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Bridging gaps in Neotropical Gripopterygidae (Plecoptera) taxonomy with focus on reproductive morphology

Mellis Layra Soares Rippel
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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-adviser: Pablo Pessacq

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Assent:

Mellis Layra Soares Rippel
Author

Frederico Falcao Salles
Adviser

To my grandparents.

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To my dear family. My wonderful parents, who have supported me from the very beginning, even though it meant living far away; and who are proud of me for pursuing a research career; for sometimes supporting me financially when my scholarship and bench fee weren't enough; for all the love constantly given. To my loving sisters who always encouraged and believed in me; for all the conversations, wishes, care, and affection. To my sister Melline for understanding my absence. To my dear grandparents, who are and were so proud of me; for always putting faith in me, for the calls, for being so thoughtful; know that I feel and felt your love all the way. I wish you could have seen me reaching the end of the doctorate, but I know you were already happy for me. I love you, guys.

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Deixando vou as terras
de minha primeira infância.
Deixando para trás
os nomes que vão mudando.
Terras que eu abandono
porque é de rio estar passando.
Vou com passo de rio,
que é de barco navegando.
Deixando para trás
as fazendas que vão ficando.
Vendo-as, enquanto vou,
parece que vão desfilando.
Vou andando lado a lado
de gente que vai retirando;
vou levando comigo
os rios que vou encontrando.
— João Cabral de Melo Neto, O Rio, in Serial e Antes (1997)

ABSTRACT

RIPPEL, Mellis Layra Soares, D.Sc., Universidade Federal de Viçosa, April, 2025. **Bridging gaps in Neotropical Gripopterygidae (Plecoptera) taxonomy with focus on reproductive morphology**. Adviser: Frederico Falcao Salles. Co-adviser: Pablo Pessacq.

The phylogenetic relationships and species delimitation within Gripopteryginae, which includes Neotropical Gripopterygidae, remain poorly understood regarding morphological characters. This thesis advances the understanding of Neotropical Gripopterygidae through an integrative taxonomic revision of Guaranyperla, anatomical and histological analyses of the reproductive system, and the first morphological characterization of the sperm cells in the family. The revision expands *Guaranyperla* to six recognized species, with at least three additional species awaiting formal description. By integrating morphological and molecular data, this study refines species delimitations, describes two new species, and provides identification keys for adults and nymphs. It also clarifies distinctions between *Guaranyperla* and *Tupiperla*, enhancing taxonomic resolution within Gripopteryginae. The reproductive system analyses reveal structural conservation in the histology of ovaries and testes across genera but significant anatomical variation among them. Comparisons with Australasian taxa suggest potential evolutionary divergence, highlighting the need for further studies, particularly in Andean lineages. The first description of sperm morphology in Gripopterygidae reveals interspecific variation that may reflect evolutionary differentiation. Intraspecific variation observed in *Gripopteryx reticulata* and *Guaranyperla puri* suggests the presence of cryptic species, reinforcing the utility of sperm morphometry as a complementary tool for species delimitation. This study addresses key gaps in Plecoptera systematics, reproductive morphology, and evolutionary biology, providing essential datasets for future phylogenetic studies and strengthening the foundation for further research on Neotropical Gripopterygidae.

Keywords: morphology; integrative taxonomy ; aquatic insects; internal anatomy; reproductive system ; Gripopteryginae

RESUMO

RIPPEL, Mellis Layra Soares, D.Sc., Universidade Federal de Viçosa, abril de 2025. **Preenchendo lacunas na taxonomia de Gripopterygidae (Plecoptera) com foco na morfologia reprodutiva**. Orientador: Frederico Falcao Salles. Coorientador: Pablo Pessacq.

As relações filogenéticas e a delimitação de espécies dentro de Gripopteryginae, que inclui os Gripopterygidae neotropicais, permanecem pouco compreendidas em relação aos caracteres morfológicos. Esta tese avança no conhecimento sobre os Gripopterygidae neotropicais por meio de uma revisão taxonômica integrativa de *Guaranyperla*, análises anatômicas e histológicas do sistema reprodutivo e a primeira caracterização morfológica dos espermatozoides na família. A revisão expande *Guaranyperla* para seis espécies reconhecidas, com pelo menos três outras aguardando descrição formal. Ao integrar dados morfológicos e moleculares, este estudo refina a delimitação de espécies, descreve duas novas espécies e fornece chaves de identificação para adultos e ninfas. Além disso, esclarece as distinções entre *Guaranyperla* e *Tupiperla*, aprimorando a resolução taxonômica dentro de Gripopteryginae. As análises do sistema reprodutivo revelam conservação estrutural na histologia de ovários e testículos entre os gêneros, mas variação anatômica significativa entre eles. Comparações com táxons australasianos sugerem uma possível divergência evolutiva, ressaltando a necessidade de mais estudos, especialmente em linhagens andinas. A primeira descrição da morfologia espermática em Gripopterygidae revela variação interespecífica que pode refletir diferenciação evolutiva. A variação intraespecífica observada em *Gripopteryx reticulata* e *Guaranyperla puri* sugere a presença de espécies crípticas, reforçando a utilidade da morfometria espermática como ferramenta complementar na delimitação de espécies. Este estudo preenche lacunas importantes na sistemática de Plecoptera, na morfologia reprodutiva e na biologia evolutiva, fornecendo conjuntos de dados essenciais para futuras análises filogenéticas e fortalecendo a base para novas pesquisas sobre os Gripopterygidae neotropicais.

Palavras-chave: morfologia; taxonomia integrativa; insetos aquáticos ; anatomia interna ; sistema reprodutor; Gripopteryginae

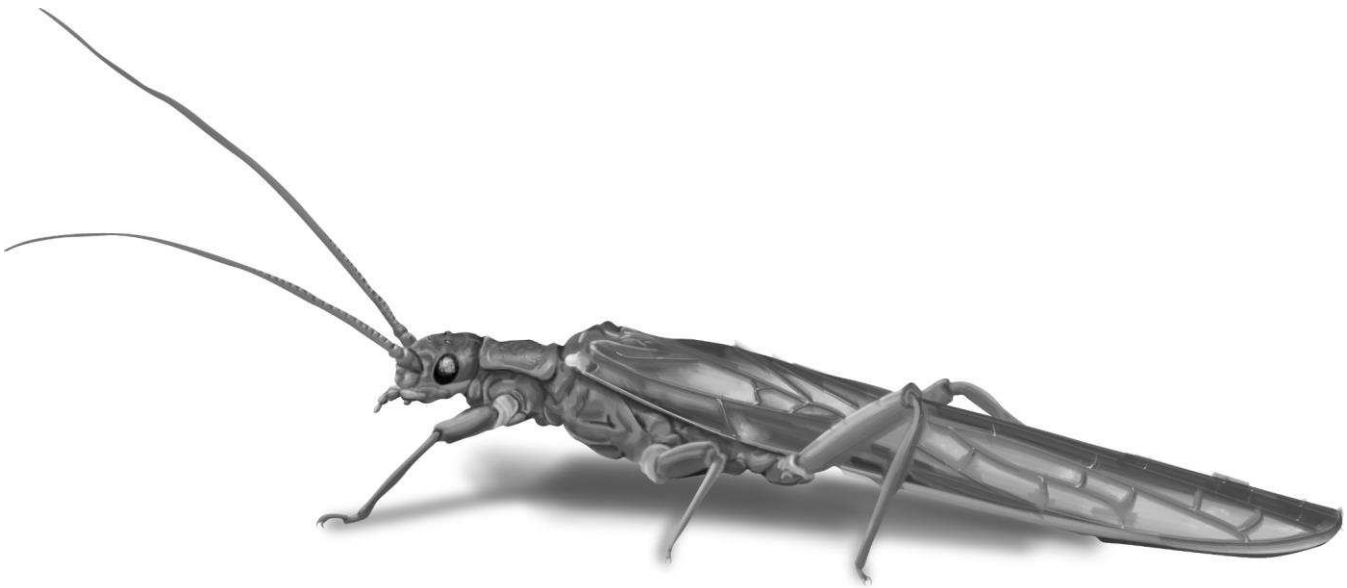

Illustration by Rodrigo Braga Gastaldo

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1 GENERAL INTRODUCTION

Plecoptera Burmeister, 1839

Plecoptera Burmeister, 1839 (stoneflies) are integral components of stream ecosystems worldwide (Fochetti; Figueroa, 2008). They serve as bioindicators of water quality, a food source for predators, and key mediators in energy flow and nutrient cycling (DeWalt; Ower, 2019). With few exceptions, their immature stages are aquatic, while adults are winged and terrestrial. Stoneflies are most diverse in cold, running waters; however, despite the relatively warmer conditions of Neotropical streams and rivers, this region harbors one of the highest species diversities globally, with over 500 described species and many more yet to be discovered (DeWalt; Ower, 2019; Pessacq; Zúñiga; Duarte, 2019). While they are primarily found in unpolluted waters, stoneflies also inhabit lakes, streams of varying sizes and thermal regimes, and even intermittent water bodies (Stark; Froehlich; Zúñiga, 2009; DeWalt; Kontradieff; Sandberg, 2014). Due to their sensitivity to environmental disturbances, such as water quality degradation and riparian deforestation, they are valuable indicators of anthropogenic impacts. Plecoptera, along with Ephemeroptera and Trichoptera, form the EPT group, widely used in freshwater biomonitoring studies (Hellawell, 1986; Brasil et al., 2020; Gomes et al., 2022).

Plecoptera belongs to the insect superorder Polyneoptera and holds a phylogenetic position as the sister group to all other Polyneoptera, except for Zoraptera + Dermaptera. The order comprises approximately 4,000 described species across 17 families (DeWalt; Ower, 2019; South et al., 2021; DeWalt et al., 2025). Stoneflies are globally distributed, occurring on every continent except Antarctica, with the highest species diversity recorded in Temperate Asia, which harbors 1,178 species (Zwick, 2000; DeWalt; Ower, 2019). The current classification system of the Plecoptera is based on Zwick (2000), who proposes two suborders: Arctoperlaria and Antarctoperlaria. The first suborder comprises 13 families and, with exception of Notonemouridae and three tribes of Perlidae, it has a Laurasian distribution. Two infraorders compose Arctoperlaria: Euholognatha, containing the families Capniidae, Leuctridae, Nemouridae, Notonemouridae, Taeniopterygidae, and Scopuridae; and Systellognatha, comprising the families Chloroperlidae, Kathroperlidae, Peltoperlidae, Perlidae, Perlodidae, Pteronarcyidae, and Styloperlidae (Zwick, 2000; South et al., 2021). Antarctoperlaria, on the other hand, has a Gondwanic distribution, and it is composed by two superfamilies: Eustheniodea, including the families Eustheniidae and Diamphipnoidae, and

Gripopterygoidea containing the families Gripopterygidae and Austroperlidae (Zwick, 2000; Letsch et al., 2021) (Fig. 1).

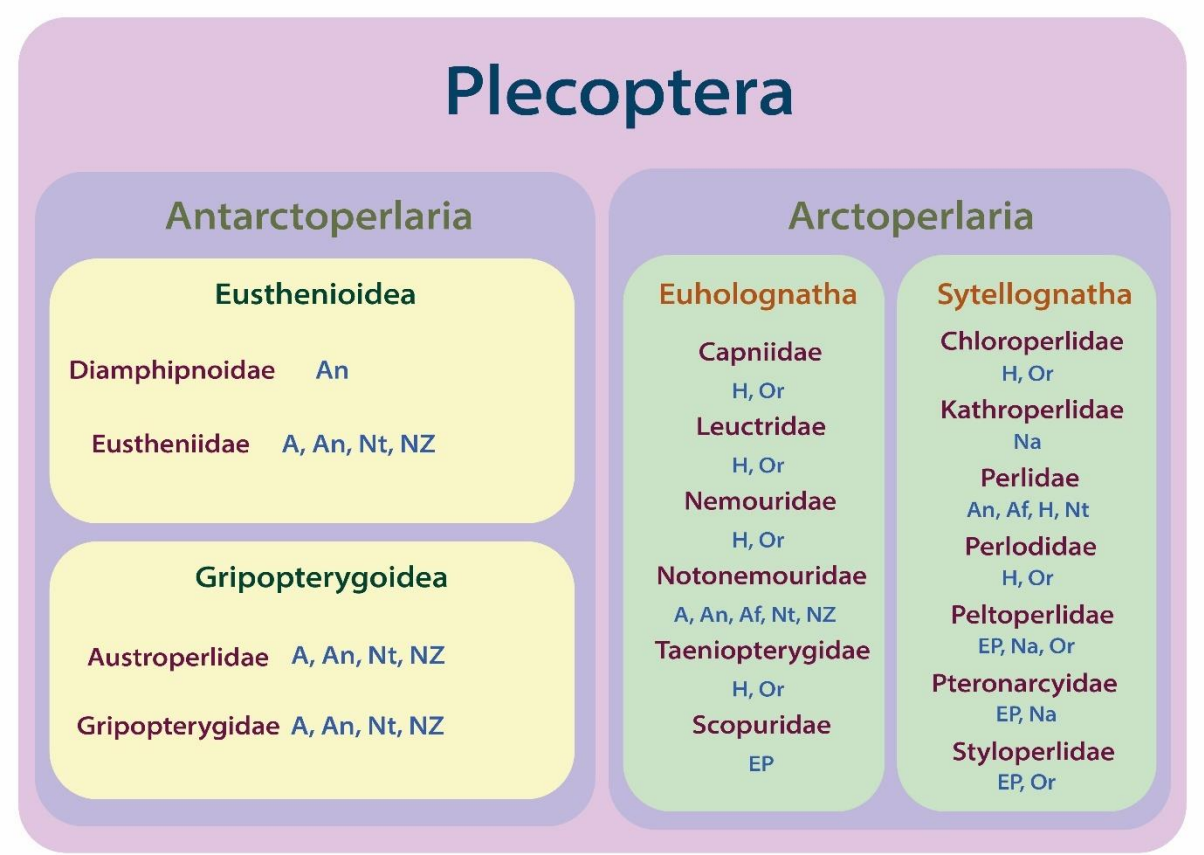

Figure 1. Schematic representations of the current classification of Plecoptera and their distribution by regions (modified from (Eichert et al., 2025)). Abbreviations: Australia (A), Afrotropical (Af), Andean (An), Holarctic (H), Eastern Palaearctic (EP), Nearctic (Na), Neotropical (Nt), New Zealand (NZ), Oriental (Or).

Recent advances have provided an updated phylogenetic framework for Plecoptera based on expanded mitogenomic sampling and site-heterogeneous models (Wang et al., 2025). Their study included representatives of all 17 families, resulting in a robust topology that confirmed the monophyly of the two suborders, Antarctoperlaria and Arctoperlaria, as well as the infraorders Euholognatha and Systellognatha, as proposed by (Zwick, 2000). Within Antarctoperlaria, Eustheniidae and Diamphipnoidae were recovered as the clade Eustheniioidea, sister to Gripopterygoidea (Gripopterygidae + Austroperlidae). Within Euholognatha, Scopuridae was placed as the earliest diverging lineage, clarifying its position relative to Nemouridae and related families. In turn, the relationships among Perlidae, Perlodidae, Chloroperlidae, and other families of Systellognatha were resolved with strong support, largely consistent with previous phylogenetic hypotheses (Letsch et al., 2021; Eichert

et al., 2025). The simplified topology presented in their graphical abstract (Fig. 2) summarizes these updated relationships.

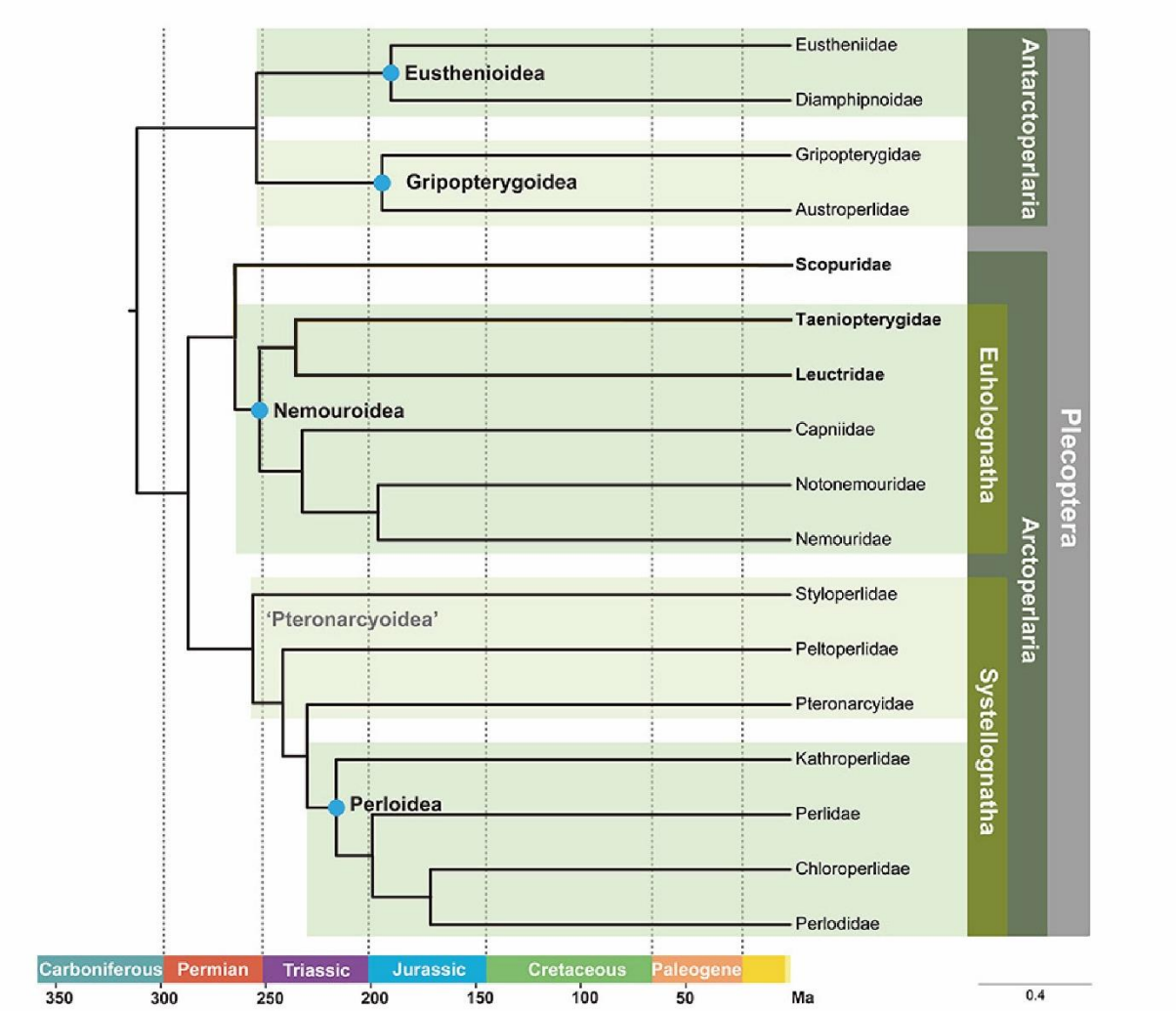

Figure 2. Simplified phylogeny of Plecoptera based on mitogenomic data (modified from Wang et al., 2025), showing the main relationships among families and divergence times, with crown-Plecoptera originating in the Pennsylvanian.

An overview of stonefly biology and morphology

A stonefly can be distinguished from other insect orders based on a combination of external characteristics (Fig. 3). These include the presence of two pairs of folded wings in most adult individuals, nymphs exhibiting two multi-articulated cerci (which are often reduced in certain families) as well as adults, a three-articulated tarsus, and two claws per leg (DeWalt; Kontradieff; Sandberg, 2014). These insects are hemimetabolous, therefore they exhibit the egg, nymph and adult life stages. The nymph goes through successive molts until it reaches the

adult stage. The number of larval instars in different stonefly groups varies between 12 and 23 (Hynes, 1976). Considering the Brazilian fauna, Froehlich (1969) estimated in laboratory 13 instars in about five months for *Paragripopteryx anga* Froehlich, 1969 (Gripopterygidae) and Dorvillé and Froehlich (2001) estimated the maximum number of 34 instars for *Kempnyia tijucana* Dorvillé & Froehlich, 1997 (Perlidae).

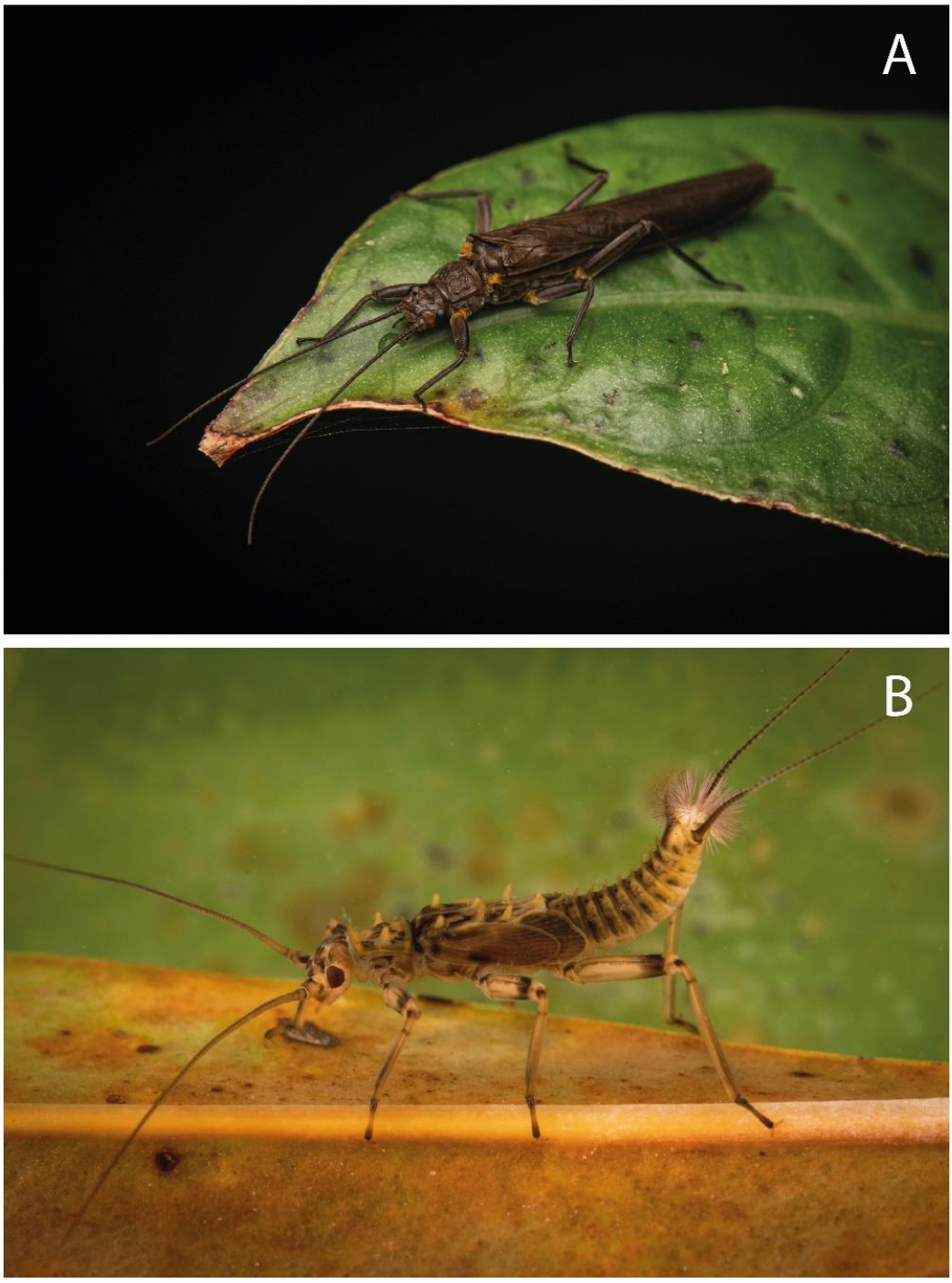

Figure 3. General illustration of Plecoptera. (A) Adult of *Gripopteryx cancellata* (Pictet, 1841). (B) Nymph (immature stage) of *Gripopteryx* sp. Modified from *Frederico F. Salles*.

According to Froehlich and Oliveira (1997), nymphs of stoneflies in streams are commonly found in riffles, taking refuge under rocks, tree trunks, moss, and leaf litter (Fig. 4). They occupy a variety of niches, such as predators (eg Eustheniidae and most Perloidea), shredders (eg. many Austroperlidae, Pteronarcyoidea, Nemouridae, Notonemouridae, Scopuridae, Gripopterygidae), collector-gatherers (eg. many Leuctridae), and scrapers (eg. some Taeniopterygidae) (Tierno De Figueroa; López-Rodríguez, 2019). While all nymphs feed, only a subset of adults does so (Figueroa; Sánchez-Ortega, 2000). During the early stages (first instars), nymphs primarily feed on fine particulate organic matter. As they mature, they adopt different feeding strategies, such as predation, shredding, or scraping (Stark; Froehlich; Zúñiga, 2009; DeWalt; Kontradieff; Sandberg, 2014).

Figure 4. A–B: Typical stream microhabitats where stoneflies occur, photographed in the Caparaó region, along the Espírito Santo–Minas Gerais border. *Credit: Frederico Falcão Salles*.

Adult stoneflies can be found either in riparian forests within lotic environments or flying close to streams (Froehlich, 2012; Lecci; Righi-Cavallaro, 2017). The life span of the adult stage of stoneflies is relatively short, typically ranging from a few days to a few weeks. They have a varied diet, including pollen, lichens, cyanobacteria, leaf buds, fruits, and other food sources (Stewart, 2009; Tierno De Figueroa; López-Rodríguez, 2019). However, it is worth noting that the majority of stonefly species do not appear to engage in feeding activities, mainly larger species. Some species, such as those belonging to Perlidae, only drink water, in which case their mouthparts are atrophied (Hynes, 1976; Tierno De Figueroa, J. M.; Fochetti, 2001; Fochetti; Figueroa, 2008).

Stoneflies exhibit a diverse array of complex behaviors related to intersexual communication, mate encounters, copulation, and mate guarding, though many aspects remain poorly understood (Tierno De Figueroa, J. Manuel; Luzón-Ortega; López-Rodríguez, 2019). In Arctoperlaria, the primary mode of mate communication is vibrational signaling transmitted through substrates (Stewart, 2009). These signals vary among species and are produced by tapping or rubbing the abdomen against the substrate or through body tremulations, which generate vibrations without direct abdominal contact (Stewart, 2009, 2001; Tierno De Figueroa; López-Rodríguez, 2019). While vibrational communication has been extensively studied, broader aspects of stonefly reproductive biology remain largely unexplored, particularly in the Southern Hemisphere, where research is still limited to a few species (Tierno De Figueroa; Luzón-Ortega; López-Rodríguez, 2019).

Males have complex internal and external primary and secondary sexual structures, which shape is commonly used to describe new species (DeWalt; Kontradieff; Sandberg, 2014). According to Brinck (1956) and Zwick (1973), the male reproductive system of Plecoptera offer a rich array of features. This morphological diversity, as most insects, includes significant modifications in the internal mesodermal organs (testes, *vasa deferentia*, and seminal vesicles) and the various forms of copulatory structures. One example is the adaptable "genital cavity" (Brinck, 1956), at the posterior edge of the sternite IX, which serves as the opening for internal organs. Typically non-sclerotized, the genital cavity evolves into a complexly structured penis in a few groups. The male reproductive system (MRS) comprises the accessory glands and an ejaculatory duct. Semen transfer during copulation is generally facilitated by the paraprocts and epiproct, the segment’s XI pleurites and sternite, respectively

(Snodgrass, 1993), often referred to as "accessory copulatory organs" to distinguish them from the mesodermal organs and genital cavity (Zwick, 1973, 1980).

The female reproductive system comprises paired ovaries, paired lateral oviducts, a common oviduct, a vagina, and typically a spermatheca (Brinck, 1956; Zwick, 1973; Rościszewska; Rzońca, 2009). In contrast to the male reproductive system, female external genitalia are generally more structurally conservative (DeWalt; Kontradieff; Sandberg, 2014), largely due to the limited development of external copulatory structures. In most cases, the vagina opens externally at the posterior edge of the sternite XIII, which is often specially formed and may develop a subgenital plate (Zwick, 1973; 1980; Rościszewska; Rzońca, 2009), a structure that the male grasps or holds with accessory copulatory organs, epiproct and paraprocts, during copulation (Stewart, 2009). Figure 5 presents illustrations of the internal reproductive systems of male and female stoneflies.

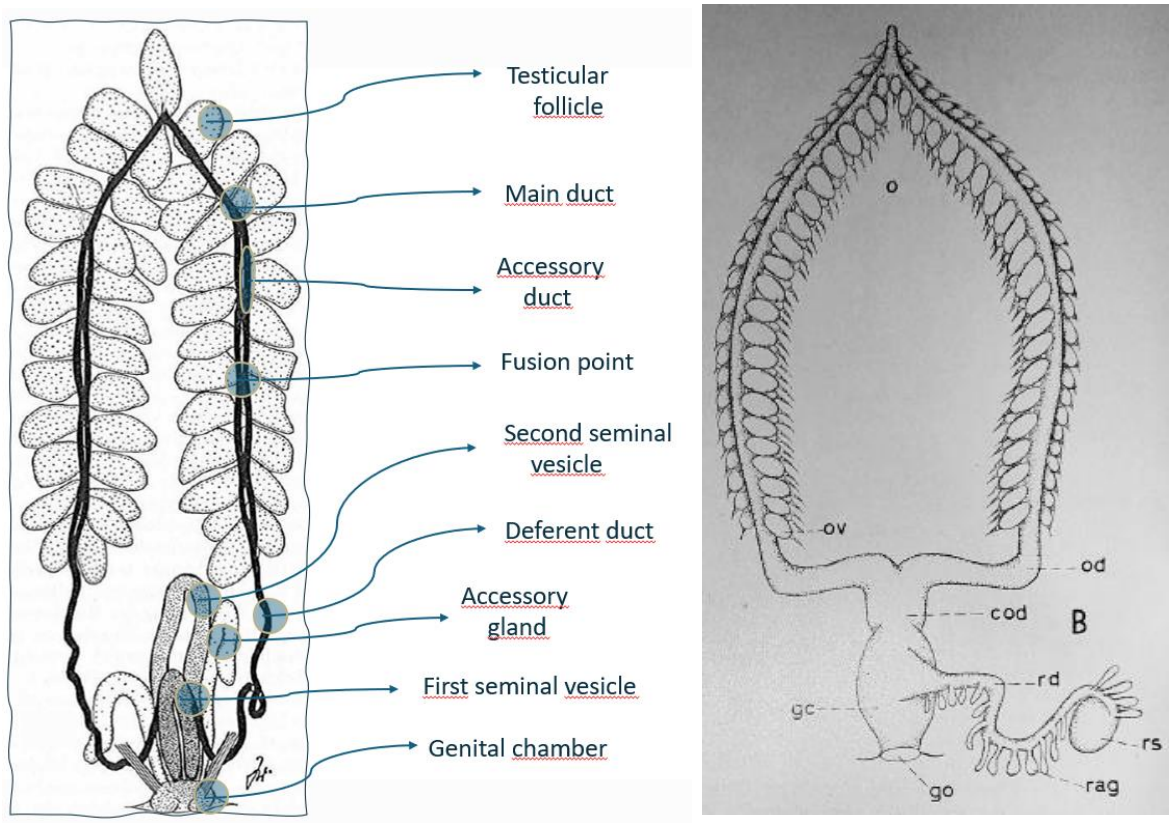

Figure 5. Schemes representing the female and male reproductive systems in stoneflies (FRS and MRS). On the left, the MRS of *Gripopteryx decorata* (modified from Zwick, 1973), and on the right, the FRS of *Isoperla grammatica* (modified from Brinck, 1956). The abbreviations used in this study correspond to the following anatomical structures: **o** ovary; **ov** ovariole; **od** oviduct; **cod** common oviduct; **rs** seminal receptacle; **rd** receptacular duct; **rag** receptacular accessory glands; **gc** genital chamber; **go** genital opening (modified from Brinck, 1956).

Plecoptera mating involves the male mounting the female and curving his abdomen around her left or right side to engage her subgenital plate, pulling it downward with his external genitalia (Stewart, 2009) (see figure 8). This action aligns her genital opening beneath the plate with a dorsal position between his cerci, where his aedeagus (penis) projects. The male's aedeagus, everted from beneath the sternite IX, expands backward and upward between the cerci to enter the female (Benedetto, 1970). In most species, sperm is transferred via this intromittent aedeagus (Stewart; Atmar; Solon, 1969). However, in some species, sperm may be delivered through a hollow male epiproct or externally deposited near the female's genital opening, later aspirated into the bursa (vagina) through telescoping abdominal movements (Stewart, 2009).

Gripopterygidae Enderlein, 1909

Gripopterygidae (Antarctoperlaria) contains about 330 described species, grouped into 57 genera, which are distributed in Australasia and South America (Pessacq; Zúñiga; Duarte, 2019; Duarte; Froehlich; Bispo, 2024; DeWalt et al., 2025). It comprises 14 genera found in Australia, 12 in New Zealand, and 28 in South America (Duarte, 2019) unpublished thesis).

Delving into the classification of Gripopterygidae

Gripopterygidae is the second most diverse family of stoneflies in South America, and comprises 112 species distributed across 28 genera (Fig. 6). The current classification was proposed by McLellan (1977) who divided Gripopterygidae into five subfamilies based on morphological characters: Antarctoperlinae Enderlein, 1909, Dinotoperlinae McLellan, 1977, Gripopteryginae Enderlein, 1909, Leptoperlinae Banks, 1913, and Zelandoperlinae McLellan, 1977. However, according to McLellan and Zwick (2007), the intergeneric relationships in Gripopterygidae are still confusing and the delimitation of genera in South America needs to be reviewed. Furthermore, most genera are delimited by character combinations, without explicit synapomorphies being stated (Pessacq; Rivera-Pomar, 2019). In a more recent phylogenetic study using molecular data, the gripopterygid taxa belonging either to Australasia or South America do not form monophyletic lineages *i.e.* the South American and Australasian

genera are interspersed across different clades within Gripopterygidae (McCulloch; Wallis; Waters, 2016). Consequently, the genera assigned to each subfamily proposed by McLellan (1977) were not recovered as monophyletic groups, but rather appeared distributed across multiple clades, leading to the polyphyly of these subfamilies. Nevertheless, Pessacq, Duarte and Epele (2020) redefined Antarctoperlinae, finding morphological evidence for the monophyly of most of its members, and Duarte (2019) (unpublished thesis) found evidence for the monophyly of Gripopteryginae also through morphological data.

Figure 6. *Habitus* of the (A) adult and (B) nymph of *Tupiperla* sp. *Credit: Frederico Falcão Salles*.

The family shows a curious disjunct distribution across South America. In the Andean Region (*sensu* Morrone, 2015), around 50 species are distributed across 24 genera (Duarte; Froehlich; Bispo, 2024; Pessacq; Duarte, 2024; Duarte et al., 2025), one genus (*Claudioperla* Illies, 1963) composed by four species is mainly found only in the South American transition zone, found in the Andes from Colombia to northern Argentina and Chile (Duarte; Froehlich; Bispo, 2024). In contrast, 62 species belonging to only four genera are found exclusively in the Neotropical Region (*sensu* Morrone, 2014). Of the Neotropical species, only three are restricted to southern Uruguay [*Gripopteryx serrei* Navás, *Paragripopteryx baratinii* Benedetto, 1983, and *Paragripopteryx munoai* (Benedetto, 1969)]. The remaining 59 species are recorded in Brazil, and to a lesser extent, Northern Argentina and Paraguay. Most of the Brazilian species are restricted to the Atlantic Forest, and a few occur in the Cerrado biome (Souza et al., 2020; Duarte; Froehlich; Bispo, 2024; Duarte et al., 2025). The genus *Guaranyperla* Froehlich is the most geographically restricted, occurring exclusively in the Atlantic Forest of southeastern Brazil (Froehlich, 2001, 2015). On the other hand, the other three genera (*Gripopteryx* Pictet, *Paragripopteryx* Enderlein, and *Tupiperla* Froehlich) exhibit a broader distribution across the Brazilian Atlantic Forest, extending inland within Brazil and into parts of Argentina, Paraguay, and Uruguay (Froehlich, 2010; Pessacq; Zúñiga; Duarte, 2019).

As mentioned before, McCulloch, Wallis and Waters (2016) recovered Gripopteryginae as polyphyletic within Gripopterygidae. However, considering the most recent classification of the family (McLellan, 1971), Gripopteryginae is considered valid (DeWalt et al., 2025) and is endemic to South America (Pessacq; Zúñiga; Duarte, 2019) with 15 genera comprising 84 species (DeWalt et al., 2025).

The phylogenetic relationships and species delimitation within Gripopteryginae remain poorly understood, particularly regarding morphological characters (McLellan; Zwick, 2007). To date, only a few studies have addressed these aspects. Lecci and Froehlich (2011) conducted a taxonomic revision of *Gripopteryx*, while Duarte, Calor and Bispo (2022) performed a systematic revision and presented the first morphology-based phylogeny of *Paragripopteryx*. However, the relationships among the remaining genera remain largely unexplored. The absence of broader phylogenetic analyses integrating all genera limits our ability to assess evolutionary patterns, define clear generic boundaries, and interpret character evolution within the subfamily. Taxonomic studies have largely relied on male genitalia for species

identification, making the association of life stages particularly challenging, especially in the presence of cryptic diversity (Duarte; Froehlich; Bispo, 2024).

Exploring the biology and morphology of gripopterygids

Most members of Gripopterygidae are winged, while a few genera are wingless; the latter occur in the alpine and Subantartic subregions in Australia and South America. In his study on the revision of the Australian Gripopterygidae, McLellan (1977) has provided a diagnosis to the family with the following morphological characteristics [updated from Duarte (2019) unpublished thesis]:

- Head: segment IV of the maxillary palp in larvae is often shortened.
- Forewing: presence of irregular crossveins in distal half; the M vein is always forked; the RA and CuA are either forked or unforked; anal region with three longitudinal veins, and with a thickened vein between the vein AA1 and AA2;
- Hind wing: similar to forewing except for the vein M3+4, close to its separation from M1+2, partially or completely fused to the CuA vein; also anal region large, containing six longitudinal veins, usually without bifurcations or crossveins; and the sixth anal vein may be fused with the posterior margin of the wing.
- Abdomen: *Male* - Tergum X (TX) is modified such that each antero-lateral part forms a distinct sclerite, which may fuse dorsally with its opposite counterpart; the posterior region of the TX may be divided from the rest forming a separate sclerite. The paraprocts are always present and usually have a sickle shape, long and curved. The epiproct is present in most of the genera with an upturned hook shape. The subgenital plate is usually ovoid. *Female* - subgenital plate usually short, but may be long in some genera. *Nymph* - usually with a rosette of anal gills (except for *Notoperla*).

The structures are shown in the images in figure 7.

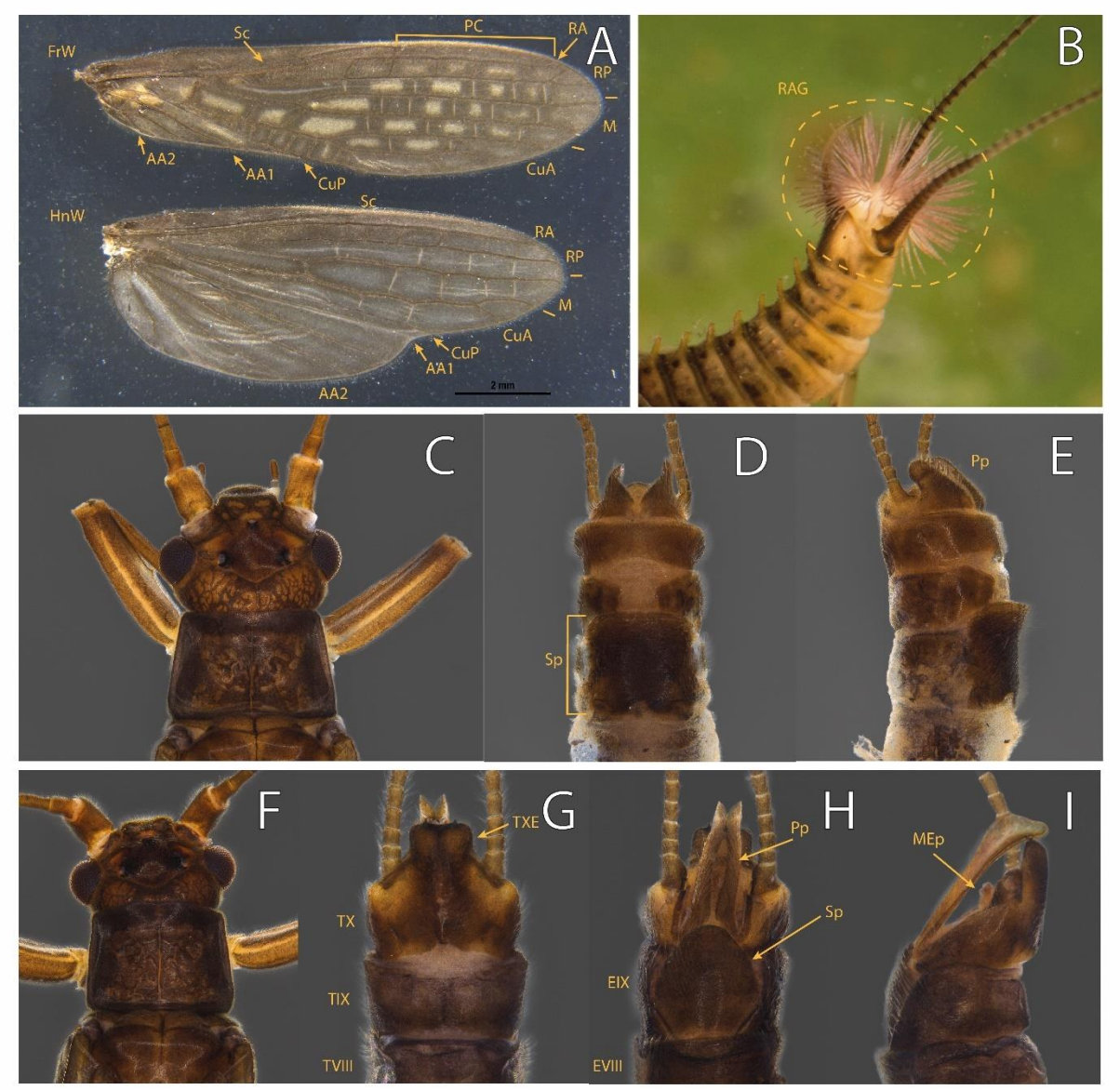

Figure 7. Morphological characters of Gripopterygidae. (A) Forewing and hindwing of *Guaranyperla* sp. (B) Posterior region of the abdomen of a *Gripopteryx* sp nymph, showing the anal gills. Female of *Tupiperla robusta* head and pronotum (C), and terminalia in ventral (D) and dorsal (E) views. Male of *T. robusta* head and pronotum (F), and terminalia in dorsal (G), ventral (H) and lateral (I) views.

Immature gripopterygids are usually scrapers and shredders, with collector-gatherer groups (Tierno De Figueroa; López-Rodríguez, 2019). They inhabit streams with well-oxygenated water, and therefore have high sensitivity to environmental changes, and are used as bioindicators for monitoring the integrity of aquatic ecosystems (Buss et al., 2002; Siegloch et al., 2017). Tierno De Figueroa, Vera and López-Rodríguez (2006) investigated the nymphal feeding habits of two Chilean gripopterygid species, *Antarctoperla michaelseni* (Klapálek,

1904) and *Limnoperla jaffueli* (Navás, 1928), revealing distinct dietary preferences. While *A. michaelseni* primarily consumed detritus, *L. jaffueli* relied mainly on diatoms as its main food source. For Neotropical gripopterygids, Froehlich (1969) reported that nymphs of *Paragripopteryx* and *Tupiperla* are primarily phytophagous, feeding mainly on decomposing leaves and occasionally on bryophytes and algae found in their habitat. Additionally, younger nymphs may consume diatoms. However, the nymphal feeding habits of other Neotropical genera remain undocumented.

In South America, the feeding behavior of stoneflies remains poorly studied (Tierno De Figueroa; López-Rodríguez, 2019; Pessacq; Duarte, 2024). Froehlich's research concluded that newly emerged adults are not sexually mature and must feed to achieve full reproductive maturity. He emphasizes the essential role of feeding in the survival of *Paragripopteryx* adults, which died within 2–3 days without food. Gut content analysis revealed a diet primarily consisting of lichens, with occasional fragments of bryophytes and higher plants (Froehlich, 1969). In laboratory conditions, adults survived up to 19 days when provided with epiphyllous vegetation; however, females did not reach full reproductive maturity even when fed (Froehlich, 1969). Similarly, according to Benetto (1970), adults of *Paragripopteryx munoai* from Uruguay, feed on algae and fungi growing on wet stones and branches. This study also found that adults deprived of food perish within 50 hours, whereas those that feed can survive for up to 20 days in females and 13 days in males (Benedetto, 1970). Tierno De Figueroa, Vera and López-Rodríguez (2006) analyzed the gut contents of two Chilean Gripopterygidae species, *Antarctoperla michaelseni* and *Limnoperla jaffueli*, revealing that their diet primarily consists of Pinaceae pollen, followed by detritus, fungi, and other pollen types.

Concerning the reproductive behavior of gripopterygids, the scenario is not different. In contrast to the species from the Northern Hemisphere (Abbott; Stewart, 1993; Stewart, 2001, 2009), knowledge on mating of members of this family, as well as in Antarctoperlaria, specially in South American species, is scarce and sometimes merely speculative (Stewart, 1994). The mechanisms of mate finding and communication in these species remain almost unknown. Vibrational signaling has not been observed in Antarctoperlaria (Stark; Froehlich; Zúñiga, 2009). The absence of drumming or other vibrational behaviors suggests that the suborder may rely on highly specific aggregation behaviors at encounter sites to locate mates. While the exact mechanisms of their mate-finding remain unknown, these adaptations likely compensate for the lack of intersexual communication through substrate vibrations, highlighting a distinct

evolutionary strategy in this group (Stewart, 2009; Tierno De Figueroa, J. Manuel; Luzón-Ortega; López-Rodríguez, 2019). To date, only a few studies have documented forms of mate finding in Antarctoperlaria (Stewart, Kenneth W., 1994; Tierno De Figueroa, J. Manuel; Luzón-Ortega; López-Rodríguez, 2019).

Benedetto (1970) has studied the behavior of adult *Paragripopteryx munoai* (Benedetto, 1969). He described that, when encountering a female, the male inspects her with his antennae, after which the female briefly moves away but ultimately allows the male to climb over her for copulation (Fig. 8), which lasts about 10 minutes. Both sexes can mate multiple times within a few hours. Approximately 12 days after mating, the female lays her light yellow, hemispherical eggs individually by submerging the tip of her abdomen in water.

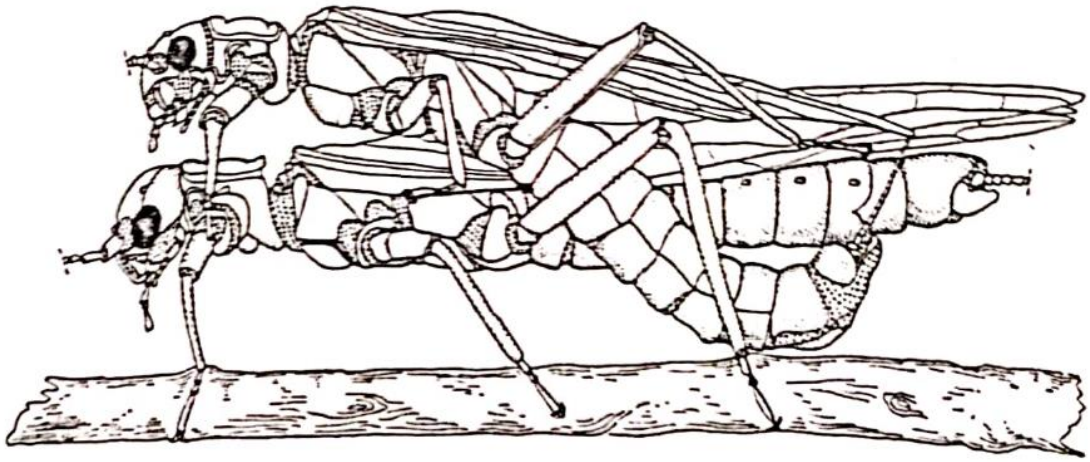

Figure 8. General diagram of *Paragripopteryx munoai* engaged on mating (modified from Benedetto, 1970).

A recent study (Kroos; Waters; McCulloch, 2021) on reproductive isolation and divergence in *Zelandoperla fenestrata* Tillyard, 1923 complex, has provided intriguing insights into the species' mating behavior. Laboratory mating trials involving male and virgin female specimens allowed researchers to observe mating duration, female preferences, and potential sexual communication between the sexes. In terms of sexual communication, males consistently displayed two distinct behaviors during the experiments. The first, termed "tapping," involved subtle inward and outward movements of the hind legs while the abdomen made contact with the ground. The second, referred to as "rubbing," involved curving the lower abdomen into an S-shape and moving it repeatedly from side to side. These behaviors were

observed in nearly half of the experiments prior to mating and in all experiments following mating (Kroos; Waters; McCulloch, 2021).

Integrative taxonomy

Integrative taxonomy involves the integration of various data types for species delineation and is increasingly recognized as a cornerstone of modern taxonomy (Wang; Nansen; Zhang, 2016). This approach combines multiple data sources—molecular, morphological, ecological, and behavioral—to provide a comprehensive understanding of species boundaries. Among these methods, DNA barcoding has gained prominence since its introduction in 2003 (Hebert et al., 2003) and has become a standard identification protocol for numerous organisms, particularly insects (Mađarić; Lešić, 2023).

In recent years, the use of integrative taxonomy has grown significantly, yielding valuable insights into insect taxonomy and classification (Jones et al., 2022; Zhu et al., 2022; Balukjian; Van Dam, 2024; Chen et al., 2024; Kasalo et al., 2025; Yang et al., 2025). The combination of molecular tools with morphological taxonomy is essential, as it enhances our understanding of insect diversity, improves identification accuracy, and supports ecological and conservation efforts (Basset et al., 2020; Lowe et al., 2022; Li; Wiens, 2023).

Morphological studies, on the other hand, remain pivotal for assessing insect biodiversity and are fundamental to the practical application of taxonomy and ecology. As noted by Trautwein et al. (2012), morphology continues to play a vital role in phylogenetic and evolutionary research. It provides independent data to validate molecular phylogenies and is indispensable for placing extinct taxa. Moreover, morphology forms the foundation for reconstructing phenotypic character evolution and developing complex evolutionary scenarios. In practice, species identification still relies primarily on morphological characters, particularly in developing countries where access to molecular tools remains limited. Identification keys and taxonomists specializing in morphology are essential to this process, as taxonomic work cannot exist without them (Engel et al., 2021). Therefore, the integration of morphological and molecular approaches ensures a robust framework for advancing our understanding of insect biodiversity and evolution.

Internal morphology is also a source of important characters in insects, as attested by Friedrich and Beutel (2010) and Randolf; Zimmermann and Aspöck (2013). Recently, studies with internal morphology in several groups of insects have proven to be important sources of data for the knowledge, not only for phylogeny (Wipfler et al., 2012; Kubiak; Beckmann; Friedrich, 2015; Richter et al., 2020) but also of different aspects, such as biology and physiology of insects (Andrade et al., 2019) and taxonomy (Lemos et al., 2005; Pires et al., 2007). Moreover, features of the reproductive system, such as spermatogenesis, sperm morphology and the ultrastructural variations of spermatozoa have commonly been used to help establish phylogenetic relationships among insects (Zwick, 2000; Englund et al., 2024; Giglio et al., 2024; Rezende et al., 2025; Silva et al., 2025).

Integrative taxonomy within stoneflies

In Plecoptera, research has been conducted on the general anatomy (e.g. Zwick, 1973) reproductive system, eggs, and spermatozoa (Fausto et al., 2001; Rościszewska; Rzońca, 2009; Li; Murányi; Yang, 2014; Mtow; Machida, 2018; Mtow; Smith; Machida, 2021), as well as on gill sensilla (Kapoor; Zachariah, 1978), chloride cells (Wichard; Eisenbeis, 1979), and antennae (Rebora; Tierno De Figueroa; Piersanti, 2016). These studies have provided valuable insights into the biology of stoneflies and their evolutionary relationships. However, such characters have never been incorporated into a quantitative cladistic analysis to assess character congruence systematically. Additionally, none of these studies have focused on Neotropical taxa.

The sperm structure of Plecoptera is relatively understudied compared to other insect orders. Only a few studies have investigated these features. Baccetti; Dallai and Rosati (1970) have studied the ultrastructure of the spermatozoon from a nemourid species, providing pioneer data on this subject. Later on, Fausto et al. (2001) compared the ultrastructure of spermatozoa from two infraorders in Arctoperlaria: euholognathan species, and systelognathan species (Fausto et al., 2002) revealing new insights for the systematics and phylogenetic relationships of stoneflies within the order and in comparison with other insect order among Polyneoptera. More recently, another study described the sperm structure, using light, scanning and transmission electron and immunofluorescence microscopy, of six Euholognatha species belonging to genera not analyzed in their previous studies, updating the evolutionary

knowledge for the order Plecoptera (Fausto et al., 2023). Nevertheless, as for the Antarctoperlarian taxa, no studies have yet been conducted.

Regarding the integration of classical morphological knowledge with that obtained from molecular data in Plecoptera, a growing number of studies have already provided the elucidation of species delimitation, the association of life stages, and the proposal of new phylogenetic and biogeographical hypotheses, due to a more complete set of information and with more precision (De Figueroa et al., 2011; Fochetti et al., 2011; Mynott; Webb; Suter, 2011; Boumans; Baumann, 2012; Gill; Sandberg; Kondratieff, 2015; McCulloch; Wallis; Waters, 2016; Chen et al., 2018; Ding et al., 2019; Letsch et al., 2021; South et al., 2021; Chen, 2022; Wang et al., 2025). These studies have helped clarify taxonomic classifications, discover cryptic species, and improve identification methods in Plecoptera. They have also provided new insights into the evolutionary history and biogeography of the order, offering a more robust framework for future research in systematics, ecology, and conservation.

