). B) Schematic drawing of U-shaped tunnels in clay sediments.

Collection and rearing of specimens

The insects were manually collected using tweezers, trays, and sieves. The nymphs of *Cs. truncatus* and *Cy. burmeisteri* were transferred alive to the Entomology Museum of the Federal University of Viçosa (UFVB) for dissection and description of the Malpighian tubules anatomy. Another set of *Cy. burmeisteri* and *Cs. truncatus* nymphs were reared in order to keep the organisms alive for a period of time. During transport from the field to the laboratory, the live nymphs of *Cy. burmeisteri* were placed in a delicate laundry washing bag submerged in water, preventing the nymphs from escaping. To maintain water temperature, the trays were stored in thermal boxes. In laboratory, nymphs of *Cy. burmeisteri* were placed in cups positioned on a floating structure that remained in an aquarium with two oxygen pumps (Velásquez, 2023).The cups had an opening covered with voile fabric at the bottom to allow water flow, and the top was also covered with voile fabric to allow air exchange and thus prevent perspiration inside the cup. Stones were placed in each cup for the nymphs to anchor themselves. For the rearing of *Cs. truncatus*, nymphs were placed in delicate laundry washing bags in aquariums with water and clay substrate. The nymphs of *Cs. truncatus* were not placed in an aquarium with oxygen pumps because these nymphs inhabited a low-oxygen environment.

Anatomy of Malpighian tubules

For the description of anatomical features of Malpighian tubules, five alive immature nymphs of *Cs. truncatus* and *Cy. burmesteri* were cold-immobilized, dissected in 0.15 M sodium phosphate buffer at pH 7.3 (PBS) under a Leica M205A stereomicroscope. Illustrations were created using Adobe Illustrator software version 2022. The anatomy of the Malpighian tubules can only be accurately depicted when the insect is alive during dissection, and among the collected species, *Cs. truncatus* and *Cy. burmeisteri* were the species in which this procedure could be performed. We were unable to keep the other species alive until dissection due to the time and transport conditions to the laboratory. However, according to Soldan (1985), there is no difference in the anatomy of the Malpighian tubules among Campsurinae species.

Dissection

The nymphs were dissected with forceps on a Petri dish containing saline solution (0.1 M NaCl + 0.1 M KH2PO4 + 0.1 M Na2HPO4), and the digestive systems with Malpighian tubules were transferred to Zamboni fixative (Stefanini et al., 1969).

Histology

Three samples of the digestive tract with Malpighian tubules from *Cs. truncatus*, *Campsurus* sp., *T. canum*, *Cy. Burmeisteri* and *H. albivitta* were dehydrated in an increasing series of ethanol (70, 80, 90, and 95%) and embedded in historesin (Leica Biosystem Nussloch GmbH, Wetzlar, Germany). Sections with a thickness of 2 μm were obtained with glass knives in a rotatory microtome (Leica). Some sections were stained with hematoxylin and eosin, while others were submitted to histochemical tests and analyzed using an Olympus BX60 optical microscope.

Histochemical tests

Periodic Acid-chiff

((PAS)

The PAS test was used to detect glycoproteins in Malpighian tubules cells. Unstained histological slides were incubated in 0.4 M periodic acid for 30 minutes. They were then washed in running water and transferred to Schiff's reagent for 1 hour in the dark. Finally, the samples were washed with running water, mounted, and analyzed under a light microscope.

Mercury Bromophenol Blue

For the detection of proteins in the unstained Malpighian tubules, samples were incubated in mercury bromophenol solution (100 mL 2% acetic acid; 0.05 g bromophenol blue; 1.5 g mercury II chloride) for 2 hours and 15 minutes. Subsequently, the samples were transferred to 0.5% acetic acid for 10 minutes, washed with running water for 15 minutes, mounted, and analyzed under a light microscope.

RESULTS

Anatomy of the Malpighian tubules of *Cy. burmeisteri* and *Cs. truncatus*

The Malpighian tubules of *Cs. truncatus* are formed by four main trunks that originate at the junction of the midgut and hindgut (Fig 4A). Each main trunk presents four ramifications that can be classified as (i) a cylindrical branch that originates from the main trunk named secondary branches (Fig 4B-D); (ii) branches from the distal portion of the secondary branch,the tertiary branches (Fig 4B-D), which varies in number, from six (Fig 5B), four (Fig 4C) or two units (Fig 4D), with no specific distribution pattern along the main trunks; (iii) filiform structures that originate from the distal portion of tertiary branches (Fig 4B-D); and (iv) a coiled structure that originates from the distal portion of the filiform structure (Fig 4B-

D).

The Malpighian tubules of *Cy. burmeisteri* are also divided into four main trunks (Fig 5A). However, these trunks originate from a distal region in the middgut and are forked at base (Fig 5A). The main trunks in *Cy. burmeisteri* present two branches; (i) the cylindrical secondary branch, that can be bifurcated (Fig 5B), trifurcated (Fig 5C), or with four ramifications (Fig 5D), without a distribution pattern, and (ii) the coiled structure which originates from the distal portion (Fig 5B-D), similar to those of *Cs. truncatus*.

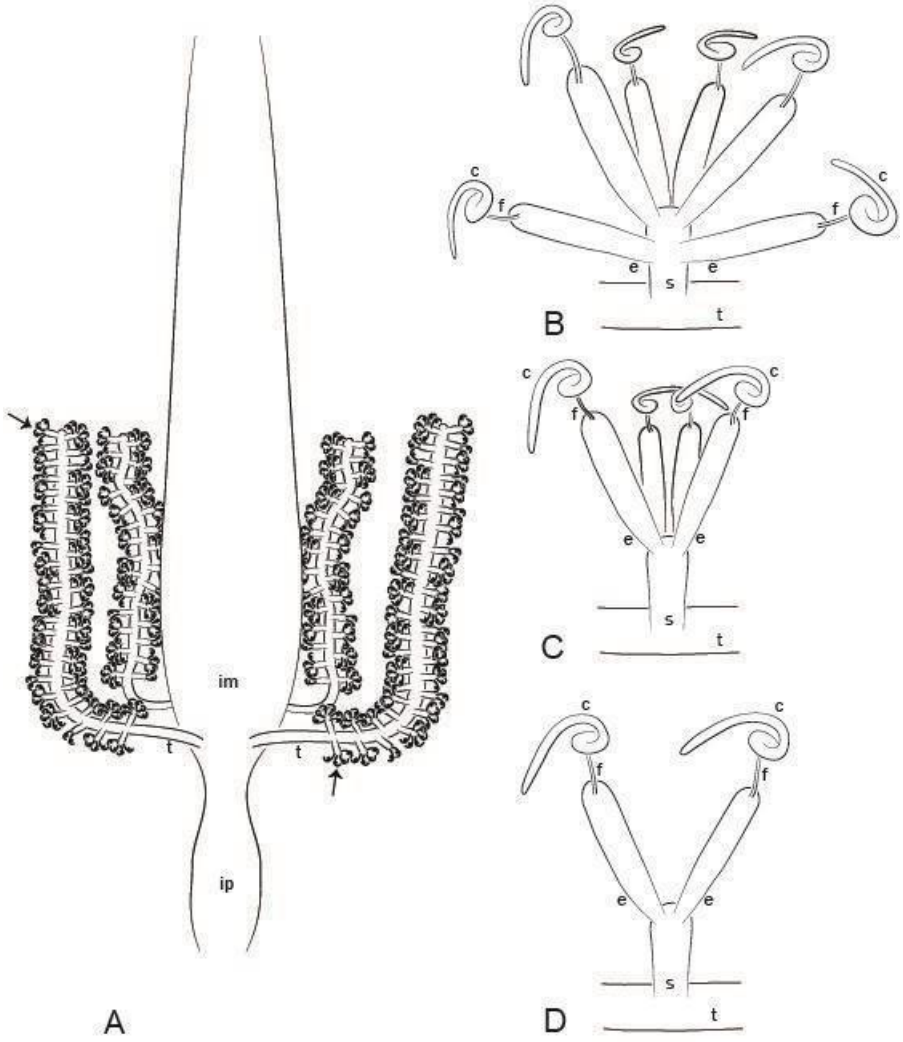

Fig 4. Anatomy of Malpighian tubules of *Campsurus truncatus* nymph. (A) General view. (B), (C) and (D) detail of shown structures. Four main trunks (t) originate at the junction of the midgut (im) and hindgut (ip). From the main trunk (t) emerges the secondary branch (s). The tertiary branch (e) can vary in number, from six (B), four (C) or two (D) units. Filiform structuture (f) at the end, linking it to coiled structure (c). Arrow: Attached forms.

