MAISA DE CARVALHO GONÇALVES

A STUDY ON PHYLOGENY, REPRODUCTIVE SYSTEM, AND SPERMATOZOA IN PLECOPTERA (INSECTA)

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Pos-Graduate Program in Entomology for the obtention of the degree of Doctor Scientiae.

Adviser: Frederico Falcão Salles

Co-advisers: José Lino-Neto
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"Além da sorte de ater-se, desde tempos imemoriais, a uma linha evolucionária privilegiada, você foi extremamente afortunado em sua ancestralidade pessoal. Considere o fato de que, por 3,8 bilhões de anos, cada um dos seus ancestrais por parte de pai e mãe foi suficientemente atraente para encontrar um parceiro, suficientemente saudável para se reproduzir e suficientemente abençoado pelo destino e pelas circunstâncias para viver o tempo necessário para isso.

Nenhum de seus ancestrais foi esmagado, devorado, afogado, morto de fome, encalhado, aprisionado, ferido ou desviado de qualquer outra maneira da missão de fornecer uma carga minúscula de material genético ao parceiro certo, no momento certo, a fim de perpetuar a única sequência possível de combinações hereditárias capaz de resultar – enfim, espantosamente e por um breve tempo – em você."

(Bill Bryson – Breve história de quase tudo)

ABSTRACT

Gonçalves, Maisa de Carvalho, D.Sc., Universidade Federal de Viçosa, November, 2023. **A study on phylogeny, reproductive system, and spermatozoa in Plecoptera (Insecta)**. Adviser: Frederico Falcão Salles. Co-advisers: José Lino-Neto and Pitágoras da Conceição Bispo.

A historical analysis of the phylogeny of Plecoptera is presented by reviewing the major phylogenetic studies of the order. The study aimed to conduct a morphologic cladistic analysis for Plecoptera, testing the monophyly of suborders, infraorders, and superfamilies proposed by the current classification using the principle of parsimony to resolve character distribution conflicts. Two sets of analyses were conducted. The first analysis recovered Systellognatha as monophyletic and did not confirm Antarctoperlaria and Euholognatha as monophyletic. Subsequent analyses identified Systellognatha as monophyletic and Antarctoperlaria as a sister group of Euholognatha, challenging the monophyly of Arctoperlaria. Despite extensive previous research, unresolved aspects persist regarding Plecoptera's phylogenetic relationships, particularly the need for additional characters. To address this, the study described, for the first time, the anatomy, histology of the reproductive system, and sperm morphology of the stoneflies of the tribe Anacroneuriini based on Neotropical fauna specimens. The male reproductive system of Anacroneuria and Kempnyia comprises a pair of medially fused testes, showing variations in testicular follicle numbers. Spermatogenesis in these genera is intermittent, suggesting readiness for mating upon emergence. Both genera exhibited long, filiform sperm with a distinctive head (acrosome and nucleus) and a flagellum. While Anacroneuria's sperm from males and females had reduced or absent acrosomes, Kempnyia's showed evident acrosomes but commonly detached nuclei from the flagella. Seminal vesicles in both genera housed spermatozoa bundles. These internal features—male and female reproductive systems, and sperm morphology—serve as valuable complementary methodologies.

Keywords: Aquatic Insects; Stoneflies; Internal Anatomy; Internal Morphology; Taxonomy; Phylogenetic Systematics; Anacroneuriini.

RESUMO

Gonçalves, Maisa de Carvalho, D.Sc., Universidade Federal de Viçosa, novembro de 2023. **A study on phylogeny, reproductive system, and spermatozoa in Plecoptera (Insecta).** Orientador: Frederico Falcão Salles. Co-orientadores: José Lino-Neto e Pitágoras da Conceição Bispo.

Uma análise histórica da filogenia de Plecoptera é apresentada por meio da revisão dos principais estudos filogenéticos da ordem. O objetivo deste estudo foi realizar uma análise cladística morfológica de Plecoptera para testar o monofileticismo das subordens, infraordens e superfamílias propostas pela classificação atual e bem aceita, usando o princípio da parcimônia para resolver conflitos na distribuição de caracteres. Foram realizados dois conjuntos de análises. Na primeira análise, Systellognatha foi recuperado como monofilético e Antarctoperlaria e Euholognathawas não foram recuperados como monofiléticos. Análises posteriores recuperaram Systellognatha como monofilético e Antarctoperlaria como um grupo irmão de Euholognatha, de modo que o monofiletismo de Arctoperlaria não é mais confirmado. Apesar da extensa pesquisa anterior, ainda há aspectos não resolvidos sobre as relações filogenéticas dos Plecoptera. Um desses aspectos é a necessidade de caracteres adicionais para elucidar melhor essas relações. Para fazer um estudo mais completo, a anatomia e a histologia do sistema reprodutivo e a morfologia dos espermatozoides dos plecópteros da tribo Anacroneuriini foram descritas pela primeira vez com base em espécimes da fauna neotropical. O sistema reprodutivo masculino dos gêneros Anacroneuria e Kempnyia inclui um par de testículos fundidos medialmente que apresentam variações no número de folículos testiculares. Notavelmente, a espermatogênese não é contínua nesses gêneros, o que sugere que os machos dessas espécies estão prontos para acasalar assim que emergem. Em ambos os gêneros, os espermatozoides eram longos, filiformes e compostos por uma cabeça distinta (com acrossomo e núcleo) e um flagelo. Os espermatozoides de machos e fêmeas de Anacroneuria exibiam acrossomos reduzidos ou ausentes, enquanto em machos e fêmeas de Kempnyia, o acrossomo era evidente, mas os núcleos eram comumente destacados do flagelo. Os espermatozoides estavam organizados em feixes nas vesículas seminais em ambos os gêneros. Essas características internas, como os sistemas reprodutivos masculino e feminino e a morfologia dos espermatozoides, podem ser metodologias complementares valiosas.

Palavras-chave: Insetos Aquáticos; Stoneflies; Anatomia Interna; Morfologia Interna; Taxonomia; Sistemática Filogenética; Anacroneuriini;

SUMMARY

1. INTRODUCTION

Order Plecoptera

Stoneflies, (Plecoptera Burmeister 1839) are hemimetabolous aquatic insects considered an ancient order among Neoptera. They are widespread and are found on almost every continent except Antarctica, occurring from sea level to 5,600 meters in the Himalayas (Theischinger, 1991). Nymphs are commonly found in current, turbulent, cold, well-oxygenated waters (Fernández & Domínguez, 2001). Approximately 3900 extant, valid species are classified into 17 families (DeWalt & Ower, 2019; South *et al*., 2021; DeWalt *et al*., 2023).

Adult stoneflies typically undergo emergence during the nighttime. They emerge from nymphs that have transitioned from aquatic habitats to terrestrial environments, often found on various streamside features like logs, debris, rocks, or riparian vegetation (Hynes, 1976; Stewart, 2009). The main communication system for finding mates is vibratory signals transmitted through substrates, a unique behavior commonly referred to as "drumming", initiated by males by tapping the substrate using the ventral part of their abdomen or specialized structures like ventral lobes, hammers, knobs, or vesicles. Females respond to the behavior by drumming back (Stewart & Maketon, 1990; Stewart, 2001; Bouchard, 2004; Stewart, 2009; Tierno de Figueroa *et al*., 2019). This allows them to find each other as they move closer, with each species having its distinct drumming pattern for species-specific recognition (Bouchard, 2004; Tierno de Figueroa *et al*., 2019). Adults possess ten abdominal segments. The male genitalia exhibits distinct features at both generic and species levels, primarily involving structures on the ninth and tenth abdominal segments, such as paired hooks, lobes (paraprocts), sclerotized stylets, and sometimes a median probe (epiproct) with varying shapes (Stewart, 2009). During copulation, the aedeagus, typically located within the abdominal cavity, is everted ventrally through the genital opening on the ninth sternum. In contrast, the external female genitalia comprise a subgenital plate that covers the genital opening on the eighth abdominal sternum. This plate is a structure for males to grasp or hold with their hooks or lobes during copulation (Stewart, 2009).

Adult Plecoptera exhibit diverse feeding habits. For example, some species have atrophied mouthparts and do not feed or only consume water, while those that do feed may consume lichens, green algae, fruits, or leaf buds (Hynes, 1976; Fenoglio & Tierno de Figueroa, 2003; Stewart, 2009). Nevertheless, for the majority of stonefly species, information regarding

their emergence, dispersal, feeding habits, periods of inactivity, and lifespan remains limited. Additionally, it's crucial to note that knowledge about individuals in the neotropical region is practically absent. Typically, adult stoneflies have a relatively short lifespan, ranging from one to a few weeks. During this brief period, their primary focus is on reproductive activities, including mate finding, copulation, and oviposition (Stewart, 2009).

Stoneflies have a disjunct biogeographical distribution with their main diversity concentrated in higher latitudes. Their limited capacity for free movement and migration makes them excellent organisms for biogeographic and phylogeographic studies (Fochetti & Tierno de Figueroa, 2008; Pessino *et al*., 2014; McCulloch *et al*., 2016; Stevens *et al*., 2018; Ding *et al*., 2019; Letsch *et al*., 2021).

Plecoptera classification and phylogeny

The current classification and most widely accepted (Zwick, 2000; Stewart & Stark, 2002; South *et al*., 2021) system of the Plecoptera is based on a series of papers by Zwick (1969, 1973, 1980, 2000), who proposes two suborders: Arctoperlaria and Antarctoperlaria.

Arctoperlaria comprises 13 families of Laurasian distribution. The only exceptions are Notonemouridae, restricted to the Southern Hemisphere, and three tribes within Perlidae (Acroneuriini, Anacroneuriini, and Neoperlini), which have expanded distributions in the Southern Hemisphere. Arctoperlaria is further divided into the infraorder Euholognatha, containing the families Capniidae, Leuctridae, Nemouridae, Notonemouridae, Taeniopterygidae, and Scopuridae, and the infraorder Systellognatha, comprising the families Chloroperlidae, Kathroperlidae, Peltoperlidae, Perlidae, Perlodidae, Pteronarcyidae, and Styloperlidae (Zwick, 2000; South *et al*., 2021, DeWalt *et al*., 2023).

Antarctoperlaria is represented by four extant families of gondwanic distribution: Austroperlidae, Diamphipnoidae, Eustheniidae, and Gripopoterygidae (Zwick, 2000; Froehlich, 2012; DeWalt *et al*., 2023). The suborder includes two superfamilies, Eusthenioidea, including the families Eustheniidae and Diamphipnoidae, and Gripopterygoidea containing the families Gripopterygidae and Austroperlidae (Zwick, 2000; DeWalt *et al*., 2023).

The family Perlidae is the most diverse among Plecoptera, with approximately 1000 described species distributed across the Nearctic, Oriental, Palearctic, Afrotropical, and Neotropical regions. Perlidae comprises two subfamilies: Acroneuriinae Klapálek, 1914, and

Perlinae Latreille, 1802, (Ricker, 1950; Illies, 1966; Zwick, 1973; Zwick, 2000; Stark *et al*., 2009; Chen & Xu, 2021; DeWalt *et al*., 2023)

Acroneuriinae consists of 35 genera and approximately 615 species distributed across the Neotropical, Nearctic, Oriental, and the eastern part of the Palearctic regions. This subfamily is divided into four tribes: Anacroneuriini Stark & Gaufin, 1976 is restricted to the Neotropics and the southern part of the Nearctic region; Acroneuriini Klapálek, 1914, occurs throughout most of the subfamily's distribution, except in the Neotropics; Kiotinini Uchida, 1990, mainly found in the Oriental and Sino-Japanese regions, and the fossil tribe Largusoperlini Chen, 2018, which occurs in the Oriental region (Zwick, 2000; Stark, 2001; Murányi & Li, 2016; Chen, 2018; DeWalt *et al*., 2023).

Anacroneuriini, the focus of this study, consists of 418 valid species. It was described by Stark & Gaufin in 1976, who originally included eight genera: *Anacroneuria* Klapálek, 1909, the dominant and most diverse genus, with about 380 species; *Kempnyia* Klapálek, 1914 with 36 species; *Eutactophlebia* Klapálek, 1914, and *Laeissa* Navás, 1934, which later became junior synonyms of *Kempnyia* (Zwick, 1983; Froehlich, 1988; 2010); *Enderleina* Jewett, 1960, with 10 species; and *Macrogynoplax* Enderlein, 1909, with 16 species. *Onychoplax* Klapálek, 1916 is indicated to have an occurrence in Brazil, but this provenance is doubtful, and it probably does not exist in the country (Froehlich, 2008, 2010). *Klapalekia* Claassen, 1936 is known to occur in Colombia, and both *Onychoplax* and *Klapalekia* are monotypic genera known only from their female holotypes (Stark *et al*., 2009; Froehlich, 2010).

The systematic study of stoneflies has been controversial and covered with classification rearrangements and conflicting phylogenetic hypotheses (Frison, 1935; Ricker, 1950, 1952; Illies, 1964; Zwick, 1980; Nelson, 1984, Zwick, 2000, Terry, 2003; Davis, 2013; McCulloch *et al*., 2016; Chen *et al*., 2018; South *et al*., 2020; Letsch *et al*., 2021; Chen, 2022). Of these, only two studies (Nelson, 1984; Chen, 2022) has employed cladistics methodologies. Recent studies have increased the knowledge regarding the diversity of Plecoptera (Stark, 2001; Fochetti & Tierno de Figueroa, 2008; Froehlich, 2010; Pessacq *et al*., 2019), but there is a lack of information on species-level identification, male and female association, and phylogenetic relationships among the genera (Gamboa & Monaghan, 2014; Avelino-Capistrano *et al*., 2018; Almeida & Bispo, 2020; South *et al*., 2021).

Species-level identification in Plecoptera can be a challenging task, as the morphology of stoneflies can be highly variable, and difficult to distinguish between closely related species

(Avelino-Capistrano *et al*., 2011; Gamboa & Monaghan, 2014; Almeida & Bispo, 2020). Recently, in addition to morphological characters, genetic analysis using DNA sequencing techniques has also been employed to identify different species within the order (Terry, 2003; Davis, 2013; Avelino-Capistrano *et al*., 2016; McCulloch et al., 2016; Elwess *et al*., 2018; Almeida & Bispo, 2020; Chen *et al*., 2020; South *et al*., 2021; Letsch *et al*., 2021; Chen, 2022).

Reproductive system

The male genitalia of Plecoptera displays distinct characteristics at both the generic and species levels, primarily involving structures on the ninth and tenth segments. These structures include paired hooks, lobes (paraprocts), sclerotized stylets, and sometimes a median probe (epiproct) with varying shapes (Stewart, 2009). During copulation, the aedeagus, which is typically located within the abdominal cavity, is everted ventrally through the genital opening on the ninth sternum. In contrast, the external female genitalia consists of a subgenital plate that covers the genital opening on the eighth abdominal sternum. This plate also serves as a structure for males to grasp or hold with their hooks or lobes during mating (Stewart, 2009). In addition, the male reproductive system comprises a pair of testes, vasa deferentia, seminal vesicles, acessory glands and an ejaculatory duct (Zwick, 1973, 1980; Rosciszewska & Soldán, 1999). The female reproductive system consists of ovaries, vitellarium, oviducts, and a spermatheca (Rościszewska, 1989, 1997, 2001). During mating, the spermatheca receives spermatozoa from the male and stores them until fertilization is required (Stewart & Stark, 1977; Zwick, 1973, 1980).