The current taxonomic status of Gripopterygidae and Gripopteryginae reflects significant gaps in our understanding of their evolutionary history and classification. Clarifying phylogenetic relationships, as well as intergeneric and interspecific delimitation, is crucial for establishing a more robust classification and understanding the evolutionary processes shaping these taxa. Identifying new morphological characters that contribute to phylogenetic studies is particularly important, and integrative taxonomy provides a powerful framework for this purpose. A well-resolved taxonomy serves as the foundation for exploring biodiversity, organismal traits, and evolutionary patterns (Fujita et al., 2012). Furthermore, integrative approaches offer valuable insights into the biology, behavior, ecological roles, and evolutionary relationships of gripopterygids, enhancing our broader understanding of Plecoptera evolution. In this context, the present study plays a key role in expanding morphological knowledge and refining phylogenetic perspectives for the group.

2 OBJECTIVES AND THESIS STRUCTURE

Faced with the need for studies that contribute to the phylogenetic knowledge of gripopterygids, this work aims to explore, describe and comparatively analyze potentially important and little explored characters of the external morphology and internal reproductive morphology of Gripopteryginae. More precisely, in the Neotropical genera, such as *Gripopteryx*, *Paragripopteryx*, *Tupiperla* and *Guaranyperla*.

This thesis is structured as follows:

Chapter I:

The purpose of the first chapter is: 1) to provide a taxonomic revision of *Guaranyperla* delving into the morphology and COI (cytochrome c oxidase subunit I) sequence variation of the genus; 2) to present a novel taxonomic perspective on the group, carrying out semaphoront (immature stage, female, male) and gender associations; 3) to redescribe and describe existing and new species, respectively; 4) to provide identification keys for both adults and immatures of the genus.

Here I present a comprehensive taxonomic revision of the genus *Guaranyperla* (Plecoptera: Gripopterygidae), focusing on species identification and classification. By analyzing morphological traits, examining both type specimens and newly collected material, and applying modern taxonomic approaches, this study aims to clarify species diversity, refine species delimitations, and resolve taxonomic ambiguities. Ultimately, it provides a more precise understanding of the genus' role within Gripopterygidae systematics and establishes a robust framework for future research.

Chapter II:

The objective of the second chapter is: 1) to describe and illustrate, for the first time, the anatomy and histology of the male and female general reproductive systems of four genera within the Neotropical Gripopterygidae; 2) to compare the new findings from this study with those in the published literature; 3) to identify a morphological pattern and/or morphological characteristics offering potential characters to resolve the internal relations among Gripopterygidae subfamilies.

In this chapter, I examine the reproductive system of Neotropical Gripopterygidae genera, providing a detailed anatomical and histological analysis of both the female and male

reproductive organs. The study aims to contribute to the understanding of the reproductive biology of this subfamily and its potential implications for phylogenetic and taxonomic studies.

Chapter III:

The goal of the third chapter is: 1) to describe for the first time the sperm morphology of four genera within the Neotropical Gripopterygidae; 2) to compare the spermatozoa of varied species within these four genera; 3) to assess whether new characters of the spermatozoa have potential use for taxonomy and phylogeny of the Gripopteryigidae.

This chapter provides the first description of sperm cells in Antarctoperlaria, with a focus on Neotropical genera of Gripopterygidae. By examining the taxonomic significance of sperm morphology, this study offers a deeper understanding of genera delimitation and reproductive strategies, while also shedding light on broader evolutionary patterns within both the family and Plecoptera as a whole. Furthermore, it lays the groundwork for future investigations into the phylogenetic relationships within Gripopterygidae.

This thesis is formatted in accordance with the guidelines of the journals in which the chapters will be published. Chapter I adheres to the *Insects Systematics and Evolution*'s guidelines, while Chapters II and III align with the ones set by *Arthropod Structure & Development*.

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CHAPTER I - TAXONOMIC REVISION OF *GUARANYPERLA* FROEHLICH (PLECOPTERA: GRIPOPTERYGIDAE): UNVEILING THE DIVERSITY AND COMPLEXITY OF A UNIQUE GENUS

Taxonomic revision of *Guaranyperla* Froehlich (Plecoptera: Gripopterygidae): unveiling the diversity and complexity of a unique genus

Taxonomic revision of *Guaranyperla* Froehlich

Mellis L. S. Rippel[1] [2]*, Lucas H. de Almeida[3], Jádila S. Prando[4], Fernanda Avelino-Capistrano[5], Daniela M. Takiya[4] & Frederico F. Salles[2]

[1]Postgraduate Program in Entomology, Federal University of Viçosa (UFV), Viçosa, MG, Brazil.

[2]Museum of Entomology, Department of Entomology, Federal University of Viçosa (UFV), Viçosa, MG, Brazil.

[3]Laboratory of Aquatic Biology, Department of Biological Sciences, São Paulo State University (UNESP), Assis, SP, Brazil.

[4]Laboratory of Entomology, Department of Zoology, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[5]Laboratory of Zoology, Biological Sciences, Health School in São José University Centre, Rio de Janeiro, RJ, Brazil.

*Corresponding author: mellisrippel@gmail.com

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Abstract

Guaranyperla Froehlich, 2001 (Gripopterygidae: Plecoptera), endemic to southeastern Brazil, includes three species (*G. guapiara*, *G. beckeri* and *G. nitens* Froehlich, 2001), but remains insufficiently studied due to challenges in collecting adults and limited type-material. Our study addresses unresolved taxonomic issues within the genus, led by incomplete semaphoront series and similarities with the related genus *Tupiperla* Froehlich, 1969 in the adult stage. Using morphological and molecular data, along with species delimitation analysis, we provide a new diagnosis for the genus and species of *Guaranyperla*, including the reclassification of

Tupiperla barbosai Avelino-Capistrano & Nessimian, 2013. All species delimitation methods based on COI suggest that nearly every *Guaranyperla* population from streams throughout Southeastern Brazil studied represents a distinct species, thus separating *G. barbosai* **comb. nov.**, *G. nitens*, *G. guapiara*, *G. froehlichi* **sp. nov**., *G. puri* **sp. nov**., and two other species that were not confidently assigned to any valid species. Additionally, it was possible to associate nymphs and adults of most studied species based on COI. We corroborate the morphological species concept of *G. beckeri*, and provide redescriptions for *G. nitens* and *G. guapiara*. Furthermore, we provide the description of two new species, updated geographical records, and images for all species. Results suggest that the diversity of *Guaranyperla* is still underestimated and further species descriptions should not rely solely on single semaphoronts or genders. Our study establishes molecular and morphological foundations for future studies on the genus, contributing to a broader understanding of Neotropical Plecoptera biodiversity.

Keywords: Gripopteryginae; integrative taxonomy; new species; species delimitation analyses; stoneflies.

Introduction

The Atlantic Rainforest was once one of the largest tropical forests in the Americas, characterized by a high heterogeneity of environmental conditions across its more than 150 million hectares (Ribeiro et al. 2009). According to estimates, the flora and fauna of this biome may represent up to 8% of the world's total species diversity (Da Silva & Casteleti 2003), with many species still unknown and awaiting description (Lewinsohn & Prado 2005). Currently, only about 11.7% of the original coverage remains preserved, mostly in small, disconnected fragments (Ribeiro et al. 2009). The largest preserved fragments are located in the Serra do Mar, a coastal region comprised of mountain ranges that are difficult for human occupation (Ribeiro et al. 2009). Despite the extensive deforestation, the Atlantic Rainforest exhibits a high level of species endemism across various groups, making it a significant global hotspot of biodiversity (Mittermeier et al. 2011).

The mountainous regions of the Atlantic Rainforest contain a rich hydrographic network with specific physical and chemical properties and high biodiversity (Padial et al. 2021). In lotic environments of low to medium orders (up to 4th order, *sensu* (Strahler 1957)), the predominant macroscopic fauna consists of aquatic insects, primarily from the orders

Coleoptera, Diptera, Ephemeroptera, Hemiptera, Megaloptera, Odonata, Plecoptera, and Trichoptera. Most of these insects have aquatic immature stages and terrestrial adults. This dual habitat makes them vulnerable to negative impacts from anthropogenic activities affecting both aquatic environments (such as reduced water quality and physical integrity of lotic habitats) and terrestrial environments, including riparian zones and watersheds (such as vegetation removal and erosion) (Merritt & Cummins 1996). Consequently, aquatic insects, particularly those from the order Plecoptera, serve as notable indicators of the conservation status of lotic ecosystems and their associated watersheds (Brasil et al. 2020; Siegloch et al. 2017).

Gripopterygidae Enderlein, 1909 belongs to the suborder Antarctoperlaria (Plecoptera), and is the most diverse group within this clade with 330 species allocated in 57 genera (DeWalt & Ower 2019; DeWalt et al. 2024). According to McLellan (1977), Gripopterygids are distributed in Australasia and South America and encompass five subfamilies: Antarctoperlinae Enderlein, 1909, Dinotoperlinae McLellan, 1977, Gripopteryginae Enderlein, 1909, Leptoperlinae Banks, 1913, and Zelandoperlinae McLellan, 1977. Gripopteryginae is a relatively diverse subfamily endemic to South America, comprising 15 genera and 83 species (Pessacq et al. 2019; DeWalt et al. 2024). In a more recent phylogenetic study using molecular data, the gripopterygid taxa belonging either to Australasia or South America do not form monophyletic lineages i.e. the South American and Australasian genera are mixed with one another in differents clades within Gripopterygidae (McCulloch et al. 2015), leading to the polyphyly of Gripopteryginae.

Within the genera that comprise Gripopteryginae, *Guaranyperla* Froehlich, 2001 (Fig. 1) is endemic to mountainous areas of the Atlantic Rainforest in Southeastern Brazil, comprising three species: *G. guapiara* Froehlich 2001, *G. beckeri* Froehlich 2001, and *G. nitens* Froehlich 2001 (Lecci & Duarte 2024). Locating and collecting individuals of *Guaranyperla*, especially the adults, poses a considerable challenge, as the traps commonly used are not efficient in capturing them, such as Malaise and light traps (Bispo & Lecci, 2011; Avelino-Capistrano & Nessimian 2013). As a consequence, there is a limited number of specimens deposited in biological collections, including type material. Also, the association between nymphs and adults of the same species is uncertain at most. Since its original description, only two studies have delved into the taxonomy of *Guaranyperla* (Froehlich 2001, 2015). The genus was initially proposed by Froehlich (2001) based on putative synapomorphies including broad paranota and vesicular body hairs on the nymph and a relatively broad

pronotum with slightly produced anterior corners in the adult. Furthermore, Froehlich (2001) added other characteristics for the genus, such as the presence of a spine on femora of nymphs and adults, pterostigmatic crossveins in forewing, absence of sclerotized epiproct in male genitalia, and extension of tergum X (TXE) short and ending in two teeth in males. Most of these features, however, are also shared with *Tupiperla* Froehlich, 1969.

Figure 1. Species of *Guaranyperla*: (A) nymph, (B) exuviae and (C) male adult of *G. puri* **sp. nov.** from Ervália, Minas Gerais State (UFVB); (D) female adult of *G. guapiara* from Ribeirão Grande, São Paulo State (UFVB). (E—G) Habitats where *Guaranyperla* specimens can be found. Photographs by ©Frederico F. Salles.

Guaranyperla and *Tupiperla* are the only genera in Gripopteryginae that share the presence of a femoral spine on all legs of nymphs and adults (Bispo & Froehlich 2007; Stark et al. 2009). Moreover, although crossveins in the pterostigmatic cell on forewings are absent in most *Tupiperla* (Froehlich 1998; Duarte et al. 2019), they are present at least in *T. serrulata* Duarte, Novaes & Bispo, 2019 (Duarte et al. 2019, figs. 2C–D) and *T. barbosai* Avelino-Capistrano & Nessimian, 2013 (personal observation), suggesting a possible misclassification of genus or a lack of diagnostic characters to define *Guaranyperla* in the adult stage. Besides that, although the diagnosis of *Tupiperla* mentions the absence of pterostigmatic crossveins as a common character state (Froehlich 1998; Bispo & Froehlich 2007), information on wing venation has been provided for only a few species (Duarte et al. 2019). Whereas nymphs of these two genera are easy to distinguish, adults are morphologically quite alike. Species from both genera are similar in size and color (varying from brown to black), and share the TXE ending in two separate teeth (Bispo & Froehlich 2007). Also, both genera lack a sclerotized epiproct, as well as in some species of *Gripopteryx* Pictet, 1841 and a few *Paragripopteryx* Enderlein, 1909 (Brazilian gripopteryginae genera) (Froehlich 1998). The absence of clear diagnostic characteristics between adults of the two genera, together with the lack of knowledge of the nymphal stage of practically all *Tupiperla* species (Froehlich 1998; table 1 in Duarte et al. 2019), could lead to erroneous allocations of species in both genera. Both *T. barbosai* and *T. serrulata*, known only from the imaginal stage, have crossveins in the pterostigmatic cell of the forewing, as commonly found in species of *Guaranyperla*, but were not placed in the latter.

Besides the above-mentioned problems, Froehlich (2001) described most of the species based on an incomplete series of semaphoronts. *Guaranyperla beckeri* was described based only on a male specimen, *G. nitens* on a female, while *G. guapiara* was the only species described based on reared material, but no males were known at that time. Furthermore, Froehlich (2001) studied, but did not associate some immatures to any of the aforementioned species, those were nymphs from Campos do Jordão State Park (São Paulo State), Serra do Japi

(Jundiaí, São Paulo State), Ouro Fino (Minas Gerais State), and Santa Teresa (Espírito Santo State).

On the second and most recent contribution to the taxonomy of *Guaranyperla*, Froehlich (2015) added the following: description of the male of *G. guapiara* based on specimens collected in areas outside the type-locality; description of the male of *G. nitens* reared from nymphs from Campos do Jordão State Park, type locality of this species; and the previously unassociated nymphs from Serra do Japi (Froehlich 2001) were identified as *G. guapiara*. However, this association was tenuous, as the nymphs from Serra do Japi were neither reared nor associated using DNA. Furthermore, the nymph was only illustrated and not formally described. Froehlich (2015) also mentioned the possibility of *G. beckeri* being a synonym of *G. guapiara* due to the similarity of males from these two species. However, no decision was made in this regard due to the necessity of more collections in type localities (Froehlich 2015).

In summary, several issues are still to be solved regarding *Guaranyperla*. Therefore, a more extensive examination of specimens is imperative to establish a reliable and thorough diagnosis for both the genus and its species. This study delves into the morphology and COI sequence variation of *Guaranyperla* to provide a taxonomic revision of the genus. As a result, we present a novel taxonomic perspective on the group, carrying out semaphoront and gender associations; species redescriptions; description of two new species; new records, illustrations, images of the species, and identification keys for both adults and immatures of the genus.

Material and methods

Taxonomic sampling

We studied material housed in the following scientific collections: Museum of Entomology of the Universidade Federal de Viçosa (UFVB), Viçosa, Brazil; Coleção Entomológica Prof. José Alfredo Pinheiro Dutra, Departamento de Zoologia of the Universidade Federal do Rio de Janeiro (DZRJ), Rio de Janeiro, Brazil; Museum of Zoology, Universidade de São Paulo (MZSP), São Paulo, Brazil; and the Aquatic Insect Collection Prof. Dr. Cláudio Gilberto Froehlich (CIACGF) at the Laboratory of Aquatic Biology, São Paulo State University – UNESP, Assism SP, Brazil. All specimens were preserved in alcohol and

are listed in the 'Examined Material' section for each species, accompanied by their respective label information.

Specimens were also collected throughout Southeastern Brazil from 2023 – 2024, with focus on type locations, in the states of Minas Gerais, Rio de Janeiro, and São Paulo (Fig. 2). Our sampling followed Brazilian laws and were authorized by the SISBIO-ICMBio (Biodiversity Authorization and Information System, Chico Mendes Institute for Biodiversity Conservation, numbers 79695-1, 55428-16, and 65213-11), also by IEF (Minas Gerais State Institute of Forests, number 058/2021) and IPA/SIMA (São Paulo State Institute of Environmental Research, number 15054/2022 and 5579/2023). Nymphs were collected with D-net and hand picking of substrate in riffles with leaf litter, and adults were obtained with malaise trap, pennsylvania (Frost 1957), and through rearing. Last instar collected nymphs were transferred to the laboratory in order to be reared until reaching the adult phase. Nymphs were kept in plastic cups in a plastic drawer (40 x 20 x 30 cm) full of water. A polystyrene plate floating in the center was placed to hold the cups, and two air compressors (model NS-F160 with a capacity of 200L/H) on opposite sides of the box provided water turbulence. Emerging adults were kept inside the cups for a few days to complete maturation, and then were fixed in either alcohol 80% or 99%, as well as all specimens collected.

For the molecular analysis, we included both newly collected specimens and museum-deposited representatives – nymphs and adults – of *Guaranyperla* from various localities in the Atlantic Rainforest across the states of Minas Gerais, São Paulo, and Rio de Janeiro. The type material of *T. barbosai*, along with other females and nymphs from this species' type locality, were also included. Taxon sampling for the molecular analysis is provided in **Table 1**.

Table 1. Species included in the *Guaranyperla* species delimitation analyses, with respective voucher specimen code, stage and gender (N= nymph, F= adult female, M=adult male), collecting locality, and GenBank accession code.

Species	**Voucher**	**Stage**	**Locality**	**GenBank**
Outgroups				
Kempnyia petersorum	ENT183	M	RJ: Macaé	KT184665

Tupiperla gracilis (Burmeister, 1839)	ENT603	M	SP: São José do Barreiro	PP947869
Gripopteryx pilosa	ENT563	M	RJ: Nova Friburgo	KT184663
Guaranyperla				
G. barbosai **comb. nov.**	ENT292	F	RJ: Nova Friburgo	PP947846
	ENT1059	F	RJ: Teresópolis	PP947847
	ENT6506	N	RJ: Teresópolis	PP947848
	ENT6513	F	RJ: Teresópolis	PP947849
	ENT6515	F	RJ: Teresópolis	PP947850
Holotype	ENT6907	M	RJ: Teresópolis	PP947851
Paratype	ENT6908	M	RJ: Teresópolis	PP947852
G. guapiara	ENT6681	F	SP: Ribeirão Grande	PP947853
	ENT6682	M	SP: Ribeirão Grande	PP947854
	ENT6683	N	SP: Ribeirão Grande	PP947856
	ENT6684	N	SP: Ribeirão Grande	PP947855
G. nitens	ENT6685	N	SP: Campos do Jordão	PP947857
	S263	N	SP: Campos do Jordão	PP947858
G. puri **sp. nov.**	ENT6507	F	MG: Araponga	PP947859
	ENT6510	M	MG: Ervália	PP947860
	ENT6511	M	MG: Ervália	PP947861
G. froehlichi **sp. nov.**	ENT2170	F	RJ: Teresópolis	PP947862
	ENT6505	N	RJ: Teresópolis	PP947863
	ENT6514	F	RJ: Teresópolis	PP947864
	ENT6953	M	RJ: Teresópolis	PV020428
Guaranyperla sp. 2	TD113	N	SP: Campos do Jordão	PP947865

	TD116	F	SP: Campos do Jordão	PP947866
Guaranyperla sp. 4	MNF110	F	SP: Jundiaí	PP947867
	MNF195	N	SP: Jundiaí	PP947868

Morphological analysis

We initially identified adults and immatures with the aid of Froehlich's articles (2001, 2015), as well as by comparison with the type material of all three species. We observed specimens using a LEICA M20 stereomicroscope. Fore- and hind wings were dry mounted on slides. We measured the head and pronotum of adults and mature nymphs; and paraprocts, fore- and hind wings were measured from available male and female adults. We also measured the antenna and cercus of mature nymphs. Moreover, we calculated proportions of pronotum and paraprocts in order to look for intraspecific variation. We obtained measurements with a Motic 5.0 camera coupled to an Olympus SZ61 stereomicroscope, using the software Motic Live Imaging Module 1.2. The method for each measurement is described in Supplementary Figure S1.

DNA sequencing and alignment

Genomic DNA from legs and abdomen of each specimen was extracted with DNeasy Blood & Tissue kit (Qiagen) with modifications of the manufacturer's protocol, where tissue was not macerated and the proteinase K digestion time was increased to 72h. The barcode region of the cytochrome c oxidase subunit I (COI) was amplified by PCR using three different primer combinations to amplify fragments of LCO-1490/HCO-2198, HCO-2198/C1-J-1718, and LCO-1490/Nancy (**Table 2**). PCR reactions had a total reaction volume of 25 µl, with 12.9 µl of DEPC H2O (Ambion), 5 µl of 5X GoTaq2 green buffer (Promega), 3.5 µl of $MgCl_2$ (25mM, Promega), 0.5 µl of dNTP solution (20mM, Promega), 0.1 µl of Go Taq2 polymerase to 5U/µl (Promega), 1.0 µl of each primer (10pmol/l, Invitrogen), and 1 µl of extracted DNA. Thermocycling protocol followed one cycle of 94 ºC for 3 min; 35 cycles of 94 ºC for 1 min, 50 ºC for 1 min, and 72 ºC for 2 min; and one final extension cycle of 72 ºC for 7 min. Successful amplification products were purified with ExoSAP-IT® (Affymetrix) and sent for capillary sequencing at Macrogen Inc. (South Korea). Consensus sequences were generated by

assembling complementary electropherograms using Geneious Prime 9.1.8 and aligned using the Geneious Aligner algorithm, in the same software.

Table 2. Primers used for amplification and sequencing of COI of *Guaranyperla*.

Primer	Direction	Sequence (5' – 3')	Reference
LCO-1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
HCO-2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
C1-J-1718	Forward	GGAGGATTTGGAAATTGATTAGTTCC	Simon et al. 1994
Nancy	Reverse	CCCGGTAAAATTAAAATATAAACTTC	Monteiro & Pierce, 2001

Phylogenetic and species delimitation analyses

Pairwise divergences were modeled with Kimura 2-Parameter (K2P) and calculated in Mega X (Kumar et al. 2018). A maximum likelihood (ML) analysis with 1000 runs was executed in IQ-TREE 2.3.5 (Nguyen et al. 2015), together with 1,000 ultrafast bootstrap replicates (UFBoot; Hoang et al. 2018) and 1,000 replicates of Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010). A Bayesian inference (BI) analysis was run in MrBayes 3.2 (Ronquist et al. 2012) sampling every 1,000 of 10,000,000 generations, in two simultaneous independent runs with four MCMC chains. Prior to these analyses, COI was partitioned by codon position and ModelFinder (Kalyaanamoorthy et al. 2017) in IQ-TREE was used twice to select with BIC the most appropriate partition scheme and substitution model for the ML (TNe+G4: COI_pos1, F81+F: COI_pos2, and TN+F+G4: COI_pos3) and for the BI analysis (SYM+G4: COI_pos1, F81+F: COI_pos2, and GTR+F+G4: COI_pos3). An ultrametric uncorrelated relaxed clock tree (UB, Drummond et al. 2006) under a birth death tree model (Gernhard 2008) was also calculated in BEAST 2.7.7 (Suchard et al. 2018). To avoid overparameterization, COI was treated as a single partition modeled by GTR+I+G and the rate prior was set fixed using the 3.54% insect COI rate estimated by

Papadopoulou et al. (2010). BEAST was run with two independent runs for 100,000,000 generations sampled every 10,000. Both Bayesian analyses were checked for convergence and appropriate parameter mixing in Tracer 1.7.1 (Rambaut et al. 2018) and 25% and 10% burnin were set at MrBayes and BEAST analyses, respectively.

Species delimitation analyses were conducted using SPdel pipeline (Ramirez et al. 2023), which performs various species delimitation methods efficiently on Python 3, based on the FASTA alignments and UB Newick tree. Methods were compared using the Poisson Tree Processes model (Zhang et al. 2013), the Generalized Mixed Yule Coalescent (GMYC) (Pons et al. 2006), the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), the Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021), the Poisson tree processes (PTP) in the original likelihood implementation, the Bayesian Poisson tree processes (bPTP), and the multi-rate Poisson tree processes (mPTP) (Kapli et al. 2017).

Geographic distribution maps and illustrations

The geographic coordinates of species occurrence were compiled from our examined material and from records of specimens studied by other authors, mainly Froehlich (2001). Points of occurrence lacking coordinates provided by the collectors were inferred using Google Maps, with the highest possible level of precision. The species distribution map was created using the QGIS Bucur 3.14.15 software and finalized using Adobe Photoshop CS3® editor.

Furthermore, we treated photographs from the live specimens with Adobe Lightroom CC® 2020. We obtained images of morphotypes and morphological characters with the aid of a Leica MC170 HD camera and edited them using the software Adobe Photoshop CC® 2020. Plates were assembled in Adobe Illustrator CC® 2020. Phylogenetic tree figure was edited in Inkscape® 1.0.2-2.

Abbreviations

Morphology. CuA–anterior cubitus vein; CuP–posterior cubitus vein; FL–forewing length; HW–head width; M–medial vein; PaL–paraproct length; PaW–paraproct width; PL–pronotum length; PmP–posteromedial projection; PP–pronotum projection; PW–pronotum width; RA–anterior radius vein; RP–posterior radius vein; SpPE–subgenital plate posterior expansion; TX–tergum X; TXE–extension of tergum X; TXEAntP–anterior portion of TXE.

For Brazilian state codes, refer to ISO 3166-2:BR.

Results

Phylogeny and molecular species delimitation

Maximum likelihood and Bayesian inference trees (Fig. 2) were congruent in recovering *Guaranyperla* as a monophyletic group with respect with the gripopteryd outgroup taxa included (*Tupiperla gracilis* and *Gripopteryx pilosa*). Trees were also congruent in the division of the genus into seven clades, each with high clade support, except for *Guaranyperla froehlichi* **sp. nov.** and *G. nitens* which were recovered with low clade support. Within *Guaranyperla*, *G. nitens* was recovered as sister to all other species in the genus. The remaining species were divided into two main clades: one comprising *G. guapiara* as sister to a group formed by *Guaranyperla* sp. 2, *Guaranyperla* sp. 4, and *G. puri* **sp. nov**.; and the other composed of *G. froehlichi* ***sp. nov.*** and *G. barbosai* as sister taxa. The phylogenetic analyses also supported the placement of *Tupiperla barbosai* within *Guaranyperla*. Thus, this species is formally transferred to *Guaranyperla* as detailed below.

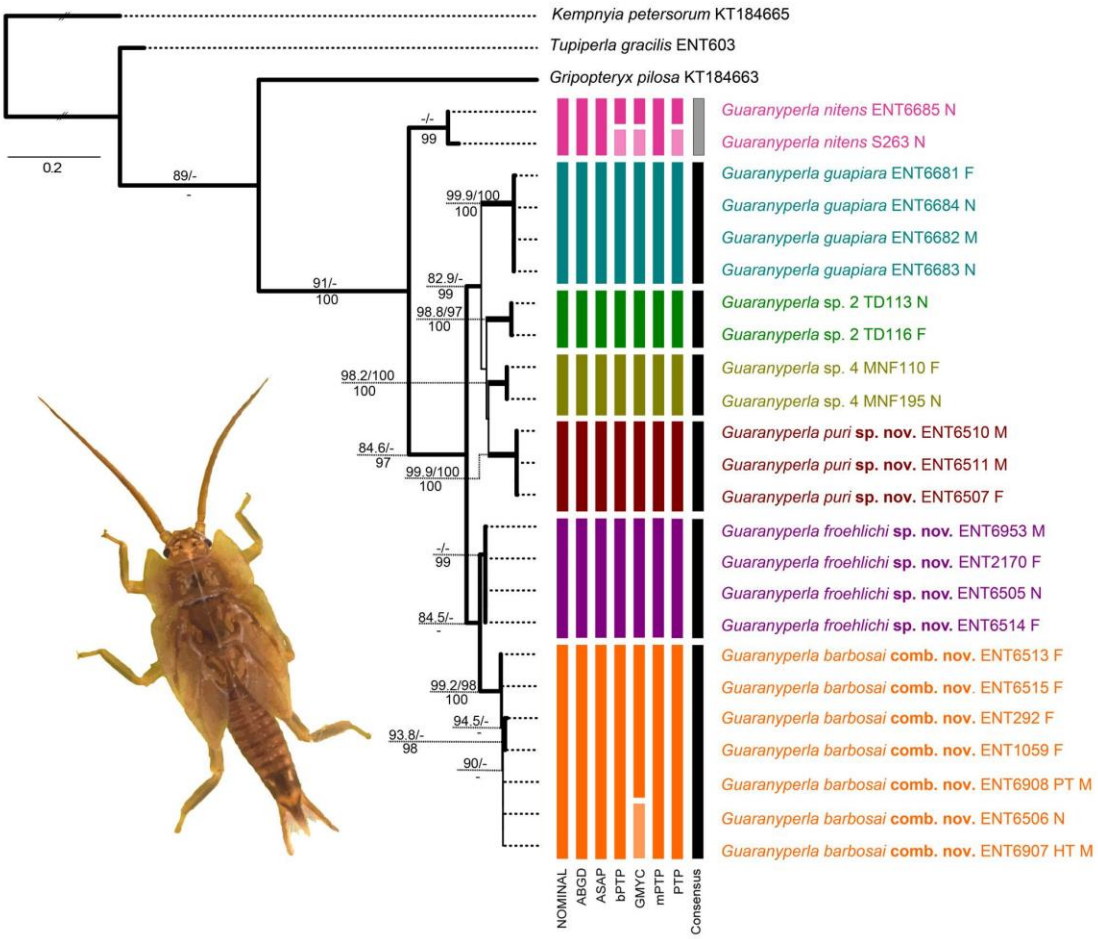

Figure 2. Maximum likelihood tree of *Guaranyperla*. Thickened branches were those also recovered by at least one of the BI analyses. Values above branches are >80 SH-aLRT / >95 UFBoot (except at nodes of two of the delimited species), and values below are Bayesian posterior probabilities >95. Vertical bars refer to results of different species delimitation analysis, and each color represents a different species.

Results of the majority of species delimitation methods (ABGD, ASAP, bPTP, GMYC, mPTP, PTP) were mostly consistent with morphology, suggesting the division of *Guaranyperla* specimens analyzed into seven or eight species (Fig. 2). The two nymphs of *G. nitens* from the type-locality were considered conspecific by half of the methods (NOMINAL, ABGD, ASAP, mPTP), while bPTP, GMYC and PTP suggested division into two species. Given this uncertainty, we are treating *G. nitens* herein as a single species. Additionally, our analyses allowed the description of two new species based on males, females, and nymphs, *G. puri* **sp. nov.** and *G. froehlichi* **sp. nov.** Results also showed that *Guaranyperla nitens* had pairwise K2P divergences of 2.3%, a high intraspecific divergence, considering that these specimens were collected in the same time and location. While for the other species maximum divergences were all lower than 0.9% even in species sampled from different municipalities (Supplementary Material S1). Interspecific K2P divergences among *Guaranyperla* species ranged from 4.3% to 16.1%. Practically every *Guaranyperla* population from streams throughout Southeastern Brazil studied belongs to a distinct species (Fig. 3). Furthermore, in a particular stream one can find at the same time at least two distinct *Guaranyperla* species, e.g., *G. froehlichi* **sp. nov.** and *G. barbosai* **comb. nov.** in Rio Paquequer, Teresópolis; and *G. nitens* complex in Rio Galharada, Campos do Jordão.

Furthermore, based on COI sequences, we were able to associate male specimens to females and/or nymphs of *G. froehlichi* **sp. nov.** and *G. puri* **sp. nov.**, *G. barbosai* **comb. nov.**, and *G. guapiara*. This allowed descriptions below of previously unknown female and nymph of *G. barbosai* **comb. nov.** and, for the first time, of a male of *G. guapiara* from the type-locality [see comments below under *G. guapiara* concerning the description of the male by Froehlich (2015)].

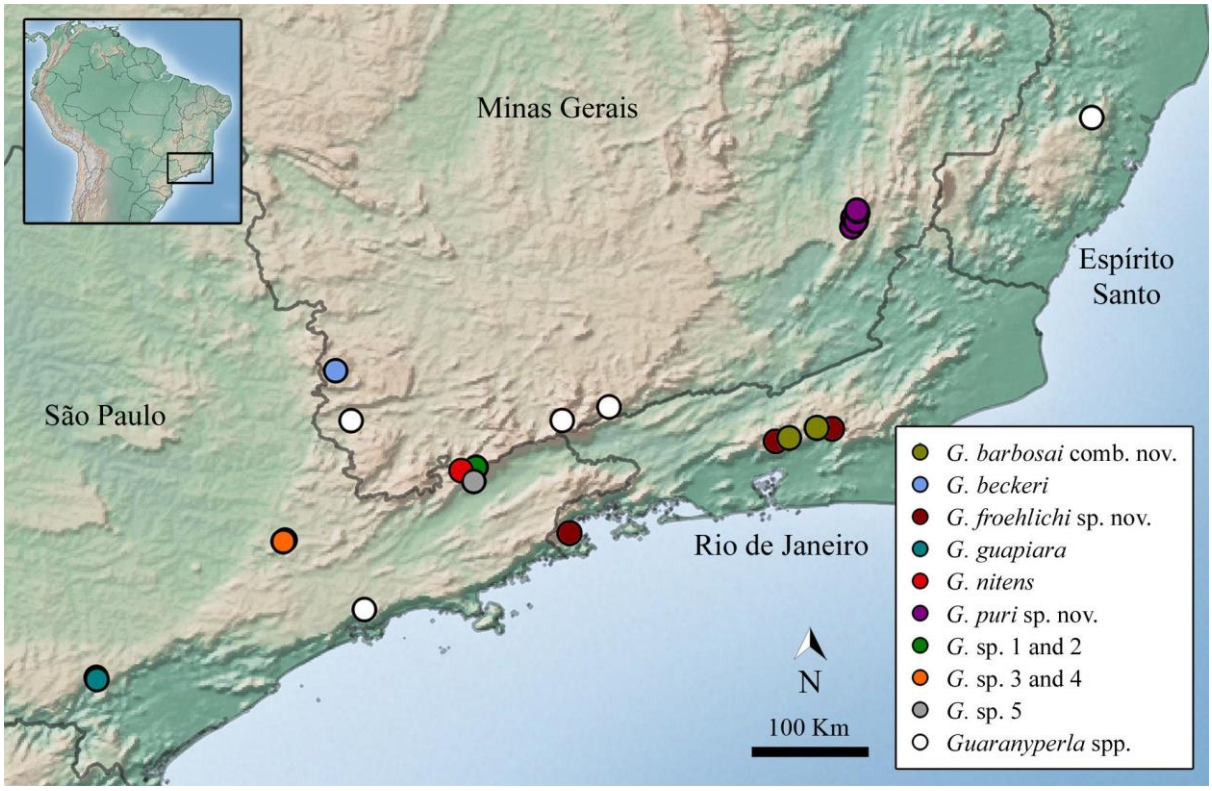

Figure 3. Map indicating sampled points in Southeastern Brazil, where *Guaranyperla* specimens were collected. Colored circles indicate localities of species and morphospecies studied herein, while white circles are records from Froehlich (2001) not accessed in the present study.

Taxonomy

***Guaranyperla* Froehlich, 2001**

Froehlich, 2001: 377 (description of genus and species); Olifiers et al., 2004 (identification key for immatures of Gripopterygidae); Stark, Froehlich & Zúñiga, 2009: 87 (comments and taxonomic notes); Froehlich 2010: 134 (catalog); Bispo & Lecci, 2011: 383 (comments); Froehlich 2011: 602 (checklist); Avelino-Capistrano & Nessiman, 2013: 186 (new record); Novaes & Bispo 2014: 440 (checklist); Froehlich, 2015: 175 (association of semaphoronts and taxonomic notes); Pessacq, Zúñiga & Duarte, 2019: 192 (catalog).

Type species. *Guaranyperla guapiara* Froehlich, 2001

Composition:

Guaranyperla guapiara Froehlich, 2001

Guaranyperla beckeri Froehlich, 2001

Guaranyperla nitens Froehlich, 2001

Guaranyperla barbosai (Avelino-Capistrano & Nessimian, 2013) **comb. nov.**

Guaranyperla froehlichi **sp. nov.** Rippel & Salles

Guaranyperla puri **sp. nov.** Rippel & Salles

Emended diagnosis

Although we did not identify unique diagnostic characters for the adults of *Guaranyperla*, only for the nymphs, its members can nevertheless be distinguished from other Atlantic Rainforest Gripopterygidae (*Paragripopteryx* Enderlein, 1909; *Gripopteryx* Pictet, 1841 and *Tupiperla* Froehlich, 1969) by the following combination of characteristics:

Adult. Head width less than 1.1× pronotum width, subquadrate (Figs. 4A–B). Femora with spine located on apical ⅔ of inner margin (Fig. 4E); forewing with pterostigmatic crossveins absent or present (Figs. 4C and 13C); RP and CuA forked; hind wing with RA unforked; CuA forked; 6th anal vein fused to the wing margin (Fig. 4D). In males, an unsclerotized cleft on lateral surface of sternite VIII (Fig. 4H); longitudinal sclerotized bar along inner dorsal margin of paraproct (Fig. 4G); sclerotized epiproct absent (Fig. 4I). In females, subgenital plate either unpigmented, or with unpigmented area centrally, never completely black (Fig. 4F).

Nymph. Body covered with short and elongate vesicular setae (Figs. 6 and 14A). Thoracic segments with paranotal expansion; pronotum with antero-lateral projection and distinctly wider than head (Fig. 5B). Spine located on the inner margin of femora. Abdomen with TX apically projected (Fig. 5D). (*As most Neotropical Gripopterygidae nymphs are still not known, any current diagnosis or characterization is not definitive).

Redescription

Adult. Anterior border of frons emarginated medially (Figs. 4A–B); medial branch of frontal suture at anterior region of vertex, lateral branch ending posteriorly to lateral ocellus (Fig. 4A). Antenna with scape larger than pedicel (Figs. 4A–B). Labrum subquadrate. Maxillary palp with first and fourth segments short, remainder longer. Head width less than 1.1× pronotum width, subquadrate (Figs. 4A–B). Femora with spine located on apical ⅔ of inner margin (Fig. 4E). Tibiae with two spurs distally. Tarsus with tarsomere I medium, tarsomere II short, and tarsomere III long; longer bristles at distal region of tarsomere III. Wings with a 1–2 costal crossveins present or absent between the humeral crossvein and the apex of the costa; forewing with pterostigmatic crossveins absent or present; RA forked or unforked; RP and CuA forked; hind wing with RA unforked; RP forked or unforked; M3+4 fused with CuA; CuA forked; 6th anal vein fused to the wing margin (Figs. 4C–D). In males, unsclerotized cleft on lateral surface of sternite VIII (Fig. 4H); elongated paraproct (Fig. 4I), sclerotized bar longitudinally along inner dorsal margin (Fig. 4H); TXE short (except in *G. nitens*) (Figs. 4G, 4I); subgenital plate on sternite IX with rounded base (Fig. 4H); sclerotized epiproct absent (Fig. 4I). In females, subgenital plate either unpigmented, or with unpigmented area centrally, never completely black; posterior margin either subquadrate or rounded; paraproct triangular, slightly elongated (Fig. 4F).

Nymph. Body covered with short and long vesicular setae (Figs. 6A–F), more numerous dorsally, present on appendages except their extremities. Anterior border of frons emarginated medially; medial branch of frontal suture at anterior region of vertex, lateral branch ending posterior to lateral ocellus (Fig. 5B). Antenna with scape larger than pedicel (Fig. 5B). Labrum subquadrate. Maxilla with palp 3-segmented; third longer. Labial palp 3-segmented. Thoracic segments with paranota (Fig. 5A); thoracic sterna with long setae present (Fig. 16C); prothoracic paranota anteriorly projected; pronotum distinctly wider than head (Fig. 5A). Femora with spine located on inner margin. Tibiae with two spurs distally. Posterior margin of sternite VIII with shallow medial notch in mature female nymphs (Fig. 14C) and regular in mature male nymphs ; TX apically projected, with medial stripe (Fig. 5C). Paraproct wide at base, tapering towards apex (Fig. 5D). Gill coloration either white or purple (Figs. 16E, 9D).

Remarks. Based on the examination of the largest series of *Guaranyperla* specimens (around 30 adults and 90 nymphs), we observed the following problems concerning the characters proposed by Froehlich (2001): the pronotal anterior corner of adults shows great intraspecific

variation, with some specimens possessing anterior corners projected, while others from the same species lack projections. In addition, the pterostigmatic crossvein of the forewing varies intraspecifically, as for a single individual can either have crossveins in only one of its forewings, being completely absent in the other, or lack it in both wings. In males, the TXE can also vary among specimens (e.g. *G. nitens*), indicating that this character should be approached with caution.

Furthermore, some species described in *Tupiperla* have characteristics that were assigned only to *Guaranyperla*. Pterostigmatic crossveins of the forewing can also be found in *T. serrulata* and the RP bifurcation is also shorter in *T. pinhoi* Duarte, Novaes & Bispo, 2019 (fig. 2B in (Duarte *et al.* 2019) and Fig. 10C, present study). Considering the lack of exclusive characters to differentiate between adults of *Guaranyperla* and *Tupiperla*, coupled with the fact that most nymphs of *Tupiperla* remain undescribed, we do not discount the possibility that, upon associating the nymphs, some species of *Tupiperla* may be transferred to *Guaranyperla*.

Nevertheless, adults of *Guaranyperla* can be distinguished from those of *Tupiperla* by the combination of the following characteristics: a subquadrate pronotum, which is either equal to the width of the head or slightly wider. In *Tupiperla*, the usual rectangular pronotum might be subquadrate in some species, but its ratio is always greater than 1.25×. The sole exception is *T. robusta*, which may overlap the narrowest width ratio in *Guaranyperla*: 1.1×. Moreover, most males of *Guaranyperla* have a short TXE and the subgenital plate of females are never completely black. Regarding the nymphs, all species of *Guaranyperla* have vesicular setae covering the whole body and paranota (Fig. 6 and Figs. 16A–E).

Based on our collection data, nymphs of *Guaranyperla* can be found in low order streams in riffles with leaves and rocks, attached to the leaves and buried in the sand, either on the surface or a few centimeters deeper in the leaf pack (Fig. 1E–G). The associated fauna can include riffle beetles (Elmidae Curtis, 1830), planarian, Perlidae Latreille, 1802 and black flies (Simuliidae Newman, 1834). Adults are active mostly in the months of July to November. The altitudinal range of localities varies from 570 to 1580 m. Collection sites for most species are in well preserved areas, mainly in National or State parks, but some species can also be found in areas surrounded with coffee plantations.

As reported by Froehlich (2001), we also found filiform setae interspersed with vesicular setae on the lateral regions of thoracic segments. In certain species, such as

Guaranyperla puri **sp. nov.**, these setae are numerous, and are often covered with sediment (Figs. 6C–D and 16C).

Geographic distribution. Atlantic Rainforest remnants in Southeastern Brazil. States of Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo.

Guaranyperla guapiara Froehlich, 2001

Figs. 4 A–I, 5A–D

Froehlich 2001:378 (original description of female and nymph); Froehlich 2010: 134 (catalog); Bispo & Lecci, 2011: 383 (identification key); Froehlich, 2011: 602 (checklist); Avelino-Capistrano & Nessiman, 2013: 186 (new record), Froehlich, 2015: 175 (description of male, new records and taxonomic notes); Pessacq, Zúñiga & Duarte, 2019: 192 (catalog).

Examined material. Holotype female, **Brazil. São Paulo. Iporanga**. Parque Estadual de Intervales, Rio das Mortes, 540m, nymph collected 06.viii.1997, adult emerged 12.viii, CG Froehlich and V Ribeiro col (MZUSP000356). Paratypes. Same data as holotype except CG Froehlich, AS Melo, V Ribeiro col, light trap, 1 female; same data except 2 females reared and emerged in cages (8.viii and 15.viii). ***Other material.*** **Brazil. São Paulo. Ribeirão Grande.** Parque Estadual de Intervales. Córrego do Carmo, 24º18'24"S 48º24'52"W, 570m, 15.viii.2023, P Taniguti, LH Almeida, ICH Cortes, MLS Rippel, FF Salles col, D-net, 2 nymphs; 1 nymph (DNA voucher ENT6683) (UFVB); 1 nymph (DNA voucher ENT6684) (UFVB); same data except FF Salles col, manual, 1 female (DNA voucher ENT6681) (UFVB); same data except 15-16.viii.2023, pennsylvania trap, 1 male (DNA voucher ENT6682) (UFVB).

Type locality. Brazil. São Paulo. Ribeirão Grande. Parque Estadual de Intervales.

Diagnosis. *Adult*: Pronotum with irregular margin (Figs. 4A–B). Male TXE wide, lateral margin broadened at its anterior portion, posterior margin almost as wide as base (Fig. 4G). Female with subgenital plate dark brown, white pentagonal unpigmented area on its center, lateral margin slightly concave, posterolateral margin convex, apex with a shallow emargination (Fig. 4F). *Nymph*: Width of paranota ⅓ the width of the pronotum, antero-lateral projection ⅓ of pronotum length. Mesothoracic paranota with anterior corner rounded and

slightly projected; apex of fore and hind wing pads rounded; outer margin of hind wing pad slightly projected laterally, apex rounded (Fig. 5B). TX with PmP with smooth lateral margin, apex acute (Fig. 5C).

Redescription.

Male. General color dark brown, almost black (Fig. 4A). ***Head***: Dark brown with a lighter spot on the center of frons (Fig. 4A). Lateral branch of frontal suture straight (Fig. 4A). Lateral ocellus and eye black; median ocellus smaller (Fig. 4A). Labrum dark brown at base and lateral margin, lighter distally. Maxilla with palp dark brown. Labium brown; labial palp dark brown; postmentum dark brown. Antenna dark brown (Fig. 4A). ***Thorax***: Pronotum dark brown, rectangular, almost as wide as head width (Fig. 4A); anterior corner projected, lateral margin irregular at mid length (Fig. 4A). ***Legs***: Dark brown (Fig. 4E). Sparse thicker bristles present, more numerous and longer on tarsus. Femora brown, darker towards apex (Fig. 4E). Tibia III with lighter region in the middle (Fig. 4E). ***Wings***: Forewing dark brown with lighter spots in the cells between RP and anal veins; unpigmented line between RP and M; crossveins of mid distal region, between RP and CuA, light; 0-1 pterostigmatic crossvein; RA distally forked; RP forked at distal ⅕ of its length; CuA forked at distal ⅔ of its length (Fig. 4C). Hind wing dark brown with unpigmented line between RP and M, lighter area between 4th and 6th anal veins; RA unforked; RP forked at distal ⅙ of its length; CuA forked at distal ⅕ of its length from fusing of M3+4 (Fig. 4D). ***Abdomen***: Brown, darker towards anterior margin of abdominal segments I–IX. Terga, sterna and paraproct densely covered with short bristles; cercus with long and sparse bristles (Figs. 4G–I). ***Terminalia***: TX sclerotized and black, anterior margin concave (Fig. 4G); TXE short and wide, lateral margin broadened at its anterior portion, posterior margin almost as wide as anterior portion and with posterolateral tooth curved ventrally (Fig. 4G). Subgenital plate black to dark brown, rounded at base, posterior margin almost triangular with tip slightly rounded (Fig. 4H). Paraproct black, apex dark brown (Fig. 4I); thin and elongated, outer surface concave along its length, constant width over most of its length, apex curved ventrally, exceeding TXE (Figs. 4G–I).

Female. Similar to male, except for: ***Wings***: RA unforked, RP forked either at distal ⅕ or ⅙ of its length (N=2), CuA forked at distal ½ of its length. Hind wing with RA unforked, RP either forked at distal ⅙ of its length or not forked (N=2), CuA forked at distal ¼ of its length from fusing of M3+4. ***Abdomen***: Dark brown, sterna almost black, sclerotized area on center of sternite VII, reaching its anterior margin; anterior and posterior margin of terga I–VII with two

lighter spots. ***Terminalia***: TX dark brown. Sternite VII with sclerotized bar on its center, along anterior margin (Fig. 4F). Subgenital plate dark brown, white pentagonal unpigmented area on its center, Sp PE 4× longer than base length, square at base, lateral margin slightly concave, latero-distal margin convex, apex with a shallow emargination, completely covering sternite IX (Fig. 4F). Paraproct triangular, slightly elongated, base 3× the size of apical region, apex slightly rounded (Fig. 4F).

Nymph. General color ochraceous to brown (Fig. 5A). ***Head***: Brown to dark brown (Fig. 5B); vertex and frons with light spots scattered over surfaces, not covered with vesicular hairs (Fig. 5B). Lateral and median ocelli brown, eye black (Fig. 5B); median ocellus smaller. Antenna brown, pedicel as wide as first flagellomeres (Fig. 5A). Labrum dark brown at base and lateral margin, lighter distally. Maxilla and labium with palpi ochraceous. Postmentum dark brown. ***Thorax***: Pronotum dark brown, paranota ochraceous, ⅓ the width of the pronotum, anterior margin straight, prothoracic paranota anteriorly projected ⅓ of pronotum length, postero-lateral margin projected (Figs. 5A–B). ***Legs***: Brown. Femora dark brown, lighter towards apex; row of sparse bristles on outer margin present; spine located on ½ inner margin of femora I, and on ⅔ of inner margin of femora II and III. ***Wing pads***: Mesothoracic paranota with anterior margin straight, corner rounded and slightly projected; apex of fore and hind wing pads rounded; metanotum with posterior margin rounded, outer margin of hind wing pad slightly projected laterally, apex rounded (Fig. 5A). ***Abdomen***: Brown; sterna I–X with central area lighter, more conspicuous on sternite IX, sternite X lighter; row of short setae along posterior margin of segments I–X (Fig. 5C–D); TX with lateral margin slightly convex (Fig. 5C); PmP subequal to TX, with smooth lateral margin, apex acute (Fig. 5C). Paraproct brown (Fig. 5A). Cerci brown, covered with short bristles (Fig. 5C–D).

Measurements. Female holotype: head width, 1.4 mm; pronotum width, 1.2 mm; pronotum length, 0.8 mm; PW/PL=1.5; forewing length, 10.6 mm; FL/PW=8.8; hind wing length, 9.6 mm; paraproct length, 0.5 mm. Male: head width, 1.2 mm; pronotum width, 1.2 mm; pronotum length, 0.7 mm; PW/PL=1.6; forewing length, 9.1 mm; FL/PW=7.8; hind wing length, 7.8 mm; paraproct width, 0.2 mm; paraproct length, 0.9 mm; PaW/PaL=0.2. Female: head width, 1.5 mm; pronotum width, 1.3 mm; pronotum length, 0.8 mm; PW/PL=1.6; forewing length, 10.8 mm; FL/PW=8.3; hind wing length, 9.8 mm; paraproct length, 0.6 mm. Nymph (N=4): body length (N=2), 7.2–8.5 mm; head width, 1.1–1.3 mm; pronotum width, 1.7–2 mm; pronotum

length, 0.8–0.9 mm; pronotum projection, 1.3–1.4 mm; PW/PL=2–2.3; PP-PL=0.5; antenna length, 6–6.7 mm; cercus length, 2.7–3.5 mm.

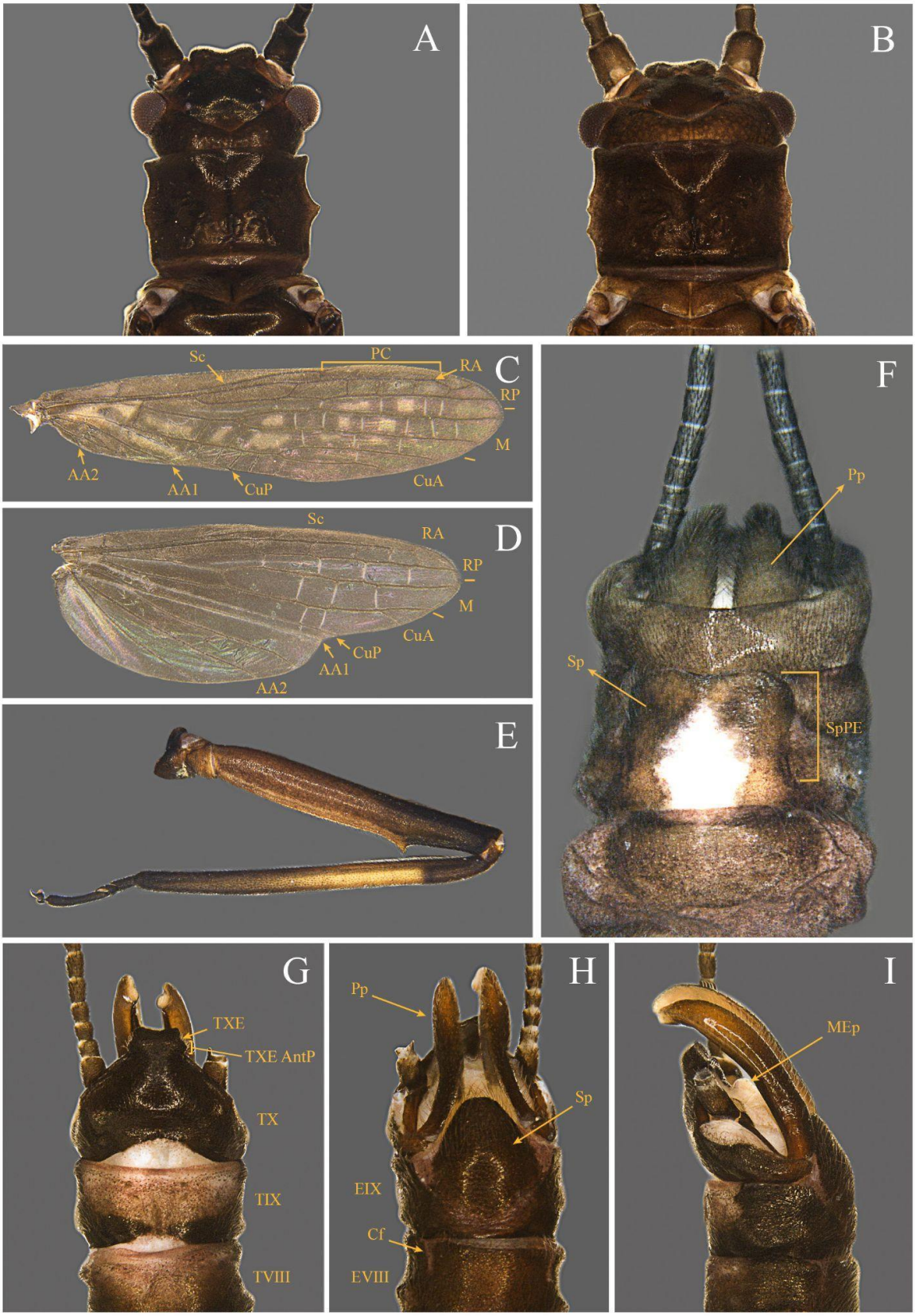

Figure 4. Adults of *Guaranyperla guapiara* from Ribeirão Grande, São Paulo State (UFVB). (A) Male, head, and pronotum, dorsal view. (B) Female, head, and pronotum, dorsal view. (C, D) Male fore- and hind wings, respectively. (E) Male mid leg. (F) Female terminalia, in ventral view. (G, H, I) Male terminalia, dorsal, ventral, and lateral views, respectively.