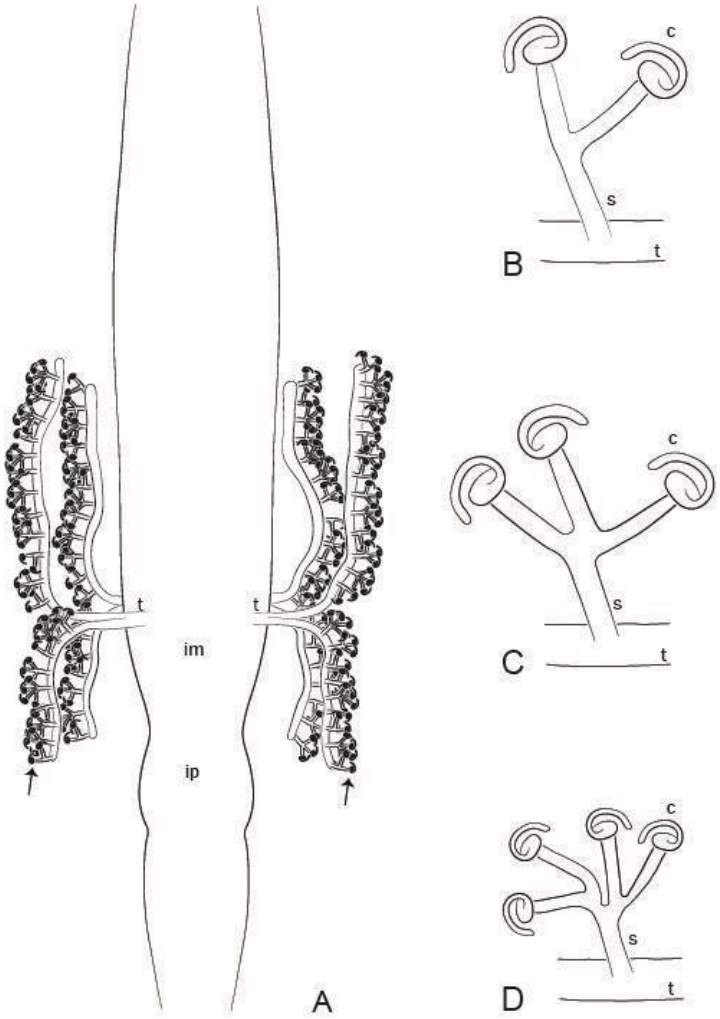

Fig 5. Anatomy of Malpighian tubules of *Campylocia burmeisteri* nymph. (A) General view. (B), (C) and (D) detail of shown structures. Main trunks (t) emerging farther from the intersection between the midgut (im) and hindgut (ip) than in *Campsurus truncatus*. From the main trunk (t) originates the secondary branch (s) which can be bifurcated (A), trifurcated (B), or with four branches (C). Coiled structure (c) at the end of the secondary branch. Arrow: Attached forms.

Histology

The Malpighian tubules of *Cy. burmeisteri* and *H. albivitta* present a single layer epithelium with cuboidal and pyramidal cells (Fig 6A-B). These cells are mononucleated, with a median oval nucleus rich in decondensed chromatin and invaginations on the basal surface in contact with the hemocoel (Fig 6A-B). Pyramidal cells are present in the coiled branch (Fig 6A) and cuboidal ones in the main trunks and secondary branches (Fig 6B). The Malpighian tubules of *Cs. truncatus*, *Campsurus* sp., and *T. canum* also present pyramidal and cuboidal cells (Fig 7A-C). Pyramidal cells are found in coiled branches and cubic cells in the main trunks

and the secondary and tertiary branches. Unlike *Cy. burmeisteri* and *H. albivitta*, the coiled branches of *Cs. truncatus*, *Campsurus* sp., and *T. canum* present columnar cells. Some of these columnar cells have non-basophilic cytoplasm (Fig 7B), whereas others have strongly basophilic cytoplasm (Fig 7B).

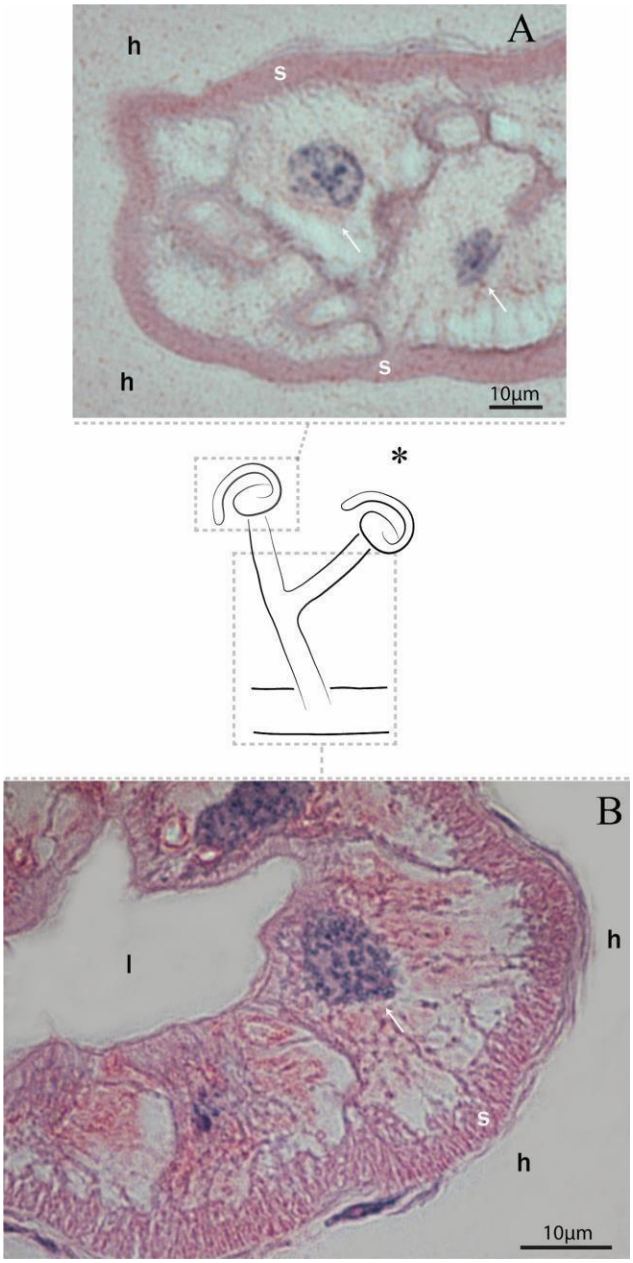

Fig 6. Light micrographs of the Malpighian tubules of *Campylocia burmeisteri* stained with hematoxylin and eosin. (A) Pyramidal cells of the coiled structure showing a median nucleus (arrow) with decondensed chromatin and basal surface (s) with invaginations; (h) hemocoel. (B) Cuboidal cells in the secondary branch with a nucleus (arrow) featuring decondensed chromatin and invaginations on the basal surface (s); (l) lumen; (h) hemocoel.

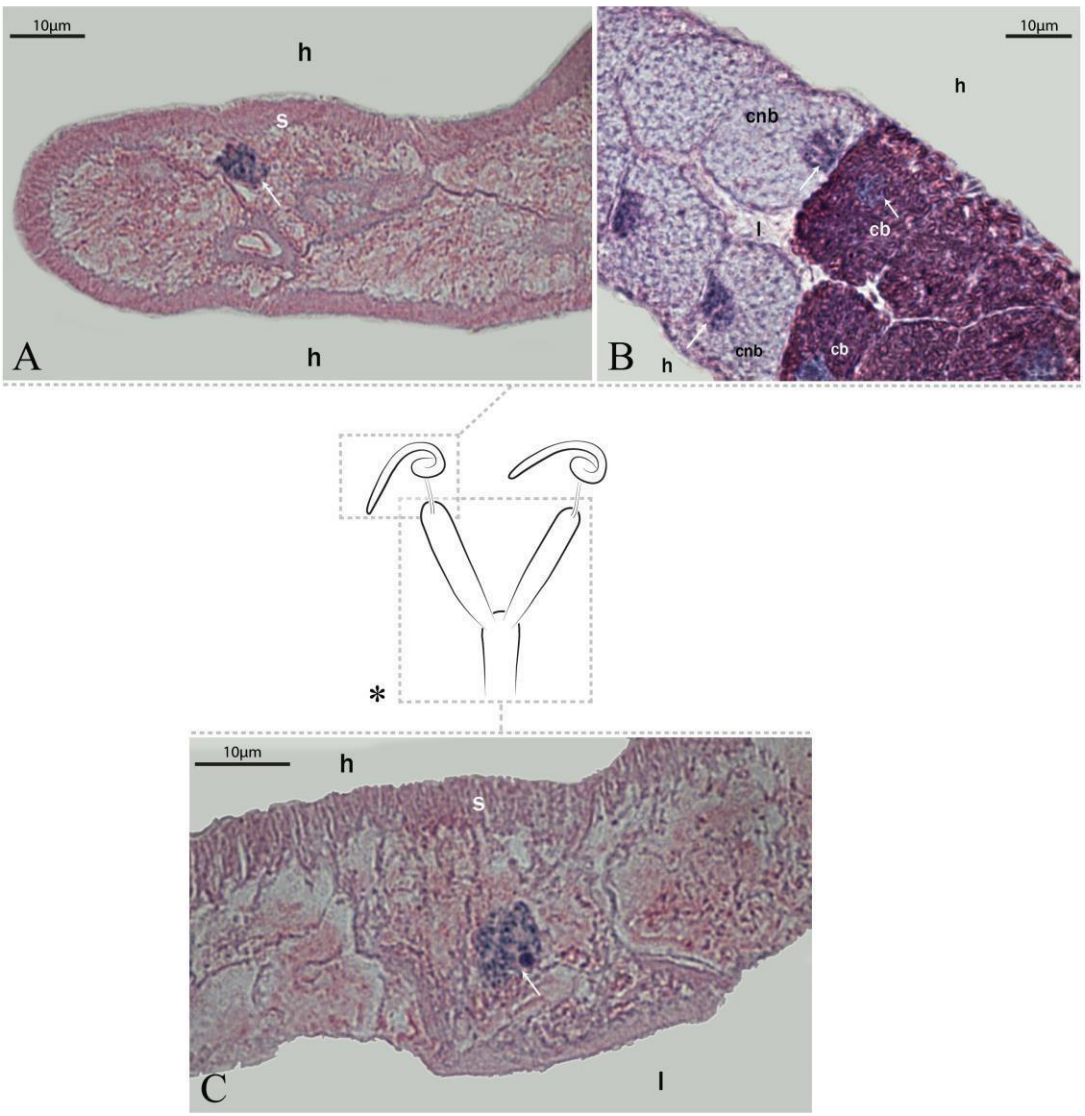

Fig 7. Light micrographs of Malpighian tubules of *Campsurus truncatus* stained with hematoxylin and eosin. (A) Pyramidal cells of the coiled structure showing a median nucleus (arrow) with decondensed chromatin and basal surface (s) with invaginations; (h) hemocoel. (B) Columnar cells with strongly basophilic (cb) and non-basophilic (cnb) cytoplasm; (l) lumen; (h) hemocoel. (C) Cuboidal cells in the secondary branch with a nucleus (arrow) featuring decondensed chromatin and invaginations on the basal surface (s); (l) lumen; (h) hemocoel. (*) Branches of Malpighian tubules of *Cs. truncatus*.