Research into the reproductive system of Plecoptera, spanning several studies, has highlighted the remarkable diversity of reproductive systems within this order of insects. Brinck's (1956) comprehensive study was fundamental in this regard, carefully examining the external and internal genitalia and proposing a standardized terminology. Zwick (1973,1980) delved even deeper into the adaptations of the male genitalia, establishing a fundamental framework for understanding the variations within the families. These adaptations in the testicular structures, vasa deferentia, and seminal vesicles contribute significantly to the diversity of the group, exploring the correlations between the structure of the genital cavity and the copulatory organs in different families, offering valuable information on the taxonomy and evolution of the Plecoptera. Stewart & Stark (1977), focusing on *Hydroperla crosbyi*, discovered an alternative method of sperm transfer, improving our understanding of reproductive behavior. Collectively, these studies have clarified the exceptional taxonomic

characters offered by the various reproductive systems of stoneflies, which have played a crucial role in inferring phylogenetic relationships within Plecoptera.

Spermatozoa

In contrast to several other insect orders, there is limited knowledge available regarding the Plecoptera spermatozoa. Insect spermatozoa display a notable morphological diversity (Phillips, 1970; Jamieson, 1987; Jamieson *et al.,* 1999; Simmons, 2002; Swallow & Wilkinson, 2002; Sasakawa, 2007). In several groups, sperm size and morphology differ even among very closely related species (Pitnick *et al*., 1999; Immler & Birkhead, 2007; Sasakawa, 2009). Insects have evolved a diverse array of reproductive strategies, and the morphology of their sperm cells can provide important insights into their biology, ecology, and evolution (Jamieson *et al*., 1999; García-González *et al*., 2005; Birkhead *et al*., 2008; Fisher *et al.*, 2023). Consequently, the structural and ultrastructural characteristics of these cells have been widely used in several taxonomic and phylogenetic studies of various animal groups, including insects (Dallai & Afzelius, 1991, 1995; Jamieson *et al*., 1999; Lino-Neto & Dolder, 2001; Alves *et al*., 2006; Mancini *et al*., 2009; Sasakawa, 2009).

Although the phylogeny of Plecoptera has been widely studied (Frison, 1935; Ricker, 1950, 1952; Illies, 1964; Zwick, 1980; Nelson, 1984, Zwick, 2000, Terry, 2003; Davis, 2013; McCulloch *et al*., 2016; Chen *et al*., 2018; South *et al*., 2021; Letsch *et al*., 2021; Chen, 2022), and despite numerous studies on sperm morphology and the implications for phylogeny (Jamieson, 1987; Jamieson *et al*.,1999; Baccetti & Dallai 1976, 1977, 1978; Dallai & Afzelius 1980,1990,1991; Dallai, 2014; Dallai *et al*., 2016; Gottardo *et al*., 2016), several aspects related to the phylogenetic relationships of Plecoptera remain unresolved, as pointed out by Nelson (1984). One of these unresolved aspects is the acquisition of additional, non-morphological characters. In this regard, comparative spermatology can make a significant contribution to phylogenetic and taxonomic problems that traditional taxonomy could not solve (Burrini *et al*., 1988).

Despite the number of studies involving phylogenetic aspects of Plecoptera, and although the classification of the group proposed by Zwick (2000) is well accepted by specialists, previous research has produced conflicting phylogenetic hypotheses. Notably, only two published studies (Nelson, 1984; Chen, 2022) have studied the phylogeny of Plecoptera using cladistic methodologies. Furthermore, it is essential to highlight the limited number of characters in phylogenetic studies. Considering these gaps in our current understanding, this

study attempted to perform a cladistic analysis of Plecoptera using Nelson's (1984) character matrix and, at the same time, to test the monophyly of the suborders, infraorders and superfamilies proposed by Zwick (1973, 1980, 2000). This analysis used the principle of parsimony to resolve discrepancies in the distribution of characters. The present study also focuses on the reproductive systems of Plecoptera, examining and comparing their reproductive structures and the characterization of spermatozoa by comparative spermatology, given that the studies undertaken on Plecoptera so far have only described these structures in individuals from North America and Europe. This information has the potential to provide valuable insights for inferring phylogenetic relationships between Plecoptera families and other insect orders. It can be used for the identification and classification of new species and the resolution of taxonomic challenges. The study efforts to discern the implications of these investigative pursuits, especially regarding the context of phylogenetic systematic analysis, have the potential to contribute significantly to our understanding of the evolutionary history, taxonomy, and reproductive biology in parallel to providing significant insights into mating behavior, including the possible presence of sperm competition, and ecological significance of Plecoptera.

2. OBJECTIVES

2.1 Main Objective: The objective of this study is to analyze the historical phylogenetic systematics of Plecoptera and propose a phylogenetic hypothesis using cladistic methodologies. It also aims to examine and describe the reproductive systems and spermatozoa of Plecoptera, providing novel data on the reproductive systems and spermatozoa of Plecoptera based on components of the Neotropical fauna.

2.2 Specific Objectives:

- Perform a cladistic analysis for Plecoptera in order to test the monophyly of the suborders, infraorders and superfamilies proposed by Zwick (1973, 1980, 2000) using the principle of parsimony to resolve conflicts in character distribution.
- To describe, for the first time, the anatomy and histology of the reproductive system of the tribe Anacroneuriini, based on specimens from the Neotropical fauna; to compare the data from this study with those in the published literature, to identify a morphological pattern and/or morphological characters which could be included in phylogenetic analyses.
- To describe, for the first time, the sperm morphology of the tribe Anacroneurini, based on specimens of neotropical fauna; to compare the data from this study with those from published literature, in order to identify possible differences and their potential utility in differentiating species and establishing associations between males and females.

This thesis is structured in accordance with the guidelines of the journals where it will be submitted for publication. Chapter 1 adheres to the standards of Zootaxa, while Chapters 2 and 3 align with the criteria set by Arthropod Structure & Development.

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CHAPTER 1 - STONEFLIES (PLECOPTERA, INSECTA) PHYLOGENY: HISTORY AND CLADISTIC ANALYSIS

3. CHAPTER 1 - STONEFLIES (PLECOPTERA, INSECTA) PHYLOGENY: HISTORY AND CLADISTIC ANALYSIS

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Abstract

The current classification system of the Plecoptera is based on Zwick (2000), who proposes two suborders: Arctoperlaria and Antarctoperlaria. A historical analysis of the phylogeny of Plecoptera is presented by reviewing the major phylogenetic studies of the order. The purpose of this study was to perform a cladistic analysis for Plecoptera based on the character matrix proposed by Nelson (1984), in addition to testing the monophyly of the suborders, infraorders, and superfamilies proposed by Zwick (1973, 1980, 2000) using the principle of parsimony to resolve conflicts in character distribution. Two sets of analyses were conducted: one containing exactly the same data as Nelson (1984) and one with additional taxa and characters. The results obtained following the same methodology as Nelson (1984) were quite similar to what he found. Subsequent analyses recovered Systellognatha as monophyletic and Antarctoperlaria as a sister group of Euholognatha. In order to make a more complete study, some criteria are proposed: It is required to use the terminal taxa as species, propose a new additional character survey with appropriate character codings, and other outgroups among Polyneoptera, which are more related to Plecoptera, favoring the study of character evolution.

Keywords: Systematic, Stoneflies, Morphology, Parsimony Analysis

3.1. INTRODUCTION

3.1.1. The order Plecoptera

Frequently known as stoneflies, Plecoptera Burmeister 1839 are classified as hemimetabolous aquatic insects and are recognized as one of the oldest orders within Neoptera. These insects are globally distributed, inhabiting nearly every continent with the exception of Antarctica, and they thrive in diverse altitudes, from sea level to heights of 5,600 meters in the Himalayas (Theischinger, 1991). Nymphs, the immature stages of stoneflies, are typically situated in swiftly flowing, cold, and well-oxygenated waters (Fernández & Domínguez, 2001). The order comprises around 3,900 currently recognized species, which are organized into 17 families (DeWalt & Ower, 2019; South *et al*., 2021; DeWalt *et al*., 2023). The limited mobility and migratory capabilities render stoneflies valuable subjects for biogeographical and phylogeographical investigations (Fochetti & Tierno de Figueroa, 2008; Pessino *et al*., 2014; McCulloch *et al*., 2016; Stevens *et al*., 2018; Ding *et al*., 2019; Letsch *et al*., 2021).

The phylogenetic study of stoneflies was preceded and significantly affected by the historical dynamics of the classification of the order (Zwick, 1973, 1980). The current classification and most widely accepted (Zwick, 2000; Stewart & Stark, 2002) system of the Plecoptera is based on a series of papers by Zwick (1969, 1973, 1980, 2000), who proposes two suborders: Arctoperlaria and Antarctoperlaria (Figure 1).

Arctoperlaria comprises 13 families of Laurasian distribution, except Notonemouridae, which is restricted to the Southern Hemisphere, and three tribes within Perlidae (Acroneuriini, Anacroneuriini, and Neoperlini), which have expanded distributions in the Southern Hemisphere. The suborder is further divided into the infraorder Euholognatha, containing the families Capniidae, Leuctridae, Nemouridae, Notonemouridae, Taeniopterygidae, and Scopuridae, and the infraorder Systellognatha, comprising the families Chloroperlidae, Kathroperlidae, Peltoperlidae, Perlidae, Perlodidae, Pteronarcyidae, and Styloperlidae (Zwick, 2000; South *et al*., 2021).

Antarctoperlaria is represented by four extant families of gondwanic distribution: Austroperlidae, Diamphipnoidae, Eustheniidae, and Gripopoterygidae (Froehlich, 2012; South *et al.*, 2021). The suborder includes two superfamilies, Eusthenioidea, including the families

Eustheniidae and Diamphipnoidae, and Gripopterygoidea containing the families Gripopterygidae and Austroperlidae.

The systematic investigation of stoneflies has been marked by controversy, extensive reclassification, and the presence of conflicting phylogenetic hypotheses (Frison, 1935; Ricker, 1950, 1952; Illies, 1964; Zwick, 1973, 1980; Nelson, 1984, Zwick, 2000). While the examination has preserved the relationship between suborders and infraorders, the specific connections at lower taxonomic levels continue to elude clarity (South *et al*., 2021). Several efforts have been made to investigate the relationships between families of Plecoptera, based on morphological characters or molecular data (Illies, 1964; Zwick, 1980; Nelson, 1984; Uchida & Isobe, 1989; Zwick, 2000; Thomas *et al.*, 2000; Stewart & Stark, 2002; Terry, 2004; Davis, 2013; McCulloch *et al.*, 2016; Chen *et al*., 2018; South *et al.*, 2021a,b).

3.1.2. An analysis of the historical phylogeny of Plecoptera

Klapálek and Enderlein presented their first definitions regarding the major systematic categories (Illies, 1964; Zwick, 2000). Klapálek (1909) and Enderlein (1909) have divided the order Plecoptera into two suborders considering the characteristics of the adult mouthparts. Klapálek named those with a sub-cylindrical maxillary palp Setipalpia, and those with filiform palp Filipalpia. Enderlein split the order differently from Klapálek, proposing as a criterion the presence or absence of mandibles in the adults, establishing two suborders, the Hologna tha (presence of mandibles in the adults) and the Systellognatha (reduction of adult mouthparts) (Zwick, 2000).

According to Illies (1964), the initial effort ("and a very successful one") to study the phylogenetic relationships between the suborders was made by Tillyard (1921). He listed 16 "important" characters of the families that are present in either “archaic” or “specialized state” and, from this, calculated the percentages of archaic features for each family. In this way, he arrived at a phylogenetic concept for all seven families known by that time (Eustheniidae, Austroperlidae, Pteronarcyidae, Perlidae, Leptoperlidae (=Gripopterygidae), Capniidae and Nemouridae), with which Illies (1964) were entirely in agreement. The higher taxa have not been named. His Phylogenetic Diagram is given in Tillyard (1921), figure 2, page 38.

Frison (1935) proposes a new classification down to genera, followed by the list of species known to occur in Illinois (USA), comprising no less than nine families from the Northern Hemisphere (Frison 1935, see classification on page 310). Additionally, he pointed

out "that this structure-based classification also accords with biological habits" (Illies, 1964). Like Klapálek (1909) and Enderlein (1909), Frison (1935) divided the order Plecoptera into two suborders: Hologнatha or Filipalpia, and Systellognatha, Sublipalpia, or Setipalpia. In his classification, Hologнatha comprises the families Pteronarcyidae, Peltoperlidae, Taeniopterygidae, Nemouridae, Leuctridae, and Capniidae, while Systelognatha includes the families Perlidae, Perlodidae, and Chloroperlidae.

Ricker (1950), in his paper on "Some Evolutionary Trends in Plecoptera", (which was slightly modified in Ricker (1952)), applied the basic concepts of Tillyard (1921). He inferred affinities based on shared derived characters, obtaining at that time, a considerably more detailed comprehension of the order, including 9 families and 16 subfamilies. In addition to the characters already used by Tillyard, he used the thoracic suture (Y-crest of the mesosternum) and the external male genitalia. Ricker (1950) also divided the families into two major groups: Hologнatha (= Setipalpia sic) including the families Eustheniidae, Austroperlidae, Leptoperlidae, Peltoperlidae, Nemouridae, Pteronarcyidae; and Systellognatha (= Filipalpia sic), with Perlodidae, Chloroperlidae, Perlidae. This scheme resembles the division proposed by Frison (1935), but with the suprafamilial names switched, Filipalpia represents what was formerly called Seti- or Subulipalpia, and vice-versa. In 1952 he made a revision and fixed the mistake.

The method conceptualized by Tillyard (1921) was applied and modified by Illies (1964) by examining not only trends that represent two evolutionary alternatives (i.e., plesiomorphic = archaic, and apomorphic = specialized state) but also anagenetic indices (calculations that involve several progressive stages of evolution) for different taxa and established a third suborder, Archiperlaria, in addition to Filipalpia and Setipalpia (Illies 1964, figure 4, page 125). Setipalpia included four families (Pteronarcyidae, Perlidae, Chloroperlidae, Perlodidae) and it is the sister group of Filipalpia, which is further divided into two uncategorized groups, one including Taeniopterygidae, Nemouridae, Leuctridae, Capniidae, and another including Austroperlidae, Scopuridae, Gripopterygidae and a uncertain placement of Peltoperlidae. Archiperlaria included only Eustheniidae and Diamphipnoidae. Still in accordance with Illies (1964), Archiperlaria were "completely primitive and defined by plesiomorphies only".

Considering this historical continuity, with long-lasting disagreement over boundaries and names of suborders (Zwick, 2000), Zwick (1969) proposed Arctoperlaria and

Antarctoperlaria as suborders, clearing up the uncertainty between the names of the suborders. Arctoperlaria corresponds to Filipalpia (suborder proposed by Klapálek, 1909), whereas Arctoperlaria comprehend the infraorder Euholognatha, which is composed of Nemouroidea (Leuctridae, Capniidae, Nemouridae and Taeniopterygidae) and Scopuridae; and the infraorder Systellognatha, composed by Subulipalpia (Perlidae, Chloroperlidae, and Perlodidae), Peltoperlidae, and Pteronarcyidae. Antarctoperlaria were also subdivided into two groups: Eusthenioidea (Diamphipnoidae and Eustheniidae) and Leptoperloidea (Austroperlidae and Gripopterygidae *s.str.*).