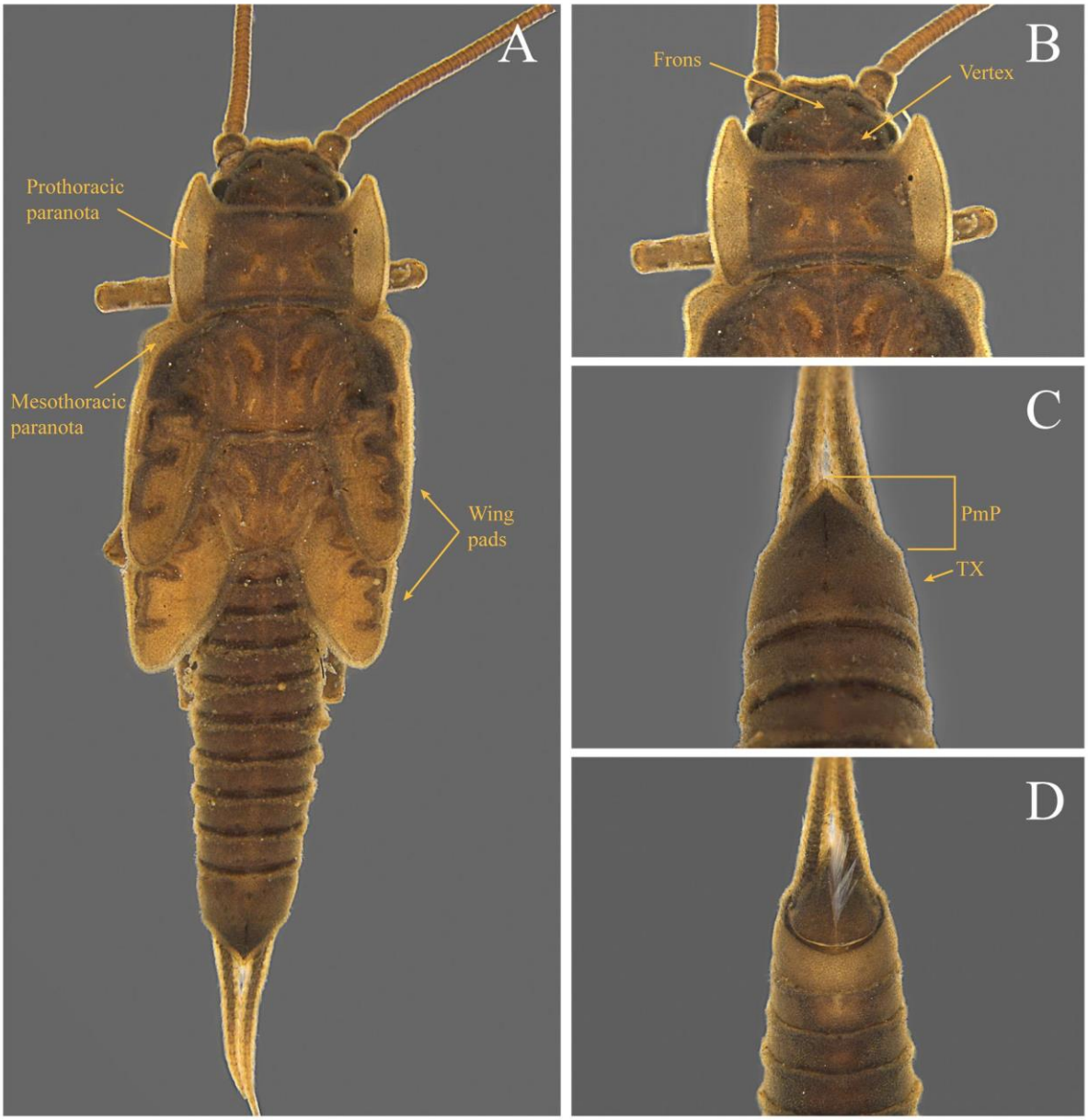

Figure 5. Nymph of *Guaranyperla guapiara* Ribeirão Grande, São Paulo State (UFVB). (A) Habitus. (B) Head and pronotum, dorsal view. (C, D) Abdomen, posterior segments, dorsal and ventral views, respectively.

Remarks. *Guaranyperla guapiara* was originally described based on a reared female from Rio das Mortes stream, Intervales State Park, in Ribeirão Grande, São Paulo, along with adult females and nymphs as paratypes from the same locality, and a nymph from Paranapiacaba Biological Station was also included as additional material (Froehlich 2001). Later on, Avelino-Capistrano & Nessimian (2013) recorded this species from Rio Paquequer stream, Serra dos Órgãos National Park, Teresópolis, in Rio de Janeiro State, based on a single reared female with corresponding exuviae, and nymphs. Both adult and nymphs were identified as *G. guapiara* due to "identical setae patterns of the body, antenna, and cerci" (Avelino-Capistrano & Nessimian, 2013). As the distribution of *G. guapiara* had supposedly been extended, Froehlich (2015) associated and described the male of the species based on material from other municipalities from São Paulo State: Campos do Jordão, Pindamonhangaba, and Jundiaí. The association was made also by rearing and comparing the immatures from these different localities.

In our study, we collected males, females, and nymphs from a stream near the type locality, in the same park, and associated these specimens as conspecific. The comparison of the topotypical associated male with males from other localities, such as, Campos do Jordão, Jundiaí and Pindamonhangaba, made it possible to restrict *G. guapiara* only to Intervales State Park. The topotypical male has the posterior margin of TXE almost as wide as its anterior portion, which has the lateral margin broadened, characteristics not observed in males studied from other localities (*Guaranyperla* sp. 1, *Guaranyperla* sp. 3 and *Guaranyperla* sp. 5). Moreover, the molecular species delimitation analysis also indicated that specimens from Campos do Jordão (*Guaranyperla* sp. 2) and Jundiaí (*Guaranyperla* sp. 4) belong to different species.

Male and female of *G. guapiara* collected and examined in our study present pronotum with irregular margins. However, upon observing other females deposited in MZUSP, it became apparent that this characteristic can vary, with some individuals having regular margins and others having irregular ones. Nymphs present paranota with rounded apices (fig. 5A and figs. 3–6 in Froehlich 2015), whilst in *Guaranyperla* sp. 4, apices are more acute (Fig. 20C). Additionally, the width of the paranota of *G. guapiara* is ⅓ the width of the pronotum, whereas in *Guaranyperla* sp. 2 and *Guaranyperla* sp. 4, they are more expanded, ½ the width of the pronotum.

The nymph from Paranapiacaba-SP, designated as additional examined material of *G. guapiara* (Froehlich 2001), could not be found. Furthermore, the nymph of *G. guapiara* illustrated in Froehlich (2001, figs. 3–6), was compared with an illustration of a nymph collected in Jundiaí and stated as conspecific to *G. guapiara* in (Froehlich 2015, fig. 5). We observed consistent morphological differences between these specimens pictured, such as paranota apices, acute in the nymph from Jundiaí (Fig. 20C) and rounded in *G. guapiara*.

The female from Serra dos Órgãos previously identified as *G. guapiara* by Avelino-Capistrano & Nessimian (2013) was associated herein, based on COI, to immatures and females collected in the same locality and determined as *G. barbosai* **comb. nov.** (Fig. 3). The female of *G. guapiara* differs from *G. barbosai* **comb. nov.** by the square subgenital plate, which is trapezoidal in *G. barbosai* **comb. nov.** Furthermore, *G. guapiara* has a white pentagonal unpigmented area on the subgenital plate (Fig. 4F), whilst *G. barbosai* **comb. nov.** has an elongated subgenital plate that has a narrow triangular unsclerotized area (Fig. 13E). Presently, according to our findings, *G. guapiara* occurs, until now, only in the type locality.

This species can be distinguished from the congeners by the male TXE, which has the lateral margin broadened at base and posterior margin nearly as wide as base; the female subgenital plate, characterized by an apex with a shallow emargination and a central white, pentagonal, unpigmented area; and by the nymph PmP of TX, featuring a smooth lateral margin, and the prothoracic and mesothoracic paranota with rounded corners.

Geographic distribution. This species is only known for Intervales State Park, Ribeirão Grande, São Paulo State, the type locality.

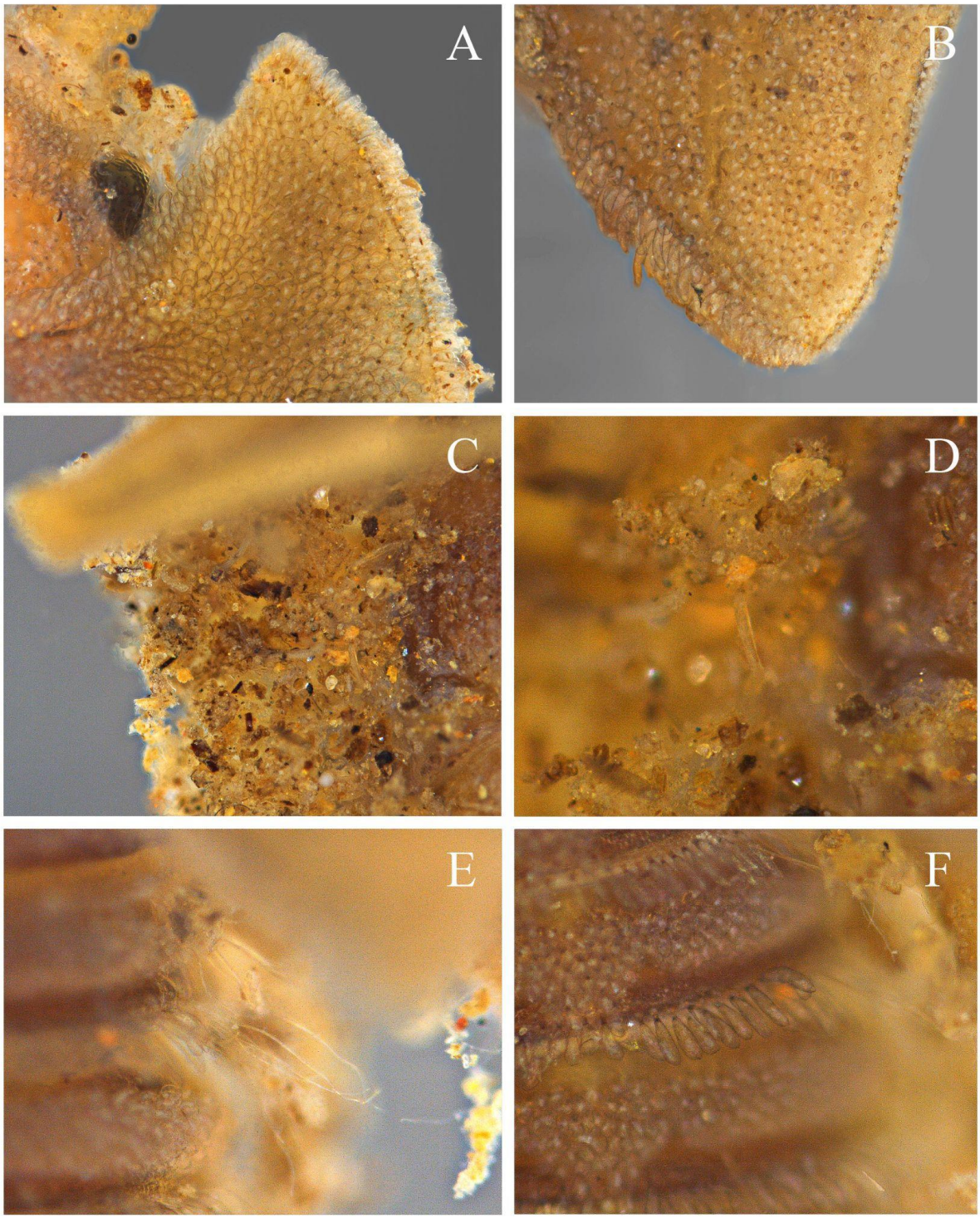

Figure 6. Details of setae in *Guaranyperla puri* **sp. nov.** Vesicular setae on pronotum paranota from the (A) left side of anterior region and (B) posterior region. Longer hair on (C–D) thorax in ventral view, and (E) abdomen in lateral view. (E) Longer vesicular setae in abdomen in dorsal view.

Guaranyperla beckeri Froehlich, 2001

Figs.7 A–D

Froehlich, 2001: 377 (original description); Froehlich, 2010: 134 (catalog); Novaes & Bispo, 2014: 440 (checklist); Pessacq, Zúñiga & Duarte, 2019: 192 (catalog).

Examined material. Holotype male, **Brazil. Minas Gerais. Poços de Caldas**, Morro do Ferro, 20.x.1963, J Becker col (MZUSP000354).

Type locality. Brazil. Minas Gerais. Poços de Caldas. Morro do Ferro.

Diagnosis. TXE short, lateral margin not broadened at its anterior portion, slightly narrowing posteriorly, posterior margin narrower than its anterior portion (Fig. 7D), apex with lateral tooth curved ventrally (Fig. 7B). Subgenital plate rounded and wider at base, elongated, slightly stubby, posterior margin rounded (Fig. 7C).

After examining the holotype and the original description, the following structures are redescribed.

Supplemental description.

Holotype male. ***Head***: Light area on frons, anteriorly to frontal suture (Fig. 7A). Lateral branch of frontal suture straight (Fig. 7A). ***Terminalia***: TX sclerotized, anterior margin concave (Fig. 7D); TXE short, lateral margin not broadened at its anterior portion, slightly narrowing posteriorly, posterior margin narrower than its anterior portion, apex with lateral tooth curved ventrally (Fig. 7D). Subgenital plate rounded and wider at base, elongated, slightly stubby, posterior margin rounded (Fig. 7C). Paraproct thin and elongated, inner surface membranous along its length, constant width over most of its length, curved ventrally, exceeding slightly TXE (Figs. 7B–D).

Measurements. Male holotype: head width, 1.2 mm; pronotum width, 1.2 mm; pronotum length, 0.9 mm; PW/PL=1.3; paraproct width, 0.1 mm; paraproct length, 0.9 mm; PaW/PaL=0.2.

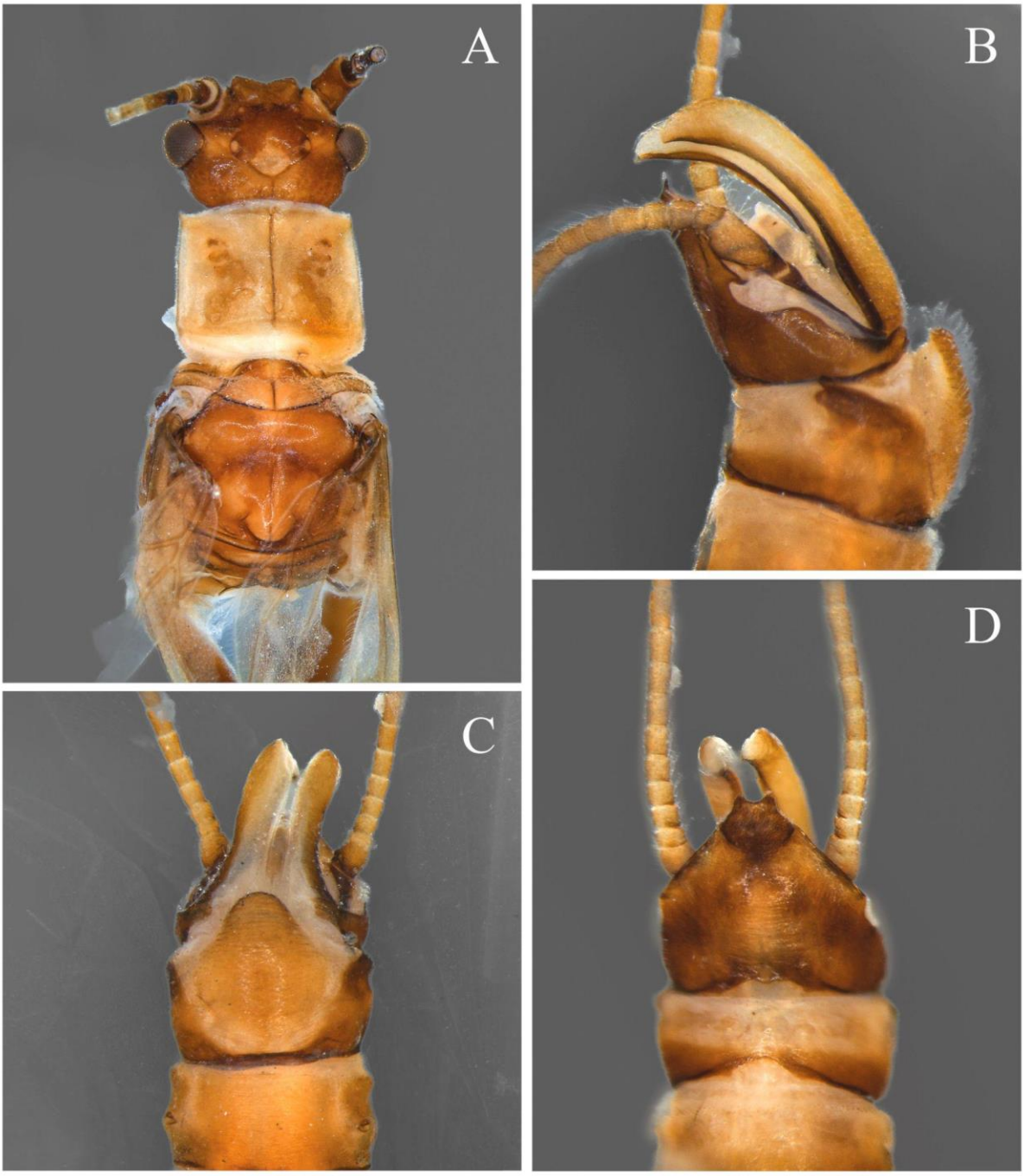

Figure 7. Male holotype of *Guaranyperla beckeri* from Poços de Caldas, Minas Gerais State (MZUSP). (A) Head and pronotum, dorsal view. (B, C, D) Terminalia, lateral, ventral and dorsal views, respectively.

Remarks. The male holotype of *G. beckeri* was collected in 1963 by Johann Becker and described by Froehlich 38 years later (Frohelich 2001). Due to a long period of preservation in alcohol, its coloration might have faded and the original description regarding the color of *G.*

beckeri (“light to medium brown") might not be accurate. Although the wings and abdomen of the holotype are damaged, the terminalia is in a good state of conservation, thus allowing comparison.

After describing what he considered the male of *G. guapiara*, Froehlich (2015) stated the possibility that *G. beckeri* may be a synonym of *G. guapiara* due to the similarity between these specimens. In the same study, he pointed out that these species differ in the shape of the subgenital plate, being “more broadly rounded” and the shape of paraprocts being “slightly larger” in *G. guapiara*. Moreover, he commented about a half-grown nymph from Ouro Fino, Minas Gerais, ca. 50-60 km south from Poços de Caldas, Minas Gerais, type locality of *G. beckeri*. This specimen was illustrated in comparison to the specimen from Jundiaí-SP, described by Froehlich (2015) as *G. guapiara* (herein, *Guaranyperla* sp. 4, Fig. 20C) (fig. 4 in Froehlich 2015). The main differences between the two nymphs lie in the paranota. The specimen from Ouro Fino has the anterior margin of the pronotum rounded, the anterior corner of mesonotum projected anteriorly, and the posterior margin of the metanotum rounded, patterns not observed either in *Guaranyperla* sp. 4 and *G. guapiara* from the type locality. Since these localities are close, Froehlich suggested the possible association of this nymph from Ouro Fino to *G. beckeri*, thus giving support for the description of the male of *G. guapiara.* Unfortunately, the specimen from Ouro Fino could not be found in MZSP. In addition, we went to Poços de Caldas, Morro do Ferro, the type locality of *G. beckeri*, but we were unable to collect any specimens of *Guaranyperla*. The region is relatively impacted with a predominance of non-native *Eucalyptus* trees. After examining the male holotype of *G. beckeri* and the male of *G. guapiara* from the type locality, it is clear that they are not conspecific. The male holotype from Poços de Caldas is the only specimen attributable to *G.beckeri*, thus far.

This species can be distinguished from the congeners by the male TXE with lateral margin not broadened at its anterior portion, and posterior margin narrower than its anterior portion.

Geographic distribution. This species is known only from the type locality, Poços de Caldas, Minas Gerais State, in the Morro do Ferro region.

Guaranyperla nitens Froehlich, 2001

Figs. 8A–F, 9A–D

Froehlich, 2001: 377 (original description, adult female); Froehlich, 2010: 134 (catalog); Froehlich, 2011: 602 (checklist); Froehlich, 2015: 177 (description of adult male and association of immature); Pessacq, Zúñiga & Duarte, 2019: 192 (catalog).

Examined material. Holotype female. **Brazil. São Paulo. Campos do Jordão,** Parque Estadual, Córrego Galharada, 30.x.1986, CG Froehlich (MZUSP000355); same data except date uncertain, between June and August, 2005, MR Spies, 2 males; same data except Córrego Campo do Meio, 08.x.2007, AE Siegloch, 1 male (MZUSP002208). ***Other material.*** **Campos do Jordão.** Parque Estadual, Córrego Galharada. 22º41'39"S 45º27'41"W, 1580 m, 18.viii.2023, P Taniguti, LH Almeida, ICH Cortes, MLS Rippel, FF Salles, D-net, 1 nymph (DNA voucher ENT6685) (UFVB); same data except 19.viii.2023, 1 nymph (DNA voucher ENT6686) (UFVB); 2 nymphs (UFVB).

Type locality. Brazil. São Paulo. Campos do Jordão. Córrego Galharada.

Diagnosis. *Adult*: Pronotum with most lateral margin regular, irregular at base (Fig. 8A). *Male*: TXE long and wide, apex projected laterally, posterior margin wider than the width of its anterior portion; short lateral clefts at about half of TX (Figs. 8C–D). *Female*: posterior margin of TX with a short rounded projection (Fig. 8B); subgenital plate trapezoidal, SpPE ⅓ shorter than its anterior portion length (Fig. 8B). *Nymph*: Width of paranota ⅔ the width of the pronotum, anterolateral projection ½ of pronotum length; mesothoracic paranota with corner slightly projected laterally and rounded; apex of fore and hind wing pads rounded (Fig. 9A). Light spot on median region of terga I–X, but on lateral region of TX; PmP longer than TX, tapering almost linearly towards a long projection, apex slightly rounded (Fig. 9C).

Supplemental description

Male. General color ochraceous to brown (the color has faded due to preservation in alcohol) (Fig. 8C–F). ***Head***: Brown, lighter centrally to frontal suture. Lateral branch of frontal suture straight. Lateral ocellus and eye black; median ocellus almost the same size as lateral ocellus. Antenna brown. Labrum ochraceous to brown, darker at base and lateral margin, lighter distally. Maxilla with palp brown. Labium ochraceous to brown; labial palp darker, postmentum brown. ***Thorax***: pronotum brown with borders lighter, rectangular, wider at base, as wide as head width; anterior corners slightly projected, lateral margin regular, but irregular

at base. ***Legs***: Brown. Sparse bristles present, more numerous and longer on tarsus. Femora brown. Tibia III with a lighter region in the middle. ***Wings***: Forewing with unpigmented line between RP and M; crossveins of mid distal region, between RP and CuA, pigmented; 2 pterostigmatic crossveins; RA distally forked; RP forked; CuA forked at distal ⅔ of its length. Hind wing brown. ***Abdomen***: Brown. Terga and sterna covered with short bristles, more dense in subgenital plate and paraproct; cercus with long and sparse bristles (Fig. 8F). ***Terminalia***: TX sclerotized and brown, anterior margin slightly concave (Figs. 8C–D); TXE long and wide, apex projected laterally, posterior margin wider than the width of its anterior portion, with posterior margin either slightly truncate (Fig. 8C) or with a smooth inward curve (Fig. 8D), lateral margin slightly curved and with posterolateral tooth curved ventrally, base about ⅔ of apex size, short lateral clefts at about half of TX (Fig. 8D). Subgenital plate ochraceous to brown, rounded at base, posterior margin broadly rounded (Fig. 8E). Paraproct ochraceous (Fig. 8E); thin and elongated, inner surface concave along its length, constant width over most of its length (Fig. 8F), curved ventrally, exceeding slightly TXE, apex rounded (Fig. 8C–F).

Holotype female. Similar to male, except for: ***Wings***: RA not forked; RP forked; CuA forked at distal ½ of its length. Hind wing with 3 pterostigmatic crossveins; RA and RP unforked. ***Abdomen***: Sternite VII with sclerotized bar on its center, along anterior margin. Terga and sterna covered with bristles, more numerous on paraproct. ***Terminalia***: TX brown, posterior margin with a short rounded projection (Fig. 8B). Subgenital plate with elliptical whitish unpigmented area on anterior margin, SpPE ⅓ shorter than its anterior portion length, trapezoidal, posterolateral margin convex, apex completely covering sternite IX (Fig. 8B). Paraproct triangular, slightly elongated, almost curved medially, base three times the size of apical region, apex slightly rounded (Fig. 8B).

Nymph. General color brown (Fig. 9A). ***Head***: Brown (Fig. 9B), with dark stripe on frons next to scape; vertex and frons with light spots scattered over surfaces, not covered with vesicular hairs; frons with irregular light spots. Lateral and median ocellus black, eye black; medium and lateral ocelli about the same size. Antenna ochraceous; pedicel as wide as first flagellomeres (Fig. 9A–B). Labrum ochraceous to brown, at base and lateral margin, lighter distally, dark brown at lateral margin. Maxilla and Labium with palpi ochraceous. Postmentum brown. ***Thorax***: Pronotum dark brown, paranota ochraceous, ⅔ the width of the pronotum, anterior margin straight, prothoracic paranota anteriorly projected ½ of pronotum length, postero-lateral margin not projected (Fig. 9A–B). ***Legs***: Ochraceous. Femora brown, lighter towards apex;

row of sparse bristles on outer margin present; spine located on ½ of inner margin of femora I, and on ⅔ of inner margin of femora II and III. ***Wing pads***: Mesothoracic paranota with anterior margin almost straight, corner slightly projected laterally and rounded; apex of fore and hind wing pads rounded; metanotum with posterior margin rounded, outer margin of hind wing pad not projected, apex rounded (Fig. 9A). ***Abdomen***: Dark brown, lighter towards apex; light spot on median region of terga I–X, but on lateral region on TX; sternite VIII with central lighter area; row of short setae along posterior margin of terga I–X; TX ochraceous with straight lateral margin (Fig. 9C); PmP longer than TX, tapering almost linearly towards a long projection, apex slightly rounded (Fig. 9C). Paraproct ochraceous (Fig. 9D). Cerci ochraceous, covered with longer bristles (Fig. 9C).

Measurements. Female holotype: head width, 1.8 mm; pronotum width, 1.8 mm; pronotum length, 1.3 mm; PW/PL=1.3; forewing length, 13,5; FL/PW=7.5; hind wing length, 12.4 mm; paraproct length, 0.7 mm. Male (N=2): head width, 1.4–1.5 mm; pronotum width, 1.3 mm; pronotum length, 1.0 mm; PW/PL=1.3; forewing length, 10.1–10.2 mm; FL/PW=7.8; hind wing length (N=1), 8.9; paraproct width (N=1), 0.2 mm; paraproct length (N=1), 1.2 mm; PaW/PaL=0.2. Nymph (N=3): body length (N=1), 10 mm; head width, 1.3–1.5 mm; pronotum width, 2.8–3.2 mm; pronotum length, 1–1.2 mm; pronotum projection, 1.8–2.3 mm; PW/PL=2.4–2.9; PP-PL=0.8–1.1; antenna length, 5.8–6.7 mm; cercus length (N=2), 3.6–3.7 mm.

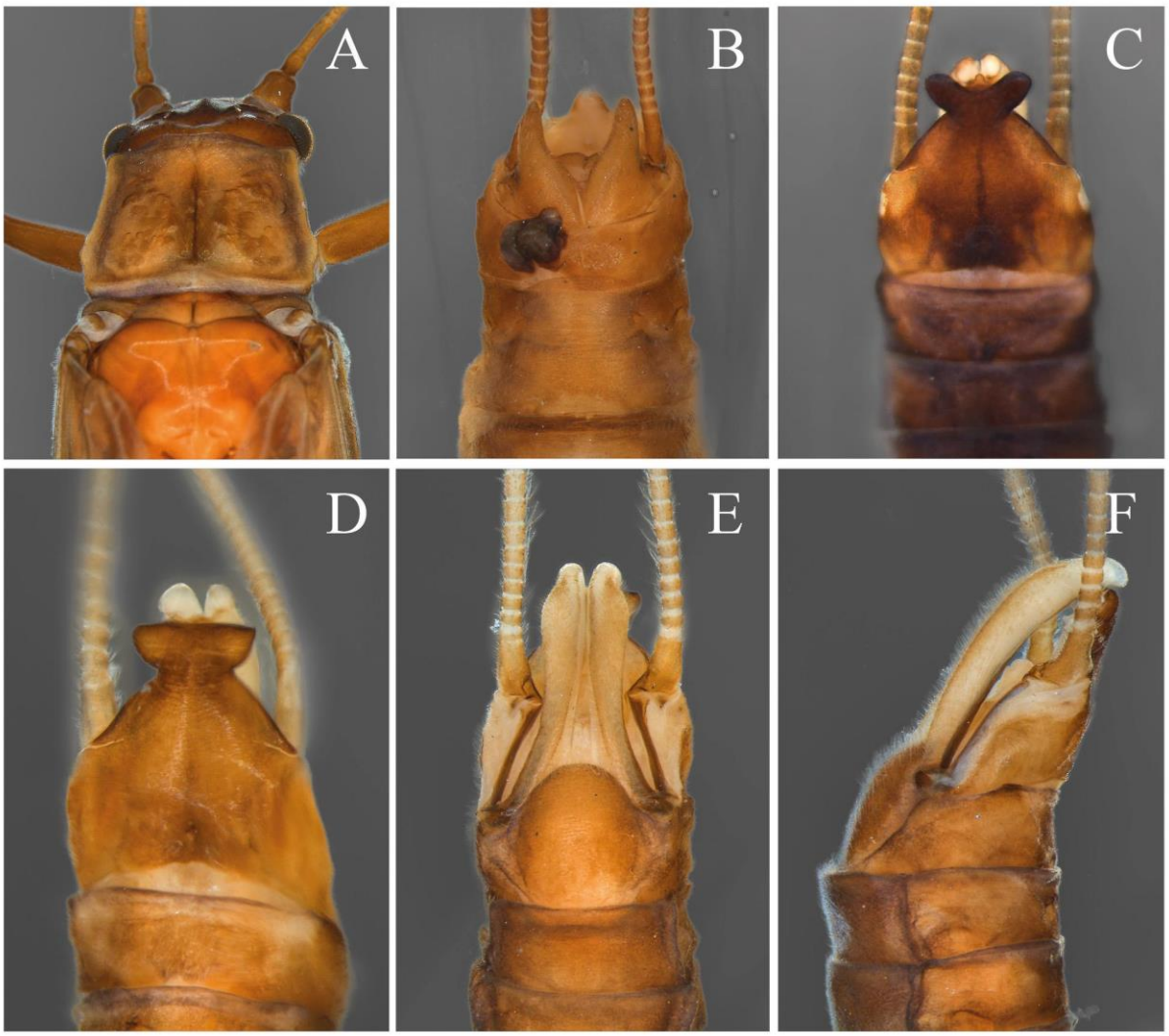

Figure 8. *Guaranyperla nitens* from Campos do Jordão, São Paulo State (MZUSP). (A) Holotype female, head and pronotum, and (B) terminalia. (C) Adult terminalia of male from Galharada stream in dorsal view. (D, E, F) Adult terminalia of male from Campo do Meio stream in dorsal, ventral, and lateral views, respectively.

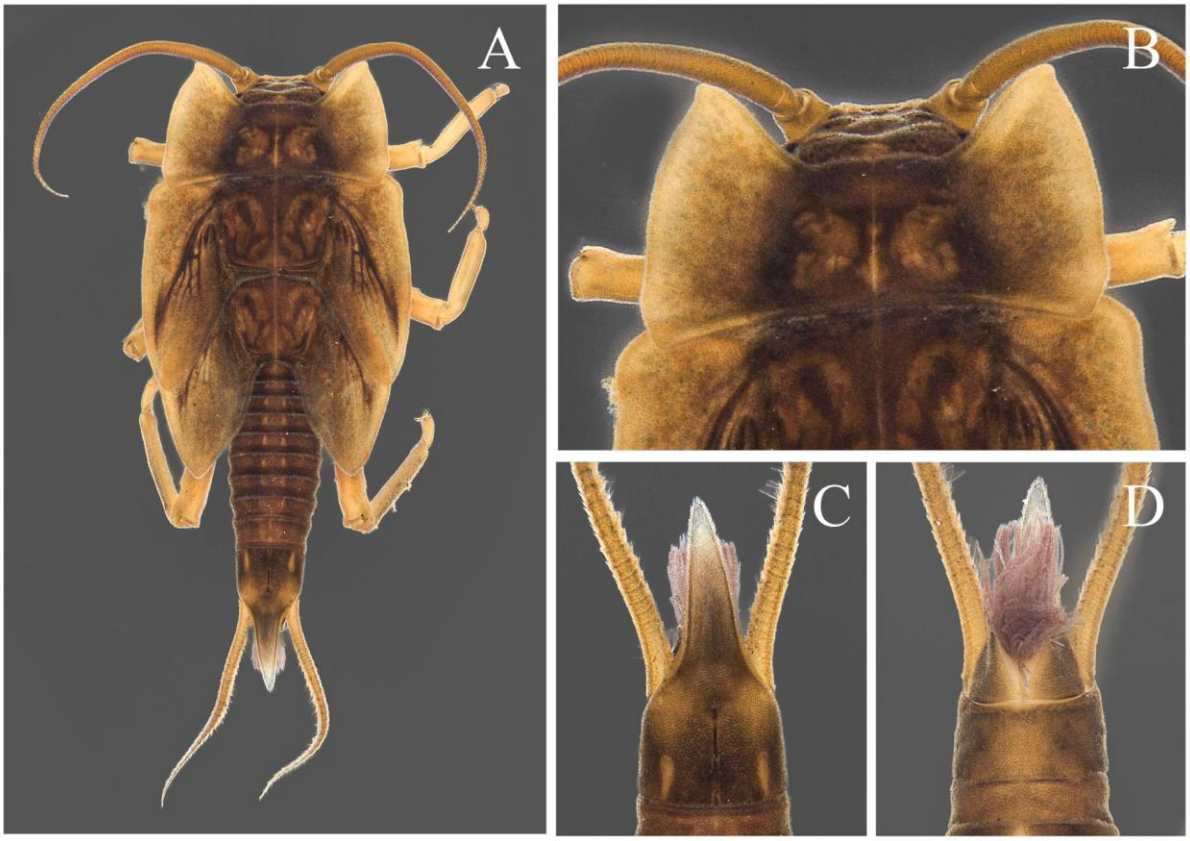

Figure 9. Nymph of *Guaranyperla nitens* from Campos do Jordão, São Paulo State (UFVB). (A) Habitus. (B) Head and pronotum. (C, D) Abdomen, posterior segments, dorsal and ventral views, respectively.

Remarks. This species was described based on a single female from Córrego Galharada stream, Campos do Jordão State Park (Figs. 8A–B), and the holotype is in a good state of conservation. In the same study, Froehlich (2001) also provides the illustration of a nymph, *Guaranyperla* sp. A, collected in the same stream as the holotype (fig. 13 in Froehlich, 2001), suggesting they could belong to the same species. Later on, a single male from another stream at the Campos do Jordão State Park, Córrego Campo do Meio, was obtained by rearing, allowing Froehlich (2015) to establish the association of nymphs and adults of *G. nitens*.

Recently, based on two nymphs collected at the type locality, Galharada stream, we were able to obtain COI sequence data. Surprisingly, our species delimitation analysis indicates that these specimens may not be conspecific. On that matter, we found a slight difference in the shape of TXE on the three males examined by Froehlich (2015) (Figs. 8C–D), two of them being lobed instead of more truncated as illustrated in Froehlich (2015) (figs. 6–8 in Froehlich

2015). Nevertheless, we are considering this difference as an intraspecific variation. DNA from more individuals, nymphs, males and females, are needed in order to ensure a more accurate status for *G. nitens*.

This species can be easily distinguished from the congeners by the male TXE trapezoidal, female with posterior margin of TX with a short rounded projection, and the long PmP of nymphs TX. In younger nymphs, the color pattern cannot be observed, and the distal margin of the wing pads is more acute.

Geographic distribution. This species is known only for the type locality, Campos do Jordão, São Paulo State, in the State Park of Campos do Jordão.

Guaranyperla barbosai (Avelino-Capistrano & Nessimian, 2013) **comb. nov.**

Figs. 10 A–E, 11 A–F, 12 A–C

Tupiperla barbosai Avelino-Capistrano & Nessimian, 2013: 185 (original description); Pessacq, Zúñiga & Duarte, 2019: 194 (catalog).

Examined material. Holotype male. **Brazil. Rio de Janeiro, Teresópolis**, Parque Nacional da Serra dos Órgãos, Rio Beija-Flor, 22º26'51''S 42º00'19''W, 1187m, 11-15.xi.2011, APM Santos, B Clarkson & JL Nessimian (DZRJ). Paratype. Rio Paquequer, Ponte na Estrada, 22º27'25''S 42º59'52''W, 1112m, 15-18.ix.2011, DM Takiya, Malaise trap, 1 male (DZRJ3465). ***Other material*****. Teresópolis**, Parque Nacional da Serra dos Órgãos, Rio Paquequer (ponte), 22º27'25''S 42º59'52''W, 1112m, 15-18.x.2011, DM Takiya, Malaise trap, 1 female (DNA voucher ENT 6513) (DZRJ3465), 1 female (DNA voucher ENT6515) (DZRJ3465A); same data except 06.ix.2022, 1 nymph (DNA voucher ENT6506) (UFVB), 1 nymph (UFVB); same data except 08.ix.2022, 1 nymph (UFVB); same data except Vale da Revolta, tributary of Rio Paquequer, 28.v.2007, 6 nymphs (DZRJ2351). **Nova Friburgo**, Rio Cascatinha, 23.x.2011, manual, DM Takiya, APM Santos, 1 female (DNA voucher ENT292) (DZRJ3472); same data except Siberia, 25.viii.2000, MH Olifiers, 8 nymphs (DZRJ0267); same data except Rio São Lourenço, 04.x.2000, 3 nymphs (DZRJ0785).

Type locality. Brazil. Rio de Janeiro, Teresópolis, Parque Nacional da Serra dos Órgãos, Rio Beija-Flor.

Diagnosis. *Adult*: head with lateral branch of frontal suture curved (Fig. 10B). Male with TXE short and wide, posterior margin wider than anterior portion, slightly pointed, posterolateral tooth absent (Figs. 11A,C); paraproct ochraceous; robust and elongated, cylindrical, with apical projection twisted dorsally, tapering abruptly to long acute apex, curved laterally (Figs. 11A–F). Female with subgenital plate brown, weakly sclerotized, a light purple region on its center, posterolateral margin rounded, SpPE ½ as long as its anterior portion length, apex not covering sternite IX completely (Fig. 10E). *Nymph*: paranota brown, ½ the width of the pronotum, row of vesicular hair along pronotum anterior and posterior margin, longer at posterior margin (Fig. 12A); TX with lateral margin slightly convex, PmP almost equal to TX, tapering towards apex, deltoid, with smooth lateral margin, apex acute (Fig. 12B).

Redescription. Paratype male. General color dark brown (Fig. 10A). ***Head***: dark brown with a lighter spot posteriorly to lateral ocellus (Fig. 10A); anterior region of frons with three lighter spots; borders of antennal sclerite and frons, darker (Fig. 10A). Lateral branch of frontal suture curved. Lateral ocellus and eye black; median ocellus smaller. Antenna ochraceous (Fig. 10A). Labrum ochraceous, lateral margin darker, distally lighter. Maxilla ochraceous with proximal outer margin darker, maxillary palp ochraceous. Labium with labial palp ochraceous; postmentum brown. ***Thorax***: pronotum dark brown, rectangular, almost as wide as head width (Fig. 10A); lateral margin regular, anterior corners slightly projected (Fig. 10A). ***Legs***: Ochraceous, darker in dorsal margin. Sparse thicker bristles present, more numerous and longer on tarsus. Femora with dorsal surface brown. ***Wings***: Forewing brown with lighter spots in the cells between RP and anal veins; unpigmented line between RP and M; crossveins of mid distal region, between RP and CuA, light; pterostigmatic cell lighter, 1 crossvein; RA unforked; RP forked at distal ⅕ of its length; CuA forked at distal ⅔ of its length (Fig. 10C). Hind wing brown with unpigmented line between RP and M, lighter area between 4th and 6th anal veins; 1 pterostigmatic crossvein; RA unforked; RP forked at distal ⅙ of its length; CuA forked at distal ⅓ of its length from fusing of M3+4 (Fig. 10D). ***Abdomen***: brown; light spot on lateral surface of terga I–VIII; sterna dark brown, darker towards posterior margin of terga V–VIII. Terga, sterna and paraproct densely covered with short bristles; cercus with long and sparse bristles. ***Terminalia***: TX sclerotized and brown, anterior margin concave (Fig. 11D); TXE dark brown, short and wide, anterior portion of TXE in acute angle with TX apex, posterior margin wider than anterior portion, slightly pointed laterally, posterolateral tooth absent. Subgenital plate brown, elongated, rounded at base, posterior margin almost triangular, tip slightly truncated (Fig. 11E). Paraproct ochraceous (Fig. 11E); robust and elongated, cylindrical, with

apical projection twisted dorsally, tapering abruptly to long acute apex, curved laterally, exceeding TXE (Fig. 11D–F).

Female. Similar to male, except for: ***Wings***: Forewing light brown with lighter spots in the center of medio-anal cells; region between C and Sc delimited by crossvein, darker; pterostigmatic cell darker, 0-1 crossvein (N=3). Hind wing light brown; region between C and Sc delimited by crossvein, darker; pterostigmatic cell darker, 0-1 crossvein (N=3); CuA forked at distal ¼ of its length from fusing of M3+4. ***Abdomen***: sternite VII with rounded sclerotized region reaching anterior and posterior margins (Fig. 10E); sternite IX with longitudinal unpigmented bar medially; sternite IX and X with unpigmented circular area laterally; segment X lighter. ***Terminalia***: TX brown, posterior margin slightly rounded, almost square. Sternite XIII angled at base, more sclerotized laterally; subgenital plate brown, weakly sclerotized, with a light purple region on its center, SpPE ½ as long as its anterior portion length, posterolateral margin rounded, apex not covering sternite IX completely (Fig. 10E). Paraproct triangular, elongated, base three times the size of apical region, apex slightly rounded and curved downwards (Fig. 10E).

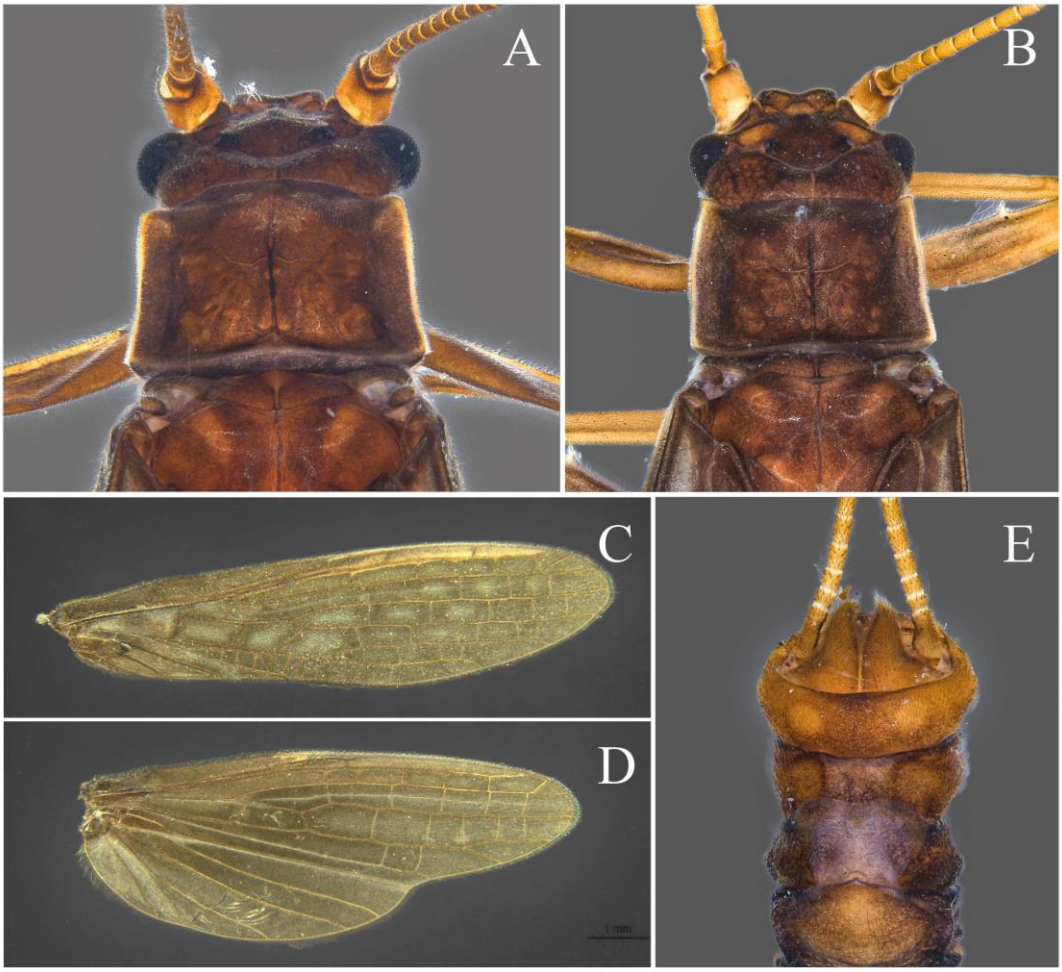

Figure 10. Adults of *Guaranyperla barbosai* **comb. nov.** from Teresópolis, Rio de Janeiro State (DZRJ). (A) Male head and pronotum, dorsal view. (B) Female head and pronotum, dorsal view. (C, D) Male fore- and hind wings, respectively. (E) Female terminalia, ventral view.

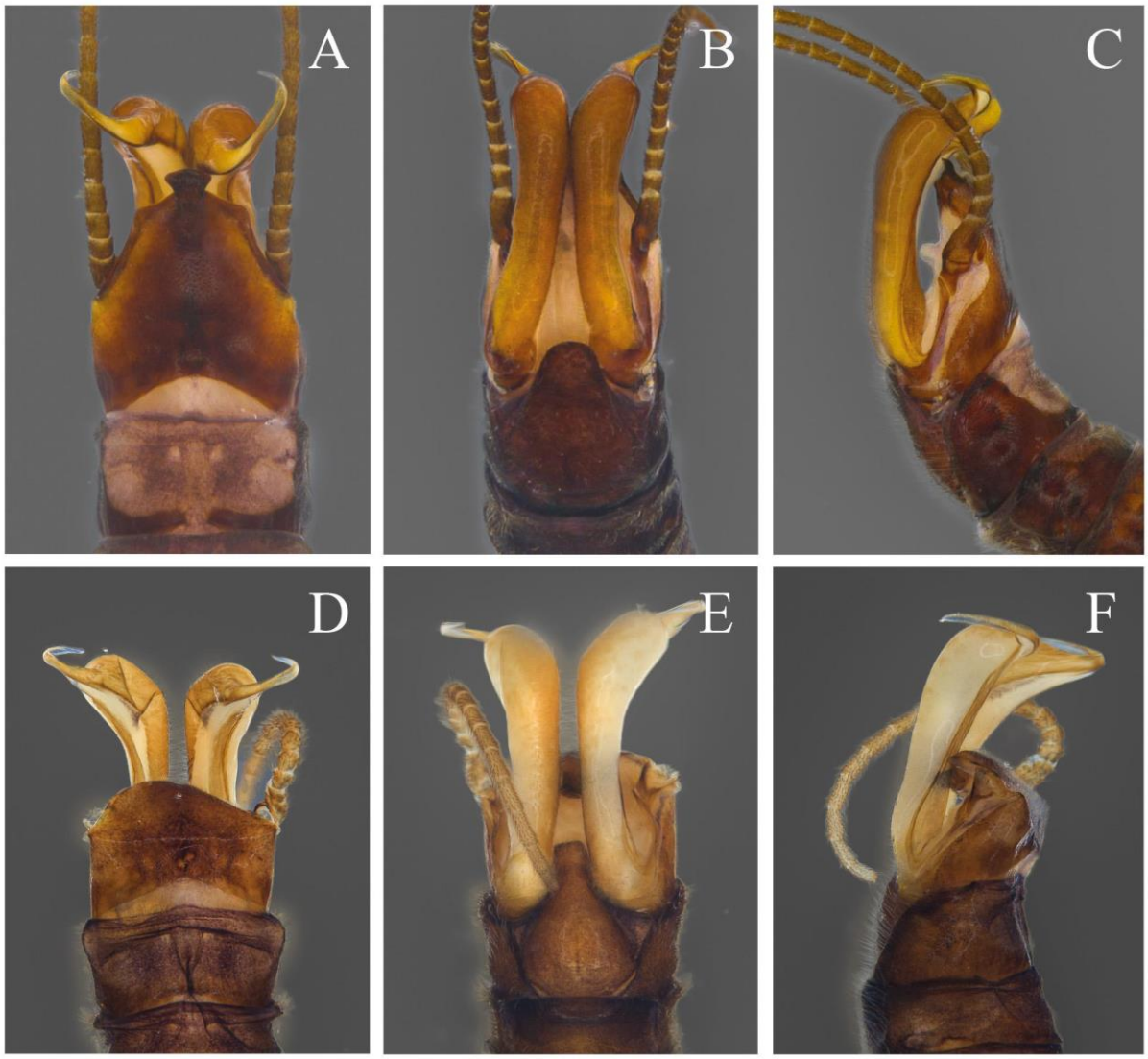

Figure 11. Adult male terminalias of *Guaranyperla barbosai* **comb. nov.** from Teresópolis, Rio de Janeiro State (DZRJ). (A, B, C) Holotype terminalia in dorsal, ventral, and lateral views, respectively. (D) Paratype terminalia, dorsal view with TXE folded, (E, F) ventral and lateral views, respectively.

Nymph. General color brown (Fig. 12A). ***Head***: vertex and frons with light spots scattered over surfaces, not covered with vesicular hairs (Fig. 12A). Lateral and median ocellus black, eye black; medium ocellus smaller. Antenna brown with pedicel narrower than first flagellomeres (Fig. 12A). Labrum ochraceous at base and lateral margin, darker distally, dark brown at lateral margin. Maxilla and Labium with palpi ochraceous. Postmentum brown. ***Thorax***: Pronotum dark brown, paranota brown, ½ the width of the pronotum, anterior margin straight, prothoracic paranota anteriorly projected ⅓ of pronotum length, postero-lateral margin projected, row of vesicular hair along pronotum anterior and posterior margin, longer at posterior margin (Fig. 12A). ***Legs***: Brown. Femora brown, lighter towards apex; bristles on outer margin absent; spine located on ½ of inner margin of femora I, and on ⅔ of inner margin of femora II and III. ***Wing pads***: Mesothoracic paranota with anterior margin slightly convex, corner projected and slightly rounded; apex of fore and hind wing pads rounded; metanotum with posterior margin rounded, outer margin of hind wing pad projected laterally, apex rounded (Fig. 12A). ***Abdomen***: Dark brown (Fig. 12B–C); sterna IX–X with lighter area medially, sternite X slightly lighter, darker band on sterna I–VIII touching posterior margin (Fig. 12C); row of setae along posterior margin of segments I–X (Fig. 12B–C); TX with lateral margin slightly convex (Fig. 12B); PmP almost equal to TX, tapering towards apex, deltoid, with smooth lateral margin, apex acute (Fig. 12B). Paraproct dark brown, lighter at base (Fig. 12C). Cerci yellow, covered with numerous longer bristles (Fig. 12B–C).

Measurements. Male holotype: head width, 1.1 mm; pronotum width, 1.0 mm; pronotum length, 0.8 mm; PW/PL=1.25; forewing length, 9 mm; FL/PW=9; paraproct width, 0.2 mm; paraproct length, 1.2 mm; PaW/PaL=0.2. Male paratype: head width, 1.2 mm; pronotum width, 1.2 mm; pronotum length, 0.7 mm; PW/PL=1.6; forewing length, 8 mm; FL/PW=6.7; paraproct width, 0.2 mm; paraproct length, 1.2 mm; PaW/PaL=0.2. Female (N=3): head width, 1.3–2.0 mm; pronotum width, 1.4–2.0 mm; pronotum length, 0.9–1.5 mm; PW/PL=1.3–1.5; forewing length, 10–10.2 mm; FL/PW=5.1–7.1; hind wing length, 8.9–9.1 mm; paraproct length, 0.5–0.7 mm. Nymph (N=1): body length, 7.4 mm; head width, 1.2 mm; pronotum width, 2.4 mm; pronotum length, 1.0 mm; pronotum projection, 1.6 mm; PW/PL=2.4; PP-PL=0.6; antenna length, 4.8 mm.