Histochemical tests

In Malpighian tubules of *Campsurus* sp., *Cs. truncatus*, and *T. canum*, histochemical tests revealed the presence of granules strongly reactive to both P.A.S and mercury bromophenol blue tests in the cytoplasm of columnar cells in Malpighian tubules of *Campsurus* sp., *Cs. truncatus* and *T. canum* (Fig 8A and 8B) which are lacking in *H. albivitta* and *Cy. burmeisteri*.

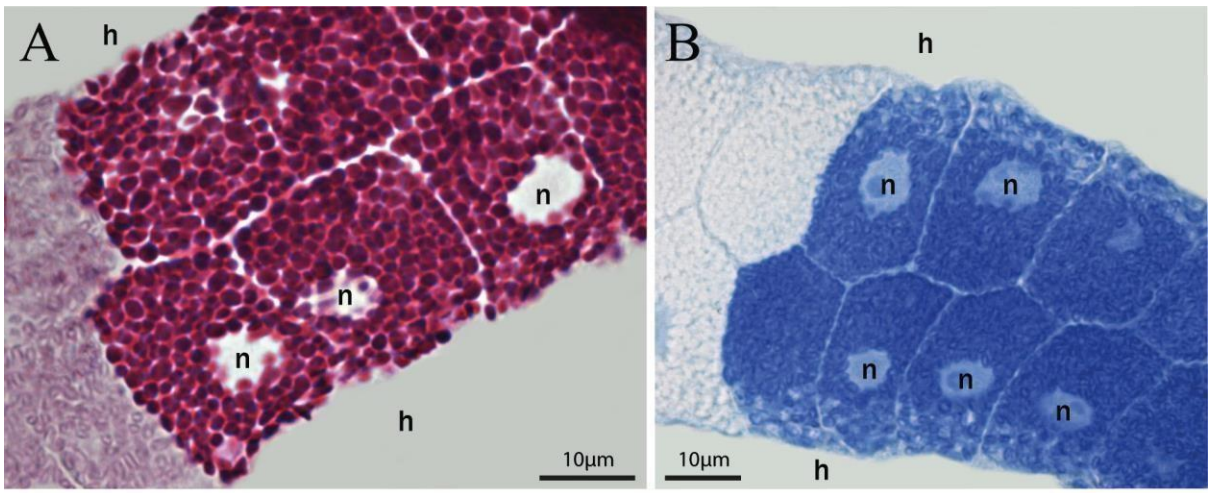

Fig 8. Light micrographs of the coiled branches of *Tortopsis canum.* (A) Granules strongly reactive to the P.A.S test in the cytoplasm of columnar cells. (B) Granules strongly reactive to the mercury-bromophenol blue test in the cytoplasm of columnar cells. (n) nucleous; (h) hemocel.

DISCUSSION

In Ephemeroptera, the Malpighian tubules emerge as individual trunks or in clusters from the posterior region of the midgut or the anterior portion of the hindgut (Soldan, 1973). Regarding the connection to the gut, the emergence of Malpighian tubules as individual main trunks is considered by Landa & Soldán (1985) as the plesiomorphic state in Ephemeroptera, as seen in both *Cs. truncatus* and *Cy. burmeisteri*. Concerning the number of trunks in Ephemeroidea, Campsurinae and Asthenopodinae have the same number as in Euthyplociidae, with four trunks. While six main trunks are present in Polymitarcyinae, one of the subfamilies of Polymitarcyidae, as in Potamanthidae, Ephemeridae and Palingeniidae. Species of Behningidae exhibit the highest number of trunks in the superfamily, with eigth trunks (Landa, 1969). *Campsurus truncatus* and *Cy. burmesteri* have one of the smallest number of main trunks (four) among Ephemeroptera species. Species of *Drunella* Needham, 1905 (Ephemerellidae) have eight trunks, species of *Hagenulus* Eaton, 1882 (Leptophlebiidae) have a pair of trunks (Landa & Soldán, 1985). According to Landa & Soldán (1985), a trend in Ephemeroptera is the reduction in the number of main trunks in the Malpighian tubules. Like in *Cs. truncatus* and *Cp. burmeisteri*, Malpighian tubules of Ephemeroptera species have secondary branches (Landa & Soldán, 1985; Gaino & Rebora, 2000). The secondary branches of Ephemeroptera seem to enhance the surface area of the tubules, thus increasing the efficiency of hemolymph osmotic regulation (Nocelli et al., 2016).

Among insects, Malpighian tubules may present different shapes, but the morphology of the cells is similar, being either cuboidal or pyramidal (Chapman, 1998; Nocelli et al., 2016).

In most species, such as the fire ant *Solenopsis saevissima* Fore, 1904, and the

grasshopper *Melanoplus differentialis* (Thomas, 1865), the tubules are formed by epithelium with a single layer of cuboidal cells (Garayoa, 1992; Arab & Caetano, 2002; Delakorda et al., 2009; Koçakoglu & Candan, 2020; Koçakoglu, 2021). Meanwhile, the Malpighian tubules of the bumblebee *Bombus morio* (Swederus, 1817) are formed by a single layer of intercalating pyramidal and cuboidal cells, without presenting regionalization of the cells (Gonçalves et al., 2014). In addition to Campsurinae nymphs, other insects also exhibit Malpighian tubules with cell regionalization, as the beetle *Melanotus communis* (Gyllenhal, 1817), which exhibits a simple tubule with cuboidal cells in the proximal and pyramidal cells in the distal region (Wilder & Smith, 1938).

In addition to cells involved in the osmoregulatory and excretory activity (Chapman, 1998), the Malpighian tubules of the three evaluated species of Campsurinae have specialized cells that exhibit secretory activity of glycoprotein evidenced by the co-localization of positive reactions to P.A.S. and mercury-bromophenol. Furthermore, these cells are columnar, a shape associated with regions with secretory activities and uncommon in Malpighian tubule epithelia (Chapman 1998, Nocelli et al., 2019). This type of cell has a larger cytoplasm than cuboidal cells and, thus, can have a greater number of organelles related to the production of glycoprotein granules, such as the Golgi complex and rough endoplasmic reticulum (Caro & Palade, 1964; Marshall, 1973). Malpighian tubules that secrete proteinaceous material have a specialized region for this function (Spiegler, 1962; Marshall, 1973; Kenchington, 1983). In other species of insects that produce silk, such as Chrysopidae (Neuroptera), Cercopidae (Hemiptera), and Curculionidae (Coleoptera), secretory cells are located in the distal portion of the Malpighian tubules (Spiegler, 1962; Marshall, 1973; Kenchington, 1983). In Campsurinae, this also occurs, and the protein-secretory cells are located in the coiled branches, in the distal portion of the secondary branches of the main trunk.

The observation of proteinaceous material in the lumen of the Malpighian tubules of Asthenopodinae (Satler, 1967), and the presence of protein-secretory cells in the tubules of Campsurinae herein studied, are strong evidence that silk secretion is widespread in Campsurinae + Asthenopodinae regardless of the species' habitat. Given the evidence here presented, the production of silk has probably evolved, at least, in the common ancestor of Asthenopodinae + Campsurinae (Molineri et al., 2015). To confirm protein secretion in Malpighian tubules as an apomorphy of Polymitarcyidae, studies on the biology and physiology of nymphs of Polymitarcyinae, the sister group of the clade Asthenopodinae + Campsurinae (Ogden, 2009), are necessary. The absence of proteinaceous material in the cells of the Malpighian tubules of *Cy. burmesteri* and *H. albivitta* is further evidence that silk production is an evolutionary novelty in Polymitarcyidae nymphs.

Currently, there are hypotheses regarding the evolutionary pressure that likely led to the silk production in Polymitarcyidae: (1) silk provides greater stability to the walls of tunnels constructed by nymphs, preventing the collapse of the shelter (Salter, 1967); (2) silk provides greater body stability for nymphs while they perform movements with legs and gills, thereby increasing the efficiency of particle filtration (Hartland-Rowe 1953); and (3) silk may reduce the size of the lumen of tunnels and, thus, decrease the possible space between suspended particles and filtering structures of the anterior legs, increasing the efficiency of particle filtration by nymphs (Hartland-Rowe 1958). The first hypothesis is weakened by our study, as nymphs of *H. albivitta* apparently do not produce silk and yet they also construct and inhabit U-shaped tunnels in substrates of lower cohesion than those in which Campsurinae nymphs build their tunnels. Framling (1943) states that *Hexagenia* nymphs keep their gills moving while inside the tunnel, similar to Campsurinae, and that when the nymph stops beating its gills or leaves the shelter, the walls begin to collapse, indicating that, in addition to substrate cohesion, water flow in the tunnel may be responsible for shelter stability.