Zwick (1973), in his book "Das Tierreich – Insecta: Plecoptera Phylogenetisches System und Katalog", has attempted to proceed with Illies catalog (1966) and presents the list of all valid and uncertain Plecoptera genera and species. According to Zwick (1973), the confusion about the names of the suborders results from the fact that an old "purely typological classification has been adopted and reinterpreted evolutionarily, although only partially adequate for this". In this paper, he maintained the proposal of the two suborders and presented a list of plesiomorphies and symplesiomorphies for each group.

Zwick (1980) sustained the proposal of the two suborders, presented new plesiomorphies and symplesiomorphies for each group, and replaced Subulipalpia to Perloidea and Leptoperloidea to Gripopterygoidea. The internal relationships among the groups remained the same.

Nelson (1984) was the first attempt to elaborate a phylogeny of Plecoptera based on modern or quantitative cladistic analysis of morphological characters. Taking into account the list of characters and states proposed by Zwick (1980), after some modification, Nelson prepared a matrix in order to run a phylogenetic analysis using the PHYLIP computer program. Despite the fact that he used a software tool to run his phylogenetic analysis, there are a few problems with the analysis performed by Nelson (1984). He defined the polarization of the characters a priori and also defined a hypothetical ancestor whose states were all plesiomorphic to root the tree. Unlike Zwick (1980), Nelson found Antactorperlaria paraphyletic, since Gripopterygoidea was found to be more related to Arctoperlaria, and Eusthenioidea as the sister group of that clade. Besides that, in Arctoperlaria, Scopuridae appears in a trichotomy with the Nemouroidea and Systellognatha. According to Nelson (1984), Zwick's morphological character data were recognized as inadequate for the resolution of several problematic

phylogenetic relationships. For this reason, he did not attempt a revised classification of the Plecoptera concordant with the phylogenetic analysis.

Uchida & Isobe (1989) revised the system of Systellognatha. They raised Styloperlinae, former in Peltoperlidae (Illies, 1966) to Styloperlidae, and proposed the new superfamily Pteronarcyoidea (with Pteronarcyidae as a sister group of Styloperlidae and Peltoperlidae). The relationship within Perloidea followed that proposed by Zwick (1980).

Zwick (2000) complemented his phylogeny of Plecoptera (Zwick, 1969; 1973; 1980), now including the new family Styloperlidae and a few additional groups. The suborder Arctoperlaria comprises the infraorder Euholognatha, which is then composed of Scopuridae as a sister group of Nemouroidea (Taeniopterygidae + Nemouromorpha). The new group Nemouromorpha is formed by Nemouridae and Notonemouridae (Nemouridae *sensu latu*) and Leuctroidea (Capniide and Leuctridae); the other infraorder, Systellognatha, is composed of Perloidea (Perlidae, Chloroperlidae, and Perlodidae) and Pteronarcyoidea (Pteronarcyidae, Styloperlidae, and Peltoperlidae). Antarctoperlaria were also subdivided into two groups: Eustheniodea (Eustheniidae and Diamphipnoidae) and Gripopterygoidea (Gripopterygidae and Austroperlidae).

From this point on, some work involving molecular phylogeny has been developed. Thomas *et al*., (2000) performed a molecular phylogenetic analysis of evolutionary trends in stonefly wing structure and locomotor behavior. Thomas *et al*., (2000) used a small subunit (18S) rRNA gene to sequence 34 stonefly species, which they judged to represent all families of Plecoptera. These results have contradicted all previous phylogenetic hypotheses of Plecoptera at the family level but recovered Perloidea (Chloroperlidae, Perlodidae, and Perlidae).

Terry (2003, PhD dissertation, unpublished) conducted a broad phylogenetic analysis using a parsimony-based investigation with six molecular markers (rrnS, rrnL, 18S, 28S, cox2, and H3) and a morphological character matrix based on the characters proposed by Zwick, 1973; 2000). The phylogenetic analysis states the monophyly of the suborders Arctoperlaria and Antarctoperlaria, and the infraorders Systellognatha and Euholognatha. Terry's analysis also supports most of the families, except for Chloroperlidae, Austroperlidae, and Gripopterygidae. Within Systellognatha, Styloperlidae is the sister group of Peltoperlidae and Pteronarcyidae, and Perloidea is a strongly supported monophyletic group with Chloroperlidae as the sister to Perlidae and Perlodidae. Megaleuctra is the sister group of the remaining

Plecoptera, thus making Leuctridae *sensu latu* paraphyletic, for which he suggests recognition of the family Megaleuctridae.

Davis (2013, MSc thesis, unpublished) utilized Illumina sequencing technology and analyzed seven plecopteran transcriptomes, representing seven (Capniidae, Chloroperlidae, Nemouridae, Perlidae, Perlodidae, Pteronarcyidae, and Taeniopterygidae) of the 16 total families, in an attempt to identify and expand the genetic marker set, identifying genes that can be used as phylogenetic markers in non-model organisms. The phylogenetic reconstruction of the transcriptome content has recalled the main relationships found in the primary Plectopteran morphological systematics studies (Zwick, 2000; Terry, 2003; Chen *et al.*, 2018). These main relationships comprise the monophyly of Perloidea, Systellognatha, and Euholognatha.

McCulloch *et al*. (2016) reconstructed the phylogenetic relationships among southern hemisphere stoneflies: Gripopterygidae, Eustheniidae, Austroperlidae, Notonemouridae, and Diamphipnoidae, using 2864 bp of mitochondrial (COI) and nuclear (18S, H3) DNA, with a calibrated relaxed molecular clock used to estimate the chronology of diversification. Their analysis suggests that the largely antitropical stonefly suborders, Arctoperlaria and Antarctoperlaria, were formed approximately 121 Ma (95% prior probability distribution 107–143 Ma), which may reflect the vicariant rifting of the supercontinent Pangaea, explaining why Notonemouridae (Arctoperlaria) has its occurrence restricted to the Southern Hemisphere.

Chen *et al*. (2018) conducted a molecular phylogeny of Systellognatha inferred from mitochondrial genome sequences. The monophyly of Perloidea (Perlidae as a sister-group of Perlodidae plus Chloroperlidae) was supported, but Pteronarcyoidea (Pteronarcyidae plus Styloperlidae as a sister-group of Peltoperlidae) was recovered as paraphyletic.

South *et al.* (2020) discussed the phylogenetic relationships of Plecoptera using molecular data, but only with families from North America. Because of that, all members of Antarctoperlaria and three families of Arctoperlaria (Scopuridae, Styloperlidae, and Notonemouridae) were excluded. The analysis recovered the monophyly of formerly accepted clades and proposed new relationships among families. The results included Perlidae as the first divergent family within Perloidea; Nemouridae plus Capniidae clade rather than the conventionally recognized Leuctridae plus Capniidae; Peltoperlidae as sister-group of Pteronarcyidae, Perlidae, Chloroperlidae, and Perlodidae; and the non-monophyly of Chloroperlidae due to the placement of the genus *Kathroperla* Banks 1920 as sister-group of

Chloroperlidae and Perlodidae, which was later raised to the family Kathroperlidae (South *et al*., 2021). The position of Taeniopterygidae and Leuctridae remained inconclusive.

Letsch *et al*. (2021) provided an evolutionary explanation for the unique distribution pattern of stoneflies by using a phylogenetic workflow that combines both transcriptomic and Sanger sequence datasets with heterogeneous taxon coverage. In this work, a phylogenetic analysis was performed to understand how the current distribution of Plecoptera has evolved. The analyses suggest that with the break-up of Pangaea around 200 Ma and the associated climatic and geographical changes, two groups of stoneflies, the Antarctoperlaria and the Notonemouridae, dispersed to Gondwana and subsequently went extinct on the northern continents. Both groups likely dispersed across Gondwana before its breakup into the modern continents. This study provided a picture of the evolution and biogeography of the major stonefly lineages, indicating that vicariance did not play a major role concerning the anti-tropical biogeographic pattern we observe today except in their ancestral northern range.

More recently, Chen (2022) discusses the larval morphology of a new extinct stonefly, *Kachinoperla zwicki*, and its systematic position in Plecoptera. The study describes the unique characters of Kachinoperlidae, a new family of stoneflies, and compares them to other stonefly families. The phylogenetic analysis was performed using two methods: parsimony and Bayesian inference. The character list and data matrix were partially derived from a previously published dataset in Nelson (1984), and the data matrix was subjected to a parsimony analysis with the program TNT 1.5 using new technology search. The Bayesian inference was performed with the software MRBAYES 3.2.7. The final matrix consisted of 41 taxa with 85 external morphological characters. The outgroup was a putative ancestor with all plesiomorphic character states. Kachinoperla shows similarities to the superfamily Perloidea in terms of mouthpart morphology, but it cannot be attributed to Perloidea due to the subequal glossae and paraglossae, which is an apomorphic character defining the monophyly of Perloidea. The study investigates the systematic placement of *Kachinoperla zwicki* and suggests that it represents a basal lineage within Systellognatha. The article also discusses the importance of the larval stage in providing biological and evolutionary information about stoneflies, as well as the challenges of studying stonefly fossils.

As demonstrated, despite the number of studies involving phylogenetic aspects of Plecoptera, and although the classification of the group is well accepted by specialists, all of these previous studies provided contradictory phylogenetic hypotheses depending on the taxa

chosen, morphological or molecular data, and the group, and/or the methods selected for tree reconstruction (Letsch *et a*l., 2021). Only two published study (Nelson, 1984; Chen, 2022) has proposed a phylogeny of Plecoptera based on cladistics methodologies. Therefore, the objective of this study was to perform a cladistic analysis for Plecoptera based on morphological character matrix proposed by Nelson (1984), in addition to testing the monophyly of the suborders, infraorders, and superfamilies proposed by Zwick (1973, 1980, 2000) using the principle of parsimony to resolve conflicts in character distribution.

3.2 MATERIALS AND METHODS

3.2.1 Matrix data

The list of characters (Appendix 1) and their states used to construct the data matrix (Appendix 2) were taken from Nelson (1984) and the references cited therein. Further information on several of these characters from additional literature was based on Zwick (1973; 1980; 2000) and original descriptions and redescriptions of the species. The characters were drawn from the external and internal morphology of adults (both males and females), nymphs, and eggs, for a total of 113 binary characters (40 from nymphs; three from eggs; 10 from females, and 60 from males) plus one additional character proposed by us to increase posterior analysis. In order to enhance our analysis, a new data matrix was constructed using a methodology inspired by Sereno (2007), as detailed in the supplementary materials (Appendix 3). This matrix included both the existing characters and the newly proposed one, providing a more comprehensive dataset for our systematic investigation.

3.2.2. Morphological phylogenetic analysis

Two sets of analyses were conducted: one containing exactly the same data as Nelson (1984) and one with additional taxa and characters. The original analysis made by Nelson (1984) included 23 terminal taxa, of which there are nine families (Peltoperlidae, Scopuridae, Perlidae, Gripopterygidae, Notonemouridae, Nemouridae, Capniidae, Perlodidae, and Austroperlidae); eight subfamilies [Stenoperlinae and Eustheniinae (belonging to Eustheniidae), Paraperlinae and Chloroperlinae (belonging to Chloroperlidae), Brachypterinae and Taeniopteryginae (belonging to Taeniopterygidae), Megaleuctrinae and Leuctrinae (belonging to Leuctridae)] and five genera [Pteronarcys, Allonarcys, and Pteronarcella (belonging to Pteronarcyidae) Diamphipnopsis and Diamphipnoa (belonging to Diamphipnoidae)] including the hypothetical ancestor (outgroup). In our analyses we added the family Kathroperlidae Banks, 1947 as a new taxon and the mayfly (Ephemeroptera)

Siphlonurus aestivalis (Eaton, 1903) as the outgroup, totaling 24 terminal taxa. *Siphlonurus aestivalis* was chosen because it is a well-studied species, with all stages described (Sartori *et al*., 1992; Kluge *et al*., 1995) and their morphological features allow character coding.

The data matrix was assembled in the program Mesquite 2.0 (Maddison & Maddison, 2007). In the data matrix, '?' indicates characters not observed, and those not applicable were coded as '–'. The polarization of the characters was executed by the criterion of rooting by external groups (Nixon & Carpenter, 1993). Cladistic analysis was performed using Tree analysis using New Technology (TNT) software version 1.5 (Goloboff & Catalano, 2016). For phylogenetic inference, the principle of parsimony was implemented to resolve conflicts in character distribution. This option aims to find the topology with the fewest number of steps (Farris, 1983). Initial analyses used equal weighting of the characters to check the distribution of the characters in the topology, and implicit weighting (implied weighting, Goloboff, 1993; Goloboff et al., 2008) was employed a posteriori, allowing the consistency of the initial results over more than one weighting model to be examined (Carpenter et al., 2000). We used k value = 3.0000 as in the default mode, and in addition, a tnt script (setk.run) written by Salvador Arias was used to calculate the appropriate value of k. The script resulted in a value of k = 2.6563 for our data set, which was then employed.

The parameters used in the analyses were as follows: space for 10,000 trees in memory; Analyze: 'Traditional Search'; Wagner trees; 'random seed' = 1 (as in the default mode); 'number of additional sequences' (replications) = 1000; swapping algorithm: none; 'tree to save per replication' = 100 and collapsing trees after the search.

3.3 RESULTS

The first analyses, replicated in order to contrast the results with the analyses made by Nelson (1984), produced 12 trees with a length of 160 steps. The topology of the strict consensus is shown in Figure 2. The final topology found in our cladogram recovered Eusthenioidea (Eustheniinae, Stenoperlinae, Diamphipnoa and Diamphipnopsis) as monophyletic group based on five synapomorphies: (1) Trochanter sternal depressor present (character 0: state 1); (2) Trochanter tergal depressor reduced (character 1: state 1); (3) Larval coxa with sensory bristle (character 6: state 1); (4) Male paraproct with bladderlike membranous region (character 7: state 1) and (5) Female genital opening extremely narrow (character 8: state 1).

Gripopterygoidea (Austroperlidae and Gripopterygidae) is supported by four synapomorphies: (1) Trochanter sternal depressor present (character 0: state 1); (2) Trochanter tergal depressor reduced (character 1: state 1); (3) Male genitalia with large accessory glands present (character 4: state 1) and (4) Female seminal receptacle present (character 5: state 1).

Nemouroidea (Taeniopteryginae, Branchypterinae, Nemouridae, Notonemouridae, Capniidae, Leuctrinae and Megaleuctrinae) is supported by eight synapomorphies: (1) Drumming present (character 2: state 1); (2) Last abdominal ganglion in segment 7 or anterior (character 16: state 1); (3) Corpus allatum paired (character 43: state 1); (4) Egg chorion gelatinous (character 44: state 1); (5) Male hammer present (character 45: state 1); (6) Spina II extended anteriorly to base of furca (character 78: state 1); (7) Sclerotic bridge uniting spina I with anterior margin of basisternum II present (character 79: state 1) and (8) Male paraproct divided into inner and outer lobes (character 80: state 1).