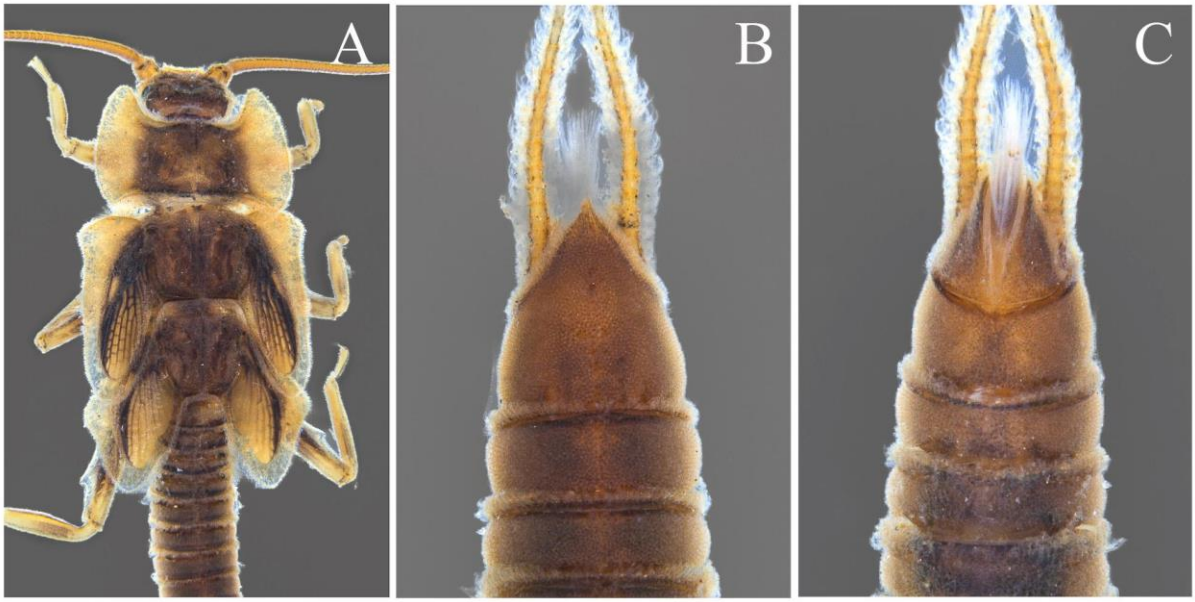

Figure 12. Young nymph of *Guaranyperla barbosai* **comb. nov.** from Nova Friburgo, Rio de Janeiro State (DZRJ). (A) Head and thorax, dorsal view. (B, C) Abdomen, posterior segments, dorsal and ventral views, respectively.

Remarks. This species was described based on the male holotype, and four male paratypes, all in a good condition of preservation. As the nymph of *Tupiperla barbosai* was not associated with any adult, and adults have some characteristics of *Guaranyperla*, such as the presence of pterostigmatic crossveins and pronotum almost as wide as head (T. Duarte, personal communication 2023), we included the holotype (DNA voucher ENT6907) and one of the paratypes (DNA voucher ENT6908) in our analysis. Avelino-Capistrano & Nessimian (2013) identified nymphs and a female of *Guaranyperla* collected in Serra dos Órgãos as *G. guapiara*. Thus, part of the examined material from the aforementioned study were assessed (6 nymphs - DZRJ2351), and the female (DZRJ 3472; DNA voucher ENT292) were incorporated in the molecular analysis. Additionally, we included other female specimens from the same collecting locality (DNA vouchers ENT292, ENT1059, ENT6513, ENT6515) and an unassociated nymph of *Guaranyperla* (DNA voucher ENT6506) from the type locality, in Serra dos Órgãos National Park, Rio de Janeiro State.

According to our results, nymph and females of *Guaranyperla* from Rio Paquequer are conspecific to the male holotype and paratype of *T. barbosai*, and this species was found nested within *Guaranyperla* in the phylogenetic analyses conducted (Fig. 3). Thus, we transfer this

species to *Guaranyperla*, herein *G. barbosai* **comb. nov.** Furthermore, the female and nymphs described as *G. guapiara* in Avelino-Capistrano & Nessimian (2013) were clustered in the same clade as *G. barbosai* **comb. nov.** Hence, no specimen of *G. guapiara* was found in this locality.

The nymph of *G. barbosai* **comb. nov.** resembles those of *G. puri* **sp. nov.** in the shape of pronotum, with paranota brown and expanded ½ the width of the pronotum. However, *G. barbosai* **comb. nov.** nymphs have a more elongated posteromedial projection of TX, similar to *G. froehlichi* **sp. nov.** (Figs. 12B and 14B). The females share similar spot patterns on the head with *G. froehlichi* **sp. nov.** Both species can be distinguished due to the shape of the subgenital plate, being shorter (posterior expansion of subgenital plate 4× shorter than base) and uniformly pigmented, with no unpigmented area, in *G. barbosai* **comb. nov.** (Fig. 10E).

Adults of both *G. barbosai* **comb. nov.** and *G. froehlichi* **sp. nov.** share the lateral branch of the frontal suture curved (Fig. 10B), being always straight in the other species of the genus. Also, both species have the unique twisted projection at the apex of paraproct. These characters may indicate that *G. barbosai* **comb. nov.** and *G. froehlichi* **sp. nov.** are sister species, as recovered in ML and UB analyses, however, due to a polytomy this relationship was not supported in the IB analysis. Additionally, we also provide new records for this species.

This species can be distinguished from the congeners by the male paraproct which has a long and twisted apical projection; the female subgenital plate, characterized by the posterior expansion ½ as long as its anterior portion length; and by the nymph featuring the lateral margin of TX slightly convex, and PmP in deltoid shape, with smooth lateral margin.

Geographic distribution. This species was known only for the type locality, Teresópolis, Rio de Janeiro State, in the National Park of Serra dos Órgãos. Herein we extend its distribution to a different locality of Rio de Janeiro State: Nova Friburgo-RJ.

Guaranyperla froehlichi Rippel & Salles **sp. nov.**

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Figs. 13 A–H, 14 A–C

Examined material. *Type material*: Holotype male, **Brazil. Rio de Janeiro. Teresópolis**, Parque Nacional da Serra dos Órgãos, Rio Beija-Flor, 22º26'50''S 43º00'19''W, 1187m, 11-15.x.2011, A Santos, B, Camisão, JL Nessimian, Malaise trap, 1 male (DNA voucher ENT6953). Paratypes: same data as holotype except Rio Paquequer (ponte), 22º27'25''S 42º59'52''W, 1112m, 15-18.x.2011, DM Takiya, Malaise trap, 1 female (DNA voucher ENT6514) (DZRJ3465B); same data except, 26.x.2013, LFL Silveira, 1 female (DNA voucher ENT2170) (DZRJ3741); same data except, 22º27'22"S 42º59'49"W, 1130m, 07.ix.2022, GM Pantoja, MLS Rippel, TYS Orlando, FF Salles, D-net, 1 nymph (DNA voucher ENT6505) (UFVB), same data except 06.ix.2022, 1 nymph (UFVB). ***Other material***: **Paraty**, Rio Parati-Cunha, 12.viii.2001, MH Olifiers, 3 nymphs (DZRJ0353). **Lumiar**, Tributário Boa Esperança, 04.viii.2001, 1 nymph (DZRJ0541).

Type locality. Brazil. Rio de Janeiro. Teresópolis, Parque Nacional da Serra dos Órgãos, Rio Beija Flor.

Diagnosis. *Adult*: Head with lateral branch of frontal suture curved (Fig. 13A). Male with TXE short and wide, posterior margin narrower than anterior portion, slightly pointed, posterolateral tooth absent (Fig. 13E, G); paraproct ochraceous; thin and elongated, constant width over most of its length, with apical projection twisted dorsally, tapering abruptly to short acute apex, curved laterally (Figs. 13E–G). Female with subgenital plate dark brown, with a narrow triangular depression weakly sclerotized located centrally; SpPE almost ½ the length of its anterior portion, square, posterolateral margin convex (Fig. 13D). *Nymph*: Body with fewer vesicular hair and forming patterns; row of vesicular hair along pronotum anterior and posterior margin, longer at posterior margin (Fig. 14A); TX with lateral margin slightly concave; PmP almost equal to TX, deltoid, tapering towards apex, with smooth lateral margin (Fig. 14B).

Description

Male. General color brown (Fig. 13A). ***Head***: Brown, darker towards center of frons, anterior region lighter; borders of antennal sclerite and frons, darker (Fig. 13A). Lateral branch of frontal suture curved. Lateral ocellus and eye black; median ocellus smaller. Antenna brown (Fig. 13A). Labrum ochraceous, lateral margin darker, distally lighter. Maxilla ochraceous with proximal outer margin darker, maxillary palp brown. Labium with labial palp brown; postmentum dark brown. ***Thorax***: Pronotum brown, rectangular, almost as wide as head width (Fig. 13A); lateral margin regular, anterior corners slightly projected (Fig. 13A). ***Legs***: Brown.

Femora brown, darker towards apex. Tibia III with a lighter region in the middle. Few sparse thicker bristles present, more numerous and longer on tarsus. Femora with dorsal surface brown. ***Wings***: Forewing brown with few lighter spots in the cells between RP and anal veins; unpigmented line between RP and M; crossveins of mid distal region, between RP and CuA, light; pterostigmatic cell lighter, crossvein absent; RA unforked; RP forked at distal ⅙ of its length; CuA forked at distal ⅔ of its length (Fig. 13C). Hind wing brown with pigmented line between RP and M, lighter area between 4th and 6th anal veins; pterostigmatic crossvein absent; RA unforked; RP forked at distal ⅙ of its length; CuA forked at distal ⅓ of its length from fusing of M3+4 (Fig. 13C). ***Abdomen***: Brown, sternite VIII darker towards its posterior margin; tergite IX darker towards its anterior margin. Terga, sterna and paraproct densely covered with short bristles; cercus with long and sparse bristles (Fig. 13E–G). ***Terminalia***: TX sclerotized and dark brown, anterior margin concave (Fig. 13F); TXE dark brown, almost black, short and wide, anterior portion of TXE in obtuse angle with TX apex, rounded, posterior margin narrower than anterior portion, slightly pointed laterally, posterolateral tooth absent (Fig. 13E, G). Subgenital plate brown, elongated, rounded at base, posterior margin almost triangular, tip slightly truncated (Fig. 13E). Paraproct ochraceous (Fig. 13E); thin and elongated, cylindrical, constant width over most of its length, with apical projection twisted dorsally, tapering abruptly to short acute apex, curved laterally, exceeding TXE (Fig. 13E).

Female. Similar to male, except for: ***Head***: Lighter spot posteriorly to lateral ocellus; anterior region of frons with three lighter spots (Fig. 13B). ***Wings***: Forewing brown with lighter spots in the center of medio-anal cells; 0-1 pterostigmatic crossvein; RP forked at distal ¼ of its length. Hind wing with CuA forked at distal ¼ of its length from fusing of M3+4. ***Abdomen***: Sternite VII with sclerotized bar on its center, along anterior margin (Fig. 13D); tergum VIII with sclerotized area on its center towards posterior margin; tergum IX with sclerotized area on its center reaching anterior and posterior margins. ***Terminalia***: TX brown, posterior margin slightly rounded, almost square. Subgenital plate dark brown, with a narrow triangular depression weakly sclerotized located centrally, Sp PE almost ½ the length of its anterior portion, square, posterolateral margin convex, apex rounded completely covering sternite IX (Fig. 13D). Paraproct triangular, elongated, base four times the size of apical region, apex slightly pointed (Fig. 13D).

Young nymph. General color yellowish ochraceous (color has faded due to preservation in alcohol); fewer vesicular hair and forming patterns (Fig. 14A). ***Head***: Vertex and frons with

light spots scattered over surfaces, not covered with vesicular hairs (Fig. 14A). Lateral and median ocellus black, eye black; medium ocellus smaller. Antenna with pedicel narrower than first flagellomeres (Fig. 14A). Labrum ochraceous at base and lateral margin, darker distally, dark brown at lateral margin. Maxilla and Labium with palpi ochraceous. Postmentum brown. ***Thorax***: Pronotum with anterior margin straight, width of paranota ⅔ the width of the pronotum, paranota anteriorly projected ¼ of pronotum length, postero-lateral margin projected, row of vesicular hair along pronotum anterior and posterior margin, longer at posterior margin (Fig. 14A). ***Legs***: Bristles on outer margin absent; spine located on ½ of inner margin of femora I, and on ⅔ of inner margin of femora II and III. Tibiae slightly expanded. ***Wing pads***: mesothoracic paranota with anterior margin straight, lateral region slightly emarginated, corner projected and rounded; apex of fore and hind wing pads rounded; metanotum with posterior margin rounded, outer margin of hind wing pad projected laterally, apex rounded (Fig. 14A). ***Abdomen***: yellowish ochraceous (Fig. 14B–C); sternum IX with central lighter area, sternum X ochraceous (Fig. 14C); row of setae along posterior margin of segments I–X (Fig. 14B–C); TX ochraceous with lateral margin slightly concave (Fig. 14B); PmP almost equal to TX, deltoid, tapering towards apex, with smooth lateral margin (Fig. 14B). Paraproct ochraceous to brown (Fig. 14C). Cerci yellow, covered with longer bristles (Fig. 14B–C).

Measurements. Male holotype: head width, 1.1 mm; pronotum width, 1 mm; pronotum length, 0.7 mm; PW/PL=1.4; forewing length, 8.5 mm; FL/PW=8.5; hind wing length, 7.5 mm; paraproct width, 0.1 mm; paraproct length, 0.8 mm; PaW/PaL=0.1. Female: head width, 1.3 mm; pronotum width, 1.2 mm; pronotum length, 0.8 mm; PW/PL=1.5; forewing length, 10.9 mm; FL/PW=9.3; hind wing length, 9.5 mm; paraproct length, 0.4 mm. Young nymph: Body length, 7.6 mm; head width, 1 mm; pronotum width, 1.9 mm; pronotum length, 0.8 mm; pronotum projection, 1.3 mm; PW/PL=2.4; PP-PL=0.5; antenna length, 5.3 mm; cercus length, 2.9 mm.

Etymology. The species is named in honor of Prof. Cláudio Gilberto Froehlich, an inspiring zoologist who dedicated most of his career to studying aquatic insects, particularly Plecoptera. He described the genus *Guaranyperla* and its three known species, making this the first new addition since its establishment. This tribute acknowledges his significant contributions to Brazilian entomology, aquatic ecology, and our understanding of biodiversity.

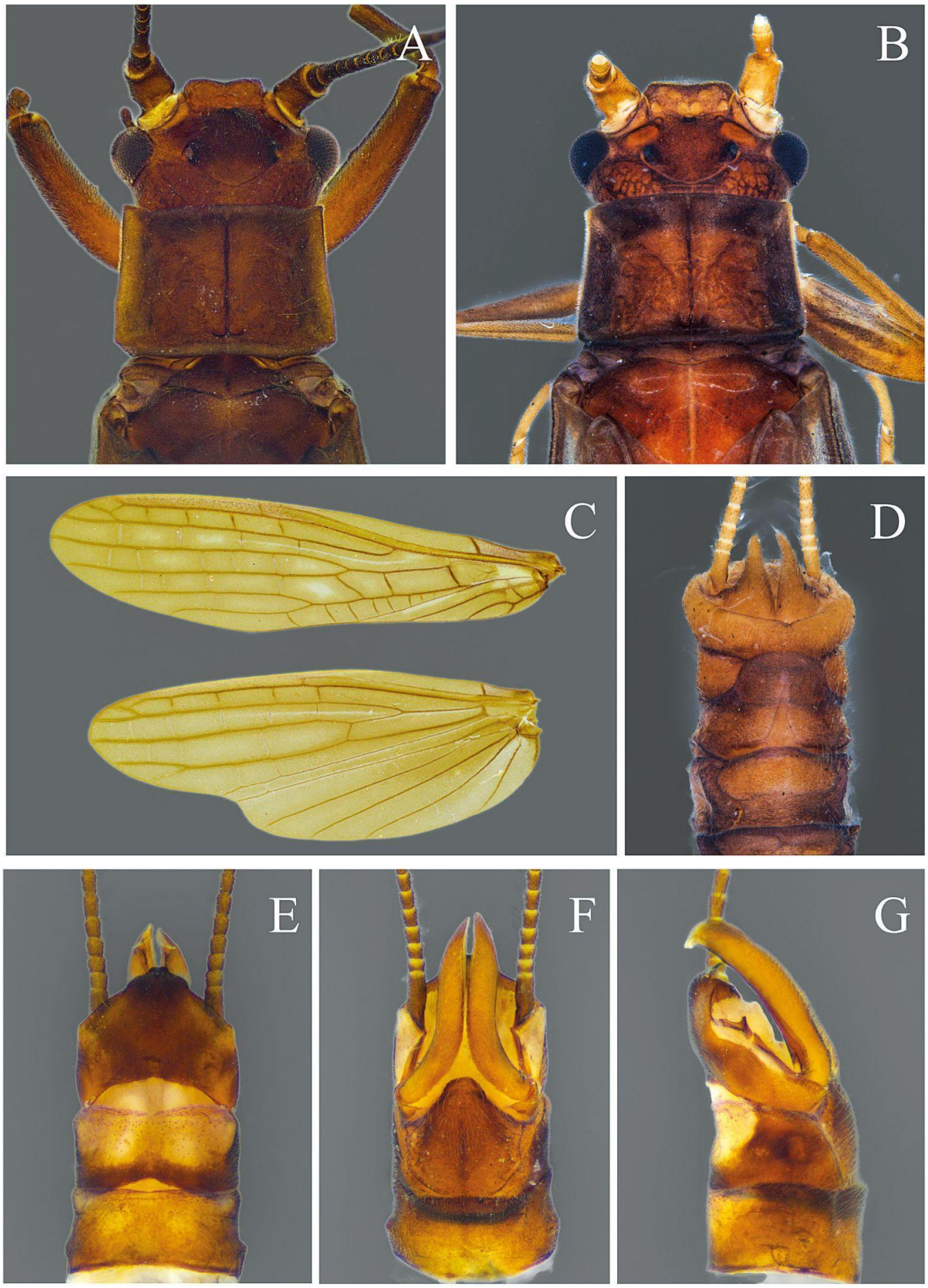

Figure 13. Adults of *Guaranyperla froehlichi* **sp. nov.** from Teresópolis, Rio de Janeiro State (DZRJ). (A) Male head and pronotum, dorsal view. (B) Female head and pronotum, dorsal

view. (C, D) Male fore- and hind wings, respectively. (E) Female terminalia, ventral view. (F, G, H) Male terminalia, dorsal, ventral and lateral views, respectively.

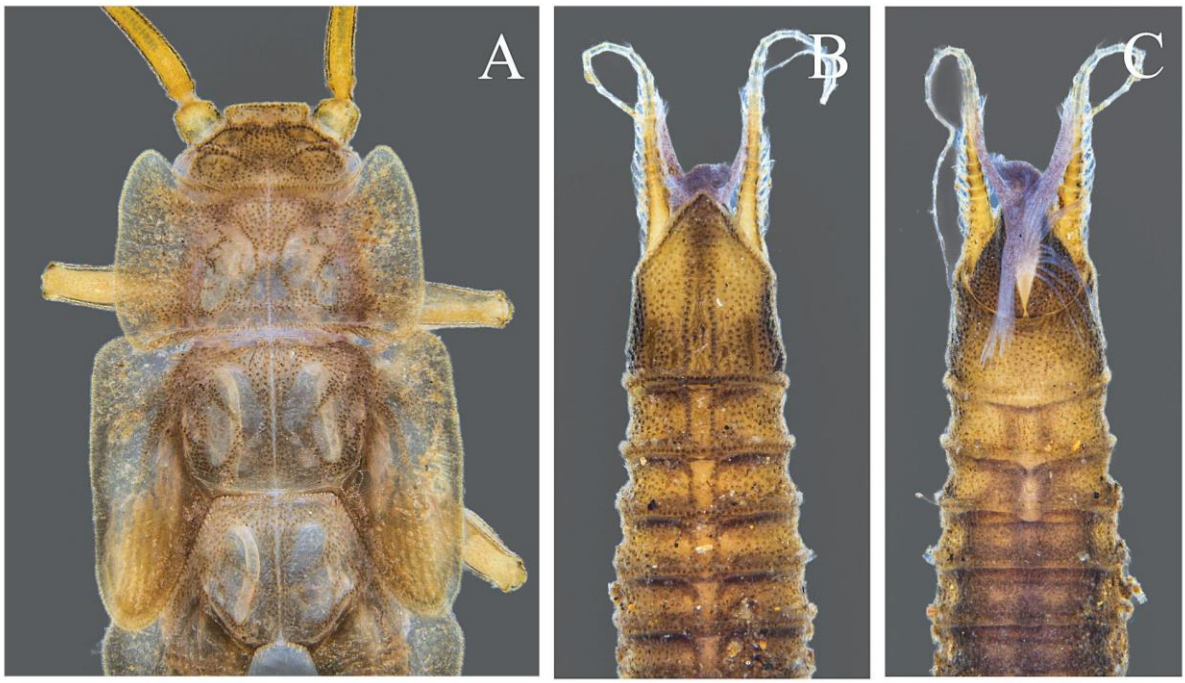

Figure 14. Young nymph of *Guaranyperla froehlichi* **sp. nov.** from Teresópolis, Rio de Janeiro State (UFVB). (A) Head and thorax, dorsal view; and (B, C) abdomen, posterior segments, dorsal and ventral views, respectively.

Remarks. Fresh material collected from the National Park of Serra dos Órgãos, Teresópolis-RJ, was also included in the analysis (ENT6505). According to our results, these specimens formed a monophyletic lineage, which also includes additional females (ENT2170 and ENT6514) and the male holotype (ENT6953). The terminalia of the male adult of *G. froehlichi* **sp. nov.** slightly resembles *G. barbosai* **comb. nov.** by the shape of the TXE as both species lack the posterolateral tooth. However, while the posterior margin of the TXE of *G. froehlichi* **sp. nov.** is narrower than the anterior portion, in *G. barbosai* **comb. nov.** it is wider. Moreover, both species share the unique twist at the apex of paraproct, but it is significantly smaller in *G. froehlichi* **sp. nov.**

The female of *G. froehlichi* **sp. nov.** can be readily distinguished from the other species by the more elongated subgenital plate that has a narrow triangular unsclerotized area (Fig. 13D). Nymphs are similar to those of *G. barbosai* **comb. nov.** (found in the same stream) in the shape of the medio-posterior projection of TX; however the body is more yellow with fewer vesicular setae, an uncommon feature in the genus (Figs. 14A–C).

Guaranyperla froehlichi **sp. nov.** was delimited as a distinct species from its sister *G. barbosai* **comb. nov.** Interestingly, these two species co-occur locally and temporally (three-day window) in Rio Paquequer, Teresópolis, as some specimens were found in the same Malaise trap sample. The male holotype is in a good state of conservation, however most of the abdomen is lost, and only the terminalia is preserved in alcohol along with the specimen.

This species can be distinguished from the congeners by the male paraproct, which has a short and twisted apical projection; the female subgenital plate, characterized by the posterior expansion almost ½ the length of its anterior portion; and by the nymph featuring the lateral margin of TX slightly concave, with PmP in deltoid shape, and body covered with fewer vesicular hair, forming patterns.

Geographical Distribution. This morphospecies is known for Rio de Janeiro State in the following localities: Teresópolis-RJ, in National Park of Serra dos Órgãos, Paraty-RJ and Lumiar-RJ.

Guaranyperla puri Rippel & Salles **sp. nov.**

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Figs. 15 A–H, 16 A–E

Examined material. *Type material*: Holotype male, **Brazil. Minas Gerais. Ervália,** Córrego Pico do Cruzeiro, Complexo Turístico do Pico do Cruzeiro, Ponte, 20°46’37”S 42º29’49”W, 1110m, nymph collected in 28.ix.2022 male emerged in laboratory 02.x, MLS Rippel, DF Nunes (DNA voucher ENT6511) (UFVB PL0060). Paratypes: same data as holotype except 1 male (UFVB PL0059). **Araponga**, Parque Estadual do Brigadeiro (PESB), Córrego da Trilha das Águas, 20º43’52”S 42º27’50”W, 1110m, 19/x/2022-03/xi/2022, malaise trap, 1 female (UFVB PL00032) (DNA voucher ENT6507); same data except Trail to Pico do Boné,

20º40’8”S 42°27’3”W, 1350m, 09.ix.2023, Salles FF, ICH Cortes, P Bonfá, I Amaral, 1 male nymph (UFVB PL00285); same data except, 1 female nymph (UFVB PL00292b). ***Other material***: **Ervália**, Complexo Turístico do Pico do Cruzeiro, Ponte, 28.ix.2022, MLS Rippel, DF Nunes, 1 nymph (UFVB); same data except 2 nymphs collected, 1 male emerged in laboratory 02.x (UFVB); same data except 1 nymph collected, 1 male emerged in laboratory 06.x (UFVB); same data except 1 nymph collected, 1 female emerged in laboratory 07.x. (UFVB) **Araponga**, Parque Estadual do Brigadeiro (PESB), Córrego da Trilha das Águas, 28.vii.2022-19.x.2022, malaise trap, 1 male and 1 female (UFVB); same data except 25.v.2022, GM Pantoja, TYS Orlando, ICH Cortes, 6 nymphs (UFVB); same data except FF Salles, JM Lopez, MLS Rippel, 1 nymph collected 19.x.2022, adult emerged in laboratory 25.x (UFVB); same data except upstream site, 20º43’43”S 42º28’13”W, 1190m, 28.vii.2022, MG Carvalho, GM Pantoja, ICH Cortes, 4 nymphs (UFVB); same data except Poço Cipó, 20º43’45”S 42º28’11”W, 1180m, 3 nymphs (UFVB); same data except Trilha das Águas - Poço do Pato, 20º43’52”S 42º27’54”W, 1120m, 1 nymph (UFVB); same data except Trail to Pico do Boné, 09.ix.2023, Salles FF, ICH Cortes, P Bonfá, I Amaral, 7 nymphs (UFVB); same data except 20°42’16”S 42º29’19”W, 1200m, 08.ix.2023, 4 nymphs (UFVB); same data except 20º43’55.04”S 42º28’0.78”W, 09.ix.2023, 8 nymphs (UFVB); same data except Fazenda Pousda Remanso (Seu Dico), 20º39’21”S 42º27’11”W, 1070m, 08.ix.2021, 1 nymph (UFVB); same data except Cachoeria do Boné, 20º38’45”S 42°27’26”W, 950m, 24.ix.2018, MLS Rippel & Castillo-Velásquez, FF Salles, Belich, Mines, 1 nymph (UFVB).

Type locality. Brazil. Minas Gerais. Ervália. Complexo Turístico do Pico do Cruzeiro

Diagnosis. *Adult*: frons with darker-brown area delimited by frontal suture, antennal sclerite and frons (Figs. 15A–B). Male with TXE short and wide, lateral margin broadened at its anterior portion, posterior margin narrower than its anterior portion (Fig. 15F). Female with subgenital plate brown, white rounded rectangular unpigmented area on its center, square, posterolateral margin convex (Fig. 15E). *Nymph*: width of paranota ½ the width of the pronotum, antero-lateral projection ⅔ of pronotum length; row of bristles along pronotum margin, more numerous on anterior margin. Mesothoracic paranota with anterior corner acute and projected; apex of fore and hind wing pads acute; outer margin of hind wing pad projected laterally, apex acute (Fig. 16A). TX with PmP tapering towards apex, acuminate shape (Fig. 16D).

Description

Male. General color dark brown (Fig. 15A). ***Head***: Brown, frons with darker-brown area delimited by frontal suture, antennal sclerite and anterior region of frons (Fig. 15A). Lateral branch of frontal suture straight (Fig. 15A). Lateral ocellus and eye black; median ocellus smaller. Antenna dark brown (Fig. 15A). Labrum ochraceous to brown, darker at base and lateral margin, lighter distally. Maxilla with palp brown. Labium ochraceous to brown; labial palp darker; postmentum dark brown. ***Thorax***: pronotum dark brown, rectangular, almost as wide as head width; anterior corners slightly projected, lateral margin regular (Fig. 15A). ***Legs***: Brown. Sparse thicker bristles present, more numerous and longer on tarsus. Femora brown, darker towards apex. Tibia III with a lighter region in the middle. ***Wings***: Forewing dark brown with weak lighter spots in the cells between RP and anal veins, unpigmented line between RP and M; crossveins of mid distal region, between RP and CuA, light; 0-1 pterostigmatic crossvein (N=6); RA either forked or unforked; RP either forked at distal ¼ or ⅕ of its length; CuA forked at distal ⅔ of its length (Fig. 15C). Hind wing dark brown, with unpigmented line between RP and M, lighter area between 4th and 6th anal veins; RA unforked; RP either forked at distal ⅕ of its length or not forked; CuA forked at distal ¼ of its length from fusing of M3+4 (Fig. 15D). ***Abdomen***: dark brown, darker towards anterior margin of abdominal segments I–IX, excepting sterna VII–IX, dark brown entirely (Figs. 15F–H). Terga and sterna densely covered with short bristles, fewer on paraproct; cercus with long and sparse bristles. ***Terminalia***: TX sclerotized and black, anterior margin concave (Fig. 15F); TXE short and wide, lateral margin broadened at its anterior portion, posterior margin narrower than its anterior portion, and with postero-lateral tooth curved ventrally (Fig. 15F). Subgenital plate dark brown, rounded and wider at base, posterior margin rounded (Fig. 15G). Paraproct brown, apex lighter (Figs. 15G–H); thin and elongated, inner surface membranous along its length, constant width over most of its length, curved ventrally, exceeding slightly TXE (Figs. 15F–H).

Female. Similar to male, except for: General color brown (Fig. 15B). ***Thorax***: Pronotum dark brown, lighter towards lateral margin, rectangular, as wide as head width; lateral margin regular, anterior corners projected (Fig. 15B). ***Wings***: Forewing dark brown with lighter spots in the cells between medio and anal veins; 0-1 pterostigmatic crossvein (N=2); RA not forked; RP either forked at distal ⅕ of its length or not forked (N=2); CuA forked at distal ½ of its length. Hind wing with RA not forked; RP either forked at distal ⅙ of its length or not forked (N=2). ***Abdomen***: Brown entirely, sternite VII with sclerotized bar on its center, along anterior margin (Fig. 15B). Terga and sterna densely covered with long bristles, more numerous on

paraproct. ***Terminalia***: TX dark brown, posterior margin slightly rounded. Subgenital plate brown, white rounded rectangular unpigmented area on its center, Sp PE 3× longer than base, square, posterolateral margin convex, apex either completely covering sternite IX, or not (Fig. 15E). Paraproct triangular, slightly elongated, curved medially, base three times the size of apical region, apex slightly rounded (Fig. 15E).

Nymph. General color brown (Fig. 16A). ***Head***: dark brown; vertex and frons with light spots scattered over surfaces, not covered with vesicular hairs. Lateral and median ocellus black, eye black; medium ocellus smaller. Antenna brown, pedicel as wide as first flagellomeres (Fig. 16B). Labrum ochraceous at base and lateral margin, darker distally, dark brown at lateral margin. Maxilla and Labium with palpi ochraceous. Postmentum brown. ***Thorax***: Pronotum dark brown, paranota brown, ½ the width of the pronotum, anterior margin straight, prothoracic paranota anteriorly projected ⅔ of pronotum length, postero-lateral margin projected, row of bristles along pronotum margin, more numerous on anterior margin (Fig. 16A). ***Legs***: Brown. Femora dark brown, lighter towards apex; row of sparse bristles on outer margin present; spine located on ½ of inner margin of femora I, and on ⅔ of inner margin of femora II and III. ***Wing pads***: mesothoracic paranota with anterior margin straight, corner projected and acute; apex of fore and hind wing pads acute; metanotum with posterior margin rounded, outer margin of hind wing pad projected laterally, apex acute (Fig. 16A). ***Abdomen***: Brown (Fig. 16A); sternite IX with central lighter area, sternite X ochraceous (Fig. 16E); row of setae along posterior margin of segments I–X (Fig. 16A); TX ochraceous with lateral margin slightly convex; PmP subequal to TX, tapering towards apex, acuminate, with slightly concave lateral margin, apex acute (Fig. 16D). Paraproct brown (Fig. 16E). Cerci brown, covered with longer bristles (Figs. 16D–E).

Measurements. Male holotype: head width, 1.3 mm; pronotum width, 1.2 mm; pronotum length, 0.9 mm; PW/PL=1.3; forewing length, 9.4 mm; FL/PW=7.8; hind wing length, 8 mm; paraproct width, 0.2 mm; paraproct length, 0.9 mm; PaW/PaL=0.2. Male (M=4): head width, 1.2–1.3 mm; pronotum width, 1.2–1.3 mm; pronotum length, 0.6–0.9 mm; PW/PL=1.3–2; forewing length, 8–9.5 mm; FL/PW=6.8–7.8; hind wing length, 6.9–8.4 mm; paraproct width, 0.1–0.2 mm; paraproct length, 0.8–1 mm; PaW/PaL=0.2. Female (N=3): head width, 1.4–1.5 mm; pronotum width, 1.4–1.5 mm; pronotum length, 0.9–1 mm; PW/PL=1.4–1.6; forewing length(N=2), 11.1–11.2 mm; FL/PW=7.4–7.7; hind wing length, 9.6–9.9 mm; paraproct length, 0.4–0.6 mm. Nymph (N=4): body length, 8.2–9.2 mm; head width, 1.2 mm; pronotum width,

2.2–2.7 mm; pronotum length, 0.9–1 mm; pronotum projection, 1.7–1.9 mm; PW/PL=2.4–2.7; PP-PL=0.8–0.9; antenna length (N=2), 6.2–6.8 mm; cercus length (N=2), 2–3.4 mm.

Etymology. The name refers to the Puri indigenous people, one of the first inhabitants of the mountainous region of the Atlantic Rainforest.

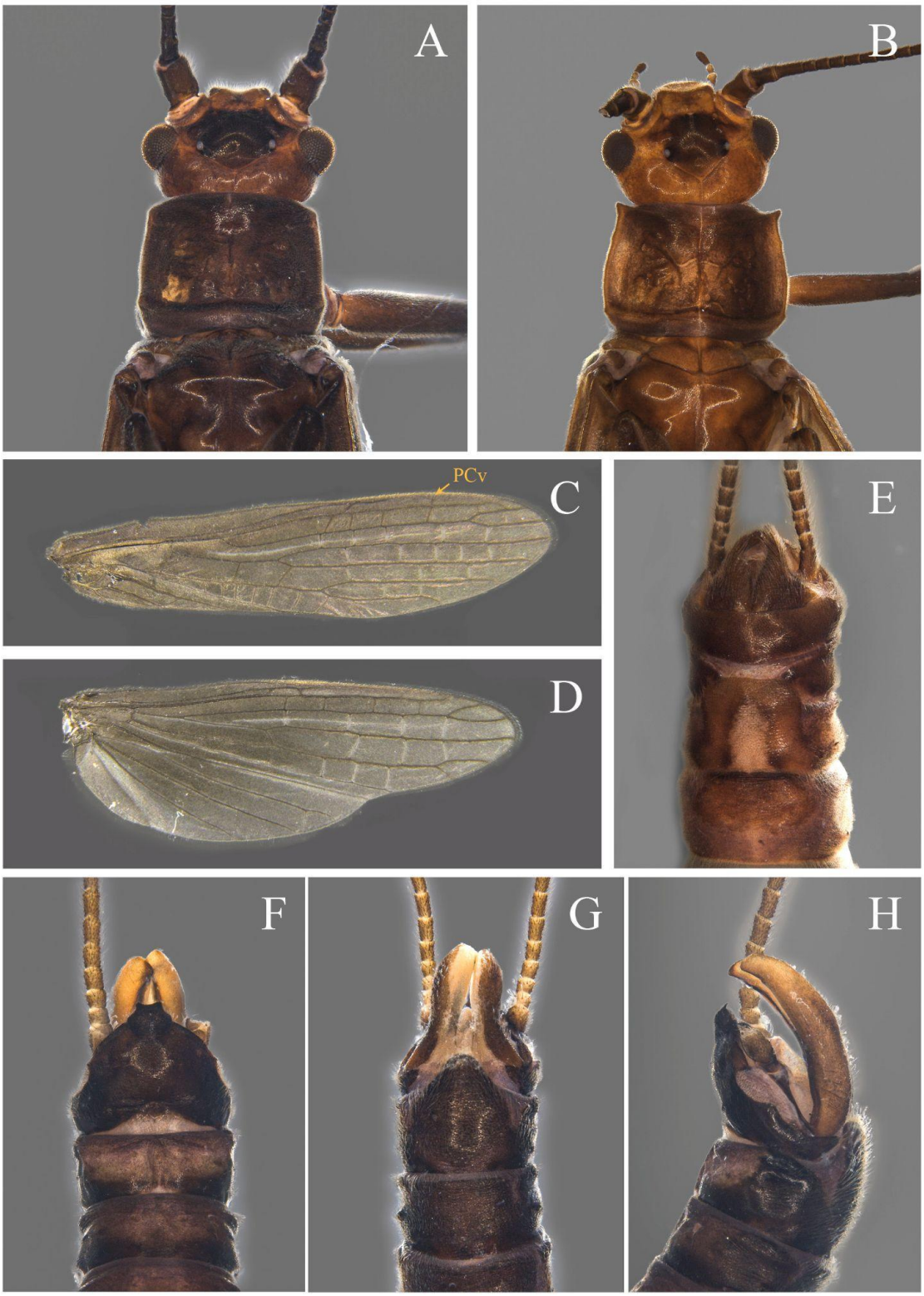

Figure 15. Adults of *Guaranyperla puri* **sp. nov.** (UFVB). (A) Male from Ervália, Minas Gerais State, head and pronotum, dorsal view. (B) Female from Araponga, Minas Gerais State, head and pronotum, dorsal view. (C, D) Male fore- and hind wings, respectively. (E) Female terminalia, ventral view. (F, G, H) Male terminalia, dorsal, ventral and lateral views, respectively.

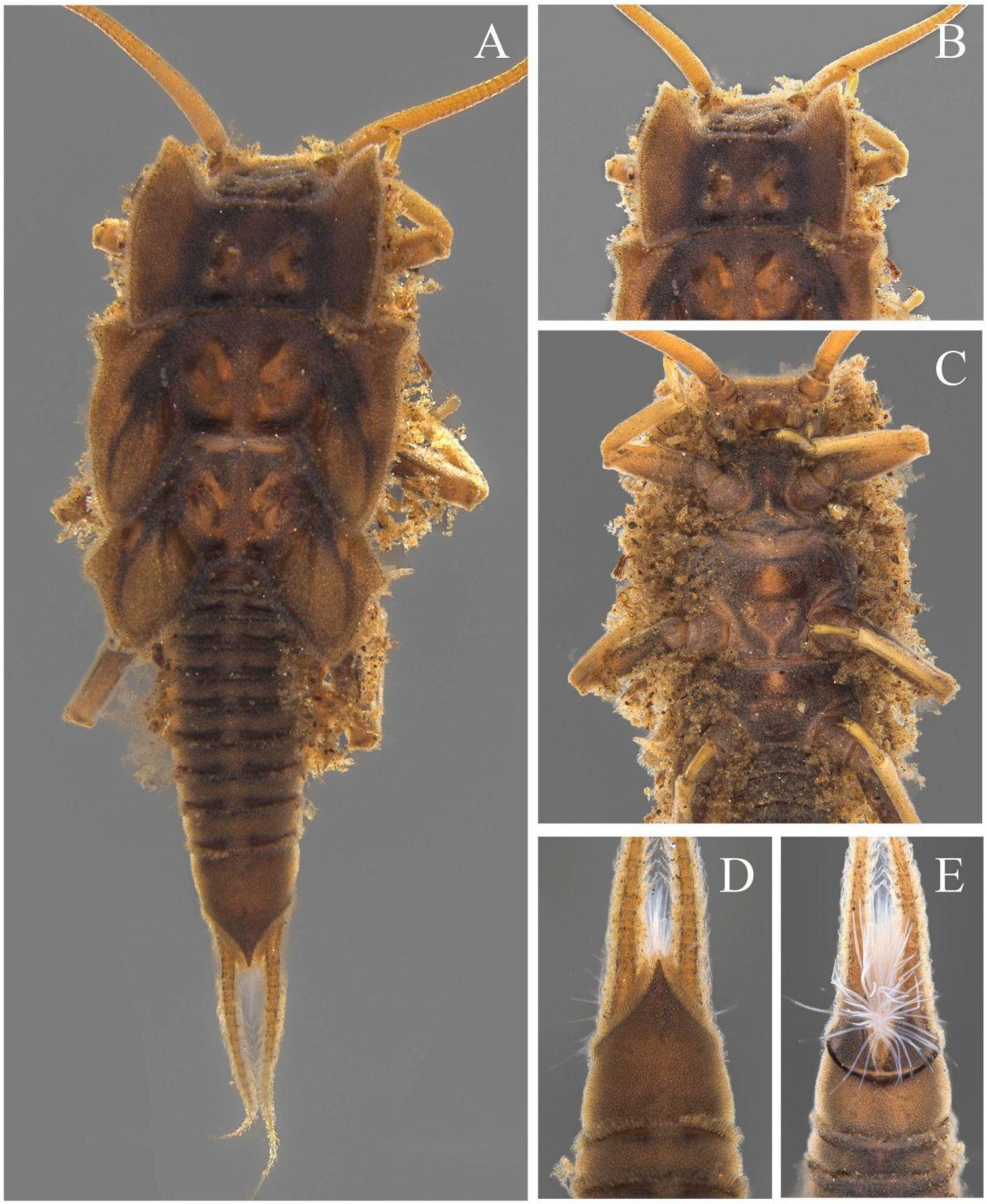

Figure 16. Nymph of *Guaranyperla puri* **sp. nov.** from Araponga, Minas Gerais State (UFVB). (A) Habitus. (B, C) Head and pronotum, dorsal and ventral views, respectively. (D, E) Abdomen, posterior segments, dorsal and ventral views, respectively.

Remarks. *Guaranyperla puri* **sp. nov.** is described based on individuals collected in Serra do Brigadeiro region, in Minas Gerais. The region is characterized by Atlantic Rainforest and high-altitude fields. According to species delimitation analyses conducted, specimens from the referred region were recovered as a monophyletic lineage. The terminalia of the male adult of *Guaranyperla puri* **sp. nov.** resembles those of *G. beckeri*, *Guaranyperla* sp. 1, and *Guaranyperla* sp. 3, however the sclerotized outer surface of the paraproct is larger (Fig. 15H). In addition, adults have a conspicuous dark brown area on frons (Fig. 15A–B), which is not observed in these other species. Our material also differs from the nymph from Ouro Fino illustrated in Froehlich (2015) mainly by the shape of the pronotum, with straight and wide anterior margin (Fig. 16B). Additionally, the anterior corner of the mesonotum is projected laterally, and not anteriorly as in the nymph from Ouro Fino-MG.

Regarding intraspecific variation, *Guaranyperla puri* **sp. nov.** can differ in size of the inner membranous surface of paraproct, being either "inflated" or almost not showing the inner surface. Furthermore, the posterior margin of TX can be triangular in shape, or slightly rounded. Nymphs can show variation in the lateral margin of the pronotum, either more straight or more rounded.

This species can be distinguished from the congeners by the male TXE which has the lateral margin broadened at its base, with posterior margin narrower; the female subgenital plate, characterized by the square shape and a white rounded rectangular unpigmented area on its center; and by the nymph PmP of TX, featuring an acuminate shape, and the prothoracic and mesothoracic paranota with acute corners.

Geographic distribution. *Guaranyperla puri* **sp. nov.** occurs in the Serra do Brigadeiro region, more specifically the municipalities of Ervália and Araponga, in Minas Gerais State.

Unidentified Material

In this section we separately report material that could not be confidently assigned to any valid species or described as new due to lack of specimens or associated semaphoronts obtained by rearing or DNA.

***Guaranyperla* sp. 1**

Guaranyperla guapiara nec Froehlich, 2015

Figs.17 A–D

Examined material. Brazil. São Paulo. Campos do Jordão, State Park, 24.x.2005, MR Spies, 1 male plus exuviae, (MZUSP002218).

Measurements. Head width, 0.8 mm; pronotum width, 0.8 mm; pronotum length, 0.6 mm; PW/PL=1.3; forewing length, 8.3 mm; FL/PW=11; paraproct width, 0.1 mm; paraproct length, 1.6 mm; PaW/PaL=0.2.

Remarks. Froehlich (2015) considered males from Serra do Japi and Pindamonhangaba as *G. guapiara*, and those specimens are considered herein as *Guaranyperla* sp. 3 and *Guaranyperla* sp. 5, respectively. The terminalia of *Guaranyperla* sp. 1 (Figs. 17B–D) resembles that of *Guaranyperla puri* **sp. nov.***, Guaranyperla* sp. 3, and *G. beckeri*. Indeed, we were unable to find consistent morphological differences among males from the aforementioned species. Therefore, we are designating this specimen as morphospecies sp. 1. Despite the existence of female and nymphs from the same locality (referred to herein *Guaranyperla* sp. 2), we could not associate them based on safe methods such as barcoding or rearing. There is no information regarding the stream in which *Guaranyperla* sp. 1 was collected.

Geographical Distribution. This morphospecies is known exclusively for Campos do Jordão, São Paulo State.

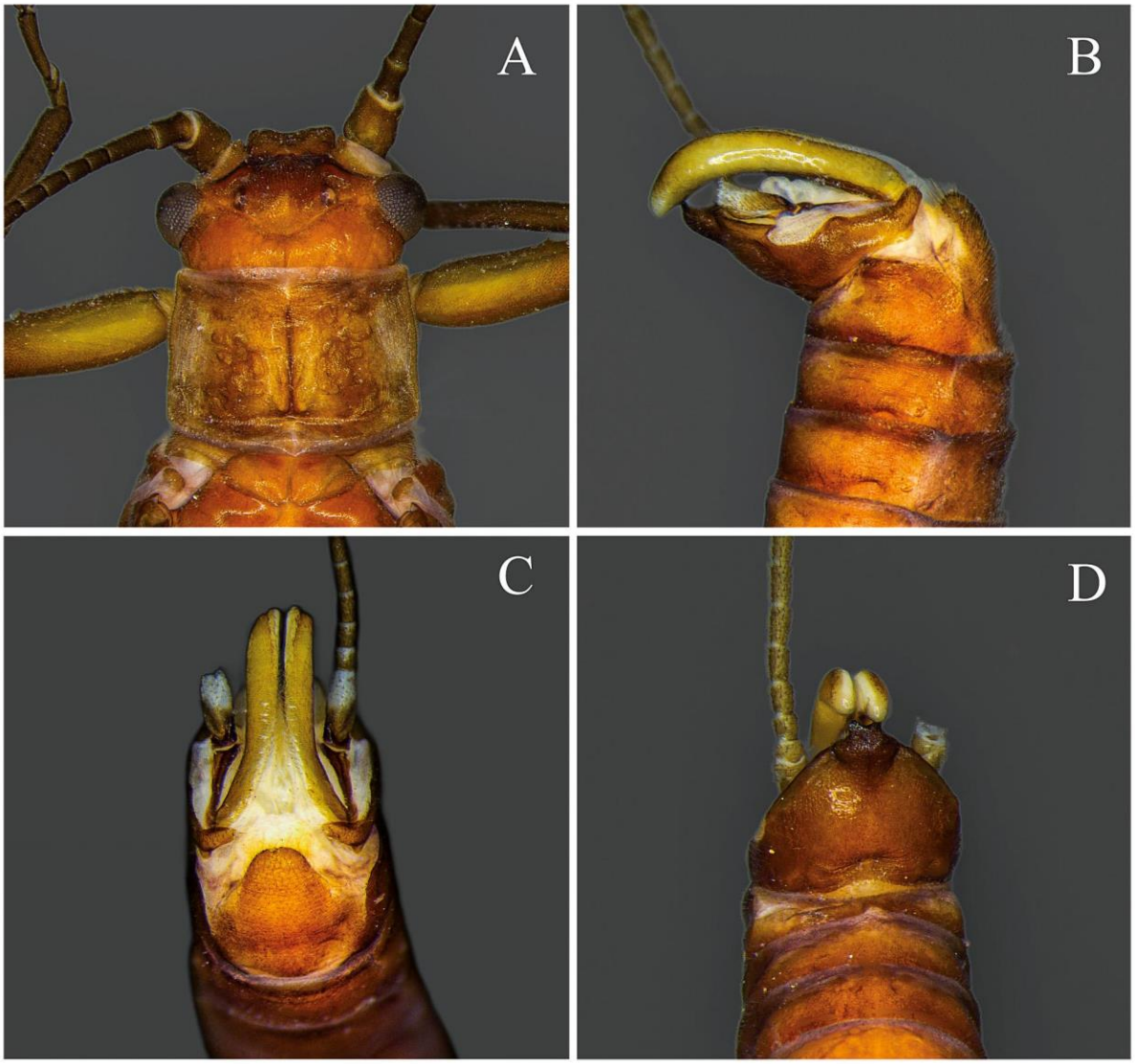

Figure 17. *Guaranyperla* sp. 1 from Campos do Jordão, São Paulo State (MZUSP). (A) Adult male, head and pronotum, dorsal view; (B, C, D) terminalia, lateral, ventral and dorsal views, respectively.

***Guaranyperla* sp. 2**

Figs. 18 A–C

Examined material. Brazil. São Paulo. Campos do Jordão, State Park, Córrego Serrote, 22º39'39"S 45º26'26"W, 18.xii.2019-15.i.2020, Malaise trap, 1 female (DNA voucher TD116) (CIACGF); same data except 03-07.x.2019, LH Almeida, RC Kobal, 1 nymph (DNA voucher TD113) (CIACGF).

Measurements. Female: head width, 0.9 mm; pronotum width, 0.9 mm; pronotum length, 0.6 mm; PW/PL= 0.9; forewing length, 10 mm; FL/PW=11; paraproct length, 0.3 mm. Nymph: body length, 4.7 mm; head width, 0.8 mm; pronotum width, 1.4 mm; pronotum length, 0.6 mm; PW/PL=2.3; antenna length, 3.7 mm.

Remarks. The nymph and female of *Guaranyperla* collected in Campos do Jordão State Park included in our analysis also appeared as a clade. The female of *Guaranyperla* sp. 2 resembles true *G. guapiara*, however the unpigmented area on the subgenital plate has a trapezoidal shape (Fig. 18B) (pentagonal in *G. guapiara*). Regarding immatures, *Guaranyperla* sp. 3 has some similarities with *G. guapiara*, such as the shape of pronotum, although its coloration is more golden and lateral margins of mesothoracic paranota are projected (Fig. 18C).

Geographical Distribution. This morphospecies is known exclusively for Campos do Jordão, São Paulo State.

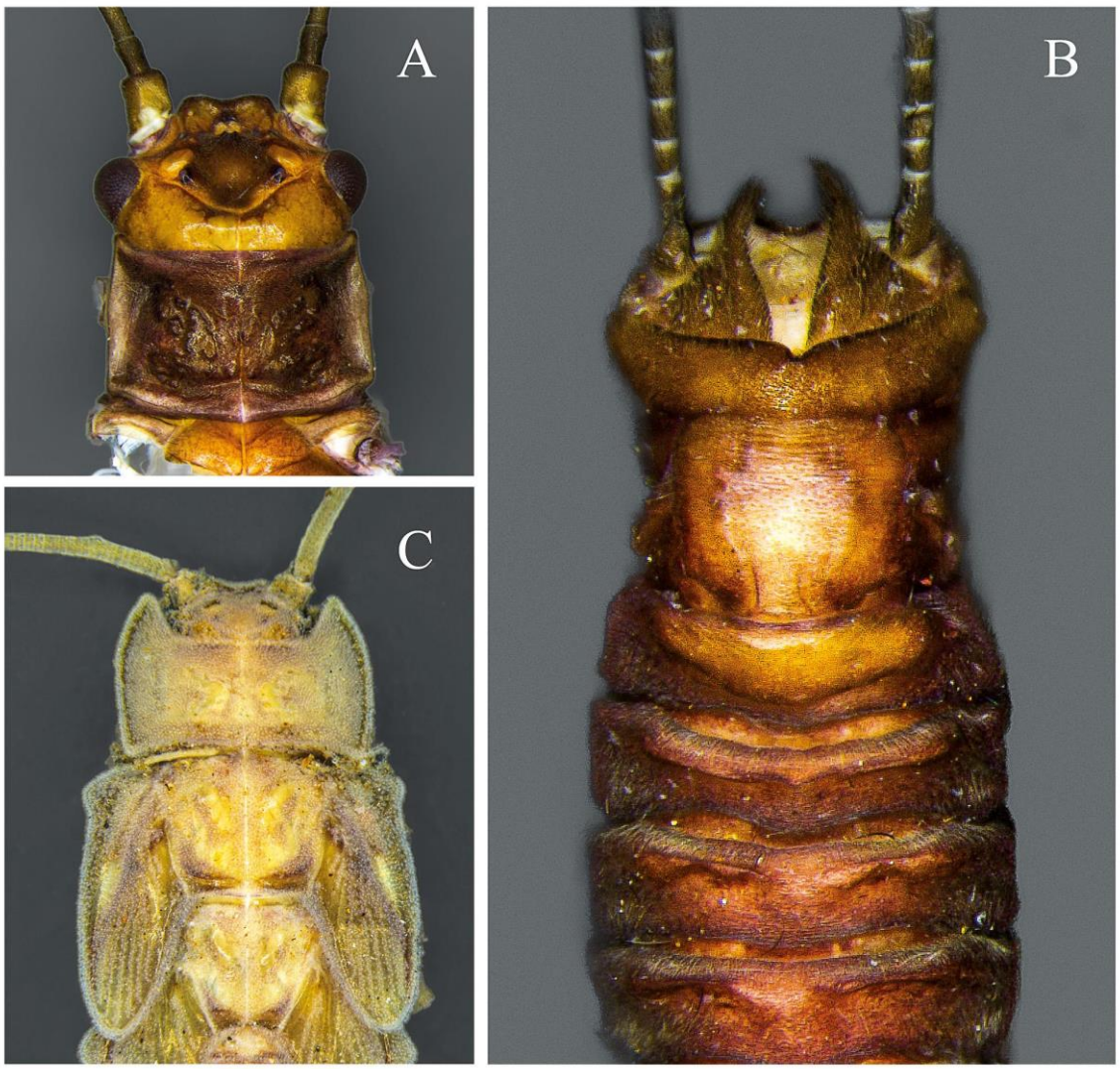

Figure 18. *Guaranyperla* sp. 2 from Campos do Jordão, São Paulo State (CLBA). (A) Adult female, head and pronotum, dorsal view; (B) terminalia, ventral view. (C) Nymph head and thorax, dorsal view.