The hypothesis proposed by Hartland-Rowe (1953), is not supported here, as nymphsof *H. albivitta*, which also filter particles inside the U-shaped tunnels, do not seem to produce silk. *Hexagenia* nymphs rhythmically beat their gills, creating a particle flow inside the shelter, and these particles are caught by filter setae on the femur and tibia of the fore legs, similar to nymphs of Polymitarcyidae (Framling, 1943; Hartland-Rowe, 1953). However, *Hexagenia* nymphs are able to efficiently filter particles without being anchored in silk on the tunnel walls.

The most likely hypothesis is Hartland-Rowe's (1958). The head, especially the tusks, is used to make the initial tunnel perforation, and thus, the tunnel lumen size is directly related to the width of the head and tusks (Keltner & McCafferty, 1986). The tusks of *Hexagenia* are slender, not heavily armed or sclerotized, and do not possess spines or crenulations (Keltner & McCafferty, 1986). On the other hand, the tusks of Polymitarcyidae are rounded basally, robust, and have spines and crenulations on the outer margin of the tusks (Bae & McCafferty, 1995). Likely, the ratio of tunnel lumen size to nymph body width is greater in Polymitarcyidae than in *Hexagenia*, making it more difficult for Polymitarcyidae nymphs to intercept suspended particles. Consequently, silk on walls of shelters and tunnels, as observed here and by Satler (1967), reduces the tunnel lumen size, allowing Polymitarcyidae nymphs to intercept suspended particles more efficiently. Behavioral studies are needed to confirm the factor that drove silk production to be an evolutionary novelty in Polymitarcyidae nymphs.

This is the first histological investigation about the silk-production in mayflies. The report of glycoprotein-secretory cells in Malpighian tubules of Campsurinae species provides strong evidence for the silk production occurring in this organ. Furthermore, the knowledge of

the anatomy of *Cs. truncatus* allowed for the placement of the glycoprotein-secretory cells in the coiled branches of the Malpighian tubules. Behavioral studies of Polymitarcyidae nymphs report the presence of silk only in the shelters of Campsurinae, and we observed that nymphs inhabiting tunnels also exhibited strong physiological evidence of silk production. This result indicates that silk production is widespread in the subfamily. To know if the silk production is a synapomorphy of Campsurinae + Asthenopodinae or its an apomorphy of Polymitarcyidae, further investigations into the behaviour and physiology of the Malpighian tubules of species Polymitarcyinae are necessary. Furthermore, ultrastructural analysis of the secretory cells can improve the knowledge about the physiology of silk production in mayflies.

REFERENCES

ANDERSON, J. M. E. Aquatic Hydrophilidae (Coleoptera). The biology of some Australian species with descriptions of immature stages reared in the laboratory. **Australian Journal of Entomology**, v. 15, n. 2, p. 219-228, 1976.

BAE, Y. J.; MCCAFFERTY, W. P. Ephemeroptera tusks and their evolution. **Current Directions in Research on Ephemeroptera,** p. 377-405, 1995.

CASE, S. T.; POWERS, J.; HAMILTON, BURTON, M. J. Silk and silk protein from two aquatic insects. In: KAPLAN, D.; ADAMS, W. W.; FARMER, B.; VINEY, C. **Silk Polymers: Materials Science and Biotechnology**, American Chemical Society, 1993, p. 80-90.

CAVASIN-OLIVERA, G. M.; CRUZ-LANDIM, C. Ultrastructure of Apoidea (Hymenoptera, Anthophorinae) salivary glands. I. Alveolar glands. **Revista brasileira de entomologia**, v. 42, n. 1-2, p. 1-6, 1998.

CHANG, J. C.; GURR, G. M.; FLETCHER, M. J.; GILBERT, G. G. Structure–Property and Structure–Function Relations of Leafhopper (Kahaono montana) Silk. **Australian journal of chemistry**, v. 59, n. 8, p. 579-585, 2006.

CRAIG, C. L. Evolution of arthropod silks. **Annual review of entomology**, v. 42, n. 1, p. 231-267, 1997.

CRAIG, L. C.; HSU, M.; KAPLAN, D.; PIERCE, N. E. A comparison of the composition of silk proteins produced by spiders and insects. **International Journal of Biological Macromolecules**, v. 24, n. 2-3, p. 109-118, 1999.

EATON, A. E. An outline of a re-arrangement of the genera of Ephemeridae. **Entomologist's Monthly Magazine**, v. 6, p. 131–132, 1868

ESBEN-PETERSEN, T. New and little-known species of Ephemerida from Argentine. (Neuropt.). **Deutsche Entomologische Zeitschrift**, 333–342, 1912.

FREMLING, C. l. R. Biology of a large mayfly, *Hexagenia bilineata* (Say), of the upper Mississippi River. **Research bulletin of Iowa**, 1960.

FREMLING, C. R.; SCHOENING, G. L. Artificial substrates for *Hexagenia* mayfly nymphs.
Proceedings of the First International Conference on Ephomeroptera, 1973. p. 208.

GAINO, E.; REBORA, M. The duct connecting Malpighian tubules and gut: an ultrastructural and comparative analysis in various Ephemeroptera nymphs (Pterygota). **Zoomorphology**, v. 120, n. 2, p. 99-106, 2000.

GONCALVES, I. C.; DA-SILVA, E. R.; NESSIMIAN, J. L. First record and new species of *Tortopsis Molineri*, 2010 (Ephemeroptera, Polymitarcyidae) from Brazil. **Zootaxa**, v. 2866, n. 1, p. 50–54, 2011.

HARTLAND-ROWE, R. Feeding mechanism of an ephemeropteran nymph. **Nature**, v. 172, p. 1109-1110, 1953.

HARTLAND-ROWE, R. The biology of a tropical mayfly Povilla adusta Navas (Ephemeroptera, Polymitarcidae) with special reference to the lunar rhythm of emergence. **Revue de Zoologie et de Botanique Africaines**, v. 58, n. 3-4, p. 185-202, 1958.

JIN, H.; KAPLAN, D. L. Mechanism of silk processing in insects and spiders. **Nature**, v. 424,

n. 6952, p. 1057-1061, 2003.

KELTNER, J.; MCCAFFERTY, W. P. Functional morphology of burrowing in the mayflies Hexagenia limbata and Pentagenia vittigera. **Zoological Journal of the Linnean Society**, v. 87, n. 2, p. 139-162, 1986.

KENCHINGTON, W. The larval silk of Hypera spp.(Coleoptera: Curculionidae). A new example of the cross-β protein conformation in an insect silk. **Journal of Insect Physiology**, v. 29, n. 4, p. 355-361, 1983.

KIEL, E.; RÖDER, T. Gelelectrophoretic studies on labial gland secretions of immature blackflies (Simuliidae, Diptera). **Limnologica**, v. 32, n. 3, p. 201-205, 2002.

KOVOOR, J. Comparative structure and histochemistry of silk-producing organs in arachnids. In: **Ecophysiology of spiders**. Berlin, Heidelberg: Springer Berlin Heidelberg, 1987. p. 160-186.

LANDA, V. A contribution to the evolution of the order Ephemeroptera based on comparative anatomy. In PETERS, W.L.; PETERS, J. G. **Proceedings of the first international conference on Ephemeroptera**. E.J. Brill, Leiden, 1973. P. 155-159.

LANDA, V.; SOLDÁN T. **Phylogeny and higher classification of the order Ephemeroptera: a discussion from the comparative anatomical point of view**. Studie CSAV, Ceskoslovenska Akademie Ved, Praha. 1985. 121 pp.

LAWRENCE, W.; FOIL, L. D. The effects of diet upon pupal development and cocoon formation by the cat flea (Siphonaptera: Pulicidae). **Journal of Vector Ecology**, v. 27, p. 39-43, 2002.

LOMMELEN, E.; SCHOETERS, E.; BILLEN, J. Ultrastructure of the labial gland in the ant Pachycondyla obscuricornis (Hymenoptera, Formicidae). **Netherlands journal of zoology**, v. 52, n. 1, p. 61-68, 2002.

LUCAS, F.; SHAW, J. T. B.; SMITH, S. G. Comparative studies of fibroins: I. The amino acid composition of various fibroins and its significance in relation to their crystal structure and taxonomy. **Journal of Molecular Biology**, v. 2, n. 6, p. 339-349, 1960.

MACKAY, R. J.; WIGGINS, G. B. Ecological diversity in Trichoptera. **Annual review of entomology**, v. 24, n. 1, p. 185-208, 1979.

MARSHALL, A. T. Protein synthesis and secretion by the Malpighian tubules of cercopoid larvae (Homoptera). **Journal of Insect Physiology**, v. 19, n. 12, p. 2317-2326, 1973.

MASCHWITZ, U.; DOROW, W. H. O.; BOTZ, T. Chemical composition of the nest walls, and nesting behaviour, of Ropalidia (Icarielia) opifex van der Vecht, 1962 (Hymenoptera: Vespidae), a Southeast Asian social wasp with translucent nests. **Journal of Natural History**, v. 24, n. 5, p. 1311-1319, 1990.

MATHEW, G.; RAI, K. S. Fine structure of the Malpighian tubule in Aedes aegypti. **Annals of the Entomological Society of America**, v. 69, n. 4, p. 659-661, 1976.

MCCAFFERTY, W. P. The burrowing mayflies (Ephemeroptera: Ephemeroidea) of the United States. **Transactions of the American Entomological Society**, v. 101, p. 447-504, 1975.

MILLER, D. B.; BARTLETT, S.; SARTORI, M.; BREINHOLT, J. W.; OGDEN, T. H. Anchored phylogenomics of burrowing mayflies (Ephemeroptera) and the evolution of tusks. **Systematic Entomology**, v. 43, p. 692-701, 2018.