Systellognatha (Pteronarcella, Allonarcys, Pteronarcys, Peltoperlidae, Perlodidae, Chloroperlinae, Paraperlinae, and Perlidae) is recovered as monophyletic and supported by seven synapomorphies: (1) Drumming present (character 2: state 1); (2) Adult mandible thin, lacking mola (character 37: state 1); (3) Adult mandible thin, not sclerotized, soft (character 38: state 1); (4) Egg with anchor and collar (character 39: state 1); (5) Male epiproct sunk into a pocket (character 41: state 1); (6) Thoracic postalar bridge present (character 42: state 1) and (7) Male hammer present (character 45: state 1).

Scopuridae appears in a polytomy with Gripopterygoidea, Nemouroidea, and Systellognatha. Further in these results, Antarctoperlaria and Euholognatha were not recovered as monophyletic.

The second analysis, with the addition of *Siphlonurus aestivalis* as an outgroup and Kathroperlidae in the data set, under equal weight resulted in two equally parsimonious trees with a length of 165 steps (fig. 3). The analysis under Implied Weighting (k = 3.0000 and k = 2.6563) produced a single tree. The tree topologies using Implied Weighting recovered the relationships within Perloidea (grayed rectangle) and are shown in the upper corner of Figure 3. The results recovered Systellognatha [Pteronarcyidae + (Pteronarcyidae + Perloidea)] as monophyletic and supported by two synapomorphies: (1) Drumming present (character 2: state 1) and (2) Egg with anchor and collar (character 39: state 1). Perloidea is supported based on three synapomorphies: (1) Larval mandible molar area slender and reduced (character 13: state

1); (2) Larval labium with glossal apices at the same level as paraglossal apices (character 14: state 1) and (3) Last abdominal ganglion in segment 7 or anterior (character 16: state 1).

Antarctoperlaria (Gripopterygoidea and Eusthenioidea) is also recovered as monophyletic based on two synapomorphies: (1) Trochanter sternal depressor present (character 0: state 1) and (2) Trochanter tergal depressor reduced (character 1: state 1).

Gripopterygoidea is supported by two synapomorphies: (1) Male genitalia with large accessory glands present (character 4: state 1) and (2) Female seminal receptacle present (character 5: state 1). Eusthenioidea is supported based on seven synapomorphies: (1) Larval coxa with sensory bristle (character 6: state 1); (2) Male paraproct with bladderlike membranous region (character 7: state 1); (3) Female genital opening extremely narrow (character 8: state 1); (4) Larval abdominal gills present on at least segments 1-4 (character 20: state 0); (5) Larval abdominal gills present on at least segments 1-3 (character 21: state 0); (6) Larval abdominal gills present on at least segments 1-2 (character 22: state 0); and (7) Larval abdominal gills absent (character 23: state 0).

Euholognatha [Scopuridae and Nemouroidea (Taeniopteryginae, Branchypterinae, Nemouridae, Notonemouridae, Capniidae, Leuctrina, and Megaleuctrinae)] is recovered as monophyletic and supported by two synapomorphies: (1) Corpus allatum paired (character 43: state 1) and (2) Egg chorion gelatinous (character 44: state 1). Nemouroidea is supported based on six synapomorphies: (1) Drumming present (character 2: state 1); (2) Last abdominal ganglion in segment 7 or anterior (character 16: state 1); (3) Male hammer present (character 45: state 1); (4) Spina II extended anteriorly to the base of furca (character 78: state 1); (5) Sclerotic bridge uniting spina I with anterior margin of basisternum II present (character 79: state 1) and (6) Male paraproct divided into inner and outer lobes (character 80: state 1).

3.4 DISCUSSION

The results obtained following the same methodology as in Nelson (1984), are quite similar to what he found. Systellognatha is recovered as monophyletic. The synapomorphies supporting Systellognatha corroborate those listed by Zwick (1973, 1980, 2000), such as drumming, or behavior derived from drumming and/or related male structures (ventral lobe, hammer, vesicle, hairbrush). In addition to these, other characters appeared as synapomorphic in our analysis such as adult mandible thin, lacking mola, not sclerotized, soft; egg with anchor and collar, and thoracic postalar bridge present.

Similarly to our analysis Nelson (1984) did not recover Antarctoperlaria, because Eusthenioidea did not appear as a sister group only to Gripopterygoidea. In our results, Antarctoperlaria was not recovered as monophyletic, since the characters (0) Trochanter sternal depressor present and (1) Trochanter tergal depressor reduced are homoplasic regarding Eusthenoidea and Gripopterygoidea. Gripopterygoidea appeared in a polytomy with Scopuridae, Nemouroidea, and Systellognatha, which also did not recover Euholognatha.

Euholognatha was not recovered as monophyletic by Nelson's analysis, which agrees with our results. The main difference is related to the position of Scopuridae, which in his analysis appears in a trichotomy with Nemouroidea and Systellognatha. In our analysis, Scopuridae appears in a polytomy with Gripopterygoidea, Nemouroidea, and Systellognatha.

By adding a real external group in the analysis, *Siphlonurus aestivalis*, as well as a new terminal, Kathroperlidae, we obtained a new topology, which was corroborated with different K values using Implied Weighting.

For Systelognatha, our analysis recovered the same topology as in Nelson (1984). The characters supporting this group are (2) Drumming present and (39) egg with anchor and collar. Additionally, unlike South (2021), in which Kathroperla was recovered as a sister group to Perloidea, in our analyses we recovered it as a sister group to Paraperlinae. In order to sustain the family status proposed by this author, Paraperlinae should be elevated to this same ranking. Alternatively, Kathroperlidae should return to the subfamily status in Chloroperlidae.

The suborder Antarctoperlaria was recovered as monophyletic, supported by (0) Trochanter sternal depressor present and (1) Trochanter tergal depressor reduced, and this monophyly is corroborated in some phylogenetic studies (Zwick, 2000; Lestch, 2021). However, its placement in the order is dissonant from most phylogenies proposed for Plecoptera, in which it appears as a sister group to Arctoperlaria (Systellognatha + Euholognatha). In our analysis, this clade appears as a sister group to Euholognatha, so the monophyletism of Arctoperlaria is no longer confirmed.

Additionally, Euholognatha was recovered as monophyletic and Scopuridae is recovered as a sister group to Nemouroidea, thus confirming the status of this infraorder (Zwick 1974, 1980, 2000; Terry 2003; Letsch, 2021).

Arctoperlaria was recovered as paraphyletic. Arctoperlaria are diverse structurally. According to Zwick (2000), the monophyly is underpinned by a complex character syndrome,

both structurally and behaviorally, concerned with pair-finding: drumming and related male structures (ventral lobe, hammer, vesicle, hairbrush) that is known among all families of Systellognatha and Nemouroidea except Scopuridae. In our analysis, these characters didn't support the monophyly of Arctoperlaria, since they were recovered only in Systellognatha and Nemouroidea, leading to the hypothesis that these characters appeared in the ancestor of Plecoptera. The characters (12) larvae strictly phytophagous and (40) egg with micropyles surrounding collar are the characters that provide support for the clade Antarctoperlaria + Euholognatha.

Our results show some differences regarding the historical phylogenetic relations proposed to the families in the order Plecoptera. However, our analysis still has some shortcomings, and in order to make a more complete study, some criteria must be followed: It is required to use the terminal taxa as species, propose a new additional character survey with appropriate character codings, and other outgroups among Polyneoptera, which are more related to Plecoptera, favoring the study of character evolution.

3.5 REFERENCES

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3.6 LIST OF ILLUSTRATIONS

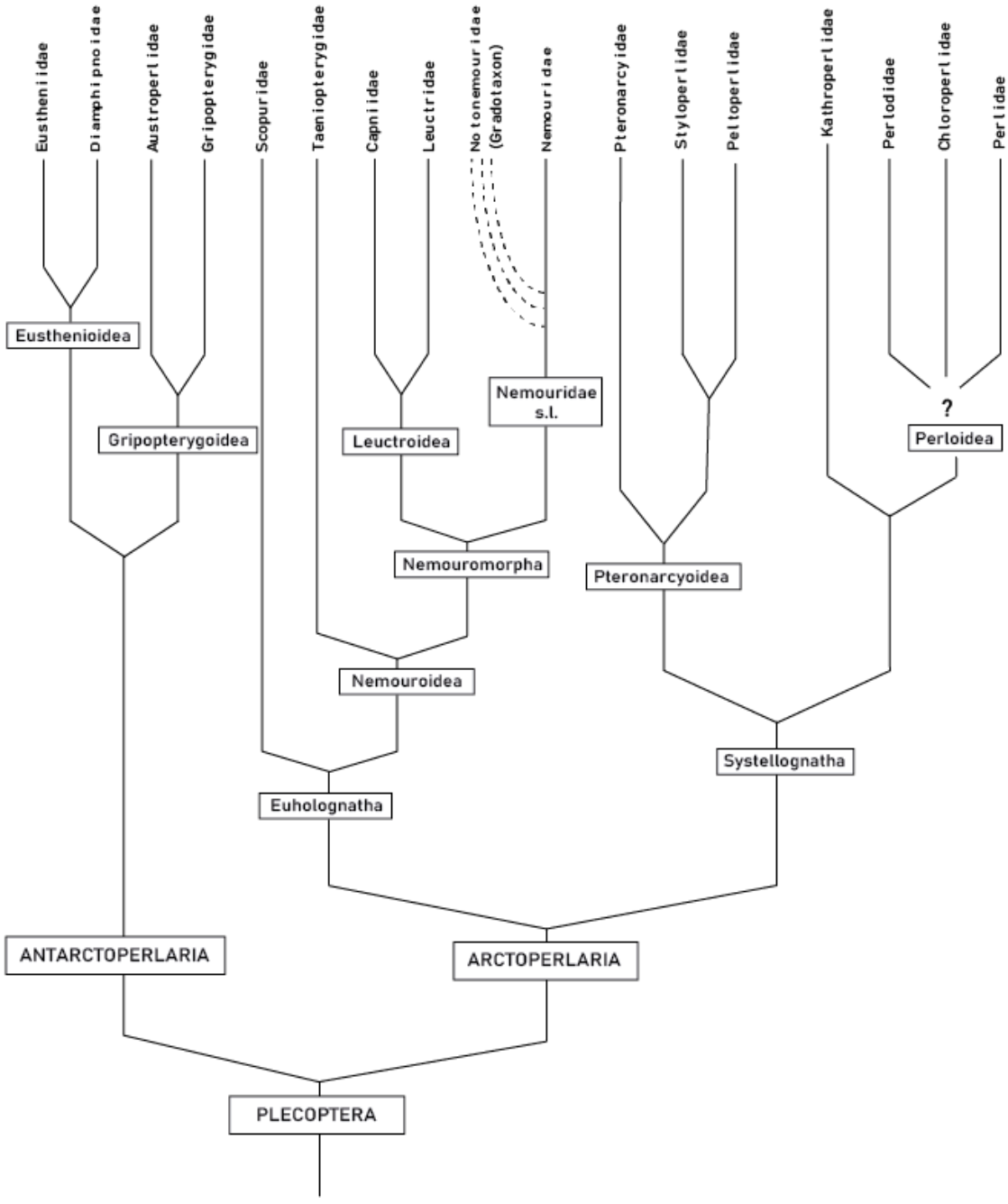

FIGURE 1. Phylogeny of extant Plecoptera adapted from Zwick (2000) by the inclusion of Kathroperlidae.

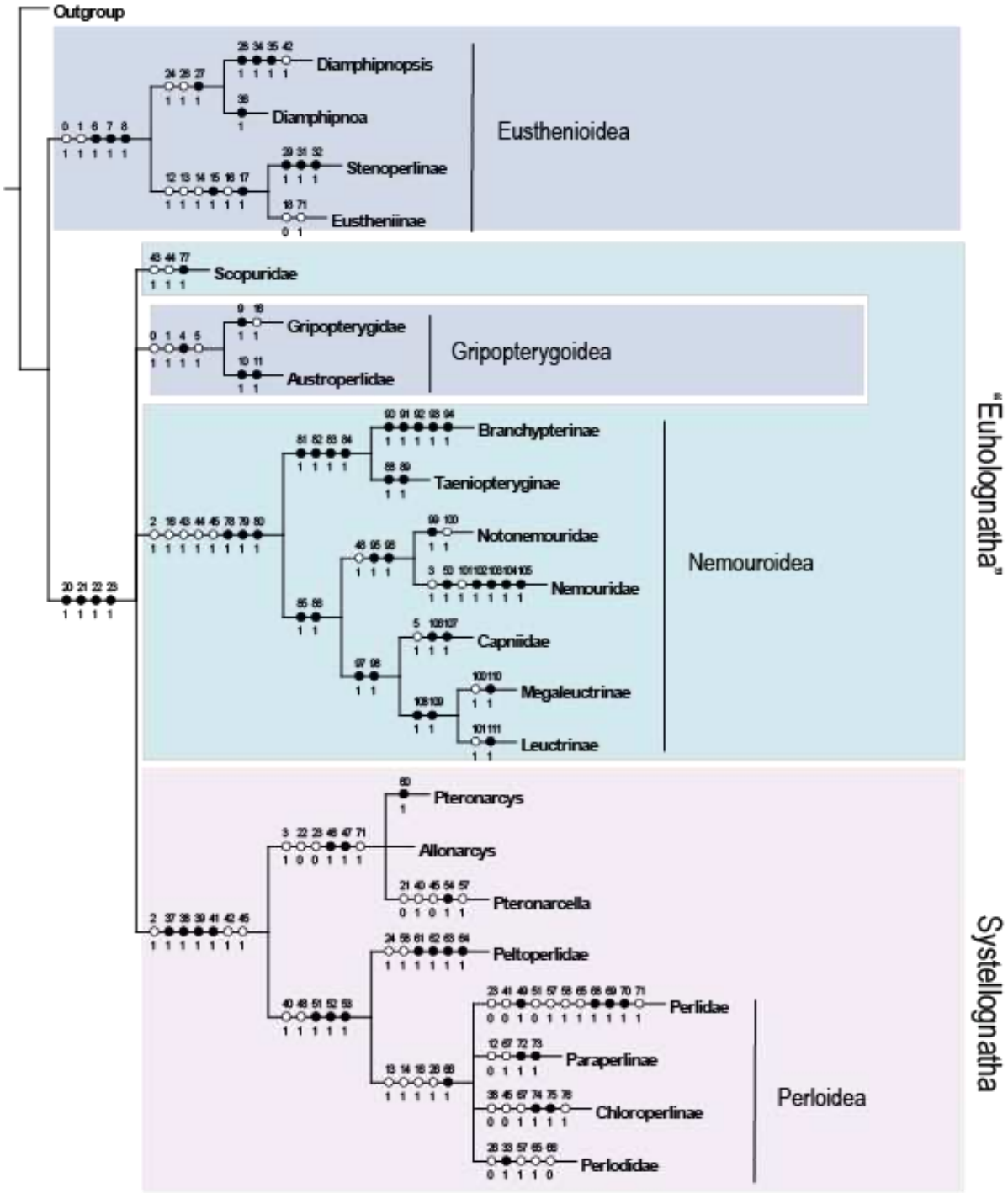

FIGURE 2. Strict consensus of the 12 most parsimonious trees with a length of 160 steps recovered with Wagner Search from the analysis of 113 morphological characters of Plecoptera. Blue boxes represent "Antarctoperlaria". Morphological characters are shown along branches as black circles (unique) and white circles (non-unique) apomorphies. Numbers above and below circles are characters and state numbers, respectively.