***Guaranyperla* sp. 3**

Guaranyperla guapiara nec Froehlich, 2015

Figs. 19 A–D

Examined material. Brazil. São Paulo. Jundiaí, Reserva Biológica da Serra do Japi, Trilha Cachoeira do Paraíso, 23°14'33"S 46°57'03"W, 28.viii.2007, aerial net, L Lecci, R Moretto, E Nascimento, 1 male (MZUSP002214).

Measurements. Head width, 0.88 mm; pronotum width, 0.8 mm; pronotum length, 0.6 mm; PW/PL=1.2; forewing length, 9 mm; FL/PW=11.7; paraproct width, 0.1 mm; paraproct length, 1.7 mm; PaW/PaL=0.06.

Remarks. Nymphs from Serra do Japi, in Jundiaí, São Paulo State, were mentioned as unidentified species, *Guaranyperla* spp., in Froehlich (2001), along with immatures from Ouro Fino, Minas Gerais State, and Santa Teresa, Espírito Santo State. Froehlich (2001) suggested that these specimens could belong to more than one species for differing in details from the nymphs of *G. guapiara*. Later on, he identified the male and nymphs from this locality as *G. guapiara* (Froehlich, 2015). However, after comparing this male with the topotypical male of *G. guapiara*, it was determined that they are not conspecific. Whilst the male of *Guaranyperla* sp. 3 shares some morphological characteristics with *G. puri* **sp. nov.**, *Guaranyperla* sp. 1, *Guaranyperla* sp. 5 and *G. beckeri*, it can be distinguished by the curved depressed line on the lateral surface of TX (Fig. 19D).

Geographical Distribution. This morphospecies is known for Serra do Japi, Jundiaí, São Paulo State.

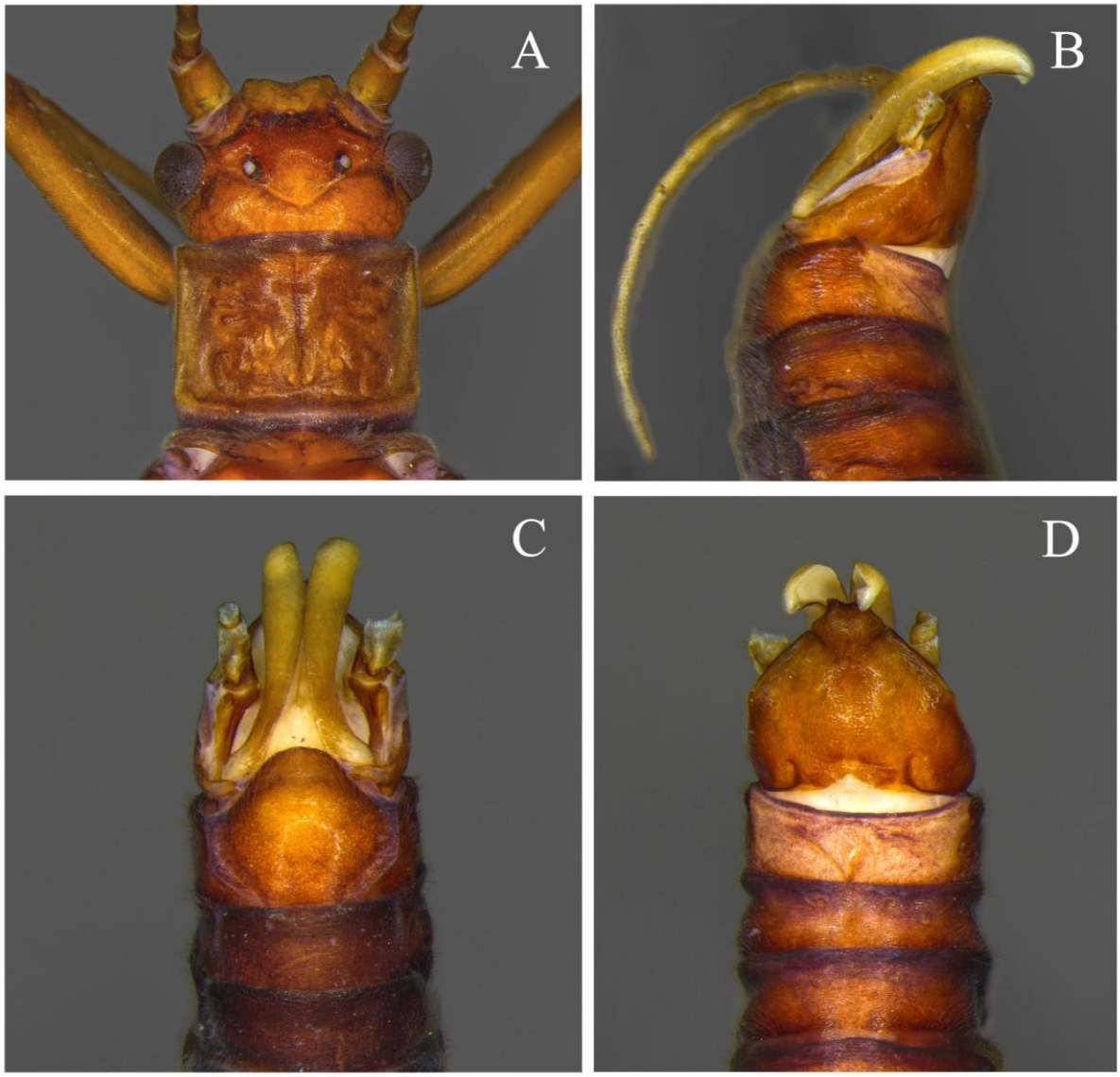

Figure 19. *Guaranyperla* sp. 3 from Jundiaí, São Paulo State (MZUSP). (A) Adult male, head and pronotum, dorsal view; (B, C, D) terminalia, lateral, ventral and dorsal views, respectively.

***Guaranyperla* sp. 4**

Figs. 20 A–C

Examined material. Brazil. São Paulo. Jundiaí, Reserva Biológica da Serra do Japi, Córrego Cachoeira do Paraíso, 23°14'35.4"S 46°56'55.1"W, 17.x-17.xi.2022, Malaise trap, 1 female (DNA voucher MNF110) (CIACGF); same data except 04.v.2023, LH Almeida, PN Taniguti,

FPR Sarmento, 1 nymph (DNA voucher MNF187) (CIACGF); same data except 02.v.2023, 1 nymph (DNA voucher MNF195) (CIACGF).

Measurements. Female: head width, 0.98 mm; pronotum width, 0.91 mm; pronotum length, 0.72 mm; PW/PL= 1.26; forewing length, 10.9 mm; FL/PW=12; paraproct length, 3.8 mm. Nymph: body length, 4.8 mm; head width, 0.8 mm; pronotum width, 1.4 mm; pronotum length, 0.64 mm; PW/PL= 2.1; antenna length, 4.4 mm.

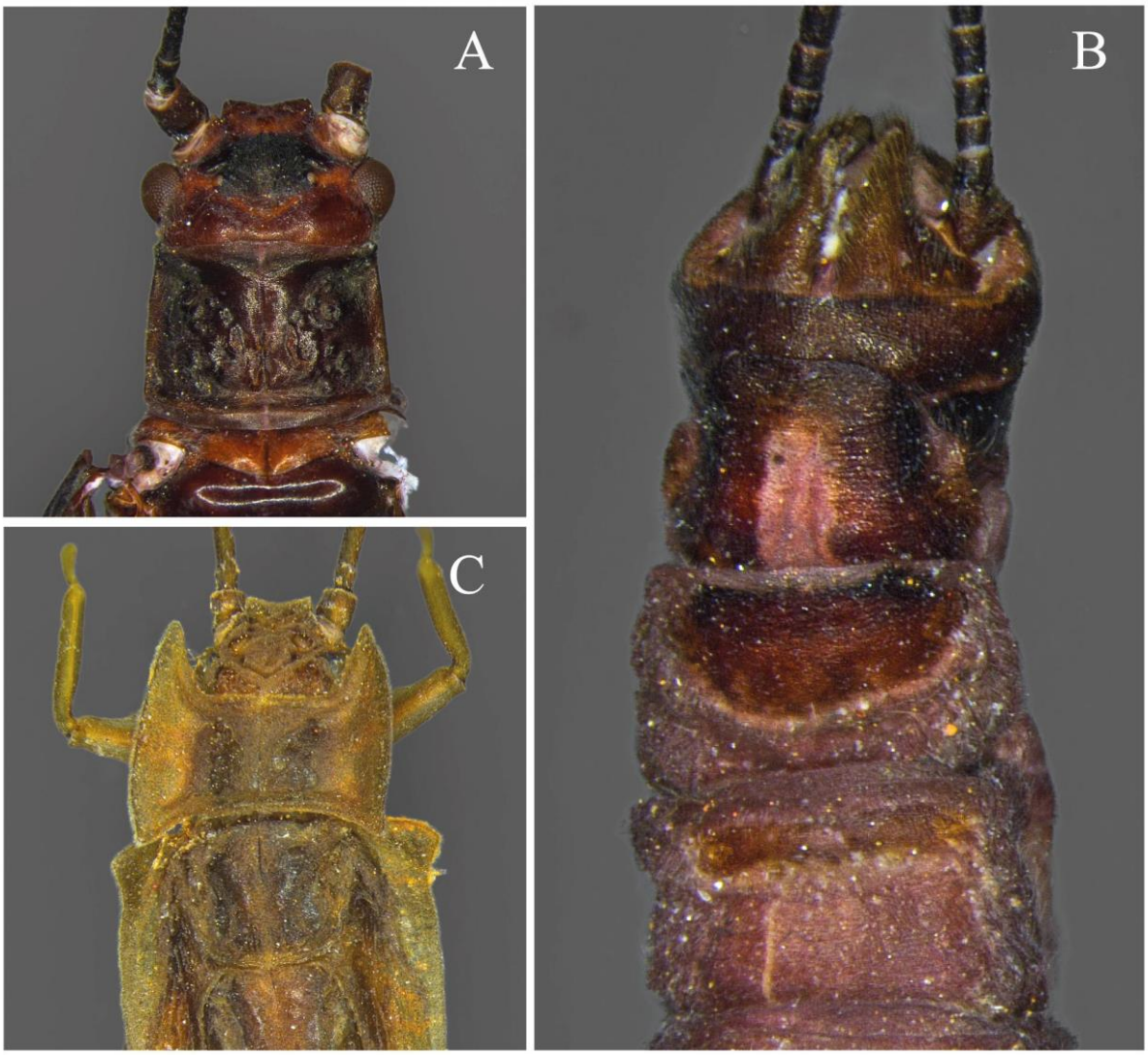

Figure 20. *Guaranyperla* sp. 4 from Jundiaí, São Paulo State (CLBA). (A) Adult female, head and pronotum, dorsal view; (B) terminalia, ventral view. (C) Nymph head and thorax, dorsal view.

Remarks. Female and nymphs from Serra do Japi were clustered in the same clade in our species delimitation analysis, also representing a distinct species from *G. guapiara*. The female of *Guaranyperla* sp. 4 (DNA voucher MNF110) is similar to *Guaranyperla puri* **sp. nov.** in the shape and color pattern of the subgenital plate, except for the lateral margin more rounded in the former (Fig. 20B). Nymphs of both species can be distinguished by the shape of pronotum, with the anterior margin of pronotum and the inner margin of the latero-anterior projection more angular in *Guaranyperla* sp. 4 (Fig. 20C). However, additional morphological characters need to be examined in nymphs to ensure a more robust diagnosis.

Geographical Distribution. This morphospecies is known for Serra do Japi, Jundiaí, São Paulo State.

***Guaranyperla* sp. 5**

Guaranyperla guapiara nec Froehlich, 2015

Figs. 21A–D

Examined material. Brazil. São Paulo. Pindamonhangaba, Fazenda São Sebastião, 22°46'19"S 45°27'29"W, Rio Cedro, 18.ix.2006, AE Siegloch, 1 male (MZUSP002220).

Measurements. Male: head width, 0.9 mm; pronotum width, 0.8 mm; pronotum length, 0.6 mm; PW/PL=1.3; forewing length, 9.5 mm; FL/PW=11.4; paraproct width, 0.1 mm; paraproct length, 1.7 mm; PaW/PaL=0.06.

Remarks. Froehlich (2015) determined the male from Pindamonhangaba, São Paulo State as *G. guapiara*, illustrating the terminalia and describing its morphology. However, since this specimen was not collected in the type-locality and the terminalia differs from the male of *G. guapiara*, we are considering it as another morphospecies. *Guaranyperla* sp. 5 has the paraproct tapering abruptly at apex (Fig. 21D), different from other males of the genus and probably represent a new species. Despite that, the terminalia of *Guaranyperla* sp. 5 also resembles *G puri* **sp. nov.**, *Guaranyperla* sp. 1, *Guaranyperla* sp. 3, and *G. beckeri*. Therefore, more semaphoronts and diagnostic characters must be assessed in order to formally describe it.

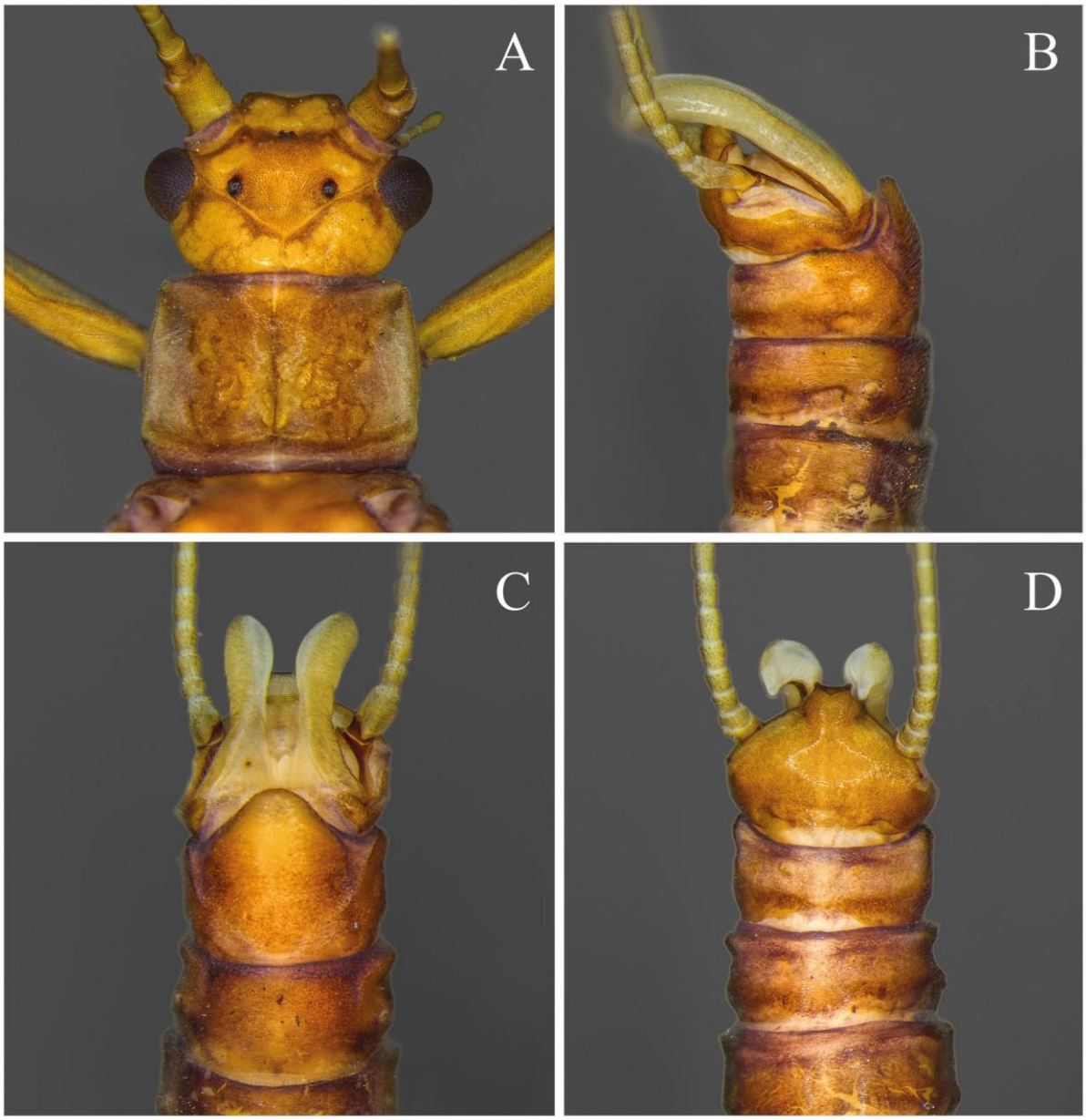

Figure 21. *Guaranyperla* sp. 5 from Pindamonhangaba, São Paulo State (MZUSP). (A) Adult male, head and pronotum, dorsal view; (B, C, D) terminalia, lateral, ventral and dorsal views, respectively.

Identification keys

Below, we provide keys to male and female adults, as well as to immatures of *Guaranyperla*. The keys do not include the unidentified material.

Males.

1. Head with frontal suture straight (Fig. 4A); paraproct without apical projection (Fig. 4I); posterolateral tooth of TXE present (Fig. 7B) ……..…..…..…… 2

1’. Head with frontal suture slightly curved (Fig. 13A); paraproct with twisted apical projection (Fig. 13E); posterolateral tooth of TXE absent (Fig. 13G) ……..……..….. 5

2 (1). TXE with posterior margin wider than the width of its anterior portion (Fig. 8D), length almost reaching the paraproct apex; TX with lateral clefts (Fig. 8D) ….. *Guaranyperla nitens*

2’. TXE with posterior margin narrower than the width of its anterior portion (Fig. 15F), or approximately the same size (Fig. 4G); TX without lateral clefts (Fig. 4G) …….…….…. 3

3 (2’). Anterior portion of TXE with lateral margin contiguous with TX lateral margin (Fig. 7D) .. ..…… *Guaranyperla beckeri*

3’. Anterior portion of TXE with lateral margin non-adjacent with TX lateral margin (Fig. 4G) ……...……..... 4

4 (3’). TXE with posterior margin almost as wide as anterior portion (posterior margin wider than 65% of anterior portion) (Fig. 4G) ...…….…… *Guaranyperla guapiara*

4’. TXE with posterior margin distinctly narrower than medial portion (posterior margin narrower than 65% of anterior portion) (Fig. 15F) ……...…. *Guaranyperla puri* **sp. nov.**

5 (1’). TXE narrow at anterior portion, wide towards apex (Fig. 11A); paraproct with strong and long apical projection (Figs.11 C, F) ……..... *Guaranyperla barbosai* **comb. nov.**

5’. TXE wide at anterior portion and narrow towards apex (Fig. 13E); paraproct with short apical projection (Figs. 13E, G) …….……...…… *Guaranyperla froehlichi* **sp. nov.**

Females.

1. Posterior expansion of subgenital plate longer than its base length (3 or 4 times longer) (Fig. 4F) 2

1’. Posterior expansion with almost the same length of its base (1½ to 1¼ times longer) or shorter (Fig. 13D) 3

2 (1). Posterior expansion of subgenital plate 4x longer than its base length, apex with a shallow emargination, with white pentagonal unpigmented area on its center (Fig. 4F) …… ……..……..*Guaranyperla guapiara*

2'. Posterior expansion of subgenital plate 3x longer than its base length, apex with an even margin, white rounded rectangular unpigmented area on center of subgenital plate (Fig. 15E) ……..… *Guaranyperla puri* **sp. nov.**

3 (1'). Posterior margin of TX with a short rounded projection present (Fig. 8B); posterior expansion of subgenital plate ⅓ shorter than its anterior portion length, trapezoidal (Fig. 8B) ……..……..…… *Guaranyperla nitens*

3'. Posterior margin of TX with a short rounded projection absent (Fig. 10E); head with frontal suture slightly curved (Fig. 13B) …….. 4

4 (3'). Subgenital plate with a narrow triangular depression weakly sclerotized centrally, posterior expansion of subgenital plate almost ½ the length of its anterior portion, apex completely covering sternite IX (Fig. 13D) ……..……. *Guaranyperla froehlichi* **sp. nov.**

4'. Subgenital plate with a light purple region on its center, posterior expansion of subgenital plate ½ as long as its base length, apex not covering sternite IX completely (Fig. 10E) …… ……..……..…… *Guaranyperla barbosai* **comb. nov.**

Nymphs.

1. Posteromedial projection longer than TX, tapering almost linearly towards a long projection (Fig. 9C). Width of paranota ⅔ the width of the pronotum (Fig. 9A) ..... …… *Guaranyperla nitens*

1'. Posteromedial projection shorter (Fig. 5C) or almost equal to TX (Fig. 12A). Width of paranota ½ (Fig. 12A) or ⅓ (Fig. 5A) the width of the pronotum ...…… 2

2 (1'). Posteromedial projection in deltoid or rhomboid shape (Fig. 12B) ……..…… 3

2'. Posteromedial projection in acuminate shape (Fig. 5C) ….…….……..……. 4

3 (2). TX with lateral margin slightly concave (14B), body yellowish ochraceous with fewer vesicular hair and forming patterns (Fig. 14A–C) …...……. *Guaranyperla froehlichi* **sp. nov.**

3’. TX with lateral margin slightly convex (12B), body brown without patterns formed by vesicular hairs (Fig. 12A–C) ……. *Guaranyperla barbosai* **comb. nov.**

4 (2’). Width of paranota ⅓ the width of the pronotum (Fig. 5B). Mesothoracic paranota with anterior corner rounded and slightly projected; apex of fore and hind wing pads rounded (Fig. 5A) ……..……. *Guaranyperla guapiara*

4’. Width of paranota ½ the width of the pronotum (Fig. 16B). Mesothoracic paranota with anterior corner acute and projected; apex of fore and hind wing pads acute (Fig. 16A) …… ……..…… *Guaranyperla puri* **sp. nov.**

Discussion

Guaranyperla comprises six species at present. However, at least three other species await formal description, but require examination of additional specimens and genetic analysis. Describing species based on an incomplete set of semaphoronts and associations made without molecular tools or by rearing is discouraged due to the cryptic diversity found in the genus. Sympatry (*e.g.*, *G. barbosai* and *G. froehlichi*), along with inconsistent morphological differences among some species, means that associations lacking confident criteria may result in taxonomic errors. Diagnostic characters for *Guaranyperla* sp. 2 and *Guaranyperla* sp. 4, for instance, were considered fragile and the molecular delimitation analysis alone is not sufficient to formally describe them. It is necessary to collect more specimens from these localities, with all semaphoronts, males and females.

Intraspecific and interspecific K2P divergences found suggest a wide barcode gap, supporting results of the other methods of species delimitation used. Intraspecific divergences had a maximum of 2.3% while interspecific divergences showed a minimum of 4.3%, even in very closely related species. Within *Guaranyperla*, specimens of *G. nitens* and *G. puri* **sp. nov.** showed the highest interspecific pairwise divergence, 16%. According to Mynott *et al.* (2011), the observed high divergences may be attributed to the isolation of populations situated at high altitudes, wherein altitude acts as a dispersal barrier for these aquatic insects. Since

plecopterans exhibit low motility (Boumans & Baumann 2012; Mynott *et al.* 2011), this suggests that populations may be isolated to specific watersheds across southern Brazil. As such, the diversity of *Guaranyperla* may still be underestimated.

This study also constitutes an effort to delimit *Guaranyperla* from other gripopterygines. Herein, we have analyzed a significant number of specimens of the genus from different regions of Southeastern Brazil. We have provided more morphological data for the studied species within the genus, as well as new DNA sequences. These additional data should help prevent future misidentifications, and enable more reliable placement of species into *Tupiperla* or *Guaranyperla*. Nonetheless, while differences between these genera have been highlighted, we encourage stonefly researchers to associate immatures from *Tupiperla* species due to the lack of nymphal descriptions for the referred genus.

We also expanded species' geographical occurrences. The genus seems to be restricted to the Atlantic Rainforest; however, more collection effort is necessary in surrounding ecoregions to confirm this distribution. Also, collections in more localities in the Atlantic Rainforest are equally important to elucidate species diversity and their respective ranges. It is still necessary to evaluate more accurately the intraspecific variation within the genus, in order to facilitate the consistent morphological species delimitation.

In this study we described two new species, provided important morphological and molecular data, and proposed nomenclatural changes. As for next steps, a phylogenetic analysis of the group, using more species of *Guaranyperla* and *Tupiperla* and more genetic markers associated with morphological characters, will create more solid foundations for the taxonomy and evolution of Neotropical Gripopterygidae.

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Supplementary material

Supplementary material is available online at:

https://doi.org/10.6084/m9.figshare.29851028

File S1 Pairwise K2P divergences of COI sequences of *Guaranyperla* and outgroups.

Figure S1 Measurements of morphological structures gathered in this study. A) Adult head width and pronotum length and width. B) Nymph head width and pronotum length and width. C) Female paraproct length. D) Male paraproct length and width. E) Antenna length. F) Body length.

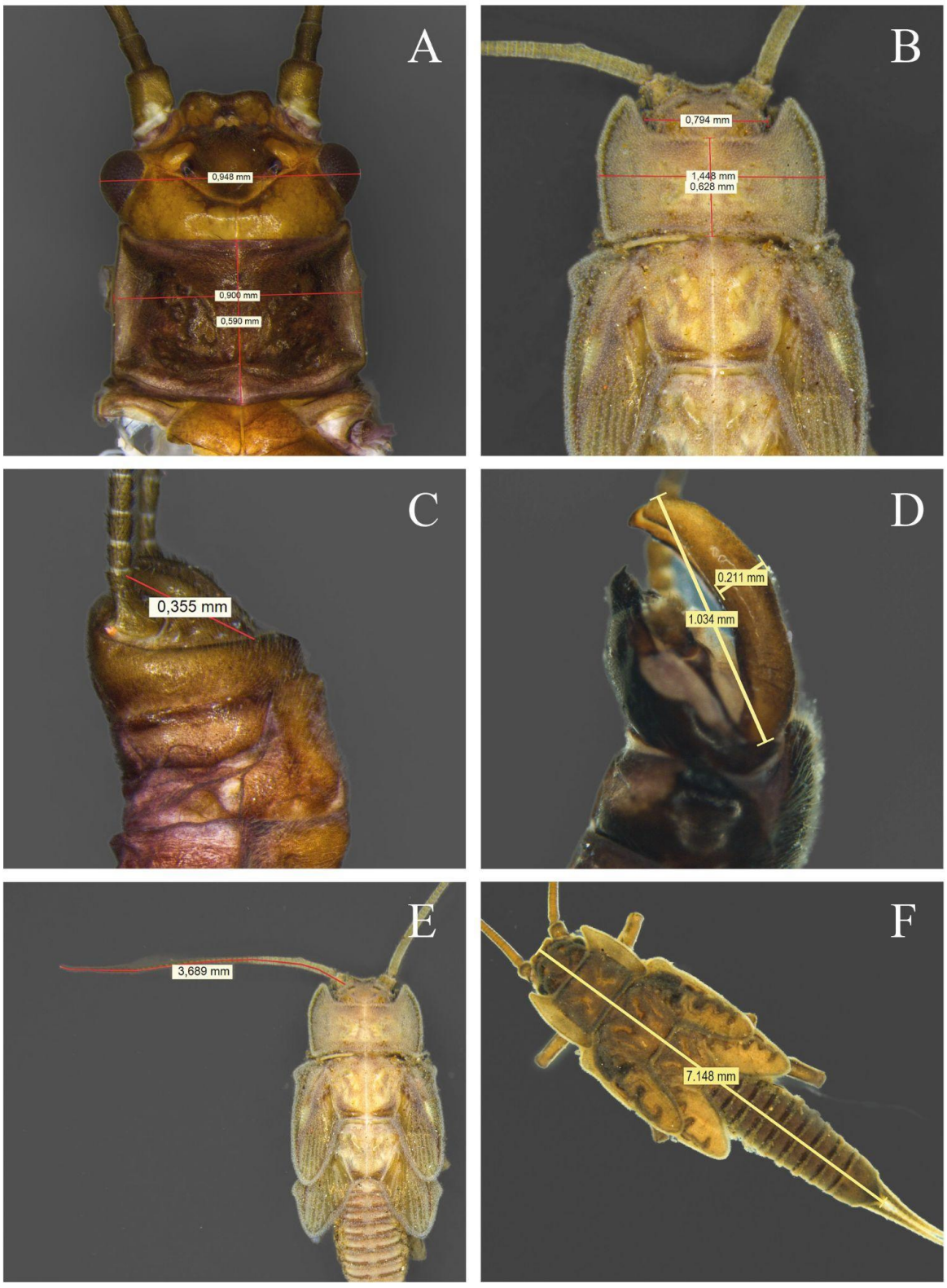


Figure S2 Bayesian consensus (MrBayes) of the analysis of COI sequences partitioned by codon position of *Guaranyperla* and outgroups. Values above branches are >80 SH-aLRT / >95 UFBoot, and values below are Bayesian posterior probabilities >95.

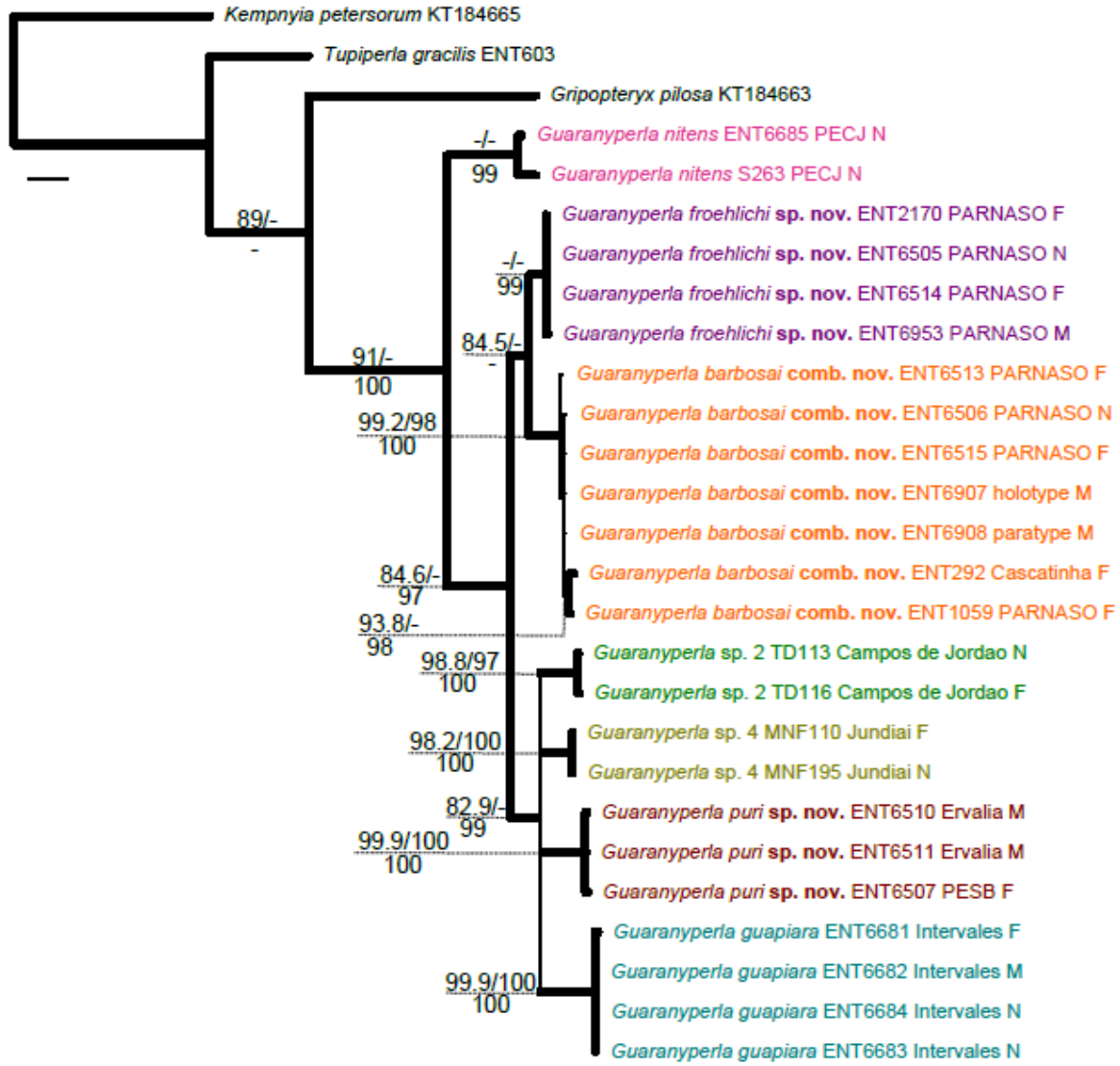
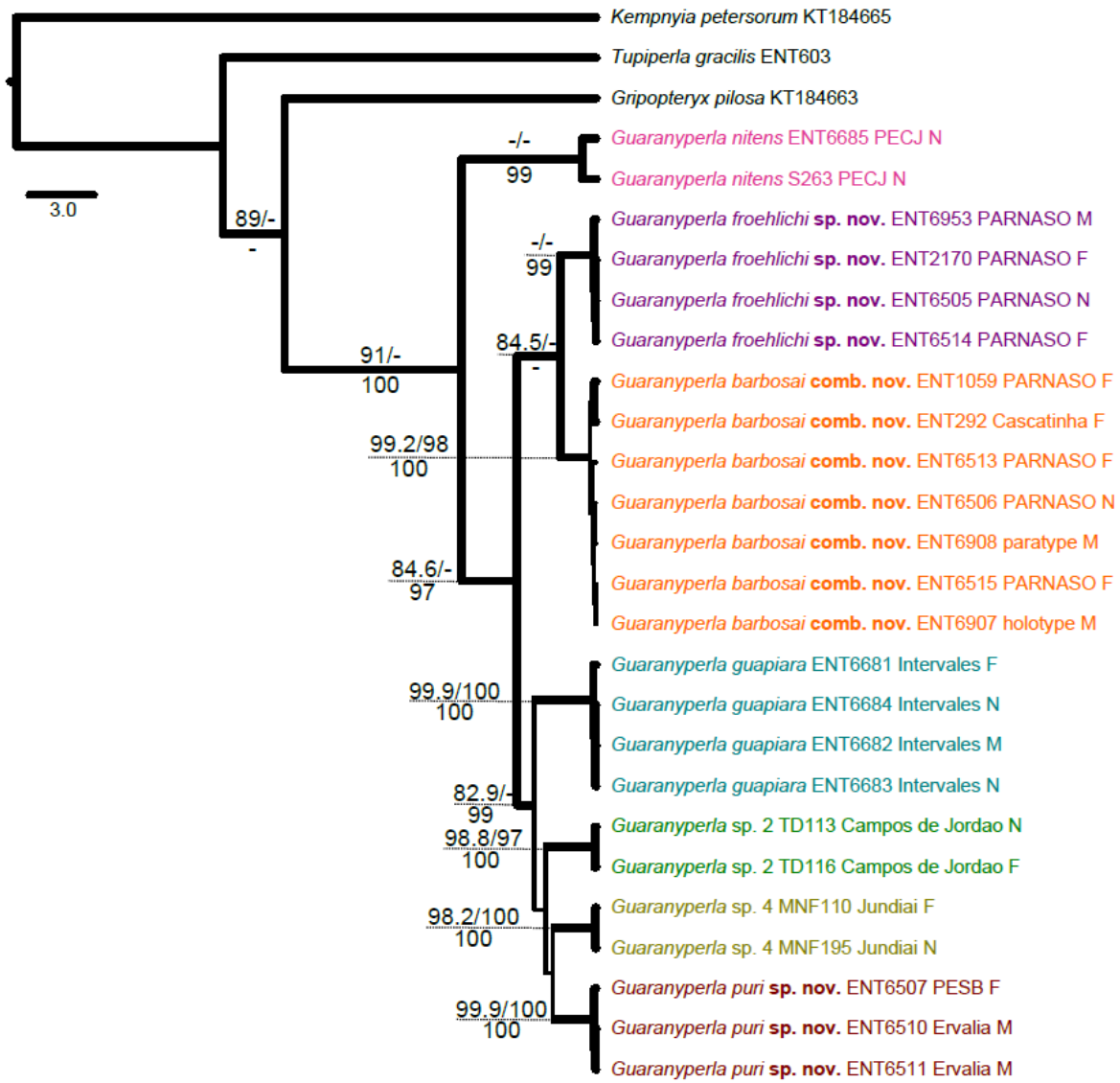


Figure S3 Bayesian ultrametric consensus (BEAST) of the analysis of COI sequences of *Guaranyperla* and outgroups. Values above branches are >80 SH-aLRT / >95 UFBoot, and values below are Bayesian posterior probabilities >95.



Author contribution Conceptualization, investigation, M.L.S.R. and F.F.S; molecular studies, J.S.P., D.M.T., L.H.A. and F.A.C.; morphology studies, M.L.S.R., F.F.S. and L.H.A; methodology, M.L.S.R., F.F.S., L.H.A., D.M.T., J.S.P. and F.A.C.; writing—original draft preparation, M.L.S.R. and F.F.S.; writing—review and editing, M.L.S.R., F.F.S., L.H.A. and D.M.T.; project administration, M.L.S.R., D.M.T. and F.F.S. All authors have read and agreed to the published version of the manuscript.

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Data availability All data are incorporated into the article and its online supplementary material. Sequence data used in this study are all available via GenBank. Phylogenetic data are deposited in TreeBASE under the link http://purl.org/phylo/treebase/phylows/study/TB2:S31613.

Statements and Declarations

Ethics approval Our sampling followed Brazilian laws and were authorized by the SISBIO-ICMBio (Biodiversity Authorization and Information System, Chico Mendes Institute for Biodiversity Conservation, numbers 79695-1, 55428-16, and 65213-11), also by IEF (Minas Gerais State Institute of Forests, number 058/2021) and IPA/SIMA (São Paulo State Institute of Environmental Research, number 15054/2022 and 5579/2023).

Conflict of interest The authors declare that they have no competing interests in relation to this work.

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CHAPTER II - ANATOMY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEM OF NEOTROPICAL GRIPOPTERYGIDAE (PLECOPTERA)

Anatomy and histology of the male and female reproductive systems of Neotropical Gripopterygidae (Plecoptera)

Mellis L. S. Rippel[1,2]; Dayvson A. Costa[1,3]; Rodrigo B. Gastaldo[1,2]; José Lino-Neto[3], Frederico F. Salles[2]

[1]Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil.

[2]Departamento de Entomologia, Museu de Entomologia, Universidade Federal de Viçosa (UFV), 36570-900, Viçosa, Minas Gerais, Brazil.

[3]Departamento de Biologia Geral, Laboratório de Ultraestrutura Celular, Universidade Federal de Viçosa (UFV), 36570-900, Viçosa, Minas Gerais, Brazil

Abstract

This study provides the first comprehensive anatomical and histological characterization of the male and female reproductive systems in Neotropical Gripopterygidae (Plecoptera), encompassing all four genera restricted to the region, represented by eight species. We identified notable anatomical differences, particularly in *Tupiperla robusta*, whose reproductive system diverges from that of congeners. In contrast, the histological organization of ovaries and testes was largely conserved across genera, indicating a shared reproductive strategy. All ovaries were panoistic, and testes displayed continuous spermatogenesis. Females lacked both spermathecae and accessory glands. Our findings include the first complete histological description of the male reproductive system in Antarctoperlaria, as well as the first detailed account of the female reproductive system in Gripopterygidae. Together, these results fill an important gap in Plecoptera reproductive biology and provide a foundation for future phylogenetic and taxonomic studies within the family.

Keywords. Stoneflies; Internal Morphology; Reproduction.

Introduction

Stoneflies (Plecoptera Burmeister, 1839) play a crucial role in stream ecosystems globally. They serve as indicators of water quality, provide food for predators, and facilitate energy flow and nutrient cycling, contributing to essential ecosystem services (DeWalt & Ower 2019). The current classification system of the Plecoptera is based on (Zwick 2000) and still

well accepted by specialists, consisting of two suborders: Arctoperlaria, predominantly found in the Holartic region, and Antarctoperlaria, with a Gondwanan distribution. This classification is supported by phylogenetic studies using morphological and molecular data (mitochondrial genome, COI, transcriptome, 18S, H3), despite presenting contradictory hypotheses (McCulloch *et al.* 2016; Ding *et al.* 2019; South *et al.* 2021; Chen 2022).

Antarctoperlaria comprises two superfamilies: Eustheniodea, including Eustheniidae and Diamphipnoidae, and Gripopterygoidea containing Gripopterygidae and Austroperlidae (Zwick 2000). In the southern region of South America, Gripopterygidae exhibits remarkable diversity in the number of genera (Pessacq *et al.* 2019). This family comprises approximately 320 described species, grouped into 55 genera, distributed across Australasia and South America (Pessacq *et al.* 2019; Duarte *et al.* 2024; DeWalt *et al.* 2025). McLellan (1977) proposed the current classification of the family without a cladistic analysis, dividing it into five subfamilies: Antarctoperlinae Enderlein, 1909, Dinotoperlinae McLellan, 1977, Gripopteryginae Enderlein, 1909, Leptoperlinae Banks, 1913, and Zelandoperlinae McLellan, 1977. Although the monophyly of these subfamilies remain untested under a cladistic framework, intergeneric relationships within Gripopterygidae are still poorly resolved. To date, no phylogenetic analysis has encompassed all groups within the family, and the delimitation of genera in South America requires revision (McLellan & Zwick 2007; McCulloch *et al.* 2016; Pessacq *et al.* 2020; Letsch *et al.* 2021).

Gripopterygidae exhibits a disjunct distribution in South America, with 50 species in 24 genera occurring in the Andean Region (*sensu* (Morrone 2015) and 62 species in four genera in the Neotropical Region (*sensu* (Morrone 2014). These genera—*Gripopteryx* Pictet, 1841, *Paragripopteryx* Enderlein, 1909, *Tupiperla* Froehlich, 1969, and *Guaranyperla* Froehlich, 2001—account for all Neotropical species, with 59 of them primarily distributed in the Brazilian Atlantic Forest (Duarte *et al.* 2024; DeWalt *et al.* 2025). All Neotropical species belong to Gripopteryginae, which is endemic to South America (Pessacq *et al.* 2019; DeWalt *et al.* 2025).

Research on the reproductive system (RS) of Plecoptera has established a solid foundation for understanding the reproductive anatomy of male and female stoneflies. These studies have advanced our knowledge of the group's internal morphology, and key aspects of the biology and phylogeny of the order (Brinck 1956; Zwick 1973, 1980; Rościszewska & Rzońca 2009; Fausto et al. 2023). Both external and internal genitalia morphology and function

among various plecopteran taxa have been explored, including mechanisms of sperm transfer (Brinck 1956; Zwick 1973, 1980). Among these contributions, the seminal work of Brinck (1956) stands out, as he provided a standardized terminology for the Plecoptera reproductive system by reviewing the taxonomic literature available at that time. His study organized the terminology for both male and female reproductive structures across several families of Arctoperlaria, unifying the nomenclature of internal and external components. This standardization not only systematized the knowledge of reproductive anatomy but also offered a consistent framework that facilitated comparative studies and contributed to a more natural classification of the group.

Despite the relevance of these contributions, most studies have remained geographically and taxonomically restricted. In fact, the vast majority have focused on species from the Northern Hemisphere (Stewart et al. 1969; Stewart & Stark 1977; Stark & Szczytko 1988; Rościszewska 1997; Rosciszewska & Soldan 1999; Rościszewska 2001; Poprawa et al. 2002), resulting in a scarcity of knowledge regarding the anatomy of RS for Antarctoperlaria (Illies 1960; Vera 2006; Rościszewska & Rzońca 2009). Moreover, only one of these studies specifically addresses the histology of the female reproductive system (FRS) in Antarctoperlaria using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), but only for larval ovarioles in one genus of the Australian Gripopterygidae (*Trinotoperla nivata* Kimmins, 1951), and adults and larvae from two Australian genera of Eustheniidae (Rościszewska & Rzońca 2009). Thus, no mature and complete FRS of Gripopterygidae has been described thus far.

Considering these constraints, we aim to describe and illustrate, for the first time, the anatomy and histology of the male and female reproductive systems of Neotropical Gripopterygidae, encompassing all four genera restricted to the region and represented by eight species. By addressing this gap, our study establishes a baseline for future research on reproductive biology in the group and provides data that may be useful for comparative and systematic studies.

Material and Methods

2.1 Collection and preparation of material

We collected male and female adults of Gripopterygidae at night using a light sheet trap along the banks of streams at different locations in the Atlantic Forest of the states of Minas Gerais and São Paulo, southeastern Brazil. Examined material is listed below:

Gripopteryx garbei Navás. São Paulo State, Ribeirão Grande municipality, Intervales State Park, 24º18’24’’S 48º24’52’’W, 15.viii.2023, two females (code. 15.viii.23-01), (code. 15.viii.23-02); Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 16–17.ii.2023, one male (code. 16-17.ii.23).

Gripopteryx pilosa Froehlich. Minas Gerais state, Espera Feliz municipality, Caparaó National Park, 20º28’19’’S 41º49’44’’W, 24.viii.2024, two females (code. 24.viii.24-01), (code. 24.viii.24-02), three males (code. 24.viii.24-01), (code. 24.viii.24-02), (code. 25.viii.24-01).

Gripopteryx reticulata Brauer. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W, 20.ix.2023, one female (code. 20.ix.23), 22.x.2022, one male (code. 01-22.x.22); Serra do Brigadeiro State Park, 20º43’52’’S 42º27’50’’W, 19.v.2024, one male (code. 19.v.24). São Paulo State, Salesópolis municipality, Boraceia Ecological Station, Rio Claro, 23º39’2’’S 45º54’42’’W, 22.iv.2024, one female (code. 22.iv.24); Venerando stream, 23º39’9’’S 45º53’26’’W, 19.iv.2024, one male (code. 19.iv.24).

Guaranyperla puri Rippel & Salles. Minas Gerais state, Ervália municipality, Complexo Turístico do Pico do Cruzeiro, 20º46’37’’S 42º29’49’’W, 07.x.2022, one male (code. 07.x.22); Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 05.x.2024, one male (05.x.24-01).

Paragripopteryx delicata Froehlich. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 15–16.ii.2023, one male (code. 15-16.ii.23).

Paragripopteryx sp. 1. São Paulo State, Salesópolis municipality, Boraceia Ecological Station, Venerando stream, 23º39’9’’S 45º53’26’’W, 22.iv.2024, one female (22.iv.24).

Paragripopteryx sp. 2. Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’23’’S 41º48’29’’, 24.viii.2024, one female (code. 25.viii.24-01).

Paragripopteryx sp. 3. Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’23’’S 41º48’29’’, 24.viii.2024, one female (code. 05.xi.24-01).

Tupiperla gracilis (Burmeister). Minas Gerais state, Ouro Preto municipality, Andorinhas Municipal Park, 20º21’30’’S 43º29’18’’W, 04.v.2024, one female (code. 06.v.24-01); Tripuí State Park, 20º23’5’’S 43º32’34’’W, 02.v.2024, two males (code. 02.v.24-01), (code. 04.v.24-01), 03.v.2024, one male (code. 06.v.24-01); Santos Dumont municipality, 21º26'45.6"S 43º40'18.6", 23–29.xii.2024, one female (code. 04.i.25).

Tupiperla robusta Froehlich. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 14.ii.2023, one female (code. 14.ii.23-01), one male (code. 14.ii.23-02), 15.ii.2023, one female (code. 15.ii.23-02), 16.ii.2023, one male (code. 16.ii.23-01), 21–22.iii.2023, one female (code. 23.iii.23). Rio de Janeiro state, Teresópolis municipality, Serra dos Órgãos National Park, Pedra do Sino trail, 22º26’53’’S 43º0’17’’W, 08.xii.2024, one male (code. 09.xii.24-01).

Tupiperla tessellata (Brauer). Minas Gerais state, Ouro Preto municipality, Itacolomi State Park, 20º27’25’’S 43º30’12’’W, 03.v.2024, one female (code. 05.v.24-01), one male (code. 04.v.24-01); Espera Feliz municipality, Caparaó National Park, 20º30’23’’S 41º48’29’’W, 24.viii.2024, one male (code. 25.viii.24-01); Santos Dumont municipality, Mantiqueira region, 21º26'45.6"S 43º40'18.6", 23–29.xii.2024, one male (code.03.i.25). Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park, 20º30’5’’S 41º49’8’’W, 21.viii.2024, one female (code. 22.viii.24-01), one male (code. 22.viii.24-01).

The captured specimens were placed alive in small vials and initially identified at the species level using identification keys from (Bispo & Lecci 2011; Lecci & Froehlich 2011), and original descriptions (Froehlich 1969, 1993, 1994, 1998; Duarte *et al.* 2014, 2019, 2022). The terminology used for the reproductive system structures was adopted from Zwick (1973) and Rościszewska & Rzonca (2009). When possible, we identified and dissected the specimens in the field, to guarantee the specimen’s survival for dissection. Alternatively, we transported the material alive to the Laboratory of Cellular Ultrastructure in the Department of General Biology, and the Entomology Museum of UFV (UFVB), both at the Federal University of Viçosa (UFV), where specimens were identified and dissected. We also transferred individuals

from each species to 90% alcohol for preservation. Images of the species were obtained with a LEICA MC170 HD camera and edited using Adobe Photoshop CC® 2024. Plates were assembled in Adobe Illustrator CC® 2024.

2.2 Anatomy and histology of the male and female reproductive systems (MRS and FRS)

We dissected at least two individuals of male and female from each species and genus, totaling 66 specimens (see Supporting Information, Table S1). For the dissection, we used 0.1 M sodium phosphate buffer (PBS), pH 7.2, and fixed for 2 – 4 h in a 2.5% glutaraldehyde solution in the same buffer. Subsequently, we transferred the samples to an excavated slide in the same solution and photographed using the equipment described in section 2.1. We also used Adobe Illustrator CC® 2024 for creating the schematic drawings.

Afterwards, we prepared the fixed samples for the histological analyses. We also post-fixed some samples in 1% osmium tetroxide for two hours, then stained them with Harris Haematoxylin for three minutes to facilitate the subsequent steps. We washed all reproductive organs in distilled water (5 times for 10 minutes each), then dehydrated them in an increasing ethanolic series (30%, 50%, 70%, and 90% and three baths in 100% alcohol) and subsequently infiltrated and blocked in historesin (Leica Historesin, Heidelberg, Germany), and finally polymerized at 60 ºC over 12 hours.

We obtained semi-thin sections (0.5 μm) on a Leica RM 2155 (Leica Corporation, Wetzlar, Germany) microtome with glass knives, and later we transferred the sections to histological slides. We then stained the resulting sections with Giemsa and covered them with coverslips using a 50% sucrose solution. We thoroughly analyzed and photographed the material with an Olympus BX-60 microscope with a QColor3 digital camera. Finally, we captured the images using 20x, 40x and 100x objectives and later assembled them using the Photomerge function of the Adobe Photoshop 2024 software.

Results

3.1 Descriptions

The male and female reproductive systems of species from *Guaranyperla*, *Gripopteryx*, *Paragripopteryx*, and *Tupiperla* (Supplementary Figure S1) were examined for comparative

analysis, photographed, measured (see Supporting Information, Tables S2 and S3) and illustrated (Figs 1A–C; 9A–C). We successfully described the FRS anatomy of three species of *Gripopteryx*, three species of *Paragripopteryx*, and three species of *Tupiperla*, as well as the MRS anatomy of two species of *Gripopteryx* (excluding *G. garbei*), one species of *Guaranyperla*, and three species of *Tupiperla*. However, we could not examine the MRS anatomy of *Paragripopteryx delicata*, which was described only histologically. We also did not collect material from females of *Guaranyperla puri*, retrieving the description of both histology and anatomy of the FRS of *Guaranyperla*. Histological analyses were not conducted for *G. reticulata* and *T. gracilis*, as our data indicated minimal variability within these genera.

The three females of *Paragripopteryx* were not identified due to the lack of definitive diagnostic characteristics and the absence of males from the same collection site, which prevented reliable association. Consequently, they are treated here as *Paragripopteryx* spp., as they represent distinct morphospecies.