MOLINERI, C.; EMMERICH, D. New species and new stage descriptions of Campsurus major species group (Polymitarcyidae: Campsurinae), with first report of silk-case construction in mayfly nymphs. **Aquatic Insects**, v. 32, n. 4, p. 265-280, 2010.

MOLINERI, C.; SALLES, F. F.; PETERS, J. G. Phylogeny and biogeography of Asthenopodinae with a revision of Asthenopus, reinstatement of Asthenopodes, and the description of the new genera Hubbardipes and Priasthenopus (Ephemeroptera, Polymitarcyidae). **Zookeys**, n. 478, p. 45, 2015.

MOLINERI, C; DIAS, L. G.; ZÚÑIGA, M. C. New insights into the phylogeny of Tortopus Needham and Murphy and Tortopsis Molineri (Ephemeroptera, Polymitarcyidae) with description of three new species. **Arthropod Systematics & Phylogeny**, 151-170, 2021.

MORTON, S. R.; RENTZ, D. C. F. Ecology and taxonomy of fossorial, granivorous gryllacridids (Orthoptera: Gryllacrididae) from arid central Australia. **Australian Journal of Zoology**, v. 31, n. 4, p. 557-579, 1983.

NAGASHIMA, T.; OKAJIMA, S.; NONAKA, T. Ultrastructure of silk gland of webspinners, Oligotoma japonica (Insecta, Embioptera). **Cytologia**, v. 56, n. 4, p. 679-684, 1991.

NOCELLI, R.; ROAT, T.C.; SILVA-ZACARIN, E.C.; MALASPINA, O. Comparative physiology of Malpighian tubules: form and function. **Insect Physiol**, v. 2016, p. 13-23, 2016.

OGDEN, T.H.; GATTOLIAT, J.L.; SARTORI, M.; STANICZEK A.H.; SOLDÁN, T.; WHITING, M.F. Towards a new paradigm in mayfly phylogeny (Ephemeroptera): combined analysis of morphological and molecular data. **Systematic Entomology**, v. 34, n. 4, p. 616- 634, 2009.

QUICKE, D. L. J.; SHAW, M. R.; TAKAHASHI, M.; YANECHIN, B. Cocoon silk chemistry of non-cyclostome Braconidae, with remarks on phylogenetic relationships within the Microgastrinae (Hymenoptera: Braconidae). **Journal of Natural History**, v. 38, n. 17, p. 2167-2181, 2004.

SATTLER, W. Über die Lebensweise, insbesondere das Bauverhalten, neotropischer Eintagsfliegen-Larven (Ephemeroptera, Polymitarcidae) Mit 14 abbildungen. **Studies on Neotropical Fauna and Environment**, v. 5, n. 2, p. 89-110, 1967.

SCOTT, C. E.; ANDERSON, A. G.; ANDRADE, M. C. B. A review of the mechanisms and functional roles of male silk use in spider courtship and mating. **The Journal of Arachnology**, v. 46, n. 2, p. 173-206, 2018.

SEHNAL, F.; AKAI, H. Insect silk glands: their types, development and function, and effects of environmental factors and morphogenetic hormones on them. **International Journal of Insect Morphology and Embryology**, v. 19, n. 2, p. 79-132, 1990.

SEIBT, U.; WICKLER, W. The protective function of the compact silk nest of social Stegodyphus spiders (Araneae, Eresidae). **Oecologia**, v. 82, p. 317-321, 1990.

SERRÃO, J.E.; CAMPOS, L. Notes on the structure of silk glands in sphecid wasps, Microstigmus and Psenulus (Hymenoptera, Sphecidae, Pemphredoninae). **Netherlands Journal of Zoology**, v. 50, n. 4, p. 479-486, 2000.

SERRÃO, J.E. Ultrastructure of the silk glands in three adult females of sphecid wasps of the genus Microstigmus (Hymenoptera: Pemphredoninae). **Revista Chilena de Historia Natural**,

v. 78, n. 1, p. 15-21, 2005.

SNODGRASS, R. E. **Principles of insect morphology**. Cornell University Press, 1935.

SOHAL, R. S. Fine structure of the Malpighian tubules in the housefly, Musca domestica. **Tissue and Cell**, v. 6, n. 4, p. 719-728, 1974.

SORENSEN, G. S.; CRIBB, B. W.; MERRITT. D.; Structure and ultrastructure of the silk glands and spinneret of Helicoverpa armigera (Hübner)(Lepidoptera: Noctuidae). **Arthropod structure & development**, v. 35, n. 1, p. 3-13, 2006.

SPIEGLER, P. E. The origin and nature of the adhesive substance in larvae of the genus Chrysopa (Neuroptera: Chrysopidae). **Annals of the Entomological Society of America**, v. 55, n. 1, p. 69-77, 1962.

SUTHERLAND, T. D.; YOUNG, J. H.; WEISMAN, S.; HAYASHI, C. Y.; MERRITT, D. J. Insect silk: one name, many materials. **Annual review of entomology**, v. 55, p. 171-188, 2010.

TASHIRO, Y.; MORIMOTO, T.; NAGATA, S. Studies on the Posterior silk gland of the silkworm, Bombyx mori: I. Growth of posterior silk gland cells and biosynthesis of fibroin during the fifth larval instar. **The Journal of cell biology**, v. 38, n. 3, p. 574-588, 1968.

TRUEMAN, J. W. H. Unusual eggshell structures in Ictinogomphus australis (Selys)(Anisoptera: Gomphidae). **Odonatologica**, v. 19, n. 3, p. 293-296, 1990.

ULMER, G. Neue Ephemeropteren. **Archiv fu¨r Naturgeschichte**, v. 85(A), p. 1–80, 1920.

VERRIER, M. L. Éphémèrọpteres. Exploration du Parc National Albert. Mission H. Damas (1935-1936) 20:1-12, 1951.

VOLLRATH, F. Biology of spider silk. **International Journal of Biological Macromolecules**, v. 24, n. 2-3, p. 81-88, 1999.

VOLLRATH, F.; KNIGHT, D. P. Liquid crystalline spinning of spider silk. **Nature**, v. 410, n. 6828, p. 541-548, 2001.

WALKER, F. **Catalogue of the specimens of neurpterous insects in the collection of the British Museum**. Edward Newman: London, 1853.

WALL, B.J.; OSCHMAN, J.L.; SCHMIDT, B.A. Morphology and function of Malpighian tubules and associated structures in the cockroach, Periplaneta americana. **Journal of morphology**, v. 146, n. 2, p. 265-306, 1975.

YANG, J. H.; MERRITT, D. J. The ultrastructure of the silk-producing basitarsus in the Hilarini (Diptera: Empidinae). **Arthropod Structure and Development**, v. 32, p. 157-65, 2003.

CHAPTER IV - SPERM ULTRASTRUCTURE OF *Campsurus violaceus* Needham & Murphy, 1924 (Ephemeroptera: Polymitarcyidae)

ABSTRACT

PANTOJA, Gabriel Martins, Federal University of Viçosa, July, 2024. **Sperm ultrastructure of *Campsurus violaceus* Needham & Murphy, 1924 (Ephemeroptera: Polymitarcyidae)**. Adviser: Frederico Falcão Salles. Co-adviser: José Eduardo Serrão.

Polymitarcyidae is the most diverse family of burrowing mayflies. Recently, the systematic classification of neotropical representatives of the family has been revised in works based on external morphological characteristics. The search for new informative characters is recurrent in phylogenetic studies, and sperm morphology can be a source of valuable information. However, knowledge of sperm morphology in Polymitarcyidae is still limited. The present study describes the ultrastructure of *Campsurus violaceus* to improve the knowledge of spermatic morphology of Polymitarcyidae and on Ephemeroptera. The sperm of *C. violaceus* exhibits some autapomorphies of mayflies, such as an axoneme with the 9+9+0 microtubule pattern and the lack of outer dynein arms in the microtubule doublets. The morphology found in *C. violaceus* sperm exhibits variations that could serve as important phylogenetic signals in evolutionary studies of mayflies, such as the pattern of protofilaments in accessory tubules, the arrangement of accessory bodies, and the number of mitochondria. The morphological variations found in the sperm of *C. violaceus* can provide useful characters for future phylogenetic studies in Polymitarcyidae as well as in Ephemeroptera.

INTRODUCTION

Insect spermatozoa display the highest structural variability among animals, exhibiting notably rapid and divergent selection (Simons, 2002). Despite the limited number of components, insect spermatozoa is a complex cell that show a great variation of characteristics that can generate valuable phylogenetic information (Gottardo et al., 2016). The evolutionary process of the general structure of spermatozoa of a particular group begins with alterations in a single component due to a cascade of events related to reproductive biology, such as sperm competition and mating strategies (Gaino & Mazini, 1991; Dallai et al., 2016). Normally, the evolution process of insect spermatozoa morphology occurs with changes in elements of the acrosome, flagellar axoneme, and centriole, with an evolutionary trend towards flagellum loss and thus sperm motility loss (Dallai et al., 2006; Dallai et al., 2016). Insect orders exhibit variations in the evolution of these characters (Dallai, 2014). In some orders, these characters may remain conserved, as in Lepidoptera and Odonata, whereas in others, spermatozoa exhibit a high evolutionary rate, as in Ephemeroptera (Gottardo et al., 2016).