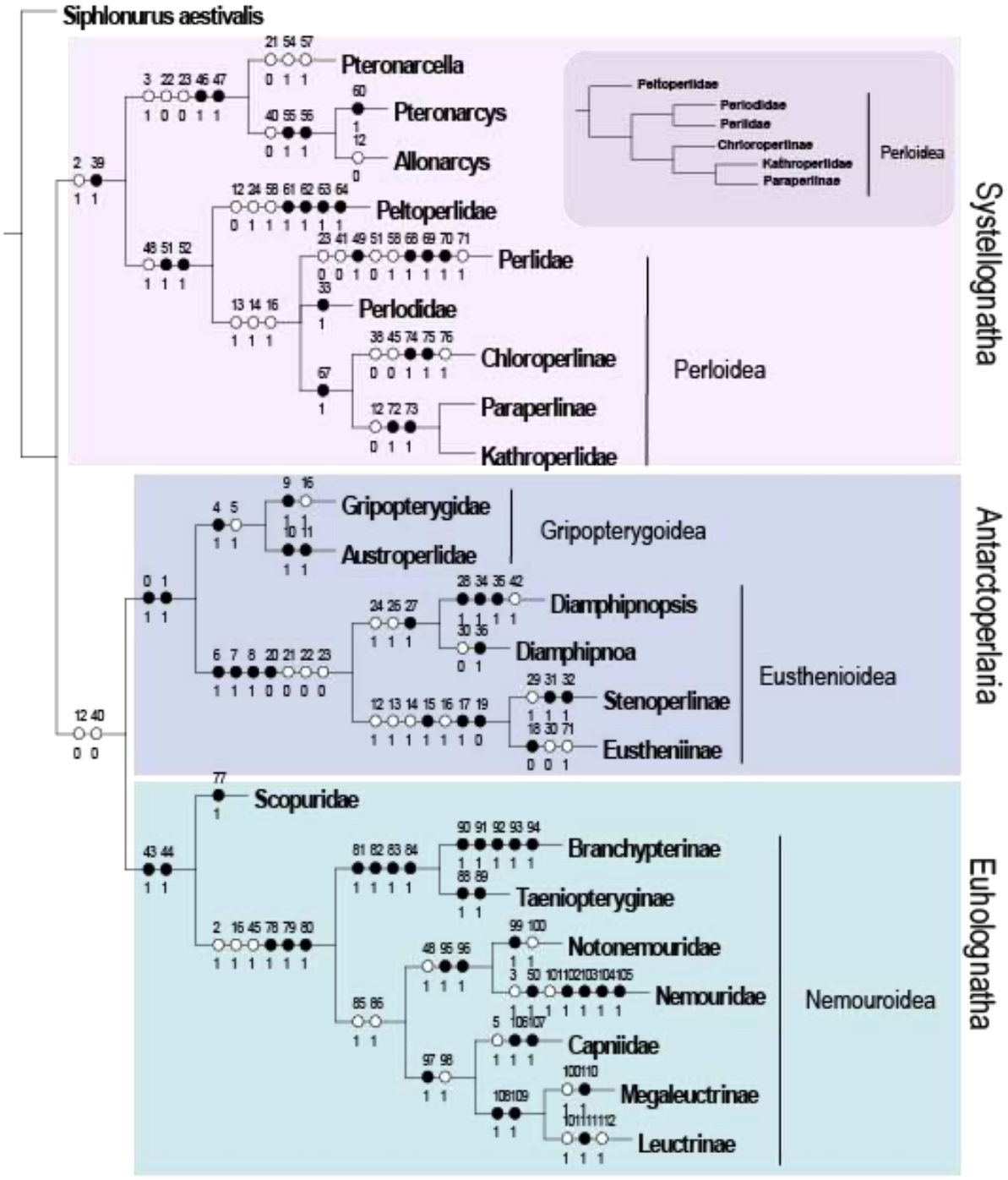

FIGURE 3. Second Analysis: Strict consensus of 2 trees with a length of 165 steps recovered with Wagner Search from the analysis of 114 morphological characters of Plecoptera. The tree topologies using Implied Weighting (k = 3.0000 and k = 2.65625) recovered the relationships within Perloidea (grayed rectangle - upper corner). Morphological characters are shown along branches as black circles (unique) and white circles (non-unique) apomorphies. Numbers above and below circles are characters and state numbers, respectively.

3.7 APPENDIX 1: Character list of major taxonomic groups of the plecoptera (Nelson, 1984)

1. Trochanter sternal depressor present
2. Trochanter tergal depressor reduced
3. Drumming present
4. Cervical gills present
5. Male genitalia with large accessory glands present
6. Female seminal receptacle present
7. Larval coxa with sensory bristle
8. Male paraproct with bladderlike membranous region
9. Female genital opening extremely narrow
10. Larval abdominal apex with retractable gill rosette
11. Larval cerci in part transformed gills or possessing gills
12. Last larval instar with separate sclerite covering developing male epiproct
13. Larvae strictly phytophagous
14. Larval mandible molar area slender and reduced
15. Larval labium with glossal apices at same level as paraglossal apices
16. Trochanter sternal depressor in front of furcal arm
17. Last abdominal ganglion in segment 7 or anterior
18. One tibial spur
19. Larval abdominal gills present on segments 1-6
20. Larval abdominal gills present on at least segments 1-5
21. Larval abdominal gills present on at least segments 1-4
22. Larval abdominal gills present on at least segments 1-3
23. Larval abdominal gills present on at least segments 1-2
24. Larval abdominal gills absent
25. Thoracic atypical longitudinal muscles reduced
26. Thoracic atypical longitudinal muscles extremely well developed
27. Sternal coxal rotator muscles present
28. Male paraproct with mediobasal appendage present
29. Male paraproct with mediobasal appendage pronounced and knife-like
30. Body form conspicuously elongate
31. Adult cerci approximately as long as abdomen
32. Larvae with spatulate spines on tarsi

33. Head postocular region extended into thorax

34. Female sternite 9 enlarged and anteriorly extended

35. Abdominal accessory trachea leading from spiracle to gill

36. Larval maxilla with radula

37. Male epiproct with basal arms deeply bent inward and extending nearly to anterior margin of tergite #10

38. Adult mandible thin, lacking mola

39. Adult mandible thin, not sclerotized, soft

40. Egg with anchor and collar

41. Egg with micropyles surrounding collar

42. Male epiproct sunk into a pocket

43. Thoracic postalar bridge present

44. Corpus allatum paired

45. Egg chorion gelatinous

46. Male hammer present

47. Trachea leading from head to cervical gills

48. Postfurcal gills present

49. Abdominal ganglia seven or fewer

50. Fusion of abdominal and metathoracic ganglia resulting in six abdominal ganglia

51. Abdominal ganglia five or fewer

52. Male epiproct with lateral stylets

53. Tarsal segments 1 and 2 together shorter than segment 3

54. Frontoclypeal suture present

55. Seminal vesicle and testes separate

56. Abdominal accessory intersegmental trachea connecting to gill present

57. Epiproct muscles with additional tergal muscle present

58. Anterior supracoxal gills present

59. Posterior supracoxal gills present

60. Epiproct sperm cup present

61. Epiproct lever arm present

62. Head bent under pronotum

63. Anterior ocellus present

64. Larval pro- and mesothorax with duplicate plate-like areas concealing furcal pits

65. Prothoracic spinasternum and furcasternum fused

66. Midgut caecal sacs present

67. Muscle ism 22 attached near base of furcal arm

68. Most distal labial palpal segment minute, conical, asymmetrically inserted on penultimate segment

69. Larval labium with inflated paraglossa and broadened mentum

70. Muscle PPM 56 attached to postalar sclerite

71. Muscle ism 24 present

72. Follicles of testes not clearly separated, organized into dense clusters

73. Head elongate, sides parallel

74. Mesosternal Y-ridge with arms complete

75. Male epiproct with distal portion pointing anteriorly

76. Epiproct lever arm broad, fused to front margin of segment 10

77. Fore- and hindwings similar, anal fan reduced

78. Gill ring in the intersegmental membrane of segments 9 and 10

79. Spina II extended anteriorly to base of furca

80. Sclerotic bridge uniting spina I with anterior margin of basisternum II present

81. Male paraproct divided into inner and outer lobes

82. Three tarsal segments, all of equal length

83. Male basal cercal segment enlarged, with remaining segments asymmetrically attached

84. Vagina minute and opening ventrally

85. Female genital opening located in middle of eighth sternite

86. Forewing with two cubital veins

87. Extrusible penis

88. Male cerci with one segment

89. Seminal receptacle with bladder-like and tube-like portion

90. Coxal gills presente

91. Female sternite 9 with postgenital plate present

92. Male paraproct asymmetrical

93. Interior bladder of male epiproct present

94. Male sternite 9 strongly produced and covering abdominal tip from beneath

95. Male basal cercal segment with process

96. Pleural arm forked

97. Ejaculatory duct paired

98. Male paraprocts with inner lobes modified into tubes for sperm transfer

99. Ovaries united, forming an arch

100. Male paraprocts with inner and outer lobes independent, inner lobes shifted to the subgenital plate

101. Female ovipositor present

102. Seminal vesicle paired

103. Testes formed into a star shaped cluster

104. Vasa opening into terminal portion of seminal vesicle

105. Most distal labial palpal segment rounded and plate-like

106. Procoxae with perpendicular insertion, enlarged, nearly touching medially

107. Cubital crossveins single or absent

108. Male paraprocts with inner and outer lobes forming fusion plate

109. Inner lobe male paraproct with bulb-shaped basal portion

110. Vasa deferentia enlarged, functioning as accessory seminal vesicles

111. Female paraprocts fused for much of their length.

112. Inner lobe male paraproct with interior sperm tube present

113. Vasa deferentia opening into base of seminal vesicle at a single point

Extra 114. Head, postocular region, length: (0) subequal to the length of the compound eye, (1) at least two times the length of the compound eye.

3.8 APPENDIX 2: Matrix ready to run.

xread

114 25

Outgroup

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Peltoperlidae

001000000000000000111111100000100000011111100100100111000010011110000
00000000000000000000000000000000000000000000?

Scopuridae

000000000000000000111111000000100000000000011000000000000000000000000
00000000100000000000000000000000000000000000?

Stenoperlinae

110000111000111111100000000000111100000000000000000000000000000000000
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Pteronarcys

001100000000100000111100010000100000011101100111000000011001100000000
00100000000000000000000000000000000000000000?

Perlidae

00100000000011101011111001100010000001111010010011001100011000000 1101
11100000000000000000000000000000000000000000?

Paraperlinae

001000000000011010111111011000100000011111100100100111000000000000110
000110000000000000000000000000000000000000000

Branchypterinae

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00000000011111110000011111000000000000000000?

Gripopterygidae

110011000100000001011111100000010000000000000000000000000000000000000
00000000000000000000000000000000000000000000?

Allonarcys

001100000000000000111100010000100000011101100111000000011000000000000
00100000000000000000000000000000000000000000?

Diamphipnopsis

1100001110000000001100001011101000110000001000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000?

Notonemouridae

0010000000000000101111110000001000000000000111001000000000000000000000000000000000000011100001110000000110011000000000000?

Taeniopteryginae

0010000000000000101111110000001000000000000111000000000000000000000000000000000000011111110011100000000000000000000000?

Nemouridae

0011000000000000101111110000001000000000000111001010000000000000000000000000000000000011100001110000000110000111110000000?

Pteronarcella

0011000000001000001110000100001000000111111000110000001001010000000000010000000000000000000000000000000000000000000000000?

Megaleuctrinae

0010000000000000101111110000001000000000000111000000000000000000000000000000000000011100001110000000000110100000011100?

Leuctrinae

0010000000000000101111110000001000000000000111000000000000000000000000000000000000010111000011100000000011001000000110 11?

Capniidae

0010010000000000101111110000001000000000000111000000000000000000000000000000000000010111000011000000000011000000011000 00?

Chloroperlinae

0010000000001110101111110110001000000101111000001001110000000000001100000011100000000000000000000000000000000000000000?

Austroperlidae

1100110000110000001111110000001000000000000000000000000000000000000000000000000000000000000000000000000000000000000000?

Diamphipnoa

1100001110000000001100001011000000001000000000000000000000000000000000000000000000000000000000000000000000000000000000?

Eustheniinae

110000111000111111000000000000000000000000000000000000000000000000000
00100000000000000000000000000000000000000000?

Perlodidae

001000000000111010111111010000100100011111100100100111000100000001000
00000000000000000000000000000000000000000000?

Kathroperlidae

001000000000011010111111011000100000011111100100100111000000000000110
000110000000000000000000000000000000000000001

Siphlonurus_aestivalis

??000??0?000100??0111111??10011000?0???01???00?0????11??00??000???0???10
???0???000?01100000?00?0?1?00000?0??00?1?

;

cnames

{0 0._Trochanter_sternal_depressor_present;

{1 1._Trochanter_tergal_depressor_reduced;

{2 2._Drumming_present;

{3 3._Cervical_gills_present;

{4 4._Male_genitalia_with_large_accessory_glands_present;

{5 5._Male_genitalia_with_large_accessory_glands_present;

{6 6._Larval_coxa_with_sensory_bristle;

{7 7._Male_paraproct_with_bladderlike_membranous_region;

{8 8._Female_genital_opening_extremely_narrow;

{9 9._Larval_abdominal_apex_with_retractable_gill_rosette;

{10 10._Larval_cerci_in_part_transformed_gills_or_possessing_gills;

{11 11._Last_larval_instar_with_separate_sclerite_covering_developing_male_epiproct;

{12 12._Larvae_strictly_phytophagous;

{13 13._Larval_mandible_molar_area_slender_and_reduced;

{14 14._Larval_labium_with_glossal_apices_at_same_level_as_paraglossal_apices;

{15 15._Trochanter_sternal_depressor_in_front_of_furcal_arm;

{16 16._Last_abdominal_ganglion_in_segment_7_or_anterior;

{17 17._One_tibial_spur;

{18 18._Larval_abdominal_gills_present_on_segments_1-6;

{19 19._Larval_abdominal_gills_present_on_at_least_segments_1-5;

{20 20._Larval_abdominal_gills_present_on_at_least_segments_1-4;

{21 21._Larval_abdominal_gills_present_on_at_least_segments_1-3;

{22 22._Larval_abdominal_gills_present_on_at_least_segments_1-2;

{23 23._Larval_abdominal_gills_absent;

{24 24._Thoracic_atypical_longitudinal_muscles_reduced;

{25 25._Thoracic_atypical_longitudinal_muscles_extremely_well_developed;

{26 26._Sternal_coxal_rotator_muscles_present;

{27 27._Male_paraproct_with_mediobasal_appendage_present;

{28 28._Male_paraproct_with_mediobasal_appendage_pronounced_and_knife-like;

{29 29._Body_form_conspicuously_elongate;

{30 30._Adult_cerci_approximately_as_long_as_abdomen;

{31 31._Larvae_with_spatulate_spines_on_tarsi;

{32 32._Head_postocular_region_extended_into_thorax;

{33 33._Female_sternite_9_enlarged_and_anteriorly_extended;

{34 34._Abdominal_accessory_trachea_leading_from_spiracle_to_gill;

{35 35._Larval_maxilla_with_radula;