Anatomy and histology of the Female Reproductive System (FRS)

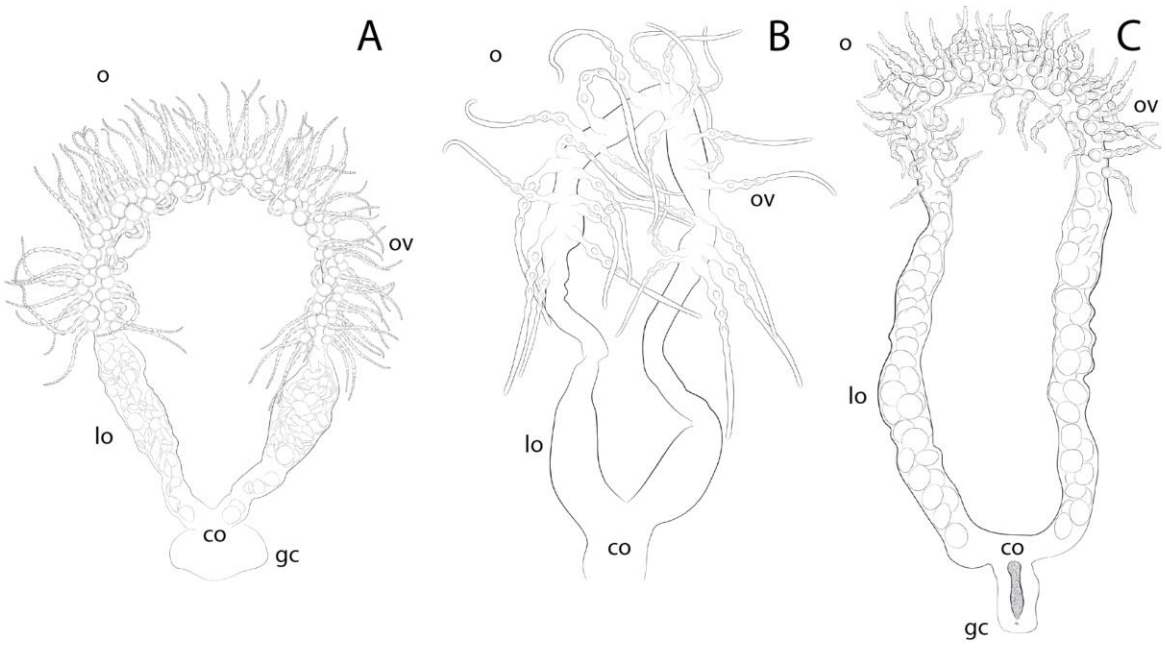

Figure 1. Illustrations of the female reproductive system of (A) *Gripopteryx pilosa*, (B) *Paragripopteryx* sp 2, and (C) *Tupiperla tessellata,* showing the paired ovaries (o) fused medially, the ovarioles (ov), lateral oviducts (lo), common oviduct (co), and the genital chamber (gc).

Gripopteryx garbei Navás, 1936

Figs. 2A–G

The FRS of *G. garbei* (n=2) comprises a pair of ovaries fused medially at the distal portion, forming an arch structure and a single tube to which the ovarioles are attached (length=0.47–0.50 mm) (Fig. 2A–B). Each ovary contains roughly 20 ovarioles (length=0.38–0.40 mm), which are composed of approximately 7 follicles in the different phases of differentiation, so oocytes mature simultaneously in all ovarioles (Fig. 2A–D). The germarium region is about 1/3 the ovariole length (Fig. 2C). The distal arch that contains the ovarioles is connected to the lateral oviduct, which terminates into a short common oviduct (Fig. 2A). The lateral oviduct is longer than the ovariole-bearing portion (length=0.70–0.95 mm), with a constant width over most of its length (Fig. 2–B). The common oviduct leads into the genital chamber, which is membranous and underlies almost the whole extension of the subgenital plate. Tracheae surround the whole ovary. Neither spermatheca nor accessory glands were observed. Eggs exhibit a dome shape, with a concave surface (Fig. 2D). The rim is bulky, tapering towards its edge, with a clear distinction between the domed center and the thicker edge. The eggs measure approximately 0.36 mm in length and 0.14 mm in height.

Histological analysis revealed that the ovaries of *G. garbei* are panoistic and consist of a germarium, a vitellarium, and a pedicel, enclosed by a thin epithelial sheath (Fig. 2E–D). Neither a terminal filament nor a peritoneal sheath was observed. The apex of the germarium could not be observed; however, the remaining region revealed oogonia arranged in a linear pattern, differentiating into oocytes, each with a centrally positioned nucleus (Fig. 2F), and surrounded by a layer of follicle cells. As they mature, increasing deposition of yolk granules becomes evident (Fig. 2E–F). The lateral oviduct consists of multiple muscular layers and an inner epithelial layer composed of columnar cells that appear more intensely stained than the surrounding tissues, suggesting heightened activity (Fig. 2G). The transition from the lateral oviduct to the common oviduct is evident, with the latter presenting a thin, wrinkled epithelial lining covered internally by a cuticle and externally by muscle tissue (Fig. 2G).

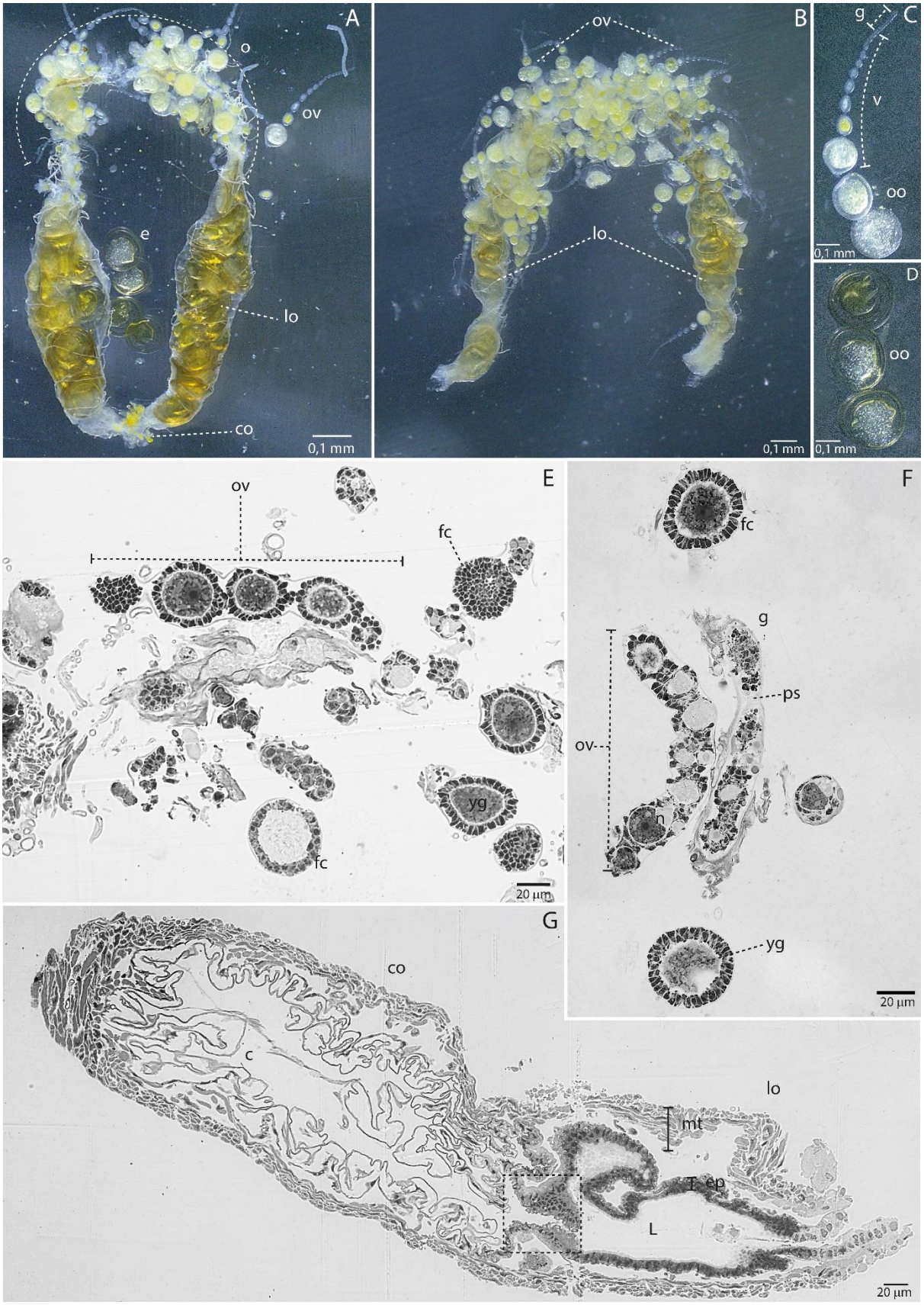

Figure 2. Anatomy and histology of the female reproductive system of *Gripopteryx garbei*. (A–B) Paired ovaries (o) fused medially, with ovarioles (ov), lateral oviducts (lo) containing eggs (e), and the common oviduct (co). (C) Ovariole structure showing the germarium (g) and

vitellarium (v), with developing oocytes (oo). (D) Detail of a mature egg and oocytes. (E) Histological section of the ovarioles (ov), displaying consecutive developmental stages of the oocytes surrounded by follicular cells (fc), with centrally located yolk granules (yg). (F) Detail of an ovariole enclosed by an epithelial sheath (es), showing the germarium (g) and oocytes at various developmental stages, with a centrally positioned nucleus (n). (G) Longitudinal section of the lateral oviduct (lo), externally surrounded by layers of muscle cells (mc), with an inner epithelial layer enclosing the oviduct's lumen (L). The dashed line marks the transition from the lateral oviduct to the common oviduct (co), which is internally lined with a thin, wrinkled epithelium containing cuticle (c).

Gripopteryx pilosa Froehlich, 1990

Figs. 1A; 3A–H

The FRS of *G. pilosa* (n=2) broadly aligns with that of *G. garbei*, except for the following differences: the ovaries length is 1.54–1.75 mm. Each ovary contains approximately 60 ovarioles, which are composed of about eight follicles and the germarium (Fig. 3A–B). The lateral oviduct is roughly the length of the ovariole-bearing portion (1.7–2.6 mm), and the width of its anterior portion is 0.35 mm. In comparison, the posterior portion is 0.30 mm (n=1) (Fig. 3A). The ovarioles measure 1.47 mm in length (n=1) (Fig. 3C). The eggs average 0.15 mm in width, while the genital chamber measures 0.74 mm in length and 1.20 mm in width (n=1).

No histological differences were observed between the FRS of *G. pilosa* and *G. garbei* (Fig 3C–H). However, examination of the germarium's apex revealed the presence of oogonia, which were not arranged in a linear pattern, along with somatic cells (Fig. 3D). Additionally, free spermatozoa were observed within the lateral oviduct (Fig. 3H).

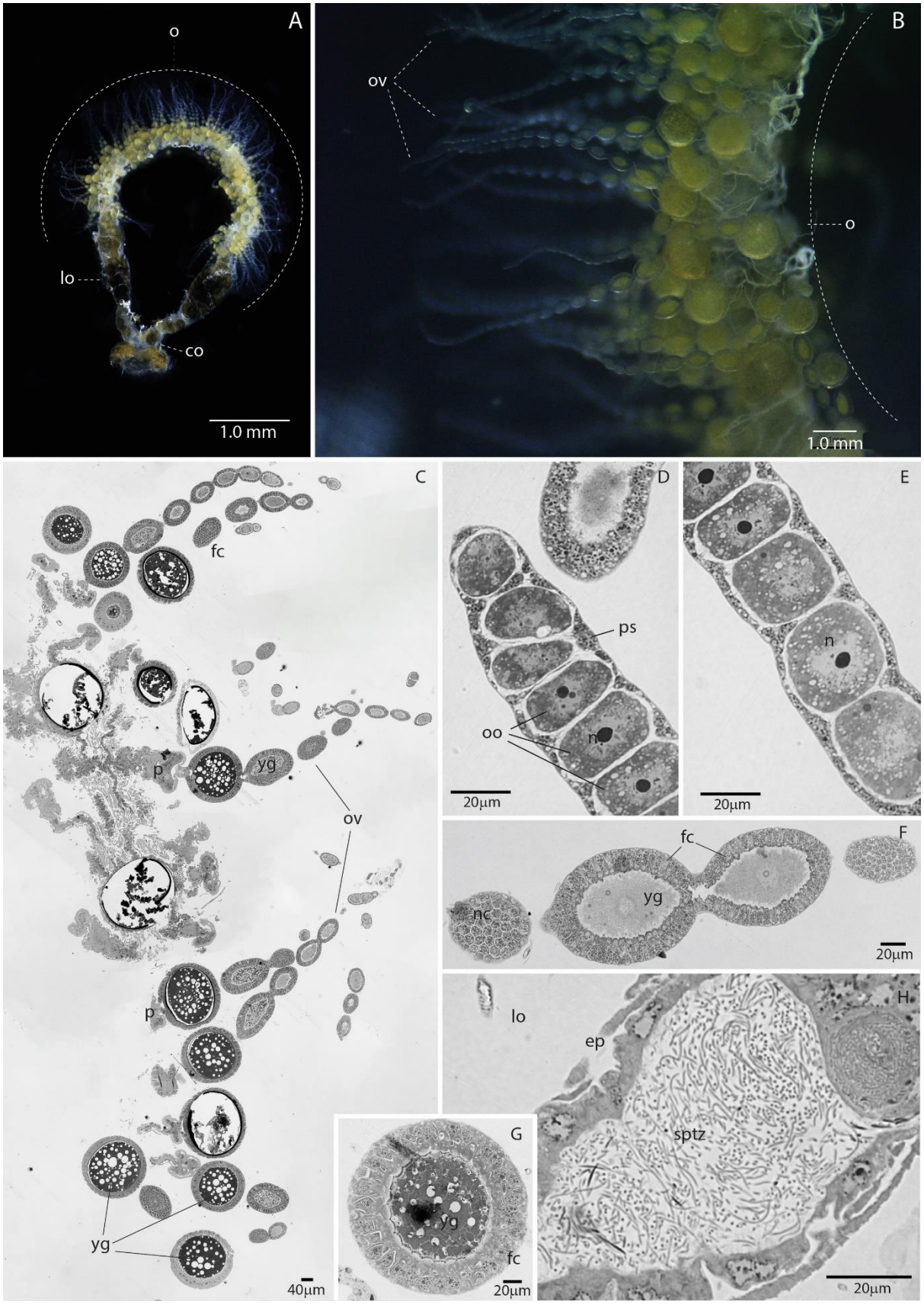

Figure 3. Anatomy and histology of the female reproductive system of *Gripopteryx pilosa*. (A) Paired ovaries (o) fused medially, the lateral oviducts (lo) containing eggs (e), and the common oviduct (co). (B) Detail of ovarioles (ov) connected to the ovary's (o) anterior portion showing

developing oocytes. (C) Histological section of the ovarioles (ov), displaying consecutive developmental stages of the oocytes (oo), surrounded by follicular cells (fc), with centrally located yolk granules (yg). Note the pedicel (p) connecting the ovariole to the lateral oviduct. (D) Germarium (g) region enclosed by an epithelial sheath (es), and containing early stages oocytes (oo). (E) Detail of the oocytes with a centrally positioned nucleus (n). (F) Detail of the vitellarium with oocytes surrounded by follicle cells (fc). (G) Magnified view of an oocyte encompassed by follicle cells (fc) containing yolk granules (yg) internally. (H) Longitudinal section of the lateral oviduct (lo), externally surrounded by a thick epithelial layer enclosing the oviduct's lumen full of free sperm cells (sptz).

Gripopteryx reticulata Brauer, 1868

Figs. 1B; 4A–D

The FRS of *G. reticulata* (n=2) is similar to that of other species in the genus, with a few distinctions. The ovaries length ranges from 1.0 to 1.81 mm, while the length of the ovarioles is 1.0 and 1.39 mm in both females (Fig. 4A–C). Each ovary contains around 60 ovarioles, which are composed of about eight oocytes and the germarium (Fig. 4C). The lateral oviducts measure 2.47 mm in length, its anterior portion has a width of 0.22 mm, and the posterior 0.30 mm (n=1) (Fig. 4A). The genital chamber has a length of 0.59 to 0.83 mm, and a width ranging from 0.18 to 1.26 mm (Fig. 4D).

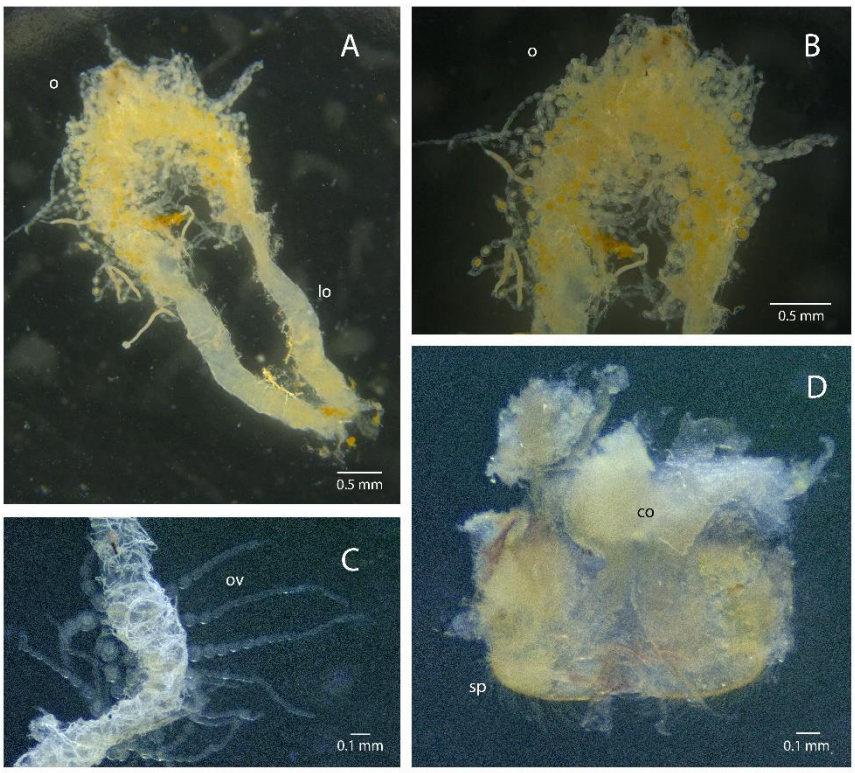

Figure 4. Anatomy of the female reproductive system of *Gripopteryx reticulata*. (A–B) Paired ovaries (o) fused medially and the lateral oviducts (lo). (C) Detail of the ovariole (ov) structure in the previtellogenic phase. (D) The common oviduct (co) still attached to the subgenital plate (sp) (ventral view).

Paragripopteryx spp.

Figs. 5A–E

The FRS of *Paragripopteryx* closely resembles that of *Gripopteryx* species. However, the three examined female specimens exhibit unique characteristics that are not observed in other genera. These include an enlarged proximal portion of the lateral oviducts that narrow distally (Fig. 5A); a relatively long pedicel with approximately the same size as its anterior follicle (Fig. 5B); and the number of oocytes varying from three to four in the same specimen. Mature oocytes were not observed. FRS measurements of the specimens examined are shown below.

- *Paragripopteryx* sp. 1 - ovaries length: 0.36 mm; lateral oviducts length: 0.54 mm, width of anterior portion: 0.04 mm and posterior portion: 0.09mm; ovariole length: 0.24 mm; genital chamber width: 0.13 mm.
- *Paragripopteryx* sp. 2 - ovaries length: 0.45 mm; lateral oviducts length: 0.72 mm, width of anterior portion: 0.05 mm, posterior portion: 0.12 mm; ovariole length: 0.54 mm; genital chamber length: 0.17 mm, and width: 0.16 mm.
- *Paragripopteryx* sp. 3 - ovaries length: 1.0mm; lateral oviducts length: 1.75 mm, width of anterior portion: 0.05 mm, and posterior portion: 0.18 mm; ovariole length: 0.76 mm; genital chamber length: 0.28 mm, and width: 0.3 mm.

The histology of the FRS in the examined *Paragripopteryx* species is largely similar to that of the *Gripopteryx* species (Fig. 5C–E). However, the oviduct has a thin outer layer of muscle tissue and a thick columnar epithelium lining its lumen (Fig. 5E).

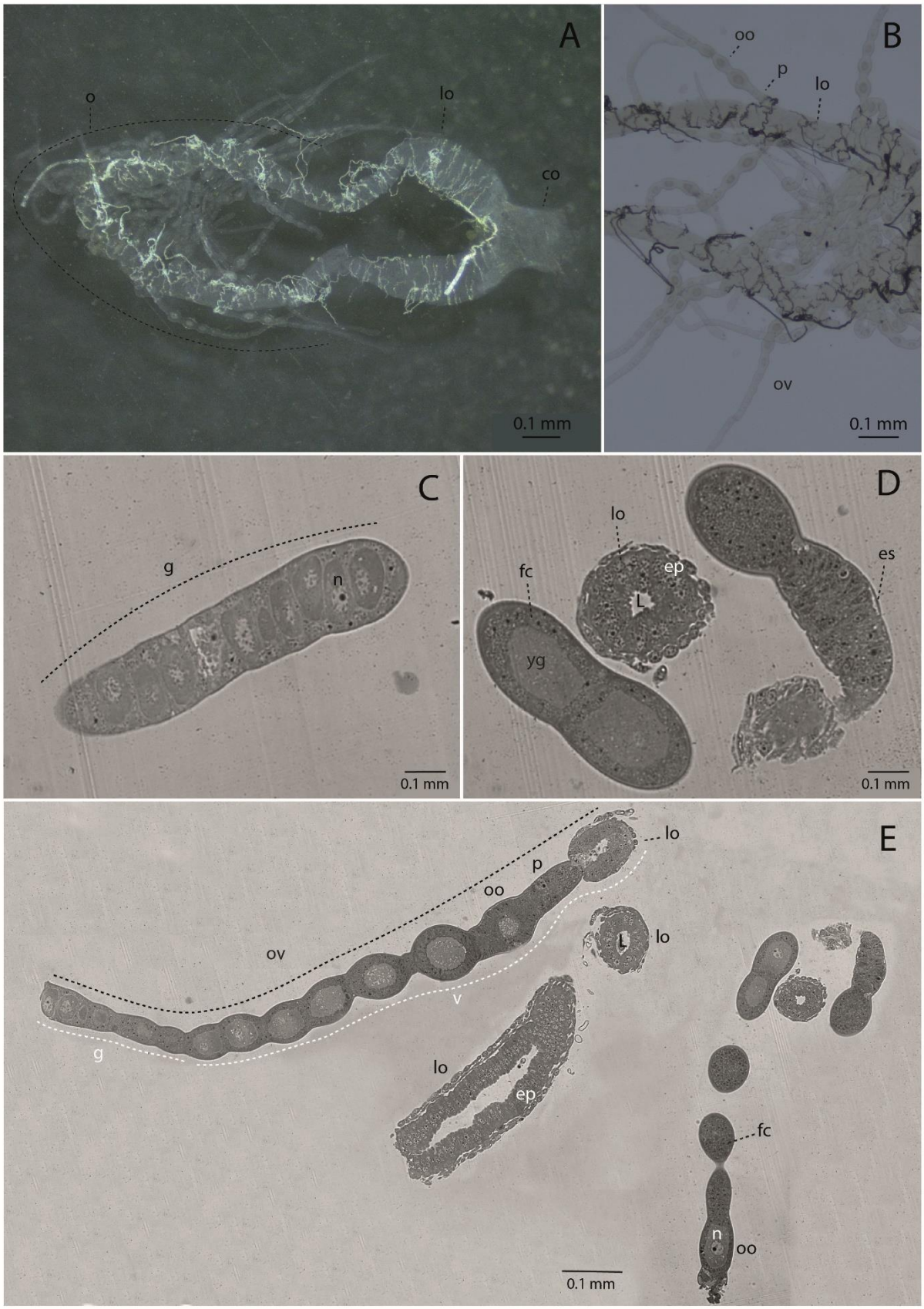

Figure 5. Anatomy and histology of the female reproductive system of *Paragripopteryx*. (A–B) Paired ovaries (o) in the previtellogenic phase, fused medially, with ovarioles (ov), lateral oviducts (lo), and the common oviduct (co). Note the long pedicel (p) connecting the ovariole

to the lateral oviduct. (C–D) Histological sections of the ovarioles (ov), showing the germarium (g) with oogonia arranged in a linear pattern, differentiating into oocytes, each with a centrally positioned nucleus. The ovariole is enclosed by an epithelial sheath (es), and the oocytes by follicle cells (fc). Note the cross section of the lateral oviduct (lo) displaying a thick layer of epithelium (ep) lining the lumen (L). (E) Longitudinal section of an ovariole showing the germarium (g), the vitellarium (v) containing the oocytes (oo), the pedicel (p) and the lateral oviduct (lo). Note that the lateral oviduct is externally surrounded by a thin layer of muscle cells (mc), with a thick inner epithelial layer (ep), composed of columnar cells, enclosing the oviduct's lumen (L).

Tupiperla gracilis (Burmeister, 1839)

Fig. 6A–C

The FRS of *T. gracilis* (n=3) contains elongated lateral oviducts, measuring from 1.0 to 2.98 mm in length, with a constant width over its length; its anterior portion ranges from 0.11 to 0.18 mm, and the posterior from 0.13 to 0.26 mm (Fig. 6A). The ovaries are about ⅖ shorter than the lateral oviducts, 0.81–1.27 mm. Each holds approximately 18 ovarioles (Fig. 6A). Each ovariole measures approximately 0.51 mm long (n=1) and is composed of about seven follicles and the germarium. The lateral oviducts connect to a very short common oviduct, which leads to the genital chamber (Fig. 6A). An elongated and narrow, longitudinal chitinous-lining is located medially to the membranous genital chamber's wall (Fig. 6A). The shape of the chitinous-lining in the anterior portion's apex is elliptic, while the posterior portion is subquadrate, presenting striae on its surface (Fig. 6A). The genital chamber underlies almost the whole extension of the subgenital plate. A paired membranous structure, most likely of glandular origin, was observed in one of the examined specimens (Fig. 6B–C). It has a subquadrate shape, with its posterior region exhibiting medial and lateral lobes. Anteriorly, it tapers along its length, terminating in an elliptical apex, slightly darker. This anterior portion is also slightly twisted, seemingly adapting to the curvature of the midgut. The structure is positioned just beneath the subgenital plate and may be connected to the short common oviduct (Fig. 6C). Unfortunately, it was impossible to analyze this structure histologically. We found tracheae surrounding the whole FRS. No accessory gland was observed. The eggs of *T. gracilis* exhibit a distinctive discoidal shape (width = 0.14 mm; height = 0.08 mm), reminiscent of classic flying saucers. They have a flattened, circular body, with one side featuring a slightly convex surface, while the opposite side is evenly flat. The rim tapers smoothly, resulting in an overall lenticular appearance.

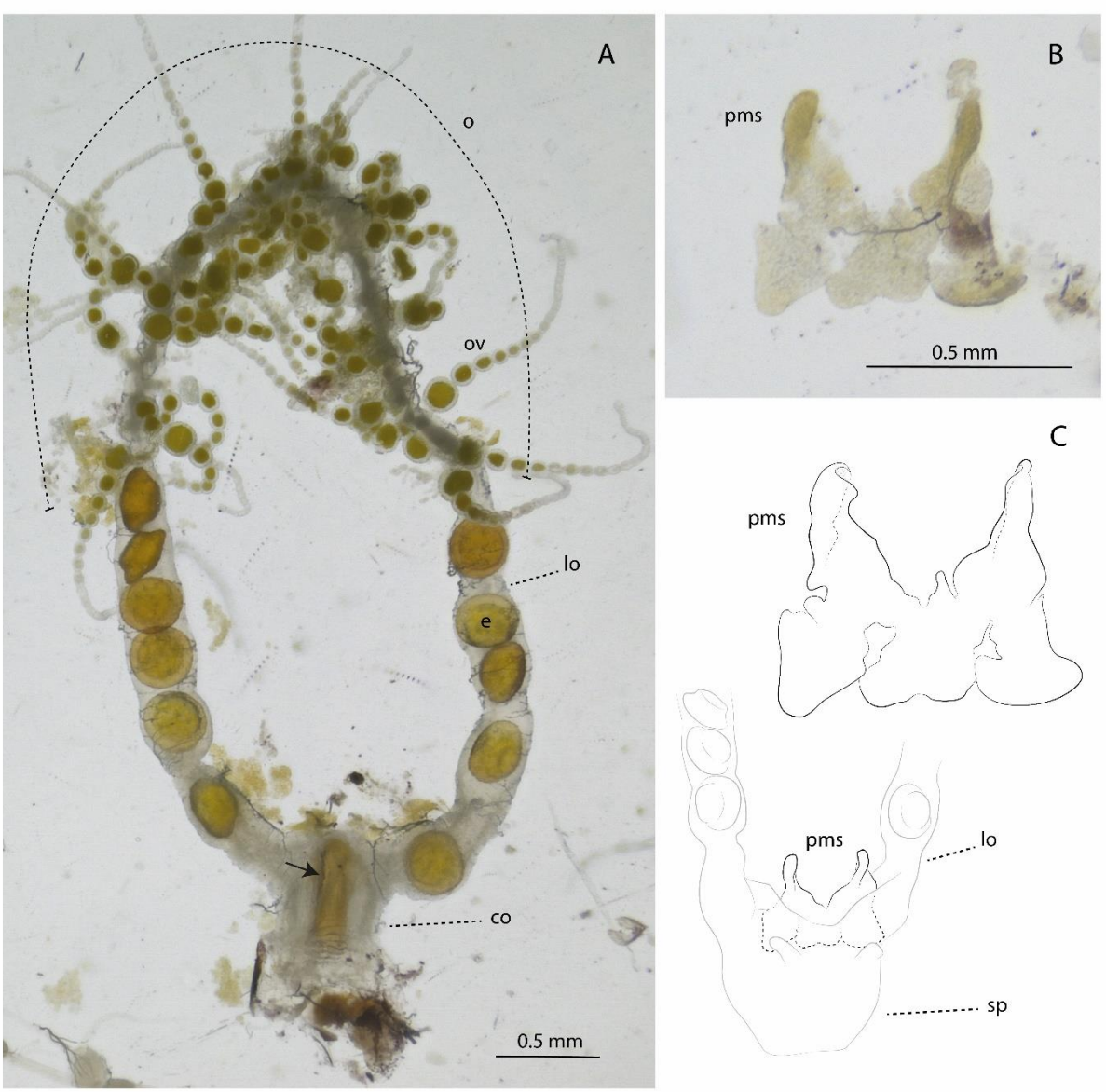

Figure 6. Anatomy of the female reproductive system of *Tupiperla gracilis*. (A) Paired ovaries (o) fused medially and the lateral oviducts (lo) containing eggs (e), and the common oviduct (co). The ovarioles (ov) are connected to the ovary's (o) anterior portion showing developing oocytes. Note the longitudinal chitinous-lining incorporated medially to the membranous genital chamber's wall. (B) The morphology of the paired membranous structure (pms) connected to the common oviduct. (C) Illustration of the paired membranous structure (pms) (dorsal view) and its position beneath the subgenital plate (sp) (ventral view).

Tupiperla robusta Froehlich, 1998

Figs. 7A–D

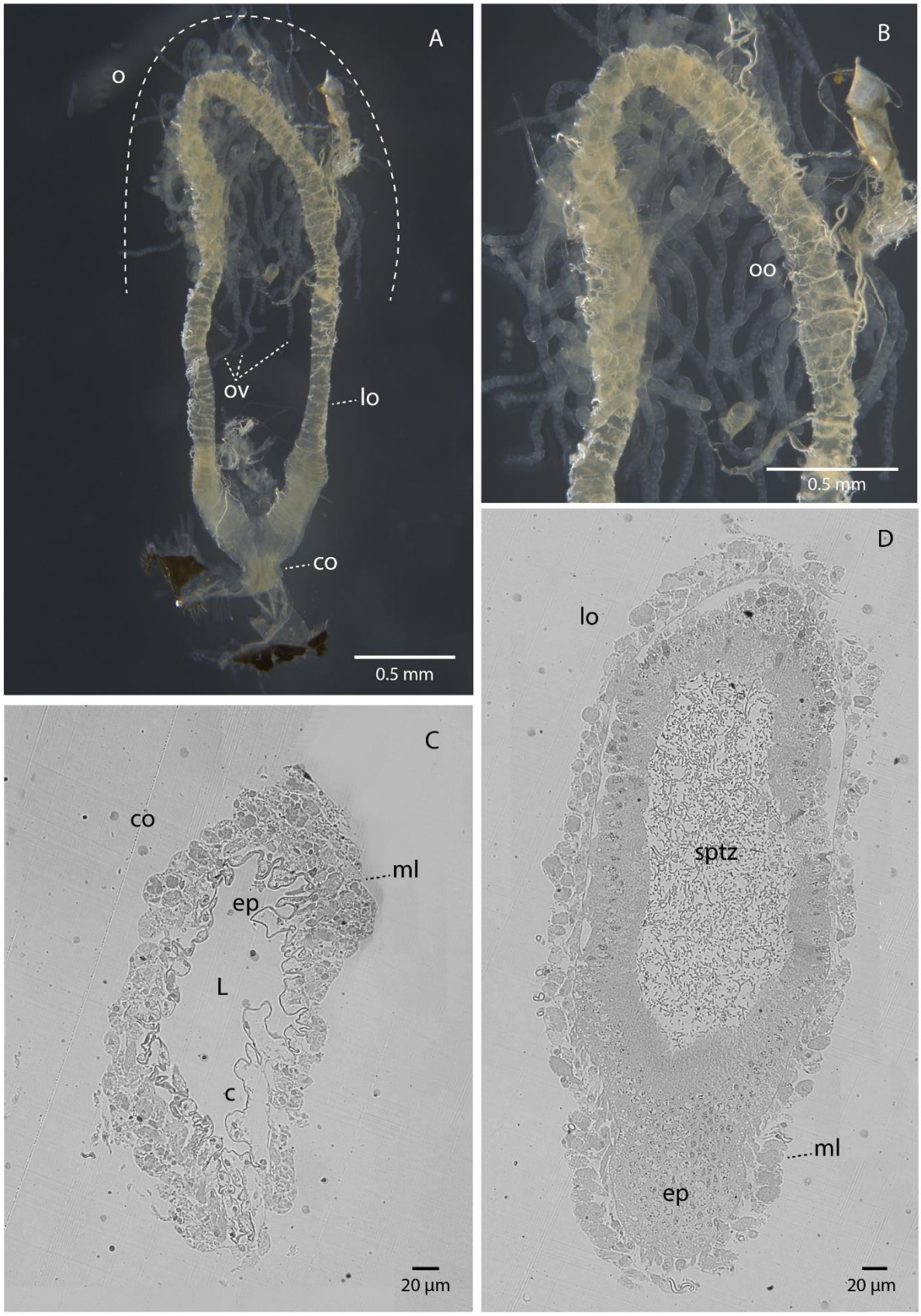

Figure 7. Anatomy of the female reproductive system of *Tupiperla robusta*. (A) Paired ovaries (o) fused medially, the lateral oviducts (lo), and the common oviduct (co). (B) Detail of ovarioles (ov) connected to the ovary's anterior portion showing oocytes (oo) in the

previtellogenic phase. (C) Histological cross section of the common oviduct surrounded by layers of muscle cells (ml) and internally lined with a thin, wrinkled epithelium containing cuticle (c). (D) Cross section of the lateral oviduct (lo), externally surrounded by a layer of muscle cells (ml), and a thick inner epithelial layer (ep), composed of columnar cells, enclosing the oviduct's lumen full of free sperm cells (sptz).

The FRS of *T. robusta* (n=3) largely aligns with that of *T. gracilis* except for the following differences: the ovaries length ranges from 0.92 mm to 1.38 mm (n=2), and the ovarioles length 0.4–0.61 mm (Fig. 7A). Each ovary contains around 25 ovarioles (Fig. 7B). The lateral oviducts measure from 1.32 to 1.64 mm in length; its anterior portion ranges from 0.1 to 0.13 mm, and the posterior 0.15–0.20 mm (n=1) (Fig. 7A). The genital chamber length is 0.14–0.22 mm, and 0.13–0.19 mm of width (n=2), and it is membranous and shorter than that of *T. gracilis*, exhibiting a bladder-like, bulbous shape rather than the elongated and flattened form seen in *T. gracilis*. The glandular structure found in *T. gracilis*, and the chitinous-lining on the genital chamber, were not observed in any female specimens of *T. robusta*. Histologically, the FRS of *T. robusta* largely corresponds to that of the other species described in this study (Fig. 7C–D). Additionally, free spermatozoa were observed inside the lateral oviduct (Fig. 7D).

Tupiperla tessellata (Brauer, 1868)

Figs. 1C; 8A–F

The FRS of *T. tessellata* (n=2) shares similarities with the other species in the genus, with some exceptions. The length of the ovaries ranges from 1.07 to 1.20 mm. Each ovary contains around 18 ovarioles (Fig. 8A), which measure from 0.59 to 0.70 mm in length (Fig. 8A). The lateral oviducts measure (length = 1.59–3.33 mm), the width of its anterior portion is 0.17–0.27 mm, while the posterior portion is 0.20 mm (Fig. 8B). The genital chamber length is 0.72–0.76 mm, and 0.31–0.34 mm of width (Fig. 8C). The examined specimens exhibit the chitinous lining on the genital chamber (Fig. 8C). Its shape is elongated and narrow, with both anterior and posterior apices slightly pointed. The surface remains smooth throughout its length. No glandular structure was observed. The histology of *T. tessellata* substantially matches the other species described in this study (Fig. 8D–F).

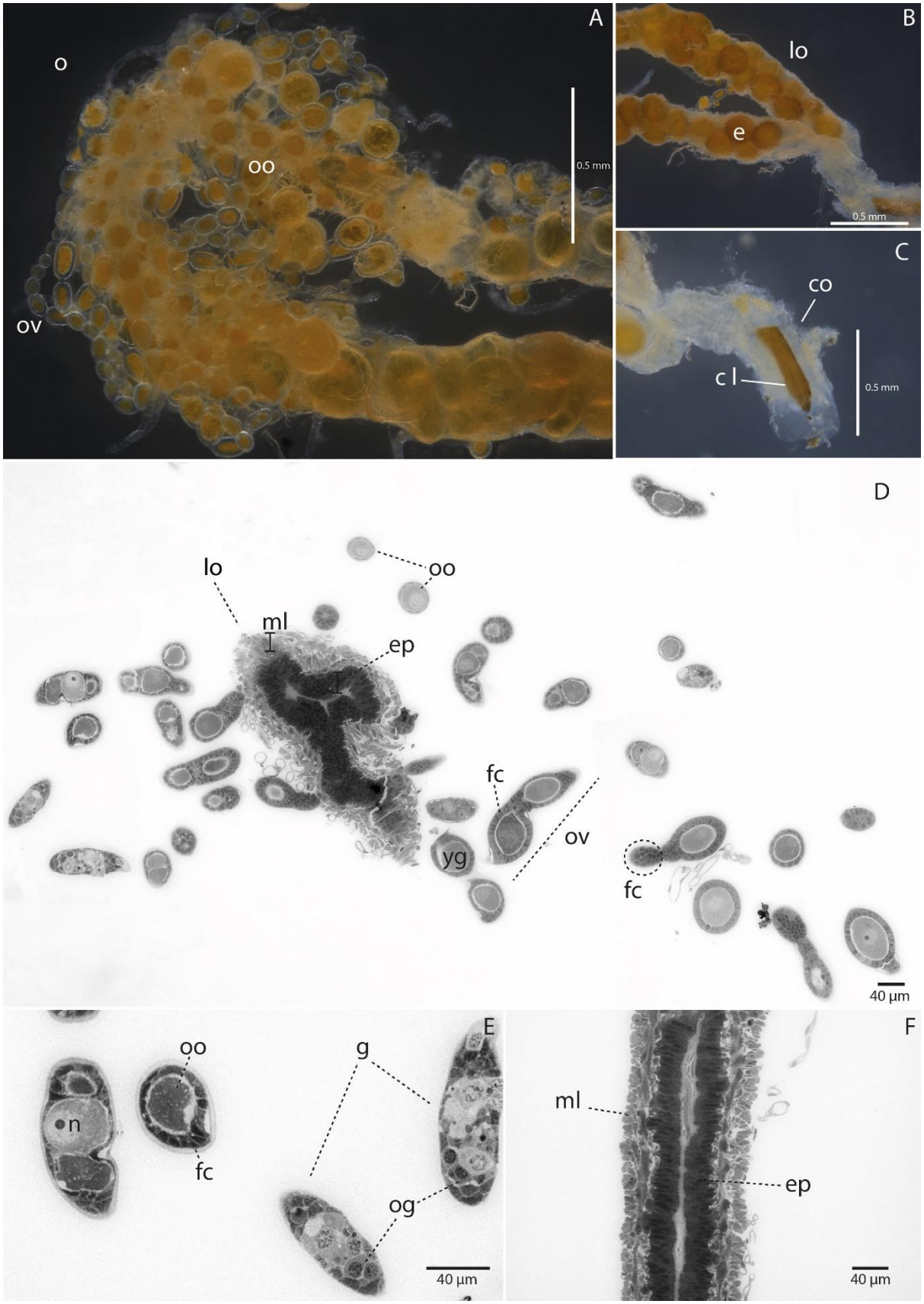

Figure 8. Anatomy and histology of the female reproductive system of *Tupiperla tessellata*. (A–C) Paired ovaries (o) fused medially, containing ovarioles (ov) showing developing oocytes (oo). The lateral oviducts (lo) containing eggs (e), and the common oviduct (co) with the

longitudinal chitinous-lining incorporated medially to the membranous genital chamber's wall. (D) Histological section of the ovarioles (ov) and lateral oviduct (lo). Oocytes (oo) surrounded by follicular cells (fc), with centrally located yolk granules (yg). Cross section of the lateral oviduct externally surrounded by a layer of muscle cells (ml), and a thick inner epithelial layer (ep), composed of columnar cells, enclosing the oviduct's lumen. (E) Detail of the oocytes with a centrally positioned nucleus (n), and the apical region of the germarium containing oogonia (og) and somatic cells. (F) Magnified view of a longitudinal section of the lateral oviduct (lo) featuring layers of muscle cells (ml) and the epithelium (ep) composed of columnar cells.

Anatomy and histology of the Male Reproductive System (MRS)

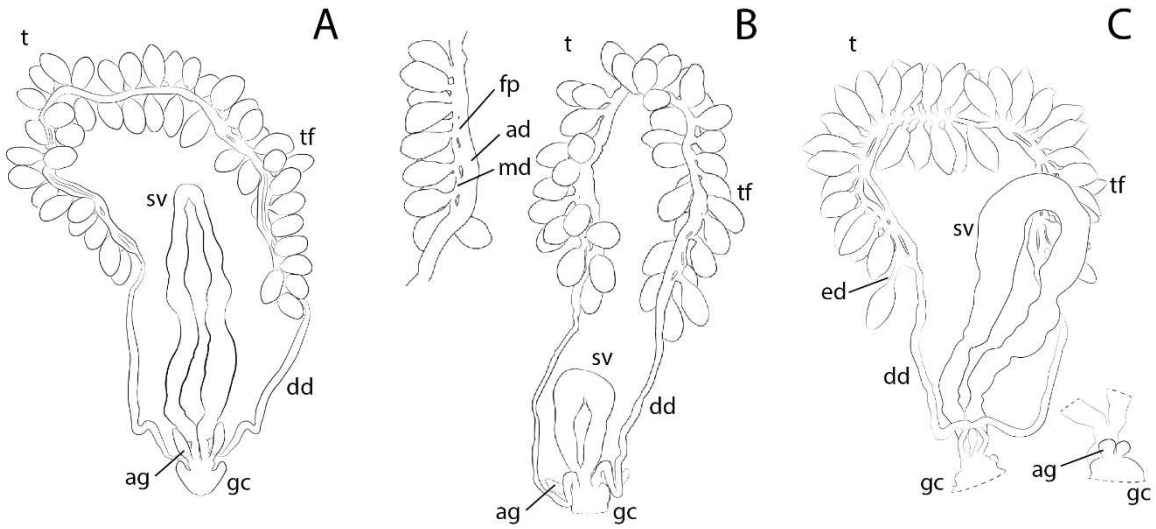

Figure 9. Illustrations of the male reproductive system of (A) *Gripopteryx pilosa*, (B) *Guaranyperla puri*. and (C) *Tupiperla tessellata*, showing the pair of testes fused medially (t), composed by the main duct (md) and the accessory duct (ad), forming four fusion points. Each testicular follicles (tf) is connected to the deferent duct (dd) by the efferent duct (ed). The deferent ducts extend to the posterior portion of the seminal vesicles (sv). A pair of accessory gland (ag) connects dorsolaterally to the genital chamber (gc).

Gripopteryx garbei Navás, 1936

Figs. 10A–C

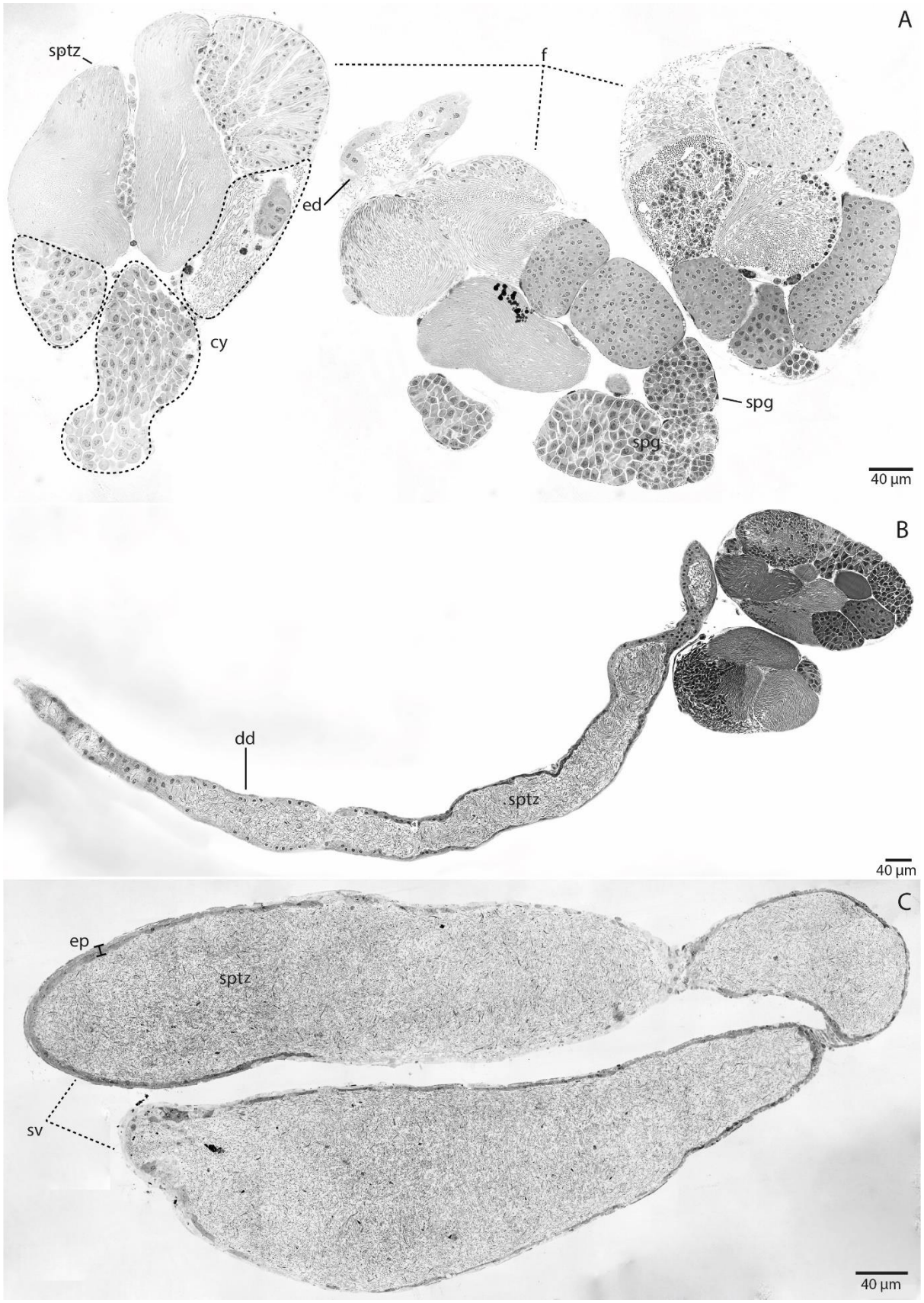

Figure 10. Histology of the male reproductive system of *Gripopteryx garbei*. (A) Longitudinal section of testicular follicles (f) filled with cysts at various stages of spermatogenesis, containing both mature sperm cells (sptz) and spermatogonia (spg). Note the small efferent

ducts (ed) composed of a cuboidal epithelium. (B) Deferent duct (dd) filled with mature sperm cells (sptz), and composed of a layer of cuboidal epithelial cells (ep). (C) Seminal vesicles (sv) filled with freely dispersed mature sperm cells (sptz). Note the cuboidal epithelium (ep) that gradually thickens along the right-left axis.

We were unable to observe the anatomy of the MRS of *G. garbei* in the single male specimen examined in this study. Thus, our description for this species is restricted to histological observations. A thin connective tissue capsule surrounds the testicular follicles, each housing approximately 10 cysts at different stages of spermatogenesis. In the same cyst, spermatogenesis occurs synchronously with all cells at the same stage (Fig. 10A). Spermatogonia are predominantly located in the apical region of the follicles, restricting the initial stage of spermatogenesis to this area. The cysts exhibit a centripetal arrangement, with those in the early stages of differentiation primarily occupying the apical region and the follicle periphery. In contrast, cysts at more advanced stages are positioned more toward the center of the follicle and close to the efferent duct (proximal region), which is short and lined by a simple cubic epithelium. The deferent ducts are lined by a simple secretory cuboidal epithelium, which maintains a consistent thickness along their length (Fig. 10B). Each follicle opens in the deferent duct by the efferent duct. The seminal vesicles are filled with freely dispersed mature sperm cells and are lined by a cuboidal epithelium that gradually thickens along the anterior-posterior axis, near its base (Fig. 10C). The ejaculatory duct, accessory glands, and the posterior portion of the MRS in the studied specimen could not be examined.

Gripopteryx pilosa Froehlich, 1990

Figs. 9A; 11A–F

The MRS of *G. pilosa* (n=3) consists of a pair of testes fused medially at the anterior ends, forming a bow-like structure (Fig. 11A). Each testis comprises two deferent ducts, the main duct and the accessory duct; however, the testicular follicles open into only the main duct (Fig. 11B). Although their primary functions may differ, both ducts contain sperm, as there are five fusion points between them, forming four loops (Fig. 11B). The loops are similar in size, but the third one (along the anterior-posterior axis) is only about 1/5 the size of the others. The main duct extends posteriorly from the first fusion point to the first fusion point of the paired testis (length = 2.52–3.1 mm), creating a transverse anterior arch, which consists solely of the

main duct (Fig. 11A–B). Each testicular follicle opens into the main duct through a very short efferent duct (Fig. 11B). The oval-shaped follicles (length = 0.48–0.60 mm; width = 0.23–0.30 mm) number 47 in both testes in *G. pilosa* (Fig. 11A–B). The deferent ducts are long and thin (length = 3.5 mm), curving upward near its connection to the basal portion of the seminal vesicle (Fig. 11A). Ventrally, the deferent ducts connect to the seminal vesicles, nearly reaching their posterior ends (Fig. 11A). The seminal vesicles are fused at the anterior apex forming an arch and extending longitudinally (length = 2.29–3.79 mm) (Fig. 11A). When filled with sperm, it becomes bulky, narrowing as it approaches the ejaculatory duct. The seminal vesicles bend ventrally in the middle in some individuals due to the large sperm quantity. Alongside the base of the seminal vesicles, there is a pair of short, elongated, oblong-shaped accessory glands (length = 0.58–0.63 mm) that are connected dorsally to the anterior end of the ejaculatory duct, which is housed within the lobed membranous penis (Fig. 11A).

The histological description of the MRS of *G. pilosa* aligns with that of *G. garbei*, with a few differences (Fig. 11C–F). The epithelium of the seminal vesicles is thicker (Fig. 11F). The accessory glands are voluminous, with the anterior region lined by a thick secretory cuboidal epithelium, while the medial-distal portion consists of epithelial tissue with secretory columnar cells (Fig. 11D). Two cell types with distinct staining affinities were observed within the accessory glands, one darker than the other. However, no secretions were detected. A thin layer of chitin lining the gland lumen can be observed, providing evidence of its ectodermal origin (Fig. 11D). The posterior portion of the deferent ducts is positioned medially within the genital chamber, surrounded by layers of muscle tissue (Fig. 11D). It gradually merges to form a single duct, the ejaculatory duct, which is encased in a retracted, lobed penis lined with a thick epithelial layer (Fig. 11D). The posterior portion of the ejaculatory duct could not be observed.