Studies on the morphology of Ephemeroptera spermatozoa began in the late 1960s and have intensified from the 1990s onwards. The first published study on the topic examined the spermatozoa ultrastructure of *Cloeon dipterum* (Linnaeus, 1761) (Baetidae) (Baccetti et al., 1969). A decade later, the sperm morphology of 11 families from Central Europe was studied using light microscopy (Soldán, 1979). This was the first study reporting aflagellate spermatozoa in Leptophlebiidae. Almost 10 years later, the spermatozoa ultrastructure of *Dolania americana* Edmunds and Traver, 1959 (Behningiidae) was analyzed by Fink & Yasui (1988). A short time later, Dallai & Afzelius (1990) analyzed the architecture of the axoneme of *C. dipterum*. One year later, Gaino & Mazzini (1991) confirmed the reduction of tail axoneme in Leptophlebiidae when they analyzed the spermatozoa ultrastructure of *Habroleptoides umbratilis* (Eaton, 1884), *Habrophlebia eldar* Jacob & Sartori 1984, and *Choroterpes picteti* (Eaton, 1871). The aflagellate sperm in Leptophlebiidae was also observed by Brito et al. (2011) in a study on the characteristics of the male reproductive system and sperm of *Massartela brieni* (Lestage), *Farrodes carioca* (Domínguez et al) and *Miroculis Mourei* (Savage & Peters). Mencarelli (2014) studied the ultrastructure of the axoneme of *C. dipterum* and *Ecdyonurus venosus* (Fabricius, 1775) (Heptageniidae). The most recent work on spermatic morphology was published by Brito et al. (2015), when the authors investigated the structure and ultrastructure in *Hexagenia albivitta* (Walker, 1853) (Ephemeridae). From these studies, the established autapomorphic characteristics of Ephemeroptera are a monolayered acrosome, a 9+9+0 axoneme microtubule pattern, accessory microtubules exhibiting 13 protofilaments, axonemal microtubules doublets exhibiting only inner dynein arms, a single

mitochondria and two crystalline accessory bodies (Dallai, 2014).

Polymitarcyidae is a group of burrowing mayflies that exhibit a worldwide distribution, except for Australia and Pacific Islands (Sartori & Brittain, 2015). *Campsurus* Eaton, 1868 is the most diverse genus of Polymitarcyidae, with 44 species, of which 39 are reported only for South America (Jacobus, 2024; Salles et al., 2024). Recently, the systematics of the Neotropical representatives of the family was revised in studies based on external morphological characters involving the genera *Campsurus* Eaton, 1868 (Molineri & Salles, 2013); *Tortopus* Needhan & Murphy, 1924 (Molineri, 2010; Molineri et al., 2021); *Tortopsis* Molineri, 2010 (Molineri et al., 2021), *Asthenopus* Eaton, 1871, and *Asthenopodes* Ulmer, 1924 (Molineri et al., 2015). Despite these recent studies, some relationships within Polymitarcyidae are still poorly understood and the addition of anatomical data may help resolve evolutionary trends in the group.

This study analyzes the spermatozoa ultrastructure of *Campsurus violaceus* Needham & Murphy 1924 (Polymitarcyidae), in order to contribute to the knowledge of the evolutionary process of spermatozoa structures in this family and to generate potential informative characters for new phylogenetic studies of Polymitarcyidae and Ephemeroidea

MATERIAL AND METHODS

Male imagos of *C. violaceus* were collected with a light trap on Santa Branca farm, Goiânia,Goiás state, Brazil (16° 25' 10.7" S; 49° 05' 20.4"W).

Alive males of *C. violaceus* were dissected in sodium phosphate buffer (0.1 M, pH 7.2) and the seminal ducts were transferred to Karnovsky's solution (2.5% glutaraldehyde and 4% paraformaldehyde). The ducts were post-fixed with 1% osmium tetroxide solution for 1 h, dehydratedin an acetone series and embedded in epoxy resin. Ultrathin sections (~70 nm) were stained with 3% aqueous uranyl acetate and 0.2% lead citrate, and analyzed with a Transmission Electron Microscope(Zeiss EM109), operating at 80 kV, at the Center for Microscopy and Microanalysis at UniversidadeFederal de Viçosa.

RESULTS

The sperm of *Campsurus violaceus* exhibits two regions: head and flagellum. The head has an acrosome and the nucleus (Fig 1A). The first proximal structure of the head is the acrosome, whichexhibits a single layered conical shape, filled with homogeneous electron dense content (Fig. 1A). The nucleus is located below the acrosome, separated from it by an electron lucent layer (Fig. 1A-B).

The nucleus has an oval shape in cross section and is filled with condensed chromatin

with some translucent areas (Fig. 1B).

The nucleus-flagellum transition exhibits an adjunct centriole filled with medium electron density content (Fig. 1C). The centriolar adjunct has a lateral extension that surrounds the initial portion of the axoneme (Fig. 1C). We could not observe the centriole in the centriolar region.

The flagellum of *C. violaceus* spermatozoa consists of three long structures: the axoneme, the accessory body and the mitochondrion. The axoneme exhibits a 9+9+0 microtubule pattern: 9 accessory microtubules, 9 axonemal microtubule doublets and the central sheath without microtubules (Fig. 1D-E). The accessory microtubules exhibit a translucent lumen and tubulin protofilaments, but the number of these structures could not be counted (Fig. 1D). Each accessory tubule is connected with an axonemal microtubule doublets by an arm. The axonemal doublets showa electron-lucent lumen and inner dynein arms (Fig. 1E). The central sheath exhibits a large electronlucent lumen and connected to the microtubules doublets by radial spokes (Fig 1D-E). The axonemeis the last structure to be disorganized in the most distal region of the flagellum (Fig. F-H). The microtubule doublets is the longest structure of the axoneme (Fig. F-H).

The accessory body is an electron-dense layer, width 0.05 μm, which surrounds nearly the whole mitochondrion, except for the region adjacent to the axoneme (Fig. 1D-E). The accessory bodyends after the mitochondrion but before the axoneme (Fig. 1F-G).

The mitochondria is arranged in a single circular matrix that is surrounded by accessory bodies(Fig. 1D-E). The mitochondria extends along the flagellum, parallel to the axoneme, and ends beforereaching the accessory body and the axoneme (Fig. 1D-F). We did not obtain a longitudinal-section of an entire flagellum, but it was possible to observe slides with more than one mitochondria along amatrix (Fig. 1I). The mitochondria exhibit an electron-dense content into the intermembrane space, and the mitochondrial matrix shows an electron-lucid material (Fig. 1D). No cristae or paracrystallinematerial were observed in the mitochondria.

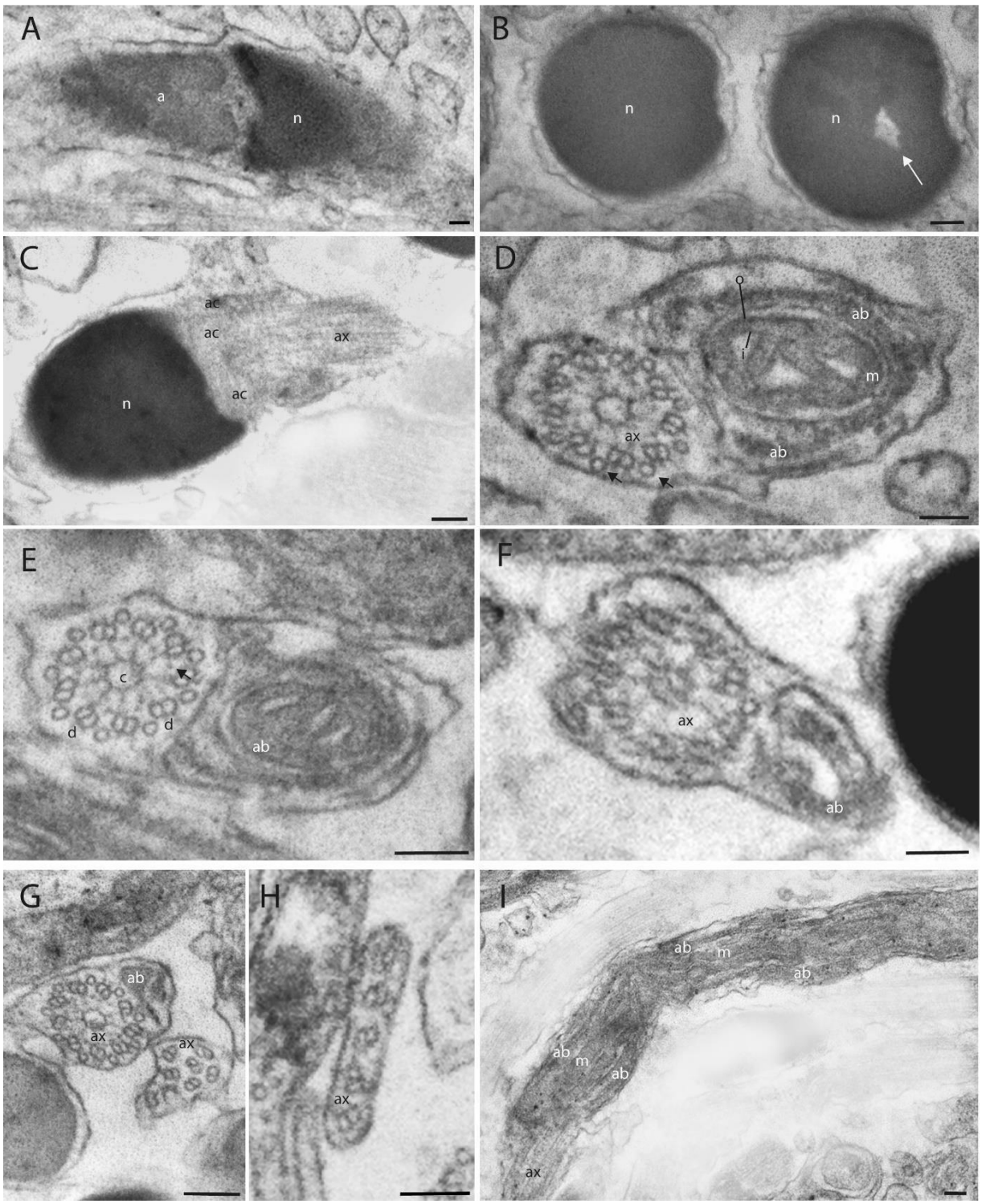

Figure 1. Transmission electron micrographs of the sperm of *Campsurus violaceus*. (A) Longitudinal section of the head, (a): acrosome, (n): nucleus; (B) Longitudinal section of the nucleus (n); arrow: translucid areas; (C) Longitudinal sectional of nucleus-flagellum region, (n): nucleus, (ca): centriolar adjunct, (ax): axoneme; (D) Cross-section of the flagellum; (ax): axoneme, (arrow): accessory tubules; (ab): accessory body, (m): mitochondria, (i) inner and (o) outer membrane of mitochondria; (E) Cross-section of the flagellum; (ab) accessory body, (d) axonemal doblets, (c) central sheath, (arrow) inner dynein arms; (F-H) Cross section in different region of the flagellum, (ax) axoneme, (ab) accessory body; (I) Longitudinal section of the flagellum, (ax) axoneme, (m) mitochondria. Scale bar = 0.1µm