{36
36._Male_epiproct_with_basal_arms_deeply_bent_inward_and_extending_nearly_to_anterior_margin_of_tergite_#10;

{37 37._Adult_mandible_thin,_lacking_mola;

{38 38._Adult_mandible_thin,_not_sclerotized,_soft;

{39 39._Egg_with_anchor_and_collar;

{40 40._Egg_with_micropyles_surrounding_collar;

{41 41._Male_epiproct_sunk_into_a_pocket;

{42 42._Thoracic_postalar_bridge_present;

{43 43._Corpus_allatum_paired;

{44 44._Egg_chorion_gelatinous;

{45 45._Male_hammer_present;

{46 46._Trachea_leading_from_head_to_cervical_gills;

{47 47._Postfurcal_gills_present;

{48 48._Abdominal_ganglia_seven_or_fewer;

{49
49._Fusion_of_abdominal_and_metathoracic_ganglia_resulting_in_six_abdominal_ganglia;
{50 50._Abdominal_ganglia_five_or_fewer;
{51 51._Male_epiproct_with_lateral_stylets;
{52 52._Tarsal_segments_1_and_2_together_shorter_than_segment_3;
{53 53._Frontoclypeal_suture_present;
{54 54._Seminal_vesicle_and_testes_separate;
{55 55._Abdominal_accessory_intersegmental_trachea_connecting_to_gill_present;
{56 56._Epiproct_muscles_with_additional_tergal_muscle_present;
{57 57._Anterior_supracoxal_gills_present;
{58 58._Posterior_supracoxal_gills_present;
{59 59._Epiproct_sperm_cup_present;
{60 60._Epiproct_lever_arm_present;
{61 61._Head_bent_under_pronotum;
{62 62._Anterior_ocellus_present;
{63 63._Larval_pro-_and_mesothorax_with_duplicate_plate-like_areas_concealing_furcal_pits;
{64 64._Prothoracic_spinasternum_and_furcasternum_fused;
{65 65._Midgut_caecal_sacs_present;
{66 66._Muscle_ism_22_attached_near_base_of_furcal_arm;
{67
67._Most_distal_labial_palpal_segment_minute,_conical,_asymmetrically_inserted_on_penultimate_segment;
{68 68._Larval_labium_with_inflated_paraglossa_and_broadened_menturn;
{69 69._Muscle_PPM_56_attached_to_postalar_sclerite;
{70 70._Muscle_ism_24_present;
{71 71._Follicles_of_testes_not_clearly_separated,_organized_into_dense_clusters;
{72 72._Head_elongate,_sides_parallel;
{73 73._Mesosternal_Y-ridge_with_arms_complete;
{74 74._Male_epiproct_with_distal_portion_pointing_anteriorly;
{75 75._Epiproct_lever_arm_broad,_fused_to_front_margin_of_segment_10;
{76 76._Fore-_and_hindwings_similar,_anal_fan_reduced;
{77 77._Gill_ring_in_the_intersegmental_membrane_of_segments_9_and_10;
{78 78._Spina_II_extended_anteriorly_to_base_of_furca;

{79
79._Sclerotic_bridge_uniting_spina_I_with_anterior_margin_of_basisternum_II_present;
{80 80._Male_paraproct_divided_into_inner_and_outer_lobes;
{81 81._Three_tarsal_segments,_all_of_equal_length;
{82
82._Male_basal_cereal_segment_enlarged,_with_remaining_segments_asymetrically_attache
d;
{83 83._Vagina_minute_and_opening_ventrally;
{84 84._Female_genital_opening_located_in_middle_of_eighth_sternite;
{85 85._Forewing_with_two_cubital_veins;
{86 86._Extrusible_penis;
{87 87._Male_cerci_with_one_segment;
{88 88._Seminal_receptacle_with_bladder-like_and_tube-like_portion;
{89 89._Coxal_gills_present;
{90 90._Female_sternite_9_with_postgenital_plate_present;
{91 91._Male_paraproct_asymmetrical;
{92 92._Interior_bladder_of_male_epiproct_present;
{93
93._Male_sternite_9_strongly_produced_and_covering_abdominal_tip_from_beneath;
{94 94._Male_basal_cereal_segment_with_process;
{95 95._Pleural_arm_forked;
{96 96._Ejaculatory_duct_paired;
{97
97._Male_paraprocts_with_inner_lobes_modified_into_tubes_for_sperm_transfer;
{98 98._Ovaries_united,_forming_an_arch;
{99
99._Male_paraprocts_with_inner_and_outer_lobes_independent,_inner_lobes_shifted_to_the
_subgenital_plate;
{100 100._Female_ovipositor_present;
{101 101._Seminal_vesicle_paired;
{102 102._Testes_formed_into_a_starshaped_cluster;
{103 103._Vasa_opening_into_terminal_portion_of_seminal_vesicle;
{104 104._Most_distal_labial_palpal_segment_rounded_and_plate-like;

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        {105
105._Procoxae_with_perpendicular_insertion,_enlarged,_nearly_touching_medially;
        {106 106._Cubital_crossveins_single_or_absent;
        {107 107._Male_paraprocts_with_inner_and_outer_lobes_forming_fusion_plate;
        {108 108._Inner_lobe_male_paraproct_with_bulb-shaped_basal_portion;
        {109 109._Vasa_deferentia_enlarged,_functioning_as_accessory_seminal_vesicles;
        {110 110._Female_paraprocts_fused_for_much_of_their_length;
        {111 111._Inner_lobe_male_paraproct_with_interior_sperm_tube_present;
        {112
112._Vasa_deferentia_opening_into_base_of_seminal_vesicle_at_a_single_point;
        {113
113._Head,_postocular_region,_length:_(0)_subequal_to_the_length_of_the_compound_eye,
_(1)_at_least_two_times_the_length_of_the_compound_eye;
        ;


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        comments 0
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3.9 APPENDIX 3: Character list of major taxonomic groups of the plecoptera proposed by Nelson, (1984) modified according to Sereno, (2007).

1. Trochanter sternal depressor present

Trochanter, muscle, sternal depressor: (0) absent; (1) present.

2. Trochanter tergal depressor reduced

Trochanter, muscle, tergal depressor, development: (0) reduced; (1) not reduced.

3. Drumming present

Behavior, drumming: (0) absent; (1) present.

4. Cervical gills present

Thorax, cervical gills (or AT1): (0) absent; (1) present.

5. Male genitalia with large accessory glands present

Male genitalia, large accessory glands: (0) absent; (1) present.

6. Female seminal receptacle present

Female genitalia, seminal receptacle: (0) absent; (1) present.

7. Larval coxa with sensory bristle

Nymphal coxa, sensory bristle: (0) absent; (1) present.

8. Male paraproct with bladderlike membranous region

Male terminalia, paraproct, bladderlike membranous region: (0) absent; (1) present.

9. Female genital opening extremely narrow

Female genitalia, genital opening, shape: (0) extremely narrow; (1) other.

10. Larval abdominal apex with retractable gill rosette

Nymphal abdomen, apex region, retractable gill rosette: (0) absent; (1) present.

11. Larval cerci in part transformed gills or possessing gills

Nymphal cerci, in part transformed gills or possessing gills: (0) absent; (1) present.

12. Last larval instar with separate sclerite covering developing male epiproct

Nymphal abdomen, last male instar, separate sclerite covering developing epiproct: (0) absent; (1) present.

Nymphal abdomen, last male instar, separate sclerite, development: (0) not covering epiproct; (1) covering epiproct.

13. Larvae strictly phytophagous

Behavior, larval feeding: (0) strictly phytophagous; (1) other.

14. Larval mandible molar area slender and reduced

Nymphal mandible, molar area, development: (0) slender and reduced; (1) other.

15. Larval labium with glossal apices at same level as paraglossal apices

Nymphal labium, glossal apices in relation to paraglossal apices: (0) at same level; (1) other.

16. Trochanter sternal depressor in front of furcal arm

Trochanter sternal depressor muscle, location: (0) in front of furcal arm; (1) other.

17. Last abdominal ganglion in segment 7 or anterior

Abdomen, last abdominal ganglion, location: (0) anterior to segment VII; (1) in segment VII.

18. One tibial spur

Leg, tibia, one tibial spur: (0) absent; (1) present

19. Larval abdominal gills present on segments 1-6

Abdomen, larval gills on segments I-VI: (0) absent; (1) present.

20. Larval abdominal gills present on at least segments 1-5

Abdomen, larval gills on segments I-V: (0) absent; (1) present.

21. Larval abdominal gills present on at least segments 1-4

Abdomen, larval gills on segments I-IV: (0) absent; (1) present.

22. Larval abdominal gills present on at least segments 1-3

Abdomen, larval gills on segments I-III: (0) absent; (1) present.

23. Larval abdominal gills present on at least segments 1-2

Abdomen, larval gills on segments I-II: (0) absent; (1) present.

24. Larval abdominal gills absent

Abdomen, larval gills: (0) absent; (1) present.

25. Thoracic atypical longitudinal muscles reduced

Thorax, atypical longitudinal muscles, length: (0) reduced; (1) other.

26. Thoracic atypical longitudinal muscles extremely well developed

Thorax, atypical longitudinal muscles, development: (0) extremely well developed; (1) other;

27. Sternal coxal rotator muscles present

Leg, coxa, sternal coxal rotator muscles: (0) absent; (1) present.

28. Male paraproct with mediobasal appendage present

Male terminalia, paraproct, mediobasal appendage: (0) absent; (1) present.

29. Male paraproct with mediobasal appendage pronounced and knife-like

Male terminalia, paraproct, mediobasal appendage, shape: (0) pronounced and knife-like; (1) other.

30. Body form conspicuously elongate

Body, shape: (0) conspicuously elongate; (1) other

31. Adult cerci approximately as long as abdomen

Adult abdomen, cerci, relative length: (0) approximately as long as abdomen; (1) other.

32. Larvae with spatulate spines on tarsi

Nymphal leg, tarsi, spatulate spines: (0) absent; (1) present.

33. Head postocular region extended into thorax

Head, postocular region, development: (0) extended into thorax; (1) other.

34. Female sternite 9 enlarged and anteriorly extended

Female abdomen, sternite 9, development: (0) enlarged and anteriorly extended; (1) other.

35. Abdominal accessory trachea leading from spiracle to gill

Abdomen, accessory trachea, position: (0) leading from spiracle to gill; (1) other.

36. Larval maxilla with radula

Nymphal maxilla, radula: (0) absent; (1) present.

37. Male epiproct with basal arms deeply bent inward and extending nearly to anterior margin of tergite #10

Male genitalia, epiproct, basal arms, position and development: (0) deeply bent inward and extending nearly to anterior margin of tergite #10; (1) other;

38. Adult mandible thin, lacking mola

Adult mandible, shape: (0) thin, lacking mola; (1) other.

39. Adult mandible thin, not sclerotized, soft

Adult mandible, shape and sclerotization: (0) thin, not sclerotized, soft; (1) other.

40. Egg with anchor and collar

Egg, anchor and collar: (0) absent; (1) present.

41. Egg with micropyles surrounding collar

Egg, micropyles around collar: (0) absent; (1) present.

42. Male epiproct sunk into a pocket

Male terminalia, epiproct, position: (0) sunk into a pocket; (1) other.

43. Thoracic postalar bridge present

Thorax, postalar bridge: (0) absent; (1) present.

44. Corpus allatum paired

Head, gland, corpus allatum: (0) paired; (1) other.

45. Egg chorion gelatinous

Egg, chorion, aspect: (0) gelatinous; (1) other.

46. Male hammer present

Male genitalia, hammer: (0) absent; (1) present.

47. Trachea leading from head to cervical gills

Thorax, trachea, position: (0) leading from head to cervical gills; (1) other.

48. Postfurcal gills present

Thorax, postfurcal gills (or PS): (0) absent; (1) present.

49. Abdominal ganglia seven or fewer

Abdomen, ganglia, number: (0) seven or fewer; (1) other.

50. Fusion of abdominal and metathoracic ganglia resulting in six abdominal ganglia

Abdomen and metathorax, ganglia, fusion: (0) resulting in six abdominal ganglia; (1) other.

51. Abdominal ganglia five or fewer

Abdomen, ganglia, number: (0) five or fewer; (1) other.

52. Male epiproct with lateral stylets

Male terminalia, epiproct, lateral stylets: (0) absent; (1) present.

53. Tarsal segments 1 and 2 together shorter than segment 3

Tarsi, length of segments 1 and 2 in relation to segment 3: (0) shorter; (1) other.

54. Frontoclypeal suture present
Head, frontoclypeal suture: (0) absent; (1) present.

55. Seminal vesicle and testes separate
Male genitalia, seminal vesicle and testes, arrangement: (0) separate; (1) other.

56. Abdominal accessory intersegmental trachea connecting to gill present
Abdomen, accessory intersegmental trachea, position: (0) connecting to gill; (1) other.

57. Epiproct muscles with additional tergal muscle present
Male terminalia, epiproct, additional tergal muscle: (0) absent; (1) present.

58. Anterior supracoxal gills present
Thorax, anterior supracoxal gills (ASC): (0) absent; (1) present.

59. Posterior supracoxal gills present
Thorax, posterior supracoxal gills (PSC): (0) absent; (1) present.

60. Epiproct sperm cup present
Male terminalia, epiproct sperm cup: (0) absent; (1) present.

61. Epiproct lever arm present
Male terminalia, epiproct, lever arm: (0) absent; (1) present.

62. Head bent under pronotum
Head, arrangement: (0) bent under pronotum; (1) other.

63. Anterior ocellus present
Head, anterior ocellus: (0) absent; (1) present.

64. Larval pro- and mesothorax with duplicate plate-like areas concealing furcal pits

Nymphal pro- and mesothorax, duplicate plate-like areas concealing furcal pits: (0) absent; (1) present.

65. Prothoracic spinasternum and furcasternum fused

Prothorax, spinasternum and furcasternum, position: (0) fused; (1) other.

66. Midgut caecal sacs present

Abdomen, midgut caecal sacs: (0) absent; (1) present.

67. Muscle ism 22 attached near base of furcal arm

Thorax, muscle ism 22, attachment: (0) near base of furcal arm; (1) other.

68. Most distal labial palpal segment minute, conical, asymmetrically inserted on penultimate segment

Labium, distal labial palpal segment, shape: (0) minute, conical, asymmetrically inserted on penultimate segment; (1) other.

69. Larval labium with inflated paraglossa and broadened mentum

Nymphal labium, paraglossa and mentum, shape: (0) inflated paraglossa and broadened mentum; (1) other.

70. Muscle PPM 56 attached to postalar sclerite

Thorax, muscle PPM 56, attachment: (0) attached to postalar sclerite; (1) other.

71. Muscle ism 24 present

Thorax, muscle ism 24: (0) absent; (1) present.

72. Follicles of testes not clearly separated, organized into dense clusters

Male genitalia, follicles of testes, arrangement: (0) not clearly separated, organized into dense clusters; (1) other.

73. Head elongate, sides parallel

Head, shape: (0) elongate, sides parallel; (1) other.

74. Mesosternal Y-ridge with arms complete

Thorax, mesosternal Y-ridge, arms: (0) complete; (1) other.

75. Male epiproct with distal portion pointing anteriorly

Male terminalia, epiproct, distal portion, shape: (0) pointing anteriorly; (1) other.