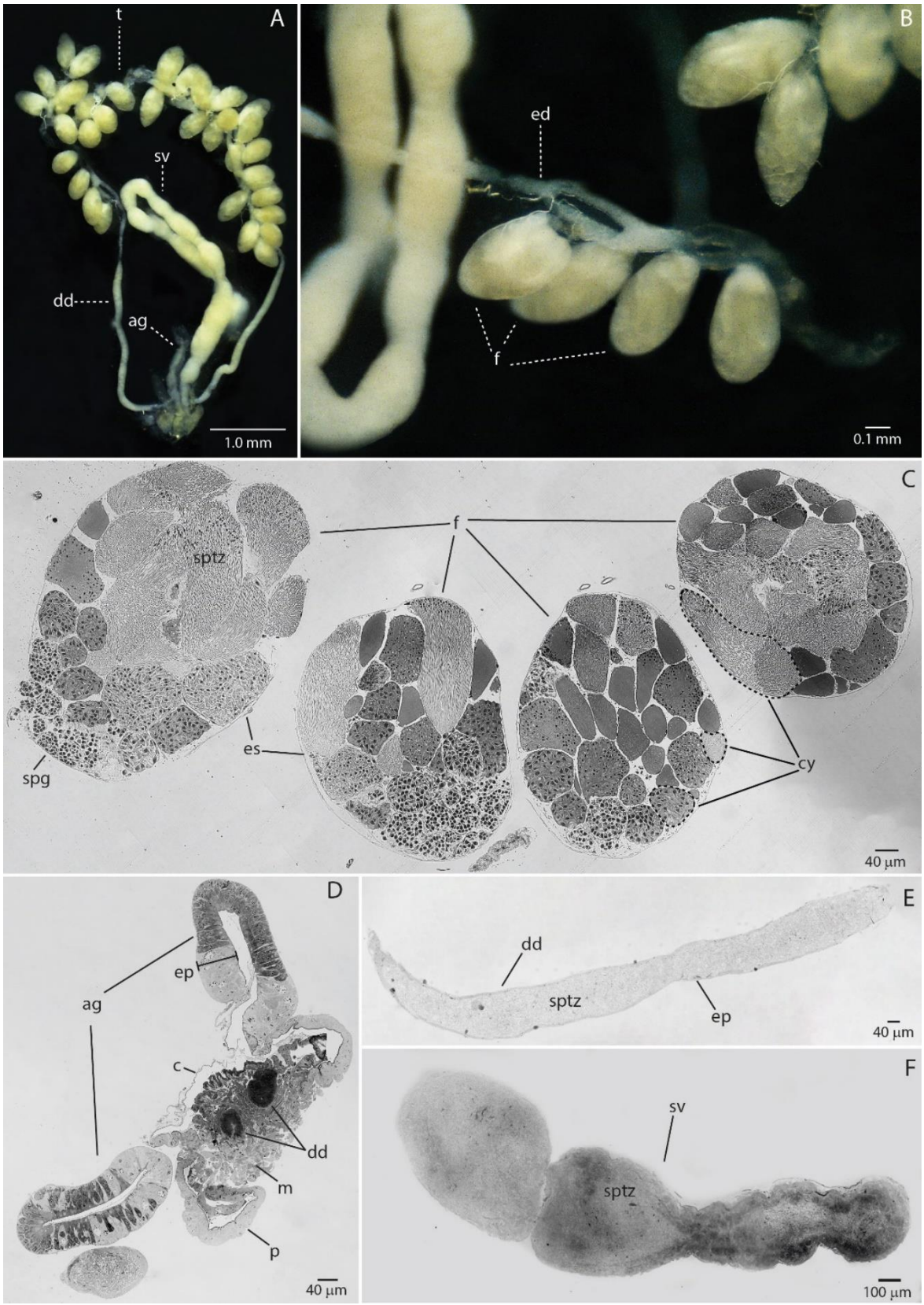

Figure 11. Anatomy and histology of the reproductive system of *Gripopteryx pilosa*. (A–B) The paired testes (t) are fused medially, containing testicular follicles (f) connected to the main duct (md), which in turn is linked to the accessory duct (ad) at fusion points (fp). The deferent

duct (dd) extends to ventrally connect with the posterior portion of a pair of seminal vesicles (sv), which are fused antero-medially. A pair of accessory glands (ag) are located dorsolaterally to the genital chamber (gc). (C) Histological longitudinal section of testicular follicles (f) filled with cysts (cy) at various stages of spermatogenesis, containing both mature sperm cells (sptz) and spermatogonia (spg). Note the thin epithelial sheath (es) enclosing each testicular follicle. (D) Cross section of the genital chamber, showing the accessory glands (ag) with an anterior region lined by thick secretory cuboidal epithelium (ep) and a medial-distal portion composed of secretory columnar cells. A thin chitinous layer (c) lines the gland lumen. The posterior portion of the deferent ducts (dd) is positioned medially within the genital chamber, enveloped by muscle layers (m), and gradually converges to form the ejaculatory duct (not seen), which is enclosed within a retracted, lobed penis (p) lined by a thick epithelial layer. (E) Deferent duct (dd) filled with mature sperm cells (sptz), and composed of a layer of squamous epithelial cells (ep). (F) Seminal vesicles (sv) composed of a thick epithelium (ep) and filled with freely dispersed mature sperm cells (sptz).

Gripopteryx reticulata Brauer, 1868

Figs. 12A–B

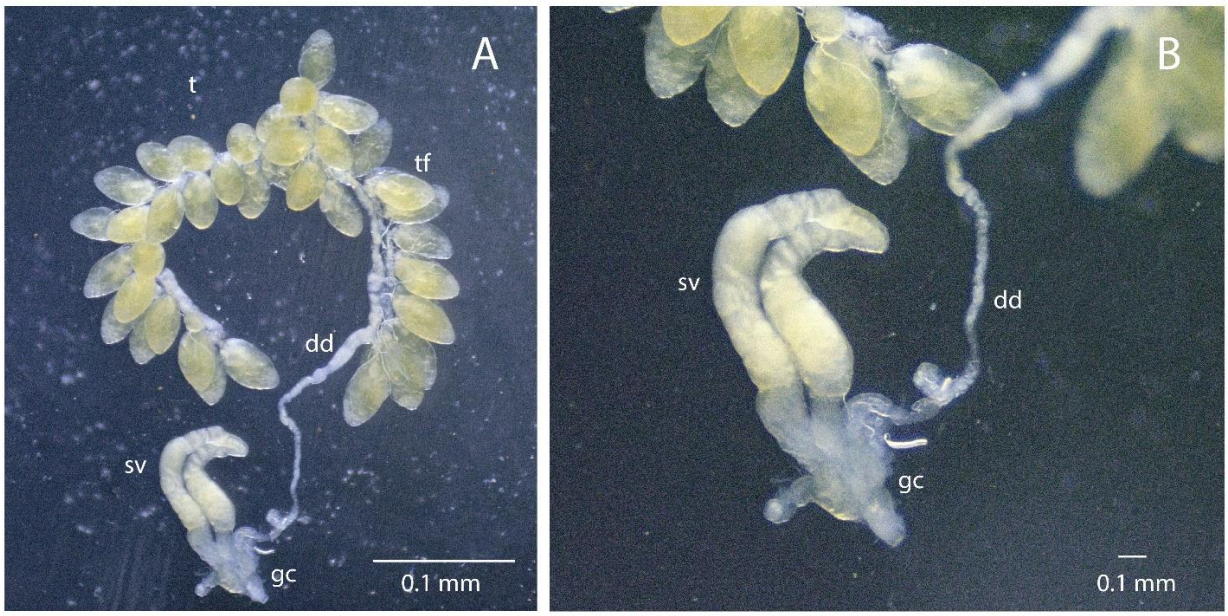

Figure 12. Anatomy of the male reproductive system of *Gripopteryx reticulata*. (A–B) Paired testes (t) fused medially, containing testicular follicles (f) that connect to the deferent duct (dd). The deferent duct extends ventrally and joins the posterior portion of a pair of partially sperm-filled seminal vesicles (sv), which are fused medially and linked to the genital chamber (gc).

The MRS of *G. reticulata* (n=3) broadly aligns with that of *G. pilosa* except for the following differences: the testes measure from 1.18 to 2.05 mm; the follicles measure 0.49–0.56 mm in length, and 0.26–0.27 mm in width (n=2), with 48 follicles present in both testes (Fig. 12A). The deferent duct is 2.52 mm long (n=1) (Fig. 12B). The seminal vesicles range from 1.57 mm in length when partially empty (Fig. 12B) to 4.10 mm when full. The accessory glands measure 0.27 mm in length and 0.08 mm in width (n=1).

Guaranyperla puri Rippel & Salles, 2025

Figs. 9B; 13A–G

The general anatomy of the MRS of *Gu. puri* (n=2) closely resembles that of *Gripopteryx* species, except for the following differences: the testes of *Gu. puri* measure 1.69–2.08 mm in length; the follicles measure 0.34–0.47 mm in length and 0.15–0.27 mm in width, with 44 follicles in both testes (Fig. 13B–C). The four loops are similar in size, but appear smaller due to the greater volume of the accessory duct, which is broader (Fig. 13B). The first loop (along the anterior-posterior axis) is notably smaller, measuring approximately ⅕ the size of the others. The seminal vesicles measure 1.3 mm in length (n=1) and become broader at the apex when full. The anatomy of the accessory glands could not be examined, preventing a detailed description. However, histological observations suggest they are elongated, elliptical, and composed of cuboidal cells (Fig. 13G). The histological analysis showed that the male reproductive organs of *Gu. puri* also align with those of *Gripopteryx* species (Fig. 13D–G), although some differences exist. The region connection between the proximal region of the follicle and the efferent ducts comprises a thick cuboidal epithelium (Fig. 13D).

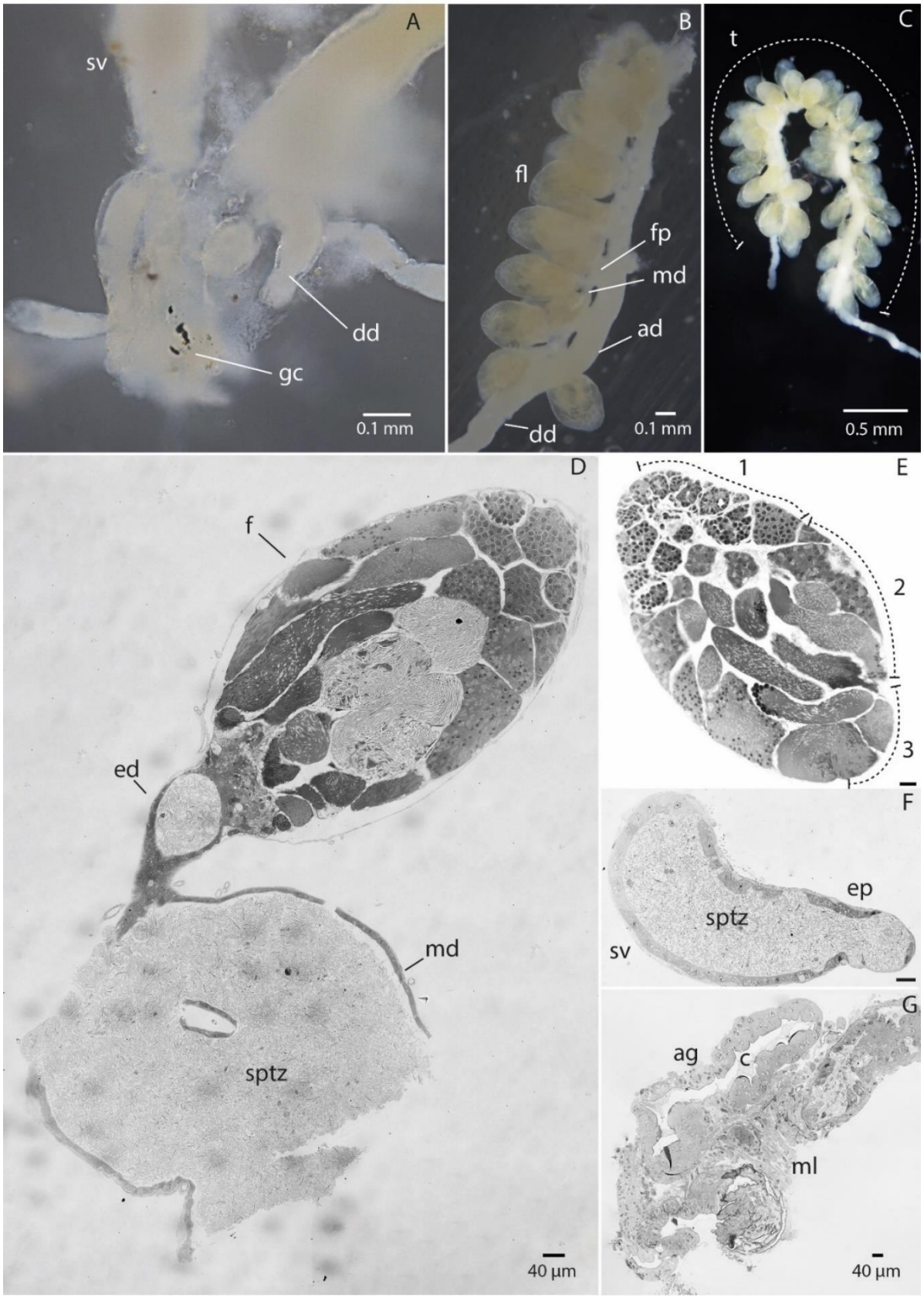

Figure 13. Anatomy and histology of the reproductive system of *Guaranyperla puri*. (A) Posterior portion of the paired seminal vesicles (sv), showing the deferent ducts (dd) opening into the genital chamber (gc). (B) Testicular follicles (f) connected to the main duct (md),

which, in turn, links to the accessory duct (ad) at fusion points (fp), merging into the deferent duct (dd). (C) The paired testes (t) are medially fused, each containing multiple testicular follicles. (D) Histological longitudinal section of a testicular follicle (f). The follicle terminates in the efferent duct, densely packed with mature sperm, which connects to the main duct (md). (E) Detailed view of a testicular follicle highlighting the sequential maturation zones of spermatozoa: (1) cysts containing spermatogonia, (2) spermatids with elongating flagella and nuclei, and (3) mature sperm cells. (F) Portion of the seminal vesicle (sv) lined with a thick epithelium (ep) and filled with freely dispersed mature sperm cells (sptz). (G) Longitudinal section of an accessory gland (ag), with an anterior region lined by thick secretory cuboidal epithelium (ep) and a medial-distal portion composed of secretory columnar cells. A thin chitinous layer (c) lines the gland lumen. Layers of muscle tissue are present within the genital chamber. Scale bars in (E – F) are 40 μm.

Paragripopteryx delicata Froehlich, 1994

Figs. 14A–D

It was not possible to observe the anatomy of the seminal vesicles, ejaculatory duct, and penis in the single male specimen examined in this study. Thus, the MRS description of *Paragripopteryx* (n=1) is restricted to testicular histology (Fig. 14A–D). The testes of *P. delicata* are similar to those of *Gripopteryx* and *Guaranyperla* species, but the follicles appear notably slenderer (Fig. 14A–C).

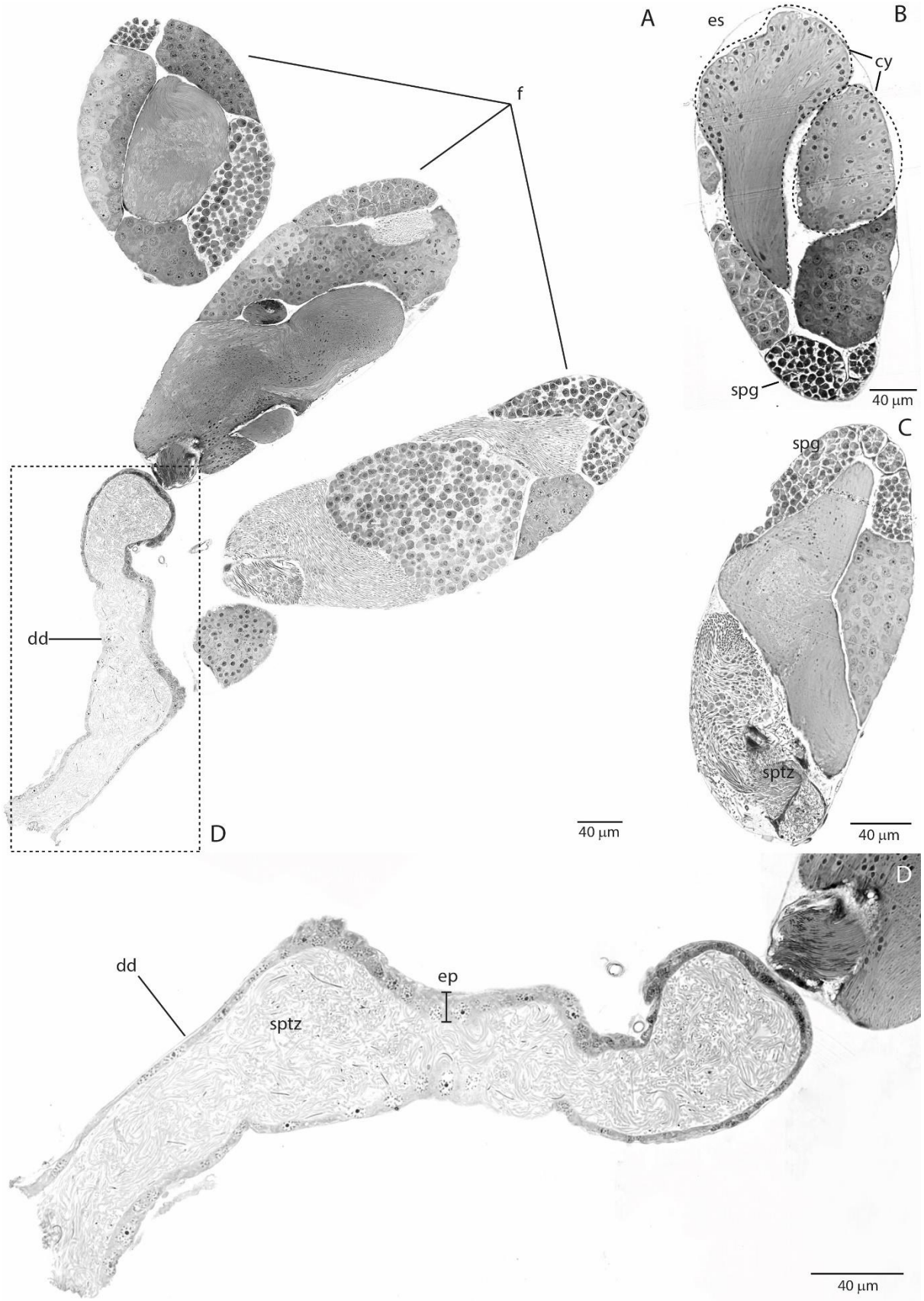

Figure 14. Histology of the male reproductive system of *Paragripopteryx delicata*. (A–C) Longitudinal section of testicular follicles (f) containing cysts at various stages of spermatogenesis, including spermatogonia (spg) and mature sperm cells (sptz). A thin

epithelial sheath (es) encloses each testicular follicle. The deferent duct (dd) is densely packed with mature sperm cells (sptz). (D) Magnified view of the deferent duct, lined with a cuboidal epithelium (ep).

Tupiperla gracilis (Burmeister, 1839)

Figs. 15A–C

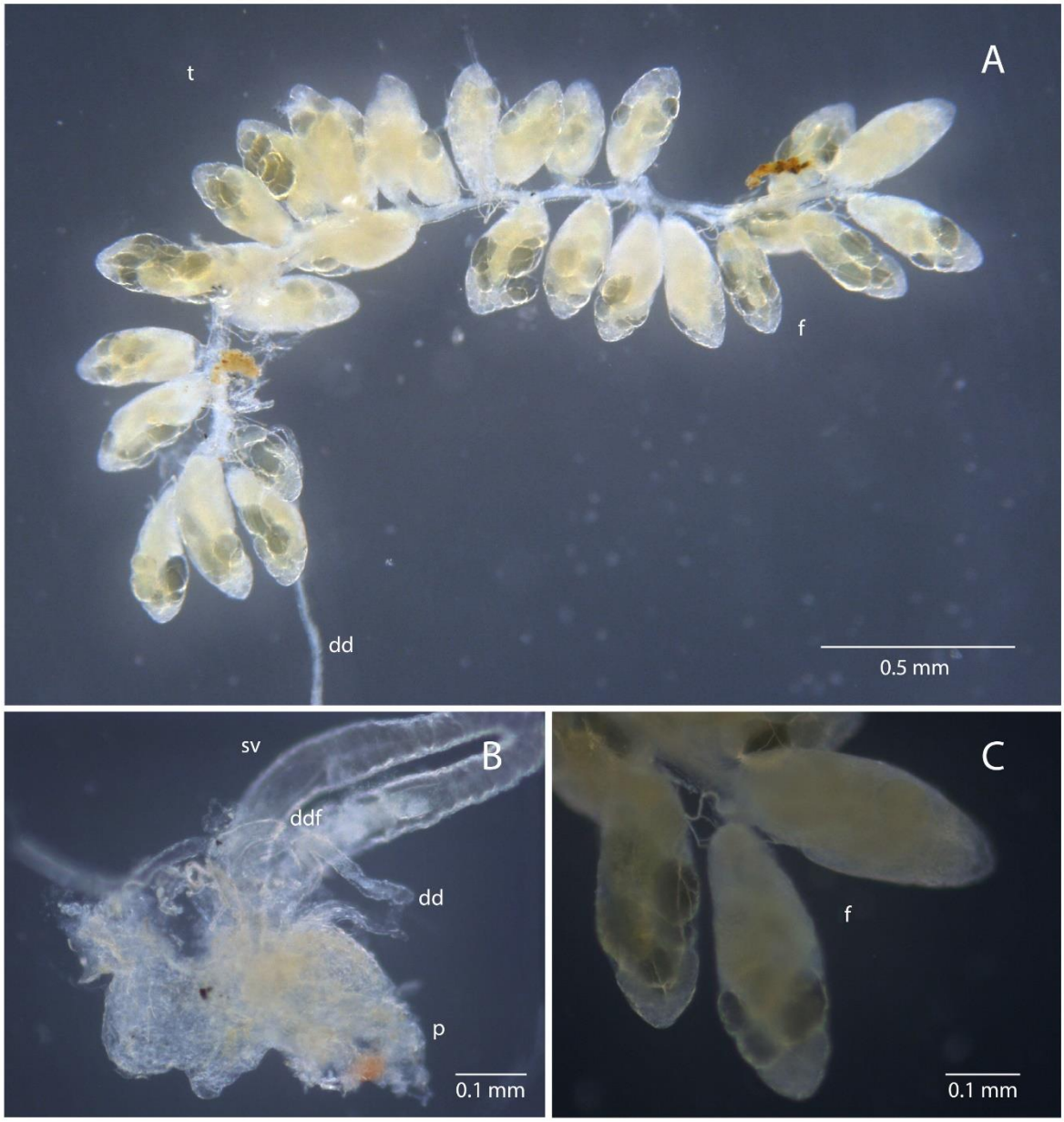

Figure 15. Anatomy of the male reproductive system of *Tupiperla gracilis*. (A) Paired testes (t) fused medially, containing testicular follicles (f) that connect to the deferent duct (dd). (B) The deferent duct (dd) bends medially to fuse at the deferent duct fusion (ddf) before curving laterally again, independently from the paired deferent duct. This arrangement forms a

distinctive figure-eight shape before merging with the terminal portion of the empty seminal vesicle (sv) and entering the penis (p). (C) Detail of the elongated and oblong testicular follicles.

The MRS of *T. gracilis* (n=3) shares similarities with other genera, but specific distinct characteristics can differentiate it. It has a pair of testes measuring 1.23 mm in length (n=1) (Fig. 15A). The loops formed at the fusion points of the accessory and main ducts are similar in size, except for the third loop, which is approximately ⅕ the size of the remaining loops. The testicular follicles are elongated and oblong, measuring 0.37–0.41mm in length and 0.14–0.20 mm in width, with 36 follicles present in both testes (Fig. 15A; C). The deferent ducts are long and slender. The ducts bend medially to fuse with each other at the deferent duct fusion (ddf) before curving laterally (Fig. 15B). This configuration creates a distinctive figure-eight shape before merging with the basal portion of the seminal vesicles. At this junction, the duct becomes slightly narrower in diameter. The seminal vesicles measure 0.55 mm long when empty (Fig. 15B) and 2.43 mm when full. The accessory glands could not be observed in the dissected male specimens. The ejaculatory duct is housed within the lobed, membranous penis, which contains a paired smaller lobe near its posterior edge.

Tupiperla robusta Froehlich, 1998

Fig. 16A–D

The MRS of *T. robusta* (n=3) resembles that of *T. gracilis* although there are differences in the length of several structures: the paired testes range from 1.65 to 2.42 mm long; the follicles vary from 0.39 to 0.47 mm long, and 0.18–0.22 mm wide (Fig. 16A); and the seminal vesicles measure from 0.9 to 1.35 mm in length (Fig. 16B). Each deferent duct connects at ¼ of the seminal vesicle length, positioned anteriorly than in *Gripopteryx, Guaranyperla*, and *T. gracilis* (Fig. 16B). The figure-eight-shaped junction of the paired ducts at their proximal portion was also not observed in any of the specimens. A pair of accessory glands are connected mediodorsally at the base of the seminal vesicles and the anterior end of the ejaculatory duct (Fig. 16B). The accessory glands have a truncated apex and gradually narrow toward their base, maintaining an overall rectangular shape. They range from 0.19 to 0.23 mm in length and from

0.07 to 0.09 mm in width (Fig. 16B). The histology of the reproductive organs of *T. robusta* males is similar to that of the other genera described in this study (Fig. 16C–D).

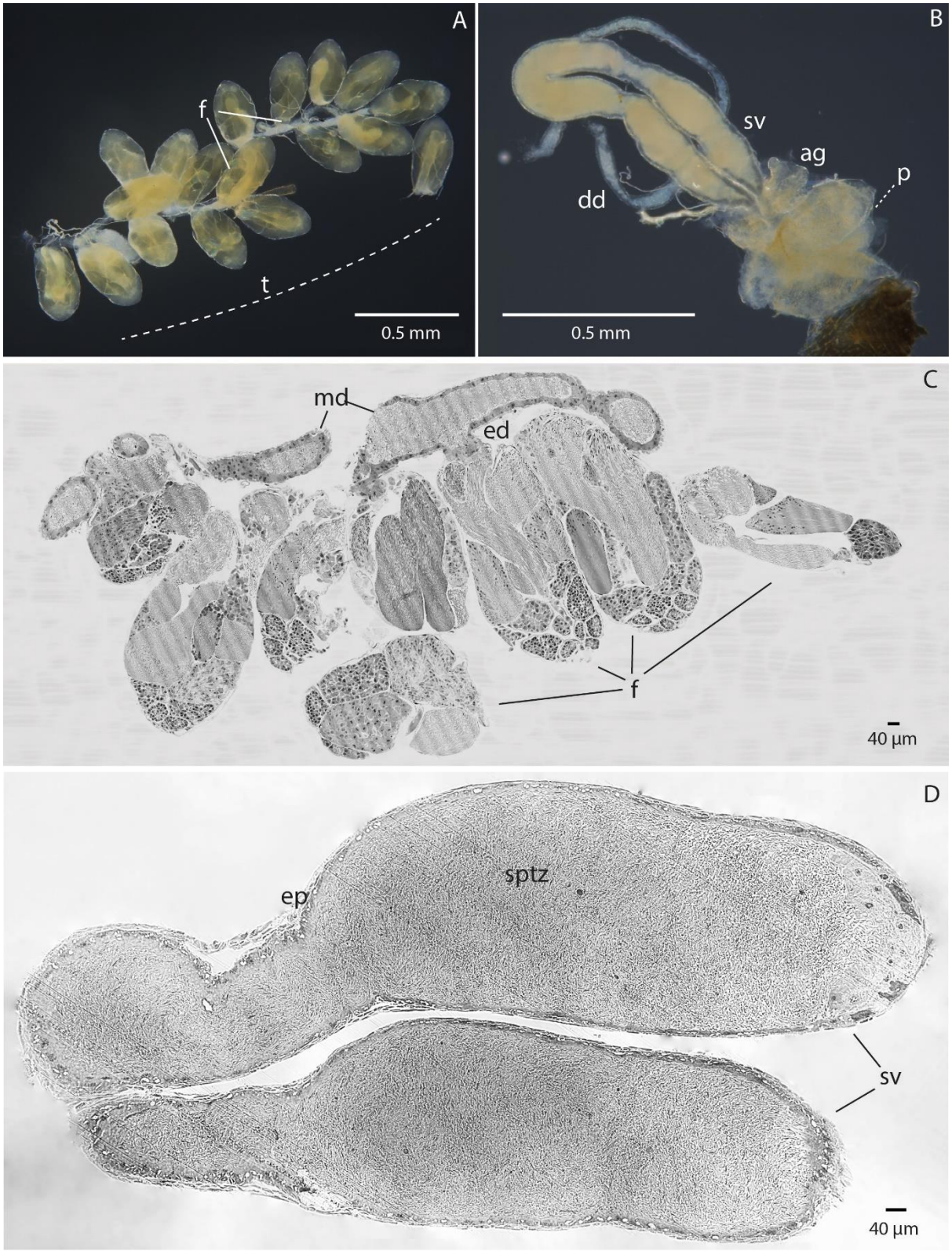

Figure 16. Anatomy and histology of the reproductive system of *Tupiperla robusta*. (A) Testis (t) with multiple testicular follicles (f) connected to the main and accessory ducts. (B) Seminal

vesicles (sv) fused anteromedially, showing the deferent ducts (dd) opening into ¼ of the seminal vesicle's length, in ventral view. A pair of rectangular shape accessory glands (ag) are located dorsolaterally to the genital chamber and penis (p). (C) Histological longitudinal section of the testicular follicles (f), terminating in the short efferent ducts (ef), which connects to the main duct (md), both ducts composed of a cuboidal epithelium, and densely packed with mature sperm. The follicles are filled with cysts at various stages of spermatogenesis, including spermatogonia (spg) and mature sperm cells (sptz). (D) Portion of the seminal vesicles (sv) lined with a thick epithelium (ep) and filled with freely dispersed mature sperm cells (sptz).

Tupiperla tessellata (Brauer, 1868)

Figs. 9C; 17A–F

The MRS of *T. tessellata* (n=4) closely resembles that of T. gracilis, with the following distinctions: the paired testes measure from 1.38 to 1.9 mm (n=3), and the follicles range from 0.40 to 0.43 mm in length and from 0.15 to 0.18 mm in width (Fig. 17A). The seminal vesicles vary in length, measuring 0.91 mm in length when partially empty, and up to 2.25 mm when full (n=3). The last efferent duct along the anterior-posterior axis is longer than the others (Fig. 9C). The accessory glands measure from 0.14 to 0.16 mm in length and 0.10 mm in width, exhibiting either an orbicular or elliptical shape (n=2) (Fig. 17B). Histological analysis of the accessory glands revealed a secretory cuboidal epithelium, and two distinct types of pigmentation, suggesting the production of different secretions (Fig. 17E). The remaining histological observations from *T. tessellata* did not differ from that of the species described herein (Fig. 17D–F).

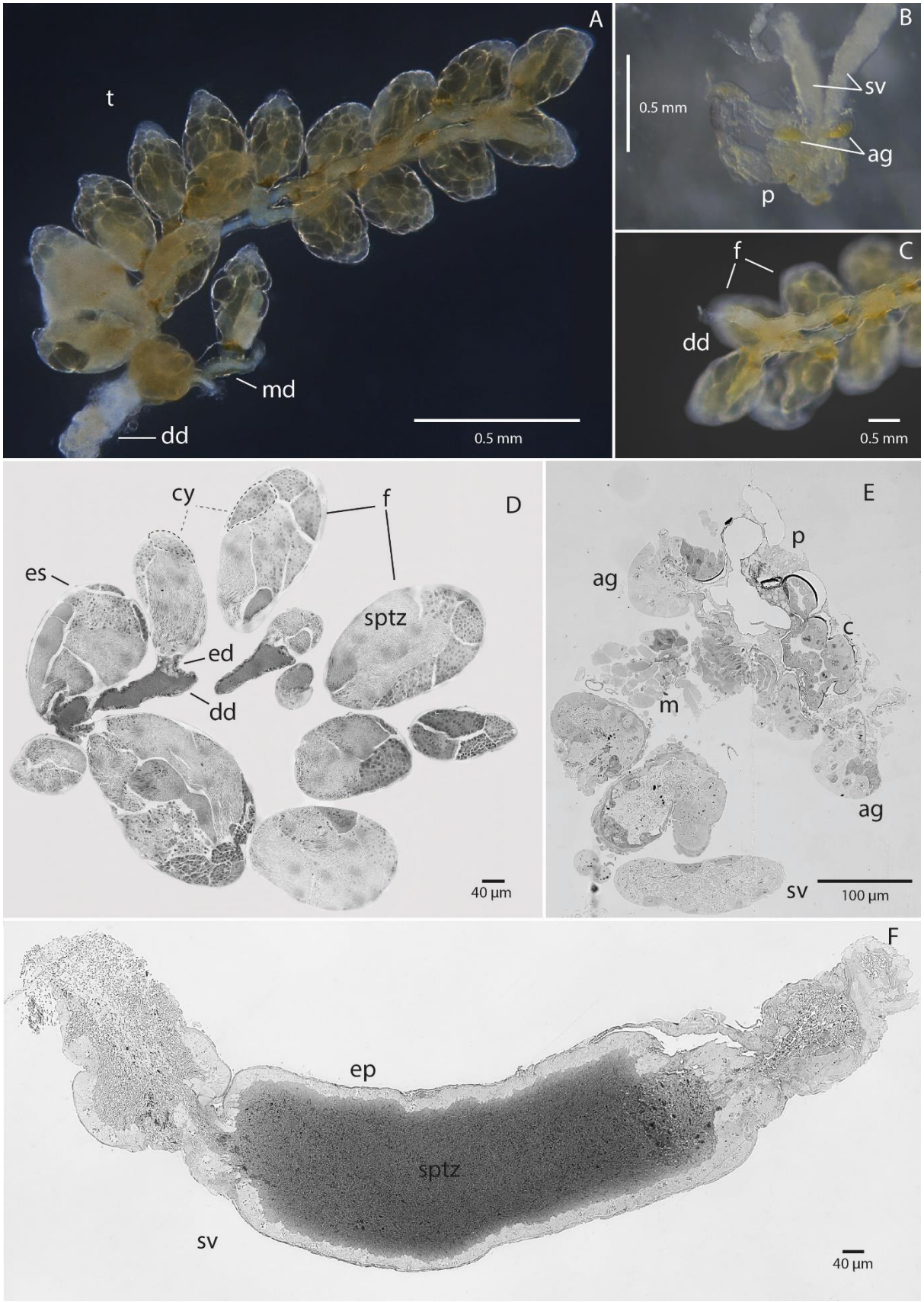

Figure 17. Anatomy and histology of the reproductive system of *Tupiperla tessellata*. (A) Testis (t) with multiple testicular follicles connected to the main (md) and accessory ducts that form the deferent duct (dd). (B) Anterior portion of the paired seminal vesicles (sv), showing

a pair of elliptical shape accessory glands (ag) are located dorsolaterally to the membranous penis (p). (C) Deferent duct (dd) bearing the testicular follicles (f). (D) Histological longitudinal section of the testicular follicles (f), terminating in the short efferent ducts (ef), which connects to the deferent duct (dd); both ducts densely packed with mature sperm. The follicles are covered by the epithelial sheath, and filled with cysts (cy) at various stages of spermatogenesis, including spermatogonia and mature sperm cells (sptz). (E) Cross section of the posterior portion of the seminal vesicles (sv) and the membranous penis, showing the accessory glands (ag) with an anterior region lined by thick secretory cuboidal epithelium (ep) and a medial-distal portion composed of secretory columnar cells. A thin chitinous layer (c) lines the gland lumen, as well as the interior of the genital chamber. Inside the genital chamber, thick epithelial and muscle layers. (F) Portion of a seminal vesicle (sv) lined with a thick epithelium (ep) and filled with freely dispersed mature sperm cells (sptz).

Discussion

The reproductive system of Neotropical Gripopterygidae exhibits the posterior ends of internal genital organs (ovaries and testes) fused, forming a loop (Figs. 1; 9), a known synapomorphy in Plecoptera (Zwick 2000). We also found anatomical diversity and histological conservatism across the studied female and male taxa. Our findings reveal that the gross morphology of both female and male reproductive systems varies at intergeneric and interspecific levels. However, as expected, these differences are more pronounced among genera than among species within the same genus. According to (Zwick 1973), the MRS in stoneflies exhibits greater anatomical diversity, whereas the FRS remains more conservative. In males, this diversity can be explained not only by the large number of modifications in the structure of mesodermal organs (testes, deferent ducts, seminal vesicles), but also the variety of forms of the copulatory organs. This low tendency of variation for stonefly females is primarily due to the limited development of external copulatory organs (Zwick 1973). Our study provides evidence that the morphology of the FRS in Gripopterygidae fauna varies in an intergeneric, and sometimes, as in *Tupiperla* species, can also vary interspecifically. These data suggest that reproductive traits may contribute to higher-level taxonomic differentiation within Gripopterygidae.

Despite these anatomical variations, histological analysis revealed high conservatism in male and female reproductive structures across the examined taxa. The uniformity in tissue organization suggests strong functional constraints that limit histological divergence,

regardless of taxonomic or geographic differences. The following sections will discuss these aspects in further detail, addressing the female and male reproductive systems separately.

4.1. Female reproductive system (FRS)

The FRS of *Gripopteryx*, *Paragripopteryx*, and *Tupiperla* aligns with the descriptions provided by (Brinck 1956) and (Zwick 1973, 1980) for groups within Arctoperlaria. In these genera, the posterior portion of the ovary consists of a single tubular structure to which the ovarioles are attached, while the anterior portion lacks ovarioles and transitions into the common oviduct. The FRS is positioned latero-ventrally to the midgut, with its posterior ends medially fused and located dorsally, consistent with these descriptions. The common oviduct then leads into a short, chitin-lined vagina (genital chamber) (Brinck 1956; Zwick 1973, 1980). Our histological analyses confirmed the presence of chitin beneath the common oviduct epithelium, supporting its ectodermal origin, as Zwick (1973) suggested. In all studied species, the membrane of the genital chamber was connected posteriorly to the margin of the subgenital plate, encompassing its posterior margin. This structural arrangement strongly suggests that the genital opening is located at this junction.

The spermatheca is one of the most variable structures in FRS. Zwick (1973; 1980) documented its presence in various families, including Leuctridae, Taeniopterygidae, Notonemouridae, and some Nemouridae. He highlighted variations in the number (one or two), their position (adjacent to the common oviduct or along the dorsal midline of the vagina), and their shape across taxa. Rościszewska and Rzońca (2009) described and illustrated a large spermatheca connected to the common oviduct in *Thaumatoperla* (Eustheniidae). However, in our study, no spermatheca was observed in any of the examined species. This suggests that sperm storage may instead occur within the oviducts and the genital chamber, as supported by the presence of sperm in these organs in *G. pilosa* and *T. robusta*. This finding aligns with the proposed synapomorphy of Gripopterygoidea (*Austroperlidae* + *Gripopterygidae*), which is the loss of a spermatheca in females (Zwick 2000). Consequently, our results indicate that the examined species lack a distinct spermatheca, and sperm is retained within the reproductive tract until fertilization, as suggested for other plecopteran taxa that do not possess a spermatheca (Brinck 1956; Zwick 1973).

Similarly, no accessory glands were observed in any of the studied specimens. However, a paired membranous structure, possibly of glandular origin, was identified in one specimen of *T. gracilis*. Zwick (1973) described a "hyaline skin sac" located dorsally on the short common oviduct in species of *Leuctra* (Leuctridae), a structure that closely resembles what we observed in *T. gracilis*. According to Zwick, this sac becomes visible only in copulated females and functions as a spermatheca. Given the lack of histological data on this structure in *T. gracilis*, we can only hypothesize about its function. It may serve as a temporary sperm storage site, provide nutrients to sperm, facilitate fertilization, or modulate sperm viability over time. Further investigation is needed to clarify its function in *Tupiperla*.

The anatomy of the FRS varied among the studied genera. In *Gripopteryx*, the size of the individuals appeared to be directly related to the number of ovarioles. For instance, *G. pilosa* and *G. reticulata*, which are relatively large, have approximately 60 ovarioles. In contrast, *G. garbei*, which has a smaller body size, possesses only 20 ovarioles. This variation is not only an indirect consequence of body size, but also has direct implications for the reproductive potential of these species, since the number and dimensions of ovarioles are key determinants of fecundity (Chapman; Simpson; Douglas, 2013). A higher ovariole count is generally associated with increased egg production capacity, whereas fewer and smaller ovarioles may indicate a lower reproductive output (Hodin, 2009). However, recent comparative studies have shown that reductions in ovariole number have evolved repeatedly across insects, indicating that reproductive potential is also shaped by developmental and life-history trade-offs (Church et al., 2021). Despite these differences, the general structure of the FRS remained similar across all three species, and all species exhibited a consistent proportional relationship between the lateral oviduct and the ovariole-bearing region.

The species of *Paragripopteryx* had the fewest ovarioles, likely reflecting its smaller body size. It differed from the other genera by possessing a notably long pedicel and an enlarged proximal portion of the lateral oviduct that gradually narrows distally. This enlarged region is likely responsible for storing mature eggs, similarly to the other species; however, no mature eggs were observed in the dissected specimens.

In *Tupiperla*, both *T. gracilis* and *T. tessellata* exhibited elongated lateral oviducts that maintained a consistent diameter along their length, even when filled with eggs. Although we did not observe oogenesis in any dissected females of *T. robusta*, all specimens displayed similarly long lateral oviducts. A notable feature in *Tupiperla* females was a slender, elongated,

chitinous structure embedded medially within the membranous wall of the genital chamber. Interestingly, the morphology of this structure varied between *T. gracilis* and *T. tessellata*, making it a potential distinguishing character between these two species.

The chitinous structure of the common oviduct possibly plays a role in guiding sperm during copulation (Eberhard 1996) or in providing structural support during egg-laying (Snodgrass 1993; Chapman *et al.* 2013). Chitinized regions within the genital chamber may provide mechanical support, helping stabilize copulation or oviposition. Studies on Orthoptera and Lepidoptera have documented hardened vaginal structures involved in sperm storage and egg-laying mechanics (Chapman *et al.* 2013; Snodgrass 1993). Another example, studies on the female reproductive system of *Bagrada hilaris* (Hemiptera: Pentatomidae) have documented a chitin-lined vagina, underscoring the structural reinforcement provided by chitin in reproductive tissues (Grodowitz *et al.* 2019). However, further histological and functional studies are necessary to elucidate the precise roles of these chitinous components.

Surprisingly, this structure was utterly absent in all *T. robusta* specimens, further highlighting its anatomical divergence from *T. gracilis* and *T. tessellata*. Additionally, *T. robusta* exhibited a significantly different genital chamber morphology, characterized by a more bulbous shape than the elongated and flattened form observed in the other *Tupiperla* species (not shown in the figures).

The histological characteristics of the ovarioles in the studied species align with the typical panoistic condition described for Plecoptera ovaries, where germ cells differentiate directly into oocytes without nurse cells (Rościszewska & and Rzońca 2009). The absence of a terminal filament and the linear arrangement of differentiating oogonia suggests a relatively simple ovarian structure. The progressive accumulation of yolk granules as the oocytes mature further supports this pattern (Büning 1994; Chapman *et al.* 2013).

The presence of intensely stained columnar cells in the epithelium of the lateral oviduct suggests high level of metabolic activity, possibly related to secretion or absorption processes. This feature, along with the muscular layers may facilitate the transport of gametes. Furthermore, the internal cuticular lining of the common oviduct, also reported in other Plecoptera (Zwick 1973), supports its ectodermal origin.

The eggs of some species could not be described because no females carrying eggs were found. We hypothesize that these females had not yet mated, as no sperm cells were observed.

Some insects require mating stimulation to initiate oogenesis, a phenomenon known as mating-induced oogenesis (Engelmann 1970). To explore this possibility, we reared female larvae of *Tupiperla tessellata* (n=3) to adulthood and maintained them in isolation for 16–18 days. Upon dissection, no signs of oogenesis were observed, supporting the hypothesis that mating may be necessary to trigger egg development. However, other forms of stimulation, such as specific environmental cues or dietary factors, could also play a role and should be considered in future studies. Furthermore, our attempts to induce mating in laboratory conditions were unsuccessful, further limiting our ability to investigate this process.

4.2. Male reproductive system (MRS)

Our study revises and updates the terminology initially proposed by Zwick (1973) regarding the structure of the *vasa deferentia* in Plecoptera. Zwick described these ducts as a "follicle-bearing strand" and an "outer strand." However, referring to these structures as the *main deferent ducts* and *accessory deferent ducts*, respectively, is more appropriate. This terminology accurately reflects their distinct functional roles. Our updated terminology provides a clearer framework for describing the organization of the male reproductive system in Gripopterygidae and facilitates comparisons across taxa.

Our findings have also revealed differences in the number of fusion points between the two types of deferent ducts. Zwick (1973) reported five fusion points in *Zelandoperla decorata* (Gripopterygidae), whereas we consistently observed four in all species examined in this study. The openings of these fused ducts varied in size across genera. In *Guaranyperla* these openings were notably smaller than in other genera, with the first opening (along the anterior-posterior axis) being significantly smaller than the remaining ones. Conversely, in *Tupiperla* and *Gripopteryx* species, the openings were more dilated overall, except for the third opening, which was consistently smaller than the others. These variations suggest potential functional differences among genera and may be associated with differences in sperm storage or transfer efficiency, although further studies are needed to explore this hypothesis.

Another important aspect of our findings is the variation in the number of testicular follicles across the different genera we examined. We recorded follicle counts of up to 47 in *Gripopteryx*, up to 44 in *Guaranyperla*, and up to 36 in *Tupiperla*. These numbers seem consistent within species and even genera, which aligns with previous observations in other

Plecoptera (Zwick 1973). However, follicle quantification remains scarce among Antarctoperlaria, with only a few reported cases, such as *Zelandoperla decorata* (approximately 49 follicles) and *Thaumatoperla robusta* (around 100 follicles) (Zwick 1973, 1980). This lack of comparative data across taxa poses challenges in identifying broader evolutionary trends. Nevertheless, our findings support Zwick's (1973) assumption that follicle count strongly correlates with insect body size. The lower follicle counts in *Tupiperla*, a genus with relatively small body size, compared to *Guaranyperla* and *Gripopteryx*, supports the idea that testicular follicle number is constrained by overall body size. In addition, we found differences in the shape of the testicular follicles across the genera, being more tapered in *Tupiperla* species, and more orbicular in *Gripopteryx* and *Guaranyperla* species.

Furthermore, one of the key characters supporting the monophyly of Plecoptera is the presence of two pairs of stacked seminal vesicles in males, which loop anteriorly (Zwick 2000). This feature, along with the anterior fusion of the testes, was referred to by Zwick (1973) as the "basic type" among stoneflies. Several lineages, including Gripopterygidae, Eusthenioidea, Pteronarcyidae, Perlodidae, Chloroperlidae, and Taeniopterygidae, retain this configuration (Zwick 1973; Rościszewska & and Rzońca 2009). In contrast, we found that the male reproductive system of Neotropical Gripopterygidae deviates from this pattern, possessing a single pair of seminal vesicles fused at the posterior end. This condition is not exclusive to the group, as it also occurs in other Plecoptera families within both Arctoperlaria (e.g., Capniidae, Notonemouridae, Nemouridae, and Leuctridae; (Zwick 1973, 1980)) and Antarctoperlaria [e.g., Diamphipnoidae, such as *Diamphipnopsis virescentipennis* (Blanchard, 1851) (Illies 1960)]. The number of seminal vesicles varies across Plecoptera lineages. Some taxa, such as Styloperlidae, Peltoperlidae, and Perloidea, have undergone a reduction, retaining only a single pair. In other families, such as Nemouridae and Leuctridae, a single unpaired seminal vesicle is present, a condition recognized as a synapomorphy for each family (Zwick 2000). The loss of one pair of seminal vesicles in Neotropical Gripopterygidae represents yet another variation, suggesting that it may be a secondary and independent modification within these lineages.

Interestingly, in the *Tupiperla* species, we observed a figure-eight-shaped segment as the paired deferent ducts connect by a fusion point near the basal portion of the seminal vesicles. In other examined genera where this structure was not detected, we suspect its apparent absence may be due to technical challenges during dissection, as the connection might have been disrupted. Supporting this hypothesis, in all dissected specimens, the deferent duct

consistently curved upward and then downward posteriorly, precisely in the region where the figure-eight structure would be expected.

Another noteworthy anatomical difference concerns the position of the connection between the deferent ducts and the seminal vesicles. In *D. virescentipennis* (Blanchard, 1851) and *Z. decorata* Tillyard, 1923 (Zwick 2000), Diamphipnoidae and Gripopterygidae, respectively, as well as in several other families within Arctoperlaria (Zwick 1973), this connection is located near the apex of the anterior region of the seminal vesicles, a feature also observed in several other families within Arctoperlaria (Zwick 1973). In all species examined in this study, the connection is positioned in the posterior region of the seminal vesicles. This divergence in reproductive anatomy may represent a significant evolutionary shift among these taxa. Notably, this posterior connection had previously been documented only in taxa of Arctoperlaria, including *Diura bicaudata*, *Perlodes dispar*, *Arcynopteryx compacta*, *Isoperla* spp. (Brinck 1956), *Hydroperla crosbyi* (Perlodidae) (Stewart & Stark 1977), as well as *Zealeuctra* sp., *Tyrrhenoleuctra* sp., *Moselia infuscata*, *Pachyleuctra benllochi*, and *Leuctra nigra* (Leuctridae) (Zwick 1973).

Structural differences among the studied genera were evident in various components of the male reproductive system, highlighting both conserved and divergent traits. The accessory gland had an elongated and oblong shape in *Gripopteryx* and *Guaranyperla*, whereas in *Tupiperla tessellata*, it was more elliptical or orbicular. Notably, *Tupiperla robusta* displayed the greatest variation, with a uniquely pair of rectangular-shaped accessory glands.

Large accessory glands in males is one of the characters supporting the monophyly of Gripopterygoidea. However, compared to the illustration of *Z. decorata* provided by (Zwick 1973, 1980), it is evident that the accessory glands in Neotropical gripopterygids are relatively small. This apparent reduction in gland size within Neotropical lineages may represent an evolutionary shift, possibly related to changes in reproductive strategy, mating behavior, or physiological constraints. Further investigation is needed to determine whether this reduction is a derived trait within Neotropical *Gripopterygidae* or if it reflects broader ecological or functional adaptations.

The seminal vesicles also varied in shape across genera. In *Gripopteryx*, they were notably long and, when filled with sperm, often bent twice downward before curving upward again. Despite this length, the vesicles maintained a consistent diameter throughout. In contrast,

the seminal vesicles of *Guaranyperla* and *Tupiperla* exhibited anterior inflation, gradually enlarging along their length. Between these two genera, *Guaranyperla* had shorter seminal vesicles compared to *Tupiperla*. Moreover, in all species examined, we observed a progressive thickening of the epithelial tissue toward the posterior region of the seminal vesicle, which may indicate increased secretory activity in this area.

The efferent ducts were relatively short, consistent with previous descriptions for most Plecoptera (Brinck 1956; Zwick 1973; Rościszewska & and Rzońca 2009). However, *T. tessellata* exhibited a distinct feature: its final efferent duct (along the anterior-posterior axis) was longer than the others. This unique characteristic may have functional significance in sperm transport or storage, although further study is needed to confirm its implications.

The MRS of *Tupiperla robusta* exhibited notable anatomical variations compared to those of other *Tupiperla* species and genera examined in this study. The shape of the testicular follicles was more orbicular, contrasting with the more narrowed ones found in *T. gracilis* and *T. tessellata*. As previously mentioned, the accessory gland morphology in *T. robusta* deviated from the pattern observed in the other species of this genus, displaying a more rectangular shape. Another key distinction was the positioning of the deferent ducts connection to the seminal vesicles, which occurred at approximately one-quarter of the vesicle's length and was positioned anteriorly than in *Gripopteryx*, *Guaranyperla*, and other *Tupiperla* species.

Histological analyses also provided new insights into the reproductive structures of the studied species. We observed two distinct stainings within the cytoplasm of the glandular epithelial cells, one darker than the other, suggesting the production of different types of secretions. However, we did not observe secretions in the lumen of the accessory glands. We also identified the presence of chitin lining the epithelial lumen indicating that the accessory gland is of ectodermal origin. This fact, along with its anatomical position, allows us to infer that it likely discharges into the ejaculatory duct. The ejaculatory duct could not be identified in any of the examined specimens, limiting our ability to further investigate its structural characteristics.

In the testes of all species, we observed undifferentiated spermatogonia capable of producing new sperm, suggesting that the four genera exhibit continuous spermatogenesis. The presence of testicular follicles with ongoing sperm production indicates that males of these species may engage in multiple mating events throughout their adult lifespan. This aligns with

previous studies reporting that these species continue to feed during adulthood (Froehlich 1969; Hynes 1976; Stewart 2009), further supporting the assumption that they sustain reproductive activity over time.

Additionally, we found that sperm cells were free-floating rather than arranged in bundles within both the deferent duct and the seminal vesicle. This characteristic may have important implications for sperm storage and transport, warranting further investigation.

Concluding remarks

Comparisons between the Neotropical Gripopterygidae analyzed in this study and Australasian species, such as the male of *Zelandoperla fenestrata* (*sensu* (Zwick 1973, 1980) and the female of *Trinotoperla nivata* (*sensu* (Rościszewska & and Rzońca 2009), highlight notable anatomical differences, potentially reflecting evolutionary divergence between these biogeographic regions. However, given the limited knowledge of the reproductive systems of Antarctoperlaria, since only a few taxa have been studied so far, further investigations, particularly in Andean lineages, are needed to clarify the extent and significance of these differences.

The histological structure of the ovaries and testes across the studied genera, including *Gripopteryx*, *Guaranyperla*, *Paragripopteryx*, and *Tupiperla*, demonstrated high structural conservation. We observed no significant interspecific variation in the reproductive organs, suggesting a relatively conserved reproductive strategy within the group. This consistency in structure supports the notion of a uniform reproductive mechanism across these genera of Gripopterygidae.

In contrast to the histological findings, the anatomy of both the male and female RS exhibited notable variation across species, especially among different genera. One example of that is *Tupiperla robusta*, which exhibited significant anatomical divergence. These differences, combined with the unique external morphology of *T. robusta*, such as the broader pronotum (Rippel et al., 2025), suggest that the classification of this species needs further investigation. The anatomical variations observed in both male and female reproductive systems imply that *T. robusta* may represent a distinct evolutionary lineage within the

Tupiperla group. However, more studies (also using molecular data) may help elucidate the systematic position of *Tupiperla* in Gripopterygidae.

Thus, our findings indicate that the anatomical divergences of the studied taxa present a valuable source of information for understanding evolutionary relationships within the family Gripopterygidae. The differences observed in RS anatomy among the genera suggest that these anatomical traits could provide insights for phylogenetic reconstruction and classification within the family. This variation in reproductive anatomy forms one of the central contributions of this study to the broader understanding of Gripopterygidae systematics.

Our study provides the first complete histological description of the male reproductive system in Antarctoperlaria, and the first detailed anatomical description of the female reproductive system in Gripopterygidae. These findings fill an important gap in our understanding of Plecoptera reproductive biology and offer valuable insights for future studies on the reproductive strategies of these and related species.

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Ethics approval

Our sampling followed Brazilian laws and were authorized by the SISBIO-ICMBio (Biodiversity Authorization and Information System, Chico Mendes Institute for Biodiversity Conservation, numbers 79695-1, 55428-16, and 65213-11), also by IEF (Minas Gerais State Institute of Forests, number 058/2021)

Author's contributions

Mellis L S Rippel: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review and Editing; **Dayvson A Costa:** Writing - Review and Editing, Methodology; **Rodrigo B Gastaldo:** Illustrations and editing, Methodology; **Jose Lino-Neto:** Writing - Review, Project administration, Methodology; **Frederico F. Salles:** Conceptualization; Writing - Review, Project administration.