DISCUSSION

The hexapod spermatozoa groundplan exhibits a three or two layer acrossome, while a monolayered one is considered a derived characteristic (Dallai, 2014; Dallai, et al., 2016). A monolayered acrosome is established as an autapomorphic character state in Ephemeroptera (Dallai,2014) and probably the bilayered acrosome arose independently in two species of Baetidae, *Callibaetis jocosus* and *Tupiara ibirapitanga* and in *Lachlania* sp. (Oligoneuriidae), which are species from phylogenetically distant families (Appendix 1) (Ogden et al., 2009; Brito, 2012). The monolayered acrosome is found in most of the mayfly species studied, including *Cloeon dipterum* (Baetidae), *Traverhyphes yuati* (Leptohyphidae), *Coryphorus aquilus* (Coryphoridae), species of Caenidae, and Leptophlebiidae (Baccetti et al., 1969 Gaino & Mazzini, 1991; Brito, 2012). In additionto *C. violaceus*, a monolayered acrosome has been observed in other species of Ephemeroidea, suchas *Asthenopus curtus* and *Campsurus* sp. (Polymitarcyidae); *Campylocia anceps* (Euthyplociidae); and in *Hexagenia albivitta* (Ephemeridae) (Brito, 2012; Brito et al., 2015), indicating that this patternhas been maintained in this superfamily.

In spermatic studies of mayflies, the observed variation in the nucleus relates to the chromatin.Chromatin is found condensed in the nucleus of *C. violaceus* and in most of the mayfly species studied. Variation is found in *Lachlania* sp. (Oligoneuridae) and *Caenis fittkaui* (Caenidae) with granular chromatin, and in *Brasilocaenis renata* (Caenidae) with decondensed chromatin (Brito, 2012). The nucleus with condensed chromatin is possibly widespread in Ephemeroptera, and the variation in chromatin condensation patterns has evolved independently in some lineages of Oligoneuriidae and Caenidae. However, more data on mayfly spermatozoa morphology are necessaryfor robust phylogenetic inferences within the order.

The centriolar adjunct is a finely granular dense material that surrounds a centriole and the presence of this structure is considered one of the characteristics supporting the clade Insecta (Dallai,2014; Gottardo et al., 2016). The function of this region remains unclear; the first hypothesis was thatits function was to connect the flagellum to the spermatozoa head (Philips, 1970), but Dallai (2014) contest this hypothesis affirming that "*...it does not explain how the two sperm regions could be connected when the centriole adjunct material is missing*.", basing this statement in eight species of Hemiptera that did not exhibit the centriolar adjunct (Dallai & Afzelius, 1980). In the early studies on spermatozoa ultrastructure in mayflies (Bacceti, 1969; Fink & Yasui, 1988; Gaino & Mazzini, 1991), the centriolar adjunct was also not observed in species of Heptageniidae, in *Cloeon dipterum*(Baetidae) and in *Dolania americana* (Behningiidae). The first observation of the centriolar adjunct was made

by Brito (2012) and Brito et al. (2015), who reported this organelle for 13 species from nine families of mayflies. The nucleus-flagellum region of *C. violaceus* corroborates the presence ofthe centriolar adjunct in spermatozoa of Ephemeroptera species. The mayfly species in which this organelle was not observed probably had a weak or scarce centriolar adjunct (Brito et al., 2015).

The flagellum of *C. violaceus* spermatozoa exhibits an axoneme with a 9+9+0 microtubule pattern, which is an autapomorphy of mayflies (Dallai, 2014). The only variation in the axoneme microtubule pattern was reported by Fink and Yasui (1988) when they observed a dark central element in the center of the axoneme of *Dolania americana* (Behningiidae), suggesting that the axoneme pattern could be 9+9+1. However, in the same article, the authors write: "*We cannot confirm, based on our present ultrastructural results, whether the central element is or is not a singlet microtubule.*" (Fink and Yasui, 1988). Therefore, except for Leptophlebiidae species which are aflagellate, all mayflies studied to date exhibit a 9+9+0 axoneme pattern. We could not observe the number of protofilaments in the accessory microtubules of *C. violaceus*, but probably this species exhibits 13 tubulin protofilaments, which is a well-established pattern for Ephemeroptera species (Dallai & Afzelius, 1990; Brito et al., 2015; Dallai, 2006). There is no subunit inside the accessory tubules of *C. violaceus* presenting a clear lumen, similar *Campurus* sp. and *Asthenopus curtus*, and the phylogenetically related *Campylocia anceps* (Euthyplociidae) (Appendix 1; Brito, 2012), whereasanother species of Ephemeroidea, *Hexagenia albivitta* (Ephemeridae), exhibits 7 subunits inside theaccessory tubules (Appendix 1; Brito, 2012). The accessory tubules with 7 subunits is considered an autapomorphy of Ephemeroptera, and probably the accessory tubules without subunit pattern is an evolutionary novelty in mayfly lineages such as Polymitarcyidae and Euthyplociidae.

The morphological groundplan of hexapod spermatozoa exhibits the flagellum with two mitochondria (Dallai et al., 2014; Dallai et al., 2016). The flagellum with one mitochondria is a derived state and is considered an autapomorphy of Ephemeroptera (Gottardo et al., 2016). *Campsurus* sp. is the only species that exhibits a flagellum with more than one mitochondria amongthe 17 species of mayflies studied to date (Baccetti et al., 1969; Fink & Yasui, 1988; Gaino & Mazzini,1991; Brito 2012; Brito et al., 2015). *Campsurus violaceus* also presents flagella with more than one mitochondria, which is an indication that this characteristic is an autapomorphy of *Campsurus* or of some species group of the genera *Campsurus*.

The arrangement of the accessory body in the flagellum is one of the most variable characteristics in Ephemeroptera spermatozoa (Bacceti et al., 1969; Gaino & Mazzini, 1991; Brito, 2012, Brito et al., 2015). Most species present the accessory body between the

mitochondrion and theaxoneme, but the arrangement of this structure can vary into a central cluster, into two lateral triangular lobes, and it can be paracrystalline or not. Some species also present the accessory body surrounding the mitochondrion, which is not a common organization among the species studied to date. This characteristic is found in *C. violaceus* and the other two species of Polymitarcyidae, as wellas in a species of Oligoneuriidae and Caenidae (Brito, 2012). The accessory body surrounding the mitochondrion likely arose independently in the Polymitarcyidae since this characteristic is observed in species of Caenidae and Oligoneuriidae, which are phylogenetically distant from Polymitarcyidae (Appendix 1). However, the sperm of other Ephemeroidea species need to be studied to understand the evolutionary history of the accessory body organization in this superfamily.

The sperm of *C. violaceus* presents some Ephemeroptera autapomorphies, such as the 9+9+0axoneme pattern and axonemal doublets with only inner dynein arms. Furthermore, the morphology of *C. violaceus* sperm exhibits characteristics that could serve as important phylogenetic signals in evolutionary studies of mayflies, such as the pattern of protofilaments in accessory tubules, the arrangement of accessory bodies, and the number of mitochondria.

Considering the highly accelerated evolutionary rate of sperm in mayflies (Gottardo et al., 2016) and the limited number of Polymitarcyidae species studied, further research on spermatic morphology in this family is necessary to reduce the gap in knowledge about mayfly sperm. Studiesof spermatic ultrastructure of mayflies could generate characters with strong phylogenetic signals forEphemeroptera systematics.

REFERENCES

BACCETTI, B.; DALLAI, R.; GIUSTI F. The Spermatozoon of Arthropoda VI. Ephemeroptera. **Journal Ultrastructure Research**, v. 29, p. 343-349, 1969.

BRITO, P.; MANCINI, K.; SALLES, F. F.; RIZZI, E. A.; DOLDER, H. The sperm of *Hexagenia (Pseudeatonica) albivitta* Walker (Ephemeroptera: Fossoriae: Ephemeridae). **Acta Zoologica**, v. 26,p. 160-166, 2015.

BRITO, P. **Morfologia do sistema reprodutivo masculino e dos espermatozoides de Ephemeroptera (Insecta) e análise do seu potencial filogenético**. Doutorado. Campinas: USP. 2012

BRITO, P.; SALLES, F. F.; DOLDER, H. Morphology of male reproductive systems in Ephemeroptera: intrinsic musculature. **Neotropical entomology**, v. 41, p. 306-310, 2012.