76. Epiproct lever arm broad, fused to front margin of segment 10

Terminalia, epiproct, lever arm, shape: (0) broad, fused to front margin of segment X; (1) other.

77. Fore- and hindwings similar, anal fan reduced

Wings, relative shape: (0) fore- and hindwings similar, anal fan reduced; (1) other.

78. Gill ring in the intersegmental membrane of segments 9 and 10

Abdomen, gill ring in the intersegmental membrane of segments 9 and 10 : (0) absent; (1) present.

79. Spina II extended anteriorly to base of furca

Thorax, spina II, position: (0) extended anteriorly to base of furca; (1) other.

80. Sclerotic bridge uniting spina I with anterior margin of basisternum II present

Thorax, sclerotic bridge uniting spina I with anterior margin of basisternum II: (0) absent; (1) present.

81. Male paraproct divided into inner and outer lobes

Male terminalia, paraproct, arrangement: (0) divided into inner and outer lobes; (1) other.

82. Three tarsal segments, all of equal length

Tarsi, segments, relative length: (0) all of equal length; (1) other.

83. Male basal cercal segment enlarged, with remaining segments asymetrically attached

Male abdomen, basal cercal segment, shape: (0) enlarged, with remaining segments asymmetrically attached; (1) other.

84. Vagina minute and opening ventrally

Female genitalia, vagina, shape: (0) minute, opening ventrally; (1) other.

85. Female genital opening located in middle of eighth sternite

Female genitalia, genital opening, location: (0) in middle of eighth sternite; (1) other.

86. Forewing with two cubital veins

Wings, forewing, cubital veins, number: (0) two; (1) other.

87. Extrusible penis

Male genitalia, penis, movement: (0) extrusible; (1) other.

88. Male cerci with one segment

Male abdomen, cerci, number of segments: (0) one segment; (1) other.

89. Seminal receptacle with bladder-like and tube-like portion

Male genitalia, seminal receptacle, bladder-like and tube-like portion: (0) absent; (1) present.

90. Coxal gills present

Thorax, coxal gills (CX): (0) absent; (1) present.

91. Female sternite 9 with postgenital plate present

Female genitalia, sternite 9, postgenital plate: (0) absent; (1) present.

92. Male paraproct asymmetrical

Male terminalia, paraproct, shape: (0) asymmetrical; (1) other.

93. Interior bladder of male epiproct present

Male terminalia, epiproct, interior bladder: (0) absent; (1) present.

94. Male sternite 9 strongly produced and covering abdominal tip from beneath

Male abdomen, sternite 9, shape: (0) strongly produced and covering abdominal tip from beneath; (1) other.

95. Male basal cercal segment with process

Male abdomen, basal cercal segment, process: (0) absent; (1) present.

96. Pleural arm forked

Thorax, pleural arm, shape: (0) forked; (1) other.

97. Ejaculatory duct paired

Male genitalia, ejaculatory duct, arrangement: (0) paired; (1) other.

98. Male paraprocts with inner lobes modified into tubes for sperm transfer

Male terminalia, paraprocts, inner lobes, shape: (0) modified into tubes for sperm transfer; (1) other.

99. Ovaries united, forming an arch

Female genitalia, ovaries, arrangement: (0) united, forming an arch; (1) other.

100. Male paraprocts with inner and outer lobes independent, inner lobes shifted to the subgenital plate

Male terminalia, paraprocts, inner and outer lobes, arrangement: (0) independent, inner lobes shifted to the subgenital plate; (1) other.

101. Female ovipositor present

Female terminalia, ovipositor: (0) absent; (1) present.

102. Seminal vesicle paired

Male genitalia, seminal vesicle, arrangement: (0) paired; (1) other.

103. Testes formed into a star-shaped cluster

Male genitalia, testes, shape: (0) star-shaped cluster; (1) other.

104. Vasa opening into terminal portion of seminal vesicle

Male genitalia, vasa, arrangement: (0) opening into terminal portion of seminal vesicle; (1) other.

105. Most distal labial palpal segment rounded and plate-like

Labium, distal labial palp segment, shape: (0) rounded and plate-like; (1) other.

106. Procoxae with perpendicular insertion, enlarged, nearly touching medially

Leg, procoxae, perpendicular insertion, shape: (0) enlarged, nearly touching medially; (1) other.

107. Cubital crossveins single or absent

Wing, cubital crossvein: (0) absent; (1) present.

108. Male paraprocts with inner and outer lobes forming fusion plate

Male terminalia, paraprocts, inner and outer lobes, shape: (0) fusion plate; (1) other.

109. Inner lobe male paraproct with bulb-shaped basal portion

Male terminalia, paraproct, inner lobe, basal portion, shape: (0) bulb-shaped; (1) other.

110. Vasa deferentia enlarged, functioning as accessory seminal vesicles

Male genitalia, vasa deferentia, shape: (0) enlarged, functioning as accessory seminal vesicles; (1) other.

111. Female paraprocts fused for much of their length.

Female terminalia, paraprocts, arrangement: (0) fused for much of their length; (1) other.

112. Inner lobe male paraproct with interior sperm tube present

Male terminalia, paraproct, inner lobe, interior sperm tube: (0) absent; (1) present.

113. Vasa deferentia opening into base of seminal vesicle at a single point

Male genitalia, vasa deferentia, opening: (0) into base of seminal vesicle at a single point; (1) other.

114. Head, postocular region, length: (0) subequal to the length of the compound eye, (1) at least two times the length of the compound eye.

CHAPTER 2 - ANATOMY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEM OF PERLIDAE (PLECOPTERA, INSECTA)

4. CHAPTER 2 - ANATOMY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEM OF PERLIDAE (PLECOPTERA, INSECTA)

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Abstract

The anatomy and histology of the reproductive system of the stoneflies of tribe Anacroneuriini were described for the first time based on specimens from the Neotropical fauna. The male reproductive system of the genera *Anacroneuria* and *Kempnyia*, includes a pair of testes fused medially which exhibit variations in the number of testicular follicles. Notably, spermatogenesis is not continuous in these genera, suggesting that the males of these species are ready to mate as soon as they emerge. This research has contributed to the understanding of Plecoptera reproductive systems, providing new insights by elucidating these structures. These features of the reproductive system, which encompass both structural elements and behaviors associated with reproduction, provide valuable information for future phylogenetic investigations. As a result, our work contributes to a deeper understanding of the evolution and diversity of this group of aquatic insects.

Keywords: internal morphology, reproductive strategies, evolutionary dynamics, anacroneuriini.

Highlights:

- Anacroneuriini' reproductive structures includes a pair of fused medially which exhibit variations in the number of testicular follicles.
- Spermatogenesis in these stoneflies is not continuous, suggesting infrequent mating or efficient sperm transfer to females in a limited number of copulations.

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4.1 INTRODUCTION

The reproductive system of Plecoptera is relatively well-known (Brinck, 1956; Stewart *et al*., 1969; Stewart & Stark, 1977; Stark & Szczytko 1982, 1984; Rościszewska 1997, 2001; Rościszewska & Soldán, 1999; Poprawa *et al*. 2002; Rościszewska & Rzońca, 2009). Brinck (1956) conducted a comprehensive study of the reproductive systems of various Plecoptera families. He carefully selected species from different locations, examined both external and internal genitalia, explored their functions, and assessed sperm transport mechanisms. This research highlighted the remarkable diversity of reproductive organs within Plecoptera. He also proposed a standardized terminology for Plecoptera genitalia by reviewing the taxonomic literature. A portion of this terminological effort was used in a glossary prepared for publication under Dr. S. L. Tuxen's editorship in Copenhagen. Stewart *et al*., (1969) described the male and female reproductive structures and the mating behavior of *Perlesta placida* (Hagen, 1861), a member of Perlidae.

Zwick (1973, 1980) conducted an extensive examination of the male genital organs of Plecoptera, emphasizing their remarkable diversity. This research established a fundamental framework for comprehending variations within different species and families. It delved into the various adaptations in testicular structures, vasa deferentia, seminal vesicles, and their contributions to the group's diversity. Furthermore, Zwick (1973, 1980) explored alterations in the genital cavity structure and its correlations with copulatory organs across various families. This study delivered a comprehensive comparative perspective on the male genital organs in Plecoptera, underscoring their importance in unraveling the taxonomy and evolution of this insect order. Stewart & Stark (1977) studied the reproductive system and mating behavior of *Hydroperla crosbyi* (Needham & Claassen, 1925), another member of Perlidae. They discovered an alternative method of sperm transfer in these insects, which involved the transfer of a spermatophore to the female. Overall, the studies cited above provide valuable insights into the morphology of the reproductive system and behavior of Perlidae. Stoneflies' reproductive system provides outstanding taxonomic characters the structure and function are incredibly diverse and have been used to infer phylogenetic relationships (Zwick, 2000, 2009).

Anacroneuriini Stark & Gaufin, 1976, is one of the most prominent tribes within the Perlidae. It is restricted to the Neotropical region and the southern portion of the Nearctic region and is represented by four genera: *Anacroneuria* Klapálek, 1909, is the most diverse, with approximately 380 recorded species; *Enderleina* Jewett, 1960 has ten registered species;

Kempnyia Klapálek, 1914, composed of 36 species and *Macrogynoplax* Enderlein, 1909, is composed by 15 species (Pessacq *et al*., 2019; DeWalt *et al*., 2023). In this study, the anatomy and histology of the male and female general reproductive system of the tribe Anacroneuriini with focus on *Anacroneuria* and *Kempnyia*, were described for the first time based on specimens from the Neotropical fauna and compared to the findings reported for Perlidae. This exploration offers insights into their evolutionary history and ecological significance, contributing to reproductive biology knowledge. Additionally, it reveals potential characters useful for future taxonomic and phylogenetic investigations in this insect group.

4.2 MATERIAL AND METHODS

4.2.1 Collection and Deposition of Material

The adult specimens analyzed were obtained using light traps equipped with white or black light fluorescent tube lamps positioned in front of white bed sheets, following the method described by Upton and Mantle (1991), at the Serra do Brigadeiro - Araponga, Minas Gerais, Brazil. A total of six adult individuals were collected, consisting of one male of *Anacroneuria flintorum*, one male of *A. itatiaiensis*, two females of *Anacroneuria* sp., one male of *Kempnyia obtusa*, and one female of *Kempnyia* sp. The females were not identified because of the difficulty in identifying them. Male specimens were identified up to the species level according to the descriptions by Zwick (1971), Froehlich (2002), Bispo & Froehlich (2004), Baldin *et al*. (2013), Bispo *et al*. (2014), Novaes & Bispo (2014), Almeida *et al*. (2018), and Almeida & Bispo (2020). The terminology used for the reproductive system structures was adopted from Brinck (1956). The specimens were then photographed using a Leica M205A stereomicroscope with an MC170 HD digital camera. Subsequently, the images were edited, and plates were generated using Adobe Photoshop CC®. These specimens were deposited at the Museu de Entomologia, Universidade Federal de Viçosa.

4.2.2 Anatomy of the reproductive system

The reproductive system of sexually mature individuals was dissected in 0.1 M sodium phosphate buffer, pH 7.2 and fixed for 2-4 hours in a 2.5% glutaraldehyde solution in the same buffer. Subsequently, they were transferred to pure buffer and photographed using a Zeiss Stemi 2000-C stereomicroscope with a Canon EOS Rebel T7I digital camera attached using the NDPL 2 (2X) adapter. Schematic drawings were created using Adobe Illustrator 2020 software, available as a free Trial version. Measurements of the length of reproductive system regions were obtained using Image-J software (http://rsbweb.nih.gov/ij/).

4.2.3 Light Microscopy

The reproductive systems of males and females of *Kempnyia* and *Anacroneuria* were were carefully dissected in 0.1 M sodium phosphate buffer, pH 7.2 (PBS) added with 3% sucrose and then fixed in a 2.5% glutaraldehyde solution for a period of 2 hours. After fixation, the samples were washed in the same buffer for 1 hour, at 10-minute intervals, and subjected to post-fixation in 1% osmium tetroxide for another 2 hours. Subsequently, they were washed again and subjected to a dehydration process in an alcoholic series (30%, 50%, 70%, 90% and 100%). The samples were then embedded in Historesin® plastic resin (Leica Historesin, Heidelberg, Germany) and polymerized at 60ºC over 12 hours.

Semi-thin sections, 0.5 µm thick, were obtained using a Leica RM 2255 automatic microtome, equipped with glass knives. The resulting sections were stained with Giemsa and covered with coverslips using a 50% sucrose solution. The material was thoroughly analyzed and photographed with the aid of an Olympus BX-60 microscope, equipped with a QColor3 digital camera. The images were captured using 40x and 100x objectives and were later assembled using the Photomerge function of the Adobe Photoshop 2022 software.

4.3 RESULTS

4.3.1 Anatomy of the reproductive system

The male reproductive system of *Anacroneuria flintorum* is primarily composed of a pair of testes fused medially which contains five follicles, two for each testis and a median follicle common to both testes. The efferent duct extends from the basal portion of the testicular follicles and connects to the vas deferens. The latter is long and thin, arch-shaped, they project from the testes, connecting to the seminal vesicles. The seminal vesicles, arranged in pairs, are longitudinally elongated and volumous, with the apical region widening and tapering as it approaches the ejaculatory duct. Below the seminal vesicles extend a pair of long accessory glands. The ducts that connect the seminal vesicles and the accessory glands connect to the ejaculatory duct. The ejaculatory duct is a short, broad, muscular structure connected to the penial armature (Figures 1 and 2).

The male reproductive system of *Kempnyia obtusa* is primarily composed of a pair of testes fused medially consisting of 25 follicles. The vasa deferentia are thin and elongated, extending to the seminal vesicles that are thin and short. Adjacent to the seminal vesicles, a pair of long and wide accessory glands can be seen with yellow-orange pigmentation. The

ejaculatory duct extends from the union of the ducts originating from the seminal vesicles and accessory glands (Figures 3 and 4).

Female reproductive systems of *Anacroneuria* (Figures 5 and 6) and *Kempnyia* (Figure 7) have anteriorly united ovaries with multiple elongated ovarioles arranged in a circumferential pattern. Each ovariole is connected to the oviduct by a pedicel. From the ovary extend long and robust paired lateral oviducts, a common oviduct, a spermatheca, and the *bursa copulatrix*. A modified sternite, the subgenital plate, protects the genital opening.

4.3.2 Histology

In *K. obtusa* adults, the different phases of differentiation of the germ cells are not observed in the testicular follicles, meaning that spermatogenesis is not continuous because it does not occur during the adult phase. It has remnants of sperm, unorganized and not in bundles (Figures 8 and 9). Testicular follicles are covered externally by a thick conjunctival capsule (Figure. 9A). The efferent duct is short and has simple cubic epithelium. The ejaculatory duct is formed by a simple cubic epithelium with rounded nuclei of partially decondensed chromatin. Externally, it is covered by voluminous muscle cells (Figure 9C). In the terminal portion of the ejaculatory duct is the aedeagus, a structure that is sclerotized. The epithelium of the accessory glands has a single layer of secretory cubic cells with rounded nuclei with a predominance of euchromatin. It is possible to observe secretory vesicles arranged along the epithelium. The lumen of the glands is vast, with secretion filling it (Figure 9, D-E). The accessory gland is of origin ectodermal, as it opens into the ejaculatory duct.