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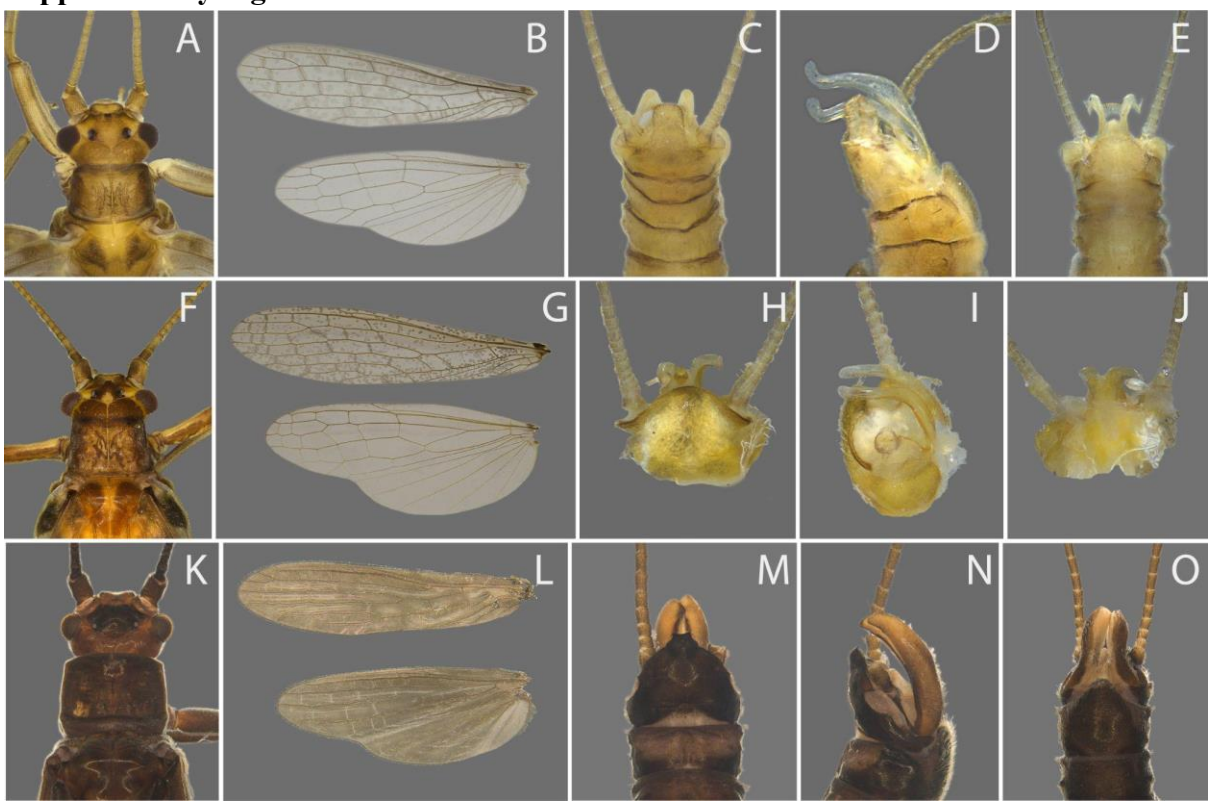
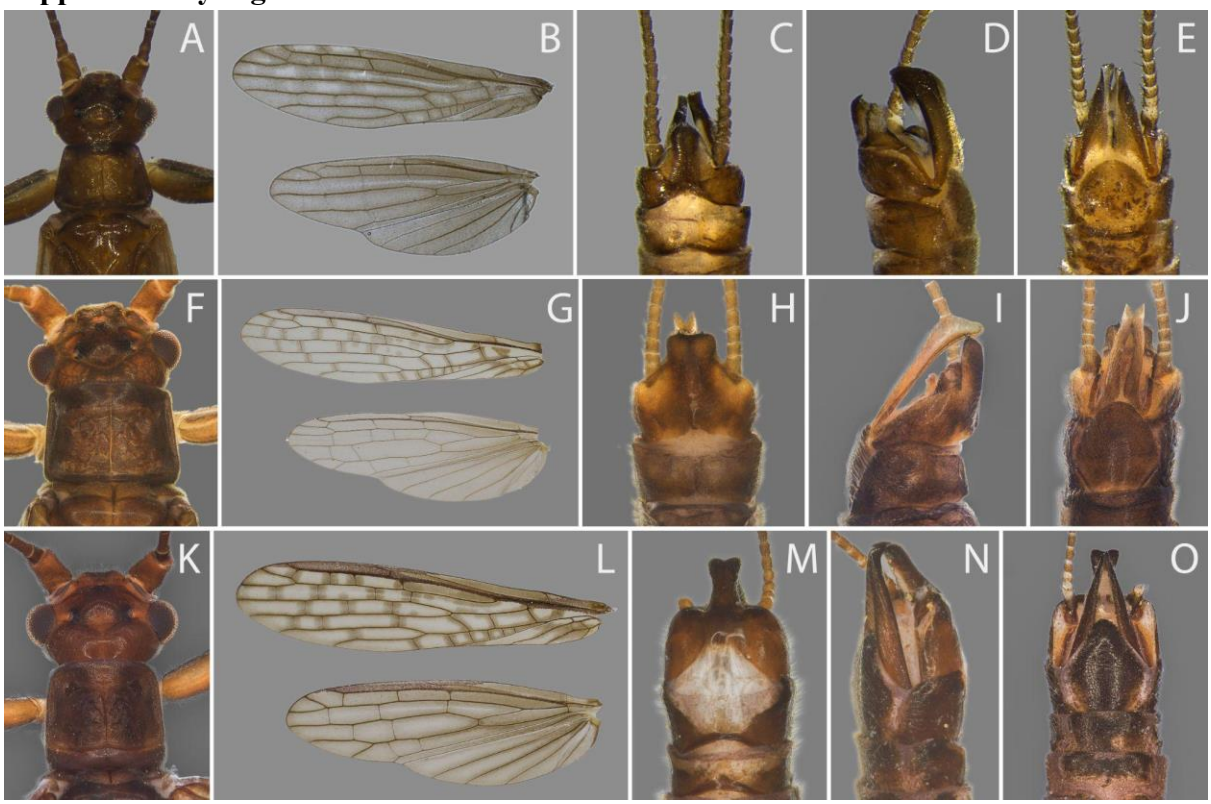
Supplementary information

Supplementary Figure S1



Head and pronotum, fore and hindwings, terminalia in dorsal, lateral and ventral views, respectively, of *Gripopteryx garbei* (A–E); *Gripopteryx pilosa* (F–J); *Guaranyperla puri* (K–O).

Supplementary Figure S2



Head and pronotum, fore and hindwings, terminalia in dorsal, lateral and ventral views, respectively, of *Paragripopteryx delicata* (A–E); *Tupiperla robusta* (F–J); *Tupiperla tessellata* (K–O).

Table S1 – Additional examined material. Some samples included here were not mentioned in the main text, however they were relevant to our conclusions.

Label code	Genus	Species	Gender	Collection	
				Location	Date
04.vii.23	*Gripopteryx*	*G. garbei*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	04.vii.2023
21.i.23-01	*Gripopteryx*	*G. reticulata*	M	São Paulo State, Salesópolis municipality, Córrego Venerando 23º39’9’’S 45º53’26’’W	21.i.2023
15-16.ii.23(1)	*Gripopteryx*	*G. pilosa*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15–16.ii.2023
23.iii.23	*Gripopteryx*	*G. pilosa*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	21–22.iii.2023
24.viii.24-03	*Gripopteryx*	*G. pilosa*	M	Minas Gerais state, Espera Feliz municipality, Caparaó National Park 20º28’19’’S 41º49’44’’W	24.viii.2024
02.v.24-01	*Tupiperla*	*T. gracilis*	F	Minas Gerais state, Ouro Preto municipality, Tripuí State Park 20º23’5’’S 43º32’34’’W	02.v.2024
03.i.25-01	*Tupiperla*	*T. gracilis*	M	Minas Gerais state, Santos Dumont municipality 21º26'45.6"S 43º40'18.6"	23–29.xii.2024
15.ii.23(1)	*Tupiperla*	*T. robusta*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15.ii.2023
16-17.ii.23	*Tupiperla*	*T. robusta*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15–16.ii.2023
14.ii.23(2)	*Tupiperla*	*T. robusta*	F	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	14.ii.2023
15.ii.23	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15.ii.2023

14.ii.23(1)	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	14.ii.2023
14.ii.23(3)	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	14.ii.2023
21-22.iii.23-01	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	21–23.iii.2023
21-22.iii.23-02	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	21–23.iii.2023
15-16.ii.23	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15–16.iii.2023
23.iii.23	*Tupiperla*	*T. robusta*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	23.iii.2023
18-19.vi.23	*Tupiperla*	*T. tessellata*	F	Minas Gerais state, Araponga municipality, Serra do Brigadeiro State Park 20º43’52’’S 42º27’50’’W	18–19.vi.2023
16.ix.24	*Tupiperla*	*T. tessellata*	F	Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’5’’S 41º49’8’’W	21.viii.2024
20.ix.24-01	*Tupiperla*	*T. tessellata*	F	Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’5’’S 41º49’8’’W	21.viii.2024
01.17.x.22	*Tupiperla*	*T. tessellata*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	17.x.2022
01.22.x.22	*Tupiperla*	*T. tessellata*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	13.x.2022
14.ii.23(1)	*Tupiperla*	*T. tessellata*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	14.i.2023
15-16.ii.23	*Tupiperla*	*T. tessellata*	M	Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W	15-16.ii.2023

20.vi.23(1)	*Tupiperla*	*T. tessellata*	M	Minas Gerais state, Araponga municipality, Serra do Brigadeiro State Park 20º43’52’’S 42º27’50’’W	18-19.vi.2023
05.v.24-01	*Tupiperla*	*T. tessellata*	M	Minas Gerais state, Ouro Preto municipality, Itacolomi State Park 20º27’25’’S 43º30’12’’W	03.v.2024
22.viii.24-02	*Tupiperla*	*T. tessellata*	M	Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’5’’S 41º49’8’’W	21.viii.2024
09.ix.24-01	*Tupiperla*	*T. tessellata*	M	Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park 20º30’5’’S 41º49’8’’W	22.viii.2024

Table S2 – Measurements of female specimens included in the study. Numbers in millimeters.

Species & Label code	Ovary length	Lateral oviducts			Ovariole length	Genital chamber		Forewing length
		Length	Anterior portion width	Posterior portion width		Length	Width	
Gripopteryx garbei								
15.viii.23-01								
15.viii.23-02	0.47	0.7	–	–	0.38	–	–	9.8
	0.5	0.95	–	–	0.40	–	–	9.9
Gripopteryx pilosa								
24.viii.2024-01								
24.viii.2024-02	1.54	2.6	–	–	–	–	–	19.4
	1.75	1.7	0.35	0.30	1.47	0.74	1.20	19.3
Gripopteryx reticulata								
20.xi.23	1.81	2.86	0.22	0.30	1.39	–	–	17.3
22.iv.24	–	–	–	–	1.0	0.59	1.18	19.0
Paragripopteryx								
P. sp. 1 22.iv.24	0.36	0.54	0.04	0.09	0.24	–	0.13	8.3
P. sp. 2 25.viii.24-	0.45	0.72	0.05	0.12	0.54	0.17	0.16	8.5
01	1.0	1.75	0.05	0.18	0.76	0.28	0.3	7.4
P. sp. 3 05.xi.24-01								
Tupiperla gracilis								
02.v.24-01	1.27	2.3	0.18	0.26	–	0.59	0.38	8.5
06.v.24-01	0.83	2.98	0.17	0.19	–	0.57	0.42	8.8
04.i.25	0.81	1.0	0.11	0.13	0.51	0.35	0.23	8.1
Tupiperla robusta								
14.ii.23-01	1.38	1.32	0.1	0.16	0.55	–	–	11.4
15.ii.23-02	0.92	1.64	0.13	0.20	0.61	0.14	0.19	11.0
23.iii.23	–	1.43	0.1	0.15	0.4	0.22	0.13	11.5
Tupiperla tessellata								
22.viii.24-01	1.21	1.59	0.17	0.20	0.59	0.76	0.34	10.1
05.v.24	1.07	3.33	0.27	0.20	0.70	0.72	0.31	9.7

Table S3 – Measurements of male specimens included in the study. Numbers in millimeters.

Species & Label code	Testes length	Testicular follicle		Deferent duct length	Seminal vesicles length	Accessory glands		Forewing length
		Length	Width			Length	Width	
Gripopteryx garbei 16-17.ii.23	–	–	–	–	–	–	–	8.8
Gripopteryx pilosa	2.74	0.60	0.30	–	2.29	0.58	–	15.5
24.viii.2024-01	3.1	0.56	0.26	3.12	3.79	0.63	–	16.4
24.viii.2024-02	2.52	0.48	0.23	2.55	–	0.60	–	17.0
25.viii.24-01								
Gripopteryx reticulata								
01.22.x.22	2.21	0.56	0.26	2.52	1.57	–	–	14.4
19.iv.24	–	0.49	0.27	–	1.18	0.27	0.08	14.8
19.v.24	–	–	–	–	4.10	–	–	15.08
Guaranyperla puri	1.69	0.34	0.15	–	–	–	–	9.3
07.x.22	2.08	0.47	0.27	–	–	–	–	9.0
05.x.2024-01								
Paragripopteryx delicata 15-16.ii.23	–	–	–	–	–	–	–	6.3
Tupiperla gracilis								
02.v.24-01	–	0.41	0.18	–	2.43	0.11	0.06	7.2
04.v.24	–	0.41	0.20	–	–	–	–	7.1
06.v.24-01	1.23	0.37	0.14	0.55	0.23	–	–	8.2
Tupiperla robusta								
14.ii.23-02	2.42	0.47	0.22	–	1.35	–	–	9.2
16.ii.23	1.76	0.39	0.19	–	0.9	0.23	0.07	9.4
09.xii.24-01	1.65	0.45	0.18	–	–	0.19	0.09	8.6
Tupiperla tessellata								
04.v.24-01	–	0.43	0.18	–	0.91	–	–	–
22.viii.24-01	1.38	0.40	0.15	–	–	–	–	8.5
25.viii.24-01	1.9	0.42	0.16	–	2.25	0.14	0.10	8.2
03.i.25-01	1.73	0.41	0.16	–	1.82	0.16	0.10	7.5

CHAPTER III - SPERM MORPHOLOGY OF ANTARCTOPERLARIA (PLECOPTERA: INSECTA): FIRST DESCRIPTION AND COMPARATIVE ANALYSIS IN NEOTROPICAL GRIPOPTERYGIDAE

Sperm morphology of Antarctoperlaria (Plecoptera: Insecta): first description and comparative analysis in Neotropical Gripopterygidae

Mellis L. S. Rippel[1,2]; Dayvson A. Costa[1,3]; Rodrigo B. Gastaldo[1,2]; José Lino-Neto[3], Frederico F. Salles[2]

[1]*Programa de Pós-graduação em Entomologia, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil.*

[2]*Departamento de Entomologia, Museu de Entomologia, Universidade Federal de Viçosa (UFV), 36570-900, Viçosa, Minas Gerais, Brazil.*

[3]*Departamento de Biologia Geral, Laboratório de Ultraestrutura Celular, Universidade Federal de Viçosa (UFV), 36570-900, Viçosa, Minas Gerais, Brazil*

Abstract

Sperm morphology provides valuable characters for phylogenetic and taxonomic studies in insects. However, detailed descriptions of spermatozoa remain limited for many lineages, including Plecoptera. Here, we present the first morphological characterization of spermatozoa in Gripopterygidae and, more broadly, Antarctoperlaria, examining eight species of the Neotropical genera *Gripopteryx*, *Paragripopteryx*, *Guaranyperla*, and *Tupiperla*. Our analyses reveal that in all examined species the sperm is filiform, conforming to the general polyneopteran structure, with an elongated nucleus and a short, apically positioned acrosome. Intergeneric differences in sperm head morphology were identified, with *Gripopteryx* and *Paragripopteryx* exhibiting slender heads, whereas *Guaranyperla* and *Tupiperla* possess more cylindrical shapes. Pairwise comparisons showed significant variation in nuclear and total sperm length across most taxa, with some exceptions. Notably, intraspecific variation in *Gr. reticulata* and *Gu. puri* suggests the presence of cryptic species. These findings provide new morphological data for understanding sperm evolution in Antarctoperlaria and underscore the need for broader taxonomic sampling, particularly from Andean and Australasian Gripopterygidae, to further assess the phylogenetic significance of sperm traits in Plecoptera.

Keywords: stoneflies; spermatozoa; comparative spermatology; integrative taxonomy.

Introduction

Spermatozoa are the most morphologically variable metazoan cell type, having undergone rapid and divergent evolution across multiple taxa (Pitnick et al., 1999). This pattern is particularly evident in insects, where sperm size and morphology can vary significantly even among closely related species (Dallai, 2014; Fitzpatrick et al., 2022). Such variability has attracted interest in taxonomic (Barcellos et al., 2015; Baffa et al., 2017) and phylogenetic studies (Dallai, 2014; Dallai et al., 2016; Dias et al., 2022; Giglio et al., 2024), as sperm traits may provide useful characters for systematics. Additionally, sperm morphology reflects reproductive strategies, particularly in species with sperm competition and cryptic female choice (Kahrl et al., 2021). Traits like increased length or specialized shape enhance fertilization success, often as adaptations to female reproductive tract morphology, highlighting the role of post-copulatory selection in shaping sperm diversity (Dallai et al., 2014; Gottardo et al., 2016).

Plecoptera (stoneflies) is a key order of insects due to its phylogenetic position within Polyneoptera, representing one of the earliest-diverging lineages of winged insects (Misof et al., 2014). It is traditionally divided into two suborders: Arctoperlaria and Antarctoperlaria. Arctoperlaria, mostly distributed across the Northern Hemisphere, includes the families Taeniopterygidae, Leuctridae, Capniidae, Nemouridae, Notonemouridae, and Scopuridae (infraorder Euholognatha), as well as Perlodidae, Chloroperlidae, Perlidae, Kathroperlidae, Peltoperlidae, Pteronarcyidae, and Styloperlidae (infraorder Systellognatha). Antarctoperlaria comprises the families Eustheniidae, Diamphipnoidae, Austroperlidae, and Gripopterygidae, which are restricted to the Southern Hemisphere (Zwick, 2000; Letsch et al., 2021; Chen, 2022).

Sperm morphology has been the subject of a few studies for stoneflies, primarily focusing on ultrastructural aspects. Fausto et al. (2001) conducted a comparative investigation of sperm structure in species of Leuctridae and Taeniopterygidae (Euholognatha), revealing interspecific variations with taxonomic and phylogenetic implications. Their findings supported a close evolutionary affinity between Plecoptera and other Polyneoptera orders. Continuing this research, Fausto et al. (2002) analyzed sperm structure in four species of Chloroperlidae and Perlodidae (Systellognatha), highlighting distinct sperm characteristics that reinforced the heterogeneity within Plecoptera. These studies underscored the diversity of

sperm morphology across species, offering insights into reproductive strategies and sperm evolution within Arctoperlaria.

More recently, Fausto et al. (2023) expanded the understanding of sperm models in Plecoptera by examining species from other euholognathan families, such as Capniidae, Leuctridae, Nemouridae, and Taeniopterygidae. Their findings indicate that sperm traits are well conserved within each family, reinforcing the potential of comparative spermatology for systematic investigations. This study further supports patterns of sperm variation and their relevance to systematics and reproductive strategies in Plecoptera, aligning with Zwick's phylogenetic proposals (Zwick, 2000) and providing a more current perspective on the order's evolutionary relationships within Polyneoptera (Fausto et al., 2023).

Gripopterygidae, the most diverse family within Antarctoperlaria, is widely distributed across the Southern Hemisphere, occurring in South America, Australia and New Zealand. It comprises approximately 330 described species classified into 57 genera (Pessacq et al., 2019; Duarte et al., 2024; DeWalt et al., 2025). Traditionally, the family has been divided into five subfamilies (Antarctoperlinae, Dinotoperlinae, Gripopteryginae, Leptoperlinae, and Zelandoperlinae) based on morphological characteristics, though these divisions were established without a cladistic framework (McLellan, 1977). As a result, the monophyly of these subfamilies remains untested, and the relationships among genera are still poorly resolved (McLellan and Zwick, 2007; McCulloch et al., 2016; Pessacq et al., 2020; Letsch et al., 2021).

In South America, Gripopterygidae displays a distinct biogeographical pattern, with species distributed across both the Andean and Neotropical regions (*sensu* (Morrone, 2014, 2015). The Andean region comprises 50 species spread across 24 genera, while the Neotropical region contains 62 species in four genera restricted to this region: *Gripopteryx* Pictet, 1841, *Guaranyperla* Froehlich, 2001, *Paragripopteryx* Enderlein, 1909, and *Tupiperla* Froehlich, 1969. The majority of Neotropical species (59) are confined to the Brazilian Atlantic Forest (Duarte et al., 2024; DeWalt et al., 2025), all of which belong to Gripopteryginae, endemic to South America (Fig. 1) (Pessacq et al., 2019; DeWalt et al., 2025).

Figure 1. Male and female of *Tupiperla* sp. mating.

Species-level identification in Plecoptera is often challenging due to high intraspecific morphological variability and subtle differences between closely related species (Vitecek et al., 2017; Almeida and Bispo, 2020). This challenge is particularly evident in Gripopterygidae, where taxonomic studies have traditionally relied on a limited set of morphological characters, primarily male genitalia. The lack of comprehensive morphological investigations complicates species delimitation and phylogenetic assessments, particularly in the presence of cryptic diversity (Duarte et al., 2024; Rippel et al., 2025). Moreover, the only morphological phylogeny proposed for the family (Duarte, 2019) remains unpublished, highlighting the challenges of constructing a robust character matrix based solely on morphological traits. Expanding the range of characters used in species delimitation is therefore crucial for developing a more comprehensive and reliable systematic framework for Gripopterygidae.

Comparative spermatology offers valuable insights into phylogenetic and taxonomic questions that traditional morphological approaches alone have been unable to resolve (Hodgson et al., 2016; Baffa et al., 2017; Fausto et al., 2023). However, sperm morphology remains poorly documented across Plecoptera, particularly within Neotropical genera, which

have received little attention. Notably, no study has described sperm morphology among taxa in Antarctoperlaria, leaving a significant gap in our understanding of their systematics and reproductive traits. Expanding this knowledge could provide important contributions to the evolutionary history, taxonomy, and reproductive biology of both Antarctoperlaria and Plecoptera as a whole. To address these gaps, this study aims to describe and compare the sperm morphology of Neotropical Gripopterygidae, analyzing species across *Gripopteryx*, *Guaranyperla*, *Paragripopteryx* and *Tupiperla*.

Material and methods

2.1. Collection and identification

We sampled male adults of Gripopterygidae collected at night using a light sheet trap along the banks of streams at different locations in the Atlantic Forest of the states of Minas Gerais and São Paulo, southeastern Brazil. The examined material is listed below:

Gripopteryx cancellata (Pictet). Brazil, border of Minas Gerais and Espírito Santo states, Caparaó National Park, 20º24’39’’S 41º50’3’’W, 20.viii.2024, one male (code. 21.viii.24-01), 20º28’57’’S 41º40’14’’W, 23.viii.2024, one male (code. 20.ix.24-01).

Gripopteryx pilosa Froehlich. Minas Gerais state, Espera Feliz municipality, Caparaó National Park, 20º28’19’’S 41º49’44’’W, 24.viii.2024, four males (code. 24.viii.24-01), (code. 24.viii.24-02), (code. 24.viii.24-03), (code. 25.viii.24-01).

Gripopteryx reticulata Brauer. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico) 20º39’24’’S 42º27’8’’W, 22.x.2022, one male (code. 01-22.x.22); Serra do Brigadeiro State Park, 20º43’52’’S 42º27’50’’W, 19.v.2024, one male (code. 19.v.24). São Paulo State, Salesópolis municipality, Boraceia Ecological Station, Venerando stream, 23º39’9’’S 45º53’26’’W, 19.iv.2024, one male (code. 19.iv.24).

Guaranyperla puri Rippel & Salles. Minas Gerais state, Ervália municipality, Complexo Turístico do Pico do Cruzeiro, 20º46’37’’S 42º29’49’’W, 07.x.2022, one male (code. 07.x.22); Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 05.x.2024, one male (05.x.24-01).

Paragripopteryx delicata Froehlich. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 15–16.ii.2023, one male (code. 15-16.ii.23).

Tupiperla gracilis (Burmeister). Minas Gerais state, Ouro Preto municipality, Tripuí State Park, 20º23’5’’S 43º32’34’’W, 02.v.2024, two males (code. 02.v.24-01), (code. 04.v.24-01), 03.v.2024, one male (code. 06.v.24-01).

Tupiperla robusta Froehlich. Minas Gerais State, Araponga municipality, Fazenda Remanso (Seu Dico), 20º39’24’’S 42º27’8’’W, 14.ii.2023, one male (code. 14.ii.23-02), 15.ii.2023, one male (code. 15.ii.23-01), 16.ii.2023, one male (code. 16.ii.23-01), 21–22.iii.2023, one male (code. 23.iii.23-01).

Tupiperla tessellata (Brauer). Minas Gerais state, Ouro Preto municipality, Itacolomi State Park, 20º27’25’’S 43º30’12’’W, 03.v.2024, two males (code. 04.v.24-01), (code. 05.v.24-01); Araponga municipality, Serra do Brigadeiro State Park, 20º43’52’’S 42º27’50’’W, 18–19.vi.2024, one male (20.vi.23-01). Espírito Santo state, Dores do Rio Preto municipality, Caparaó National Park, 20º30’5’’S 41º49’8’’W, 21.viii.2024, one male (code. 22.viii.24-01).

The collection, handling, and identification of specimens followed the methodology described in Rippel et al. (2025). Specimens were initially identified to the species level using identification keys (Bispo and Lecci, 2011; Lecci and Froehlich, 2011) and original descriptions (Froehlich, 1969, 1993, 1994, 1998; Duarte et al., 2014, 2019, 2022). Whenever possible, dissections and sperm slide preparations were performed in the field. Otherwise, live specimens were transported to the Laboratory of Cellular Ultrastructure and the Entomology Museum of UFV (UFVB) at the Federal University of Viçosa (UFV) for identification and dissection. Sperm slides were prepared for 22 male specimens representing eight species: *Gripopteryx cancellata* (n = 2), *G. pilosa* (n = 4), *G. reticulata* (n = 3), *Guaranyperla puri* (n = 2), *Paragripopteryx delicata* (n = 1), *Tupiperla gracilis* (n = 3), *T. robusta* (n = 4), and *T. tessellata* (n = 4). Specimen imaging was conducted using a LEICA MC170 HD camera, with images processed in Adobe Photoshop CC® 2024 and plates assembled in Adobe Illustrator CC® 2024.

2.2 Sperm mosphology and morphometry

We prepared histological slides by smearing sperm from testicular follicles, deferent ducts, and seminal vesicles. For nuclear measurements, we stained the slides with 0.2 mg/ml DAPI (4',6-diamidino-2-phenylindole) for 20 minutes, rinsed them in running water, and allowed them to air-dry before photographing them with an Olympus BX60 photomicroscope (Olympus Corporation, Tokyo, Japan). We then stained the same slides with Giemsa, let them rest for 20 minutes, washed them in running water, and dried them at room temperature before photographing them again with the same equipment. To obtain the total length of spermatozoa, as well as the length of their flagella and nuclei, we measured at least 15 spermatozoa per individual using the free software Sperm Sizer 1.6.6. For nuclei measurements, we processed the DAPI-stained sperm images in Adobe Lightroom CC® 2024 to invert colors, enabling the software to accurately measure the nuclei. We prepared the graphs using R version 4.2.2. The packages used were *tidyverse* (Wickham et al., 2019), *RColorBrewer* (Neuwirth, 2022) and *colorspace* (Zeileis, 2020).

Results

The spermatozoa of all examined species are long, filiform, and composed of a distinct head (acrosome and nucleus) and a flagellum (Fig. 2). The spermatozoa are not organized in bundles in either the deferent ducts or the seminal vesicles.

Among *Gripopteryx* species, the average total spermatozoon length is 104.6 µm, with *G. cancellata*, *G. pilosa*, and *G. reticulata* measuring 92.6 µm, 99.8 µm, and 119.5 µm, respectively. In all species, the head features a short, conical acrosome, measuring 11.1 µm in *G. cancellata*, 8.7 µm in *G. pilosa*, and 9.3 µm in *G. reticulata*. Notably, in two *G. reticulata* males from Venerando Stream, São Paulo State, we observed two distinct sperm size classes: one individual had an average sperm length of 86.3 µm, while the other averaged 137.0 µm, the same size observed in a third specimen from PESB, Minas Gerais State (Fig. 3). Nucleus length variation in *G. reticulata* could not be assessed due to the availability of only one specimen for DAPI measurements. All measurements are presented in **Table 1**.

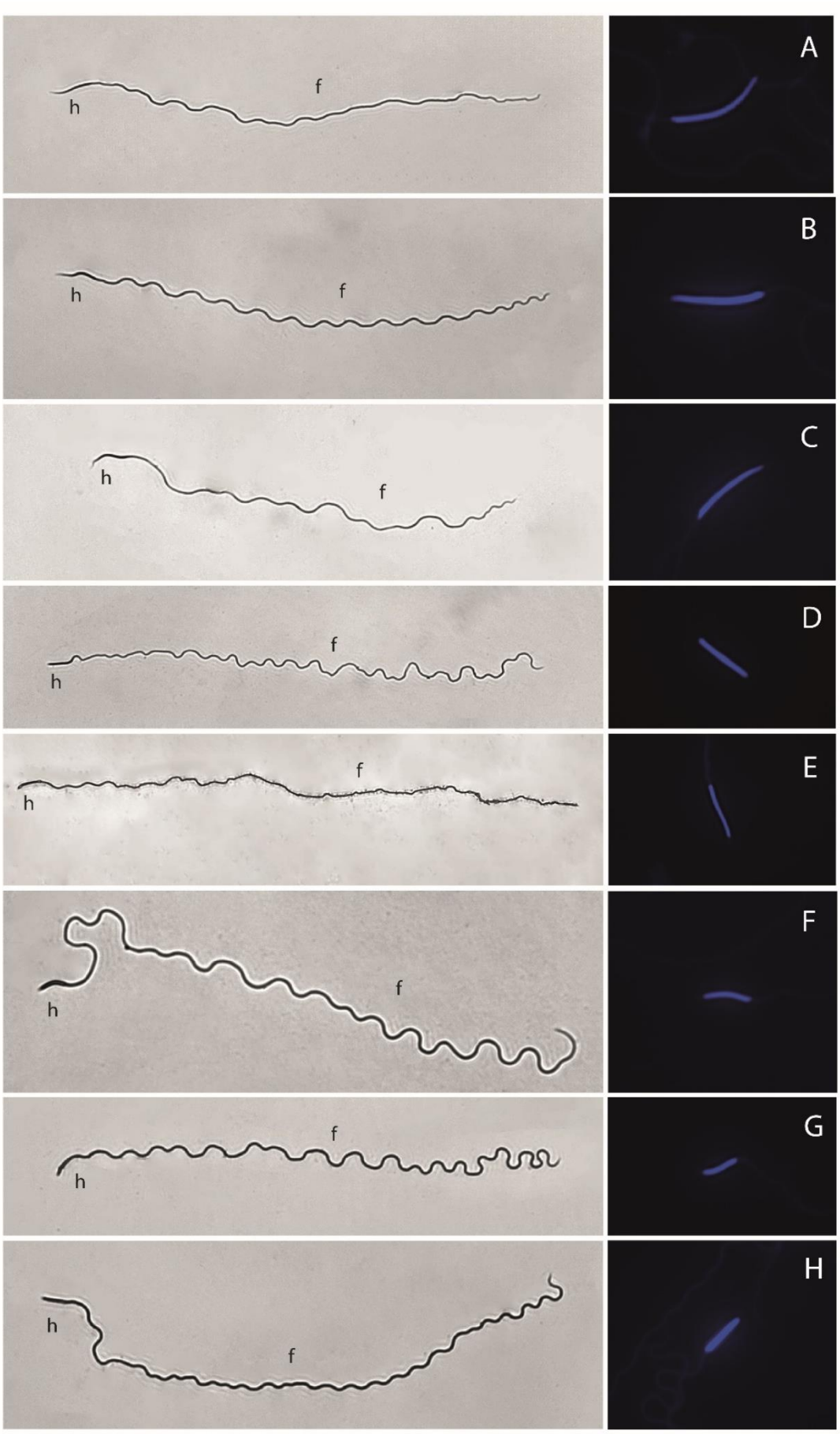

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Figure 2. Spermatozoon through Giemsa staining method (left), and DAPI staining method for nucleus visualization (right) of (A) *Gripopteryx cancellata*, (B) *G. pilosa*, (C) *G. reticulata*, (D) *Guaranyperla puri*, (E) *Paragripopteryx delicata*, (F) *Tupiperla gracilis*, (G) *T. robusta*, (H) *T. tessellata.* Abreviations: h = head and f = flagellum. Scale bars: 20 μm.

Table 1. Maximum, minimum, average and standard deviation measurements of each species through both DAPI and GIEMSA staining methods.

Species	DAPI staining			GIEMSA staining		
	Nucleus			Total spermatozoon length		
	Max	Min	$\bar{x} \pm SD$	Max	Min	$\bar{x} \pm SD$
Gripopteryx cancellata	13.6	8.4	11.1 ± 1.3	100.0	67.1	92.6 ± 5.0
Gripopteryx pilosa	9.8	7.7	8.7 ± 0.5	106.3	89.8	99.8 ± 3.4
Gripopteryx reticulata	10.6	8.3	9.3 ± 0.5	144.4	81.1	119.5 ± 24.6
Guaranyperla puri	9.6	5.2	7.3 ± 1.1	192.1	99.1	138.6 ± 34.1
Paragripopteryx delicata	11.0	7.2	8.3 ± 0.7	–	–	–
Tupiperla gracilis	11.2	4.7	6.2 ± 0.1	122.2	98.8	111.6 ± 6.0
Tupiperla robusta	6.1	3.1	4.0 ± 0.4	121.1	94.8	110.9 ± 5.1
Tupiperla tessellata	5.6	3.6	4.6 ± 0.4	121.9	86.2	104.8 ±6.7

In *Tupiperla* species, the average total spermatozoon length is 108.4 μm, with *T. gracilis*, *T. robusta*, and *T. tessellata* average measuring 111.6, 110.9, and 104.8 μm, respectively. Unlike *Gripopteryx*, *Tupiperla* spermatozoa have a reduced acrosome, and the head is cylindrical. The nuclei length averaged 6.2 μm in *T. gracilis*, 4.0 μm in *T. robusta*, and 4.6 μm in *T. tessellate* (Table 1).

Due to specimen limitations in *Paragripopteryx delicata* and the availability of only *Gu. puri* (two specimens) for *Guaranyperla*, direct comparisons with the previously described genera were not possible. Therefore, we provide only descriptions. In *Gu. puri*, spermatozoa had an average total length of 138.6 µm, with nuclei measuring 7.3 µm. The head displayed a reduced acrosome and a cylindrical shape. While nuclear length remained consistent between specimens, flagellar length exhibited two distinct classes, with one male exhibiting flagella measuring twice the length of the other male (Fig. 3). The single male of *P. delicata* had spermatozoa with a slender nucleus, averaging 8.3 µm, with a short acrosome. We could not measure the total length of *P. delicata*'s spermatozoa.

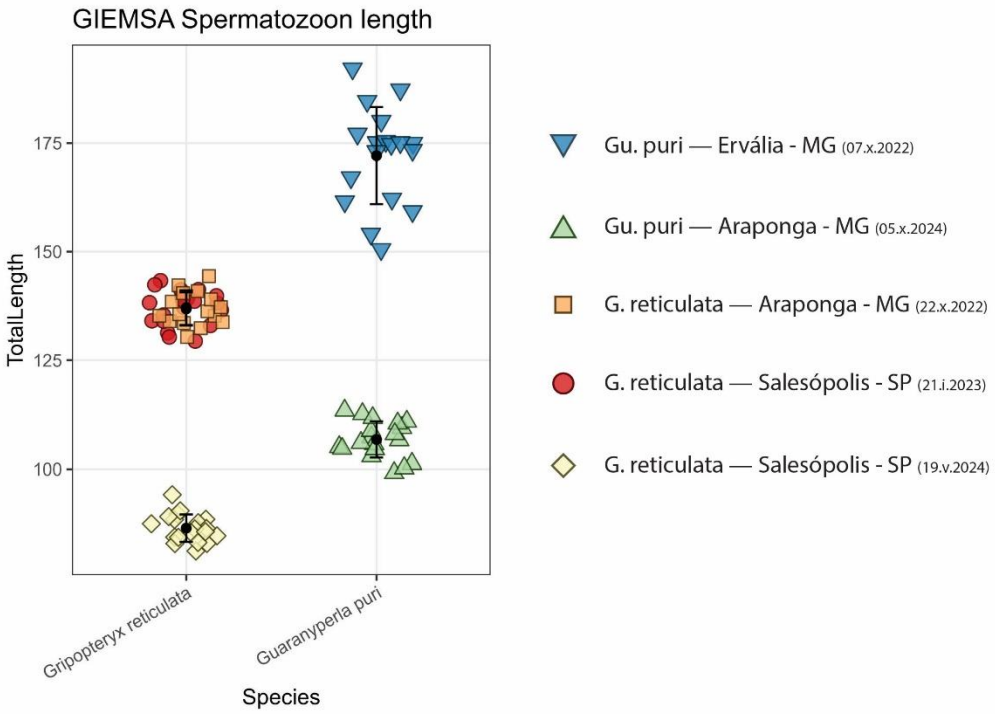

Figure
3. Variation of sperm total length (µm) in specimens within *G. reticulata* and *Gu. puri*. Markers in different colors and shapes indicate different specimens distinguished in the legend aside.

Differences in both total length and nucleus length were evident among genera, with the exception of the *Guaranyperla – Paragripopteryx* pair, which appeared similar in nucleus length (Fig. 4). This similarity should be viewed cautiously, as it may reflect the limited number of species and specimens available for these genera. Within genera, most species showed distinct nucleus lengths, except for *G. cancellata* and *G. reticulata* (Fig. 4). A similar pattern

was observed for total length, where most species pairs appeared different, apart from the *G.* cancellata – *G. reticulata* and *T. robusta* – *T. gracilis* pairs (Fig. 5).

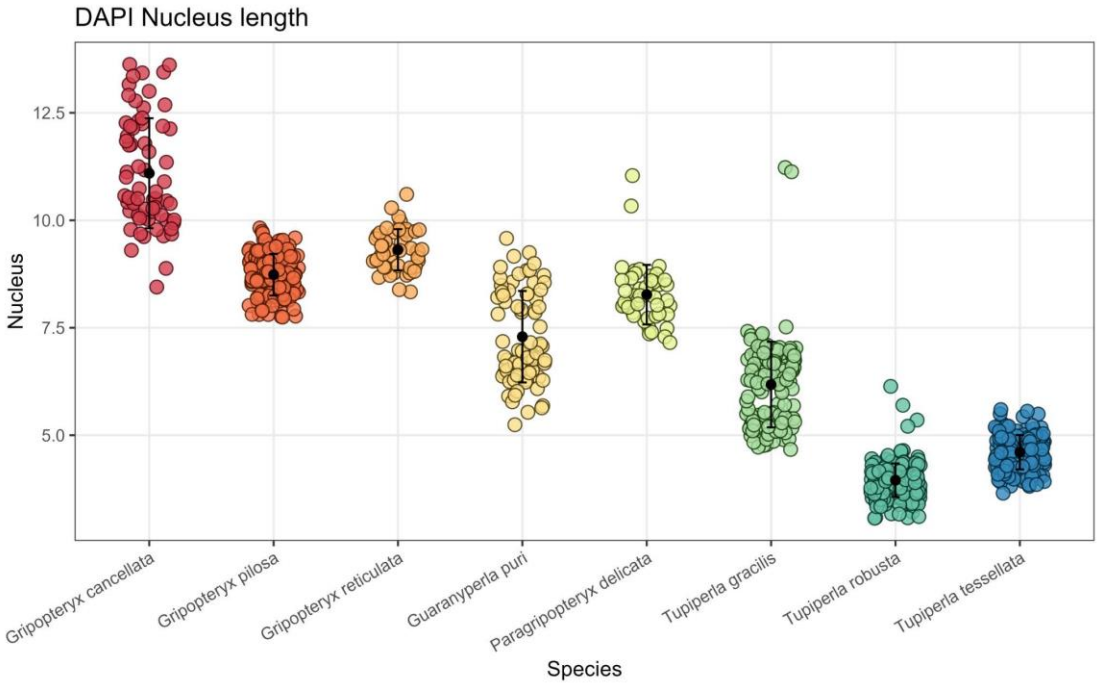

Figure 4. Comparison of species' spermatozoa nuclei length (μm) distribution through DAPI staining.

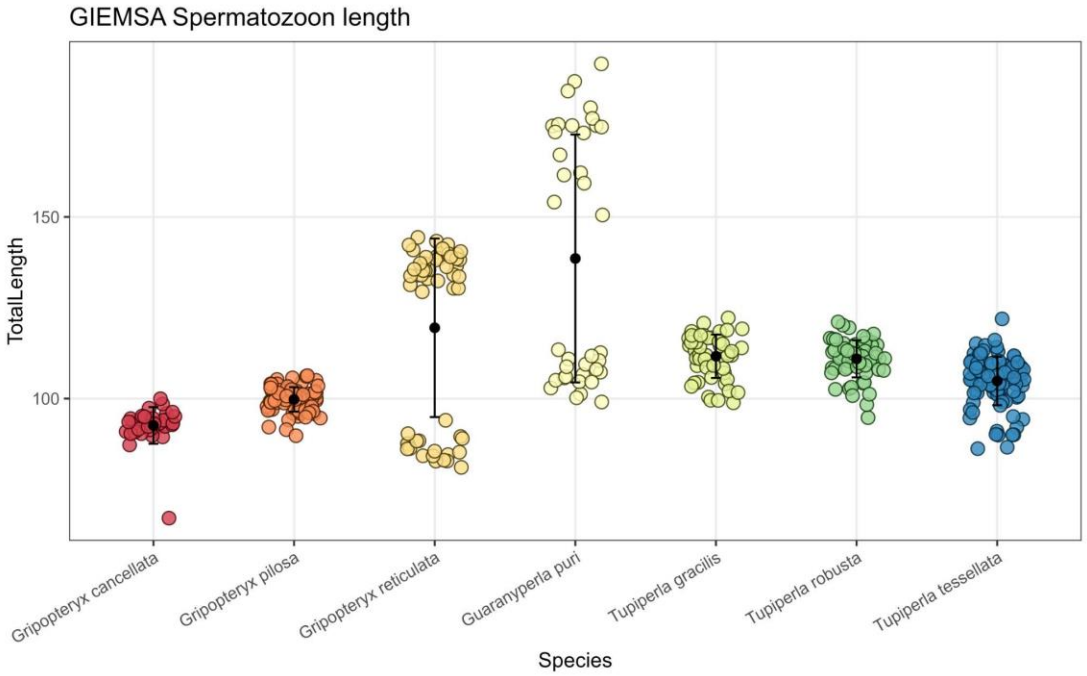

Figure 5. Comparison of species' spermatozoon length (μm) distribution through GIEMSA staining.

Discussion

The spermatozoa of Neotropical Gripopterygidae genera are long and filiform, resembling those of Arctoperlaria [see Table 1 in (Fausto et al., 2023)]. Fausto et al. (2023) reported that the sperm morphology of Arctoperlaria aligns with the typical polyneopteran structure and the groundplan sperm of insects, characterized by an elongated cell with an apical acrosome inserted in the anterior nuclear region, a long nucleus occupying the anterior portion, and a posterior flagellum extending along most of its length (Dallai et al., 2014, 2016). No prior studies have examined sperm morphology in Antarctoperlaria, making direct comparisons within this clade impossible. Therefore, throughout this discussion, we rely on available data from Arctoperlaria as a reference framework to contextualize our findings within Gripopterygidae and the broader phylogenetic landscape of Plecoptera.

Our observations indicate that *Guaranyperla puri* and *Tupiperla* species have proportionally shorter nuclei compared to the elongated nuclei reported for Arctoperlaria (Fausto et al., 2023), whereas in *Gripopteryx*, nuclear proportions are more similar to those described for Arctoperlaria. In *Gu. puri* and *Tupiperla* species, the head region accounts for an average of 5.27% and 4.52% of the total sperm length, respectively, contrasting with the lengths reported for Euholognatha taxa (Nemouridae: 11–12.72%; Capniidae: 11%; Leuctridae: 15–17.60%; Taeniopterygidae: 65–67.7%) (Fausto et al., 2023)]. Conversely, in *Gripopteryx*, the nucleus comprises approximately 9.5% of the total sperm length, closely resembling the proportions observed in the Systhelognatha Perlidae, Chloroperlidae, and Perlodidae, where the nucleus accounts for 9.2% of total sperm length (Fausto et al., 2023).

The relatively longer nuclear proportion in *Gripopteryx* compared to *Guaranyperla puri* and *Tupiperla* suggests that *Gripopteryx* and the *Tupiperla*+*Guaranyperla* group may represent distinct evolutionary lineages within Gripopterygidae. At a broader scale, the differences in sperm morphology between these Antarctoperlaria genera and those of Arctoperlaria reinforce the evolutionary distinction between these two major Plecoptera lineages. The significantly smaller nuclear proportion in *Guaranyperla puri* and *Tupiperla*, in contrast to the values reported for Euholognatha within Arctoperlaria, may reflect independent

evolutionary trajectories to Antarctoperlaria. However, the similarity between *Gripopteryx* and some Systhelognatha families raises intriguing questions about potential convergent evolution.

Using conventional Giemsa staining, we successfully distinguished the head and tail of the spermatozoon in all examined genera, except for *Paragripopteryx*, where this distinction remained inconclusive. Interestingly, SEM images from Fausto et al. (2023) suggest that Arctoperlaria spermatozoa may lack a clearly demarcated head and tail. However, the authors do not explicitly address this aspect, as their study primarily focuses on ultrastructural characteristics.

The head morphology of *P. delicata* closely resembles that of *Gripopteryx* species, both exhibiting a slender and elongated form. In contrast, *Guaranyperla puri* and *Tupiperla* species possess a more cylindrical head shape, highlighting another distinct difference between these lineages. Across all examined genera, we observed a short acrosome positioned in the anterior nuclear region, a characteristic consistent with previously described sperm structures in Plecoptera (Fausto et al., 2023). Although the acrosome's size appeared to vary among genera, precise measurements were not feasible due to the unclear structural boundaries in our preparations. Further investigation using ultrastructural techniques, such as transmission electron microscopy (TEM), would be necessary to confirm potential species-specific differences in acrosome dimensions.

Nucleus length allowed us to distinguish all genera and species, except for *P. delicata* and *Gu. puri*. However, this result is inconclusive due to the limited number of specimens available for comparison. In contrast, nucleus length effectively differentiated all other species within *Gripopteryx* and *Tupiperla*. Total spermatozoon length was also useful for distinguishing both genera and species. With *P. delicata* excluded from this analysis, *Gu. puri* was significantly different from the other genera. Nevertheless, some species remained indistinguishable from each other, particularly the pairs *G. cancellata – G. reticulata* and *T. gracilis – T. robusta*. Hence, *G. pilosa* was significantly different from the other two *Gripopteryx* species, as was *T. tessellata* within *Tupiperla*.

In *G. reticulata*, one specimen exhibited spermatozoa approximately half the length of those observed in other individuals. Notably, this male was collected from the same stream (Córrego Venerando, EBB, São Paulo State) as another individual with larger spermatozoa, whose sperm size matched that of a male from a different locality (Fazenda Remanso, Minas Gerais State). The specimens from Córrego Venerando were collected in different years, with

a three-month interval between them. Since *Gripopteryx* males generally exhibit minimal variation in terminalia morphology, this discrepancy raises the possibility that the individual with shorter spermatozoa belongs to a different species, as found in other studies (Pereira et al., 2008; Barcellos et al., 2015; Cursino and Duarte, 2016). Indeed, upon closer examination, we identified morphological differences in its terminalia that may support this hypothesis, though additional specimens are needed for confirmation.

Variation in total spermatozoon length was also observed in *Gu. puri*, with one specimen exhibiting significantly longer sperm than the other. Both males were collected from the same region (Serra do Brigadeiro) but from different localities, approximately 25 km apart, and in different years, though within the same month. Given that only two specimens were available for examination, drawing definitive conclusions remains challenging. This species was recently described by Rippel et al. (2025) based on morphology and species delimitation analysis using COI. The authors reported morphological intraspecific variation in male terminalia as well as in the nymphs' pronotum. To better assess the taxonomic status of these specimens, examining sperm from additional males from these localities would be essential. Moreover, studying other species of *Guaranyperla* could provide valuable comparisons with related genera, offering further insights into the genus's phylogenetic placement within Gripopterygidae.

Our findings suggest that sperm morphometrics is a reliable tool for distinguishing genera and may also aid in species differentiation, with some exceptions. Likewise, nuclear morphology and total sperm length provide evidence of a possible affinity between *Gripopteryx* and *Paragripopteryx*, as well as between *Guaranyperla* and *Tupiperla*. Further investigation using TEM would be valuable for confirming these relationships and refining ultrastructural comparisons among Arctoperlaria taxa. TEM could reveal additional diagnostic features, such as acrosomal structure, axonemal organization, and mitochondrial derivatives, which may further support or refine our current interpretations into the phylogenetic relationships within Gripopterygidae.

Our study presents the first morphological characterization of sperm in Gripopterygidae and, more broadly, in Antarctoperlaria, providing useful data for studies on Plecoptera evolution and reproductive biology. Future research should expand taxonomic sampling to include additional Gripopterygidae species, particularly those from the Andean and Australasian regions, as these lineages represent key components of the family's diversity.

Their inclusion could provide critical insights into the evolutionary patterns of sperm morphology within Antarctoperlaria. A broader comparative framework integrating both morphological and molecular data from these taxa would be essential for a more comprehensive understanding of sperm structure evolution in Plecoptera.

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Ethics approval

Our sampling followed Brazilian laws and were authorized by the SISBIO-ICMBio (Biodiversity Authorization and Information System, Chico Mendes Institute for Biodiversity Conservation, numbers 79695-1, 55428-16, and 65213-11), also by IEF (Minas Gerais State Institute of Forests, number 058/2021)

Author's contributions

Mellis L S Rippel: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review and Editing; **Dayvson A Costa:** Writing - Review and Editing, Methodology; **Rodrigo B Gastaldo:** Formal analysis, Methodology, Visualization, Writing - Review and Editing; **Jose Lino-Neto:** Writing - Review, Project administration, Methodology, Resources; **Frederico F. Salles:** Conceptualization; Writing - Review, Project administration, Funding Acquisition, Supervision, Resources.

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CONCLUSIONS

The taxonomic revision of *Guaranyperla* revealed a greater diversity than previously recognized, expanding the genus to six described species, with at least three additional species awaiting formal description, pending further specimen examination and genetic analysis. This study reinforces the importance of integrative taxonomy, as conventional morphology alone is insufficient for delimiting species within the genus, given the presence of cryptic diversity and sympatric occurrences. Two new species were described combining adult and nymphal morphology with molecular data. We also provided identification keys for all species, including adult male and female, and nymphs. Furthermore, our analyses contributed to a clearer delimitation of *Guaranyperla* from *Tupiperla* by expanding morphological diagnostic dataset, facilitating more accurate species identification and classification.

The second chapter presents the first complete histological description of the male reproductive system in Antarctoperlaria and the first detailed anatomical characterization of the female reproductive system in Gripopterygidae. While the histological structure of the ovaries and testes was largely conserved across the analyzed genera, the anatomy of both male and female reproductive systems exhibited notable variation among genera. Comparisons with Australasian Gripopterygidae suggest potential evolutionary divergence between these biogeographic regions. These findings highlight the potential of reproductive anatomical traits as informative characters for phylogenetic reconstruction and classification within the family.

In the third chapter we provided the first morphological characterization of sperm in Gripopterygidae and, more broadly, in Antarctoperlaria. Gripopterygidae sperm follows the typical polyneopteran structure, being filiform, with an elongated nucleus and a short, apically positioned acrosome. Intraspecific variation observed in *Gripopteryx reticulata* and *Guaranyperla puri* suggests the presence of cryptic species, reinforcing the usefulness of integrative taxonomy and highlighting sperm morphometry as a complementary tool for assessing species delimitation. Expanding taxonomic sampling, particularly to Andean and Australasian lineages, will be essential for refining our understanding of sperm evolution within Antarctoperlaria and assessing its phylogenetic significance.

These findings address significant gaps in our understanding of Plecoptera, particularly in species diversity and reproductive biology, laying a strong foundation for future research on reproductive strategies and species delimitation. This thesis highlights the presence of cryptic

species within Neotropical Gripopterygidae and contributes to their clarification through an integrative approach. By exploring previously understudied reproductive morphological traits, it demonstrates the taxonomic and phylogenetic value of internal morphology. Ultimately, this work reinforces the role of integrative taxonomy in systematics, showing that the combination of external and internal morphology, histology, and molecular data strengthens taxonomic and phylogenetic conclusions.

Furthermore, this thesis provides a dataset for future phylogenetic analyses of Gripopterygidae while bridging critical knowledge gaps regarding the reproductive system of Antarctoperlaria. This is particularly relevant given that many internal morphological characters have been shown to support the monophyly of groups within Plecoptera, especially in Antarctoperlaria. Thus, this dataset further supports phylogenetic analyses in Plecoptera, shedding light on the evolutionary history of stoneflies—an ancient lineage of singular and exciting insects that are both biologically intriguing and ecologically essential.

As for next steps, comparing the data presented here with the Andean lineages will enhance our understanding of the Gripopteryigidae systematics. Moreover, further field collections and broader taxonomic sampling, particularly of *Paragripopteryx* and *Guaranyperla*, are essential for refining species delimitation and deepening our understanding of the taxonomy and evolution of Neotropical Gripopterygidae.