DALLAI, R.; AFZELIUS, B. A. Microtubular diversity in insect spermatozoa: results obtained witha new fixative. **Journal of Structural Biology**, v. 103, n. 2, p. 164-179, 1990.

DALLAI, R.; GOTTARDO, M.; BEUTEL, R. G. Structure and evolution of insect sperm: new interpretations in the age of phylogenomics. **Annual Review of Entomology**, v. 61, p. 1-23, 2016.

DALLAI, R.; LUPETTI, P.; MENCARELLI, C. Unusual axonemes of hexapod spermatozoa. **International Review of Cytology**, v. 254, p. 45-99, 2006.

DALLAI, R. Overview on spermatogenesis and sperm structure of Hexapoda. **Arthropod Structure& Development**, v. 43, n. 4, p. 257-290, 2014.

FINK, T.J.; YASUI, L.S. Ultrastructure of the sperm of Dolania americana Edmunds and Traver (Ephemeroptera: Behningiidae). **International Journal of Insect Morphology and Embryology**, v.17, n. 6, p. 447-454, 1988.

GAINO, E.; MAZZINI, M. Spermatozoon of mayflies (Ephemeroptera): an ultrastructural approach. **Overview and strategies of Ephemeroptera and Plecoptera**, p. 27, 1991.

GOTTARDO, M.; DALLAI, R.; MERCATI, D.; HORNSCHEMEYER, T.; BEUTEL, R. G. The evolution of insect sperm– an unusual character system in a megadiverse group. **Journal of Zoological Systematics and Evolutionary Research**, v. 54, n. 4, p. 237-256, 2016.

JACOBUS, L. M. (2024) **Mayfly Central**. Availabe from: ttps://www.entm.purdue.edu/mayfly/index.php (access 31 Jan 2024)

MOLINERI, C.; DIAS, L. G.; ZÚNIGA, M. C. New insights into the phylogeny of *Tortopus* Needham & Murphy and *Tortopsis* Molineri (Ephemeroptera, Polymitarcyidae) with description of three new species. **Arthropod Systematics & Phylogeny**, v. 79, p. 151-170, 2021.

MOLINERI, C.; EMMERICH, D. New species and new stage descriptions of Campsurus major species group (Polymitarcyidae: Campsurinae), with first report of silk-case construction in mayfly nymphs. **Aquatic Insects**, v. 32, n. 4, p. 265-280, 2010.

MOLINERI, C.; SALLES, F. F.; PETERS, J. G. Phylogeny and biogeography of Asthenopodinae with a revision of Asthenopus, reinstatement of Asthenopodes, and the

BACCETTI, B.; DALLAI, R.; GIUSTI F. The Spermatozoon of Arthropoda VI. Ephemeroptera. **Journal Ultrastructure Research**, v. 29, p. 343-349, 1969.

BRITO, P.; MANCINI, K.; SALLES, F. F.; RIZZI, E. A.; DOLDER, H. The sperm of *Hexagenia (Pseudeatonica) albivitta* Walker (Ephemeroptera: Fossoriae: Ephemeridae). **Acta Zoologica**, v. 26,p. 160-166, 2015.

BRITO, P. **Morfologia do sistema reprodutivo masculino e dos espermatozoides de Ephemeroptera (Insecta) e análise do seu potencial filogenético**. Doutorado. Campinas: USP. 2012

BRITO, P.; SALLES, F. F.; DOLDER, H. Morphology of male reproductive systems in Ephemeroptera: intrinsic musculature. **Neotropical entomology**, v. 41, p. 306-310, 2012.

DALLAI, R.; AFZELIUS, B. A. Microtubular diversity in insect spermatozoa: results obtained witha new fixative. **Journal of Structural Biology**, v. 103, n. 2, p. 164-179, 1990.

DALLAI, R.; GOTTARDO, M.; BEUTEL, R. G. Structure and evolution of insect sperm: new interpretations in the age of phylogenomics. **Annual Review of Entomology**, v. 61, p. 1-23, 2016.

DALLAI, R.; LUPETTI, P.; MENCARELLI, C. Unusual axonemes of hexapod spermatozoa. **International Review of Cytology**, v. 254, p. 45-99, 2006.

DALLAI, R. Overview on spermatogenesis and sperm structure of Hexapoda. **Arthropod Structure& Development**, v. 43, n. 4, p. 257-290, 2014.

FINK, T.J.; YASUI, L.S. Ultrastructure of the sperm of Dolania americana Edmunds and Traver (Ephemeroptera: Behningiidae). **International Journal of Insect Morphology and Embryology**, v.17, n. 6, p. 447-454, 1988.

GAINO, E.; MAZZINI, M. Spermatozoon of mayflies (Ephemeroptera): an ultrastructural approach. **Overview and strategies of Ephemeroptera and Plecoptera**, p. 27, 1991.

GOTTARDO, M.; DALLAI, R.; MERCATI, D.; HORNSCHEMEYER, T.; BEUTEL, R. G. The evolution of insect sperm− an unusual character system in a megadiverse group. **Journal of Zoological Systematics and Evolutionary Research**, v. 54, n. 4, p. 237-256, 2016.

JACOBUS, L. M. (2024) **Mayfly Central**. Availabe from: ttps://www.entm.purdue.edu/mayfly/index.php (access 31 Jan 2024)

MOLINERI, C.; DIAS, L. G.; ZÚNIGA, M. C. New insights into the phylogeny of *Tortopus* Needham & Murphy and *Tortopsis* Molineri (Ephemeroptera, Polymitarcyidae) with description of three new species. **Arthropod Systematics & Phylogeny**, v. 79, p. 151-170, 2021.

MOLINERI, C.; EMMERICH, D. New species and new stage descriptions of Campsurus major species group (Polymitarcyidae: Campsurinae), with first report of silk-case construction in mayfly nymphs. **Aquatic Insects**, v. 32, n. 4, p. 265-280, 2010.

MOLINERI, C.; SALLES, F. F.; PETERS, J. G. Phylogeny and biogeography of Asthenopodinae with a revision of Asthenopus, reinstatement of Asthenopodes, and the description of the new genera Hubbardipes and Priasthenopus (Ephemeroptera,

Polymitarcyidae). **Zookeys**, n. 478, p. 45, 2015.

NEEDHAM, J. G.; MURPHY, H. E. Neotropical mayflies. **Bulletin of the Lloyd Library**, v. 22, 1–79, 1924.

OGDEN, T. H., GATTOLLIAT, J. L., SARTORI, M., STANICZEK, A. H., SOLDÁN, T., Whiting, M. F. Towards a new paradigm in mayfly phylogeny (Ephemeroptera): combined analysis ofmorphological and molecular data. **Systematic Entomology**, v. 34, n. 4, p. 616-634, 2009.

SIMMONS, L. W. **Sperm competition and its evolutionary consequences in the insects**. Princeton: University Press, 2002. 434 p.

GRIMM V. R. Vergleichend-anatomische Untersuchung der abdominalen Muskulatur und der Gonoducte männlicher Ephemeroptera-Imagines (Insecta). **Stuttgarter Beiträge zur Naturkunde (Serie A)**, v. 59, p. 1-59, 1985.

SALLES, F. F., MOLINERI, C., NIETO, C., LIMA, L. R. C., DIAS, L. C., BOLDRINI, R., MARIANO, R. & DOMÍNGUEZ, E. **Ephemeroptera da América do Sul**. 2024. Available from:http://ephemeroptera.com.br/ (access 31 Jan 2024).

SARTORI, M., BRITTAIN, J. E. Order Ephemeroptera. In: THORP, J.; ROGERS, D. C. (Eds.), **Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates**. Academic Press,2015. p. 873–891.

SOLDÁN, T. A comparative study of spermatozoa of some Central European Ephemeroptera. **Actaentomologica bohemoslovaca**, v. 76, p. 223-230, 1979.

CONCLUSIONS

Chapter I - Knowledge of the morphology of *Tortopsis canum* can contribute to phylogenetic studiesof Campsurinae by highlighting important character states for the phylogeny of this group. Of the 10described species of Tortopsis, nymphs are known for only five. Therefore, studies describing the nymphs of *Tortopsis* are necessary to diminish the gap between the number of described species andthe known nymphs.

Chapter II – Diversity of Neotropical Polymitarcyidae is much greater than currently known. The description of the new *Campsurus* species reduces the Linnean deficit of Polymitarcyidae and contributes to ecological and biogeographical studies involving mayflies.

Chapter III - Our study is the first to report secretory cells in the Malpighian tubules of Ephemeroptera. The presence of these secretory cells with lumens filled with glycoprotein material provides a strong evidence that the Malpighian tubules of Campsurinae are responsible for silk production in nymphs. Additionally, Malpighian tubules with secretory cells were found not only innymphs of species that construct tunnels in clay but also in nymphs of species that build shelters withmineral material, indicating that the physiology of silk production is widespread among Campsurinaespecies. To better understand the functioning of these cells, ultrastructural studies are necessary to observe organelles related to glycoprotein synthesis, such as the Golgi complex. Furthermore, behavioral studies on the function of silk in mayflies can elucidate the evolutionary aspects of the emergence of cells derived from Malpighian tubules in Polymitarcyidae lineages.

Chapter IV - Our study confirms the presence of unique characteristics in *Campsurus* sperm compared to other mayflies, such as the accessory body surrounding the mitochondrial matrix, the presence of multiple mitochondria, and the accessory tubules without subunit. These characteristics can be useful for phylogenetic analysis in Polymitarcyidae, as well as in Ephemeroptera. Further studies on the sperm ultrastructure of Polymitarcyidae species can highlight characters with valuable phylogenetic signals and enable greater precision in evolutionary inferences regarding sperm characteristics in this family.