Spermatogenesis is also not continuous in adults of *A. itatiaiensis*, but it is possible to note sperm in bundles (Figure 10), possibly because it is a younger individual. The nucleus of cystic cells is large and rounded, with decompressed chromatin and an evident nucleolus (Figure 10A). The follicles are also covered by a conjunctival capsule, with flattened cells and nuclei (Figure 10B). The efferent duct, in the basal region of the testicular follicle, has epithelium without a regular shape, with nuclei of different sizes (Figure 10A, B, D).

Oogenesis in *Anacroneuria* sp. is divided into zones based on the growth of oocytes within the ovarioles. Each ovariole consists of a germarium, vitellarium, and a pedicel. In the apical region of the ovarioles, the germarium is predominantly composed of somatic cells and oogonia. Slightly below, in the central portion of the ovarioles, the vitellarium is characterized by oocytes, spherical follicular cells with large central nuclei, enveloped by a follicular epithelium. The pedicel is short and contains interfollicular cells. During oocyte maturation,

there is a successive increase in lipid droplets in the cytoplasmic region. Within the lateral oviducts, oocytes in the 'patency' stage can be found, which are free oocytes covered by a distinct tunic, surrounded by follicular cells, and internally featuring a dense layer of lipids and a small, spherical nucleus located at the periphery (Figure 11).

4.4 DISCUSSION

4.4.1 Male Reproductive System:

According to Zwick (2000), the monophyly of Plecoptera is supported by very few uniquely derived character expressions, one of which is the testes medially fused. In this study, we demonstrated that the morphology of the adult male reproductive system of *A. flintorum* and *K. obtusa* consists of pair of testes fused medially. This arrangement was also found in two families of Perloidea, as described by Brinck (1956) *Diura bicaudata* (Linnaeus, 1758), *Isoperla difformis* (Klapálek, 1909), *I. grammatica* (Poda, 1761) (Perlodidae); *Perla cephalodes* Curtis, 1827 and by Stewart *et. al.,* (1969) in *Perlesta (placida)* (Hagen, 1861) (Perlidae). Although Brinck (1965) describes the testes in *P. cephalodes* as an unpaired and arched structure. Also within this superfamily, it is possible to find unfused paired testes, such as in *Chloroperla apicalis* Newman, 1836 (Chloroperlidae), *Perlodes dispar* (Rambur, 1842), *Arcynopteryx compacta* (McLachlan, 1872) (Perlodidae) reported by Brinck (1956) and *Hydroperla crosbyi* (Needham & Claassen, 1925), another member of Perlodidae, which was reported by Stewart & Stark (1977).

Additionally, the examination of testicular follicles revealed substantial variability in the number of these structures across different genera and species. In general, the number of follicles is large, for example, in *Diura, Perlodes, Hydroperla, Arcynopteryx, Isoperla* and *Perla* this number varies between 30 to 60 follicles, but it can be quite small, as in certain cases of *Isoperla, Chloroperla* and *Perlesta*, which varies between 10 to 18 follicles (Brinck, 1956; Stewart *et. al.,* 1969; Stewart & Stark, 1977). Notably, our study reported five testicular follicles in *Anacroneuria flintorum* and 25 in *Kempnyia obtusa*, contributing to the understanding of the diversity of this trait within Plecoptera.

Our results also showed comparative differences with the studies by Brinck (1956), where he describes the seminal vesicle of *Diura, Perlodes, Arcynopteryx* and *Isoperla* as a common seminal vesicle, being fused anteriorly; and by Zwick, (1973, 1980, 2000), which described that Styloperlidae, Peltoperlidae and Perloidea show a reduction of one pair of

seminal vesicles, and the Leuctridae have lost a pair of seminal vesicles and the mature sperm are stored in enlarged vas deferens. However, Zwick's (1973, 1980, 2000) definitions are unclear and he misdefined these structures, as the enlarged vas deferens he describes are the seminal vesicles. In order to understand the structures described by Zwick (1973, 1980, 2000), it is necessary to conduct a histological analysis of the reproductive systems of these families. In our findings, we describe a pair of seminal vesicles non-fused, seen in *Anacroneuria* and *Kempnyia*, similar to those found in Stewart *et. al*. (1969) and Stewart & Stark (1977).

Regarding the accessory glands, our findings obtained through histological analysis are consistent with the observations made by Brinck (1965) in *Diura bicaudata*. Brinck (1965) documented that the lumen of the structure is significantly wide, filled with secretions, and that the accessory gland derives from the ectodermal tissue, eventually discharging into the ejaculatory duct, where we could also observe it in *Kempnyia obtusa*.

In adults of *A. itatiaiensis* and *K. obtusa*, it is not possible to observe undifferentiated spermatogonia that can produce new sperm. Testicular follicles without continuous spermatogenesis indicate that the males of these species are ready to mate as soon as they emerge. Species that do not feed as adults also mate early. They perform a few copulations and manage to transfer the sperm content to the females (Hynes, 1976; Stewart, 2009).

4.4.2 Female Reproductive System:

The female reproductive system of *Anacroneuria* and *Kempnyia*, is composed of a large number of ovarioles forming anteriorly united ovaries, similar arrangement also found in *Perlodes dispar*, *Arcynopteryx compacta*, I*soperla difformis*, I*soperla grammatica, Chloroperla apicalis* (Brinck 1956), and very similiar to *Perlesta placida* (Stewart et al. 1969) and *Hydroperla crosbyi* (Stewart & Stark, 1977); and differs considerably from *Diura bicaudata*, which exhibits unjoined ovaries (Brinck, 1956).

In *Anacroneuria* sp., as observed in *Perla* sp. and *Perla marginata* (Rościszewska, 1989, 1997), the ovarioles exhibit a division based on the growth and maturation of oocytes. This sequential cell differentiation and maturation facilitate the development of nutritive reserve substances throughout the vitellogenic process, ensuring proper growth for the embryos (Rościszewska, 1989). According to Rościszewska (2003), the propensity to produce numerous ovarioles during the reproductive period is reported in Arctoperlaria and Antarctoperlaria. However, the presence of secretory cells within the ovarioles has only been identified in Arctoperlaria, including the species under investigation. The presence of these secretory cells

aids in the passage of eggs through the lateral oviduct, especially in species lacking accessory glands (Rościszewska, 2001, 2003).

This mass of eggs found in the lateral oviducts has also been reported in *Brachyptera risi* Morton, 1896 (Arctoperlaria) and *Thaumatoperla flaveola* Burns & Neboiss, 1957 (Antarctoperlaria). These egg masses can be ordered or disordered and have also been found in other Anacroneuria species: *A. talamanca* Stark 1988 and *A. starki* Fenoglio & Morisi, 2002, located near the genital opening (Zwick 1973; Hinton, 1981; Rościszewska 2001; Fenoglio & Rosciszewska, 2003). This configuration is intrinsically related to copulation in Plecoptera because species that lay eggs ready for fertilization possess these egg masses, unlike species that require primary copulation for ovariole maturation and subsequent egg mass development (Yoshimura, 2001).

In conclusion, the anatomical characteristics of the testes, testicular follicles, and seminal vesicles in Plecoptera offer valuable characteristics for phylogenetic analysis. Their presence, fusion, or variation in number can serve as distinctive features to distinguish and understand the evolutionary relationships between different groups of stoneflies. Our study has contributed to advancing the understanding of Plecoptera's reproductive systems by providing new insights through the description of these structures. Reproductive system characters, encompassing both structural characteristics and behaviors related to reproduction, offer valuable insights for species differentiation and phylogenetic analyses. This work enhances our comprehension of the evolution and diversity within this group of aquatic insects.

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4.6. LIST OF ILLUSTRATIONS

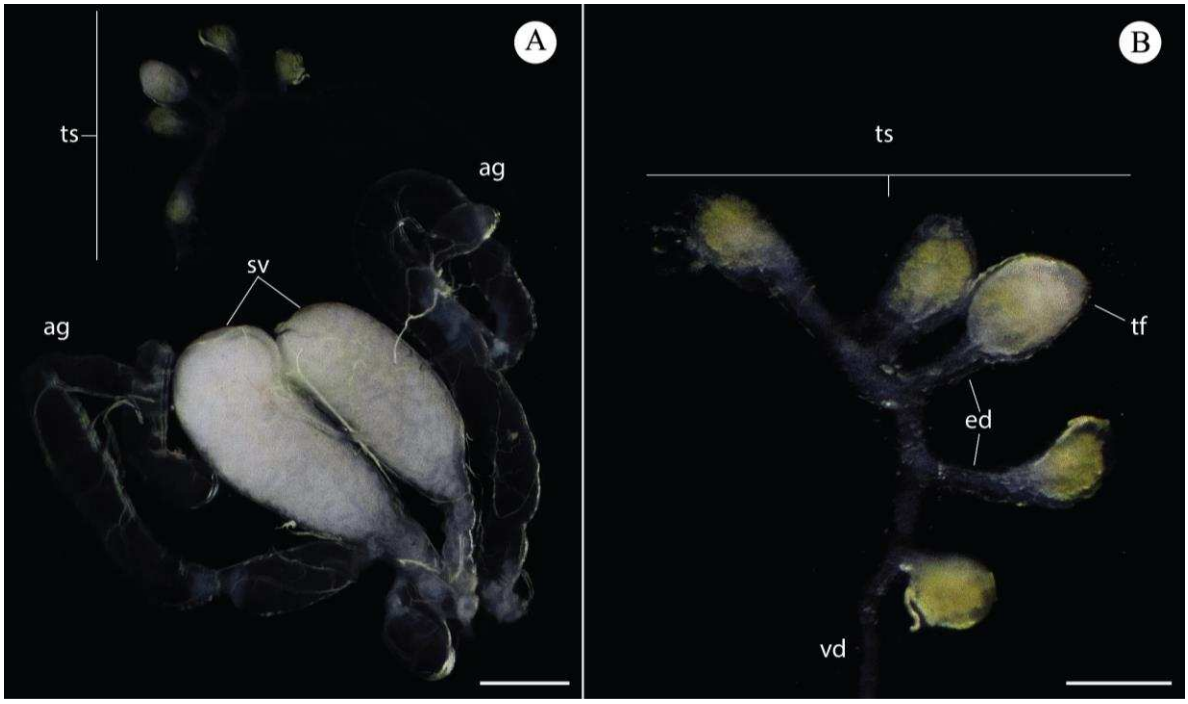


FIGURE 1. The male reproductive system of *Anacroneuria flintorum*. A - The male reproductive system of *Anacroneuria flintorum* consists of pair of testes fused medially (ts) with five testicular follicles (tf), a pair of wide seminal vesicles (sv), and accessory glands (ag) located on the sides. B - Detailed view of the testicular region highlighting the efferent duct (ed) connecting each testicular follicle (tf) to the vas deferens (vd). Scale bars: A - 0.5mm; B - 0.3mm.

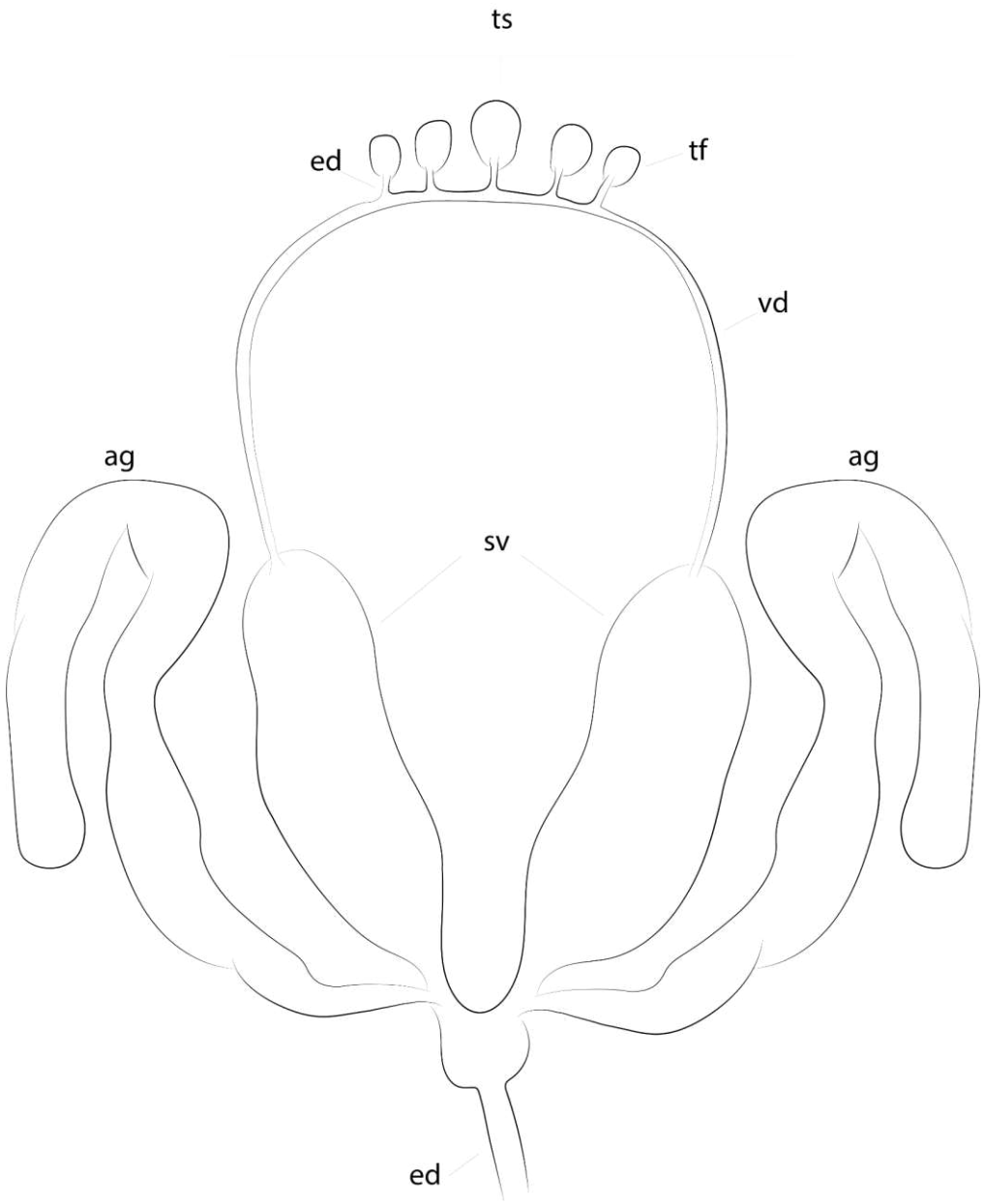


FIGURE 2. Illustration of the male reproductive system of *Anacroneuria flintorum* showing the pair of testes fused medially (ts), the testicular follicles (tf) connected to the vas deferens (vd) by the efferent ducts (ed). The arc-shaped vas deferens extend to the seminal vesicles (sv). The accessory gland (ag) elongates laterally, emptying its contents into the ejaculatory duct (ed) and the sperm content stored in the seminal vesicles.

FIGURE 3. The male reproductive system of *Kempnyia obtusa* is formed by a pair of testes fused medially (t) with 25 testicular follicles (tf). The vas deferens (vd) are thin and elongated, extending to the seminal vesicles (sv), which are long and large. The accessory gland (ag) is also long in this species, where it empties its contents into the ejaculatory duct (ed). Barras: 0,5mm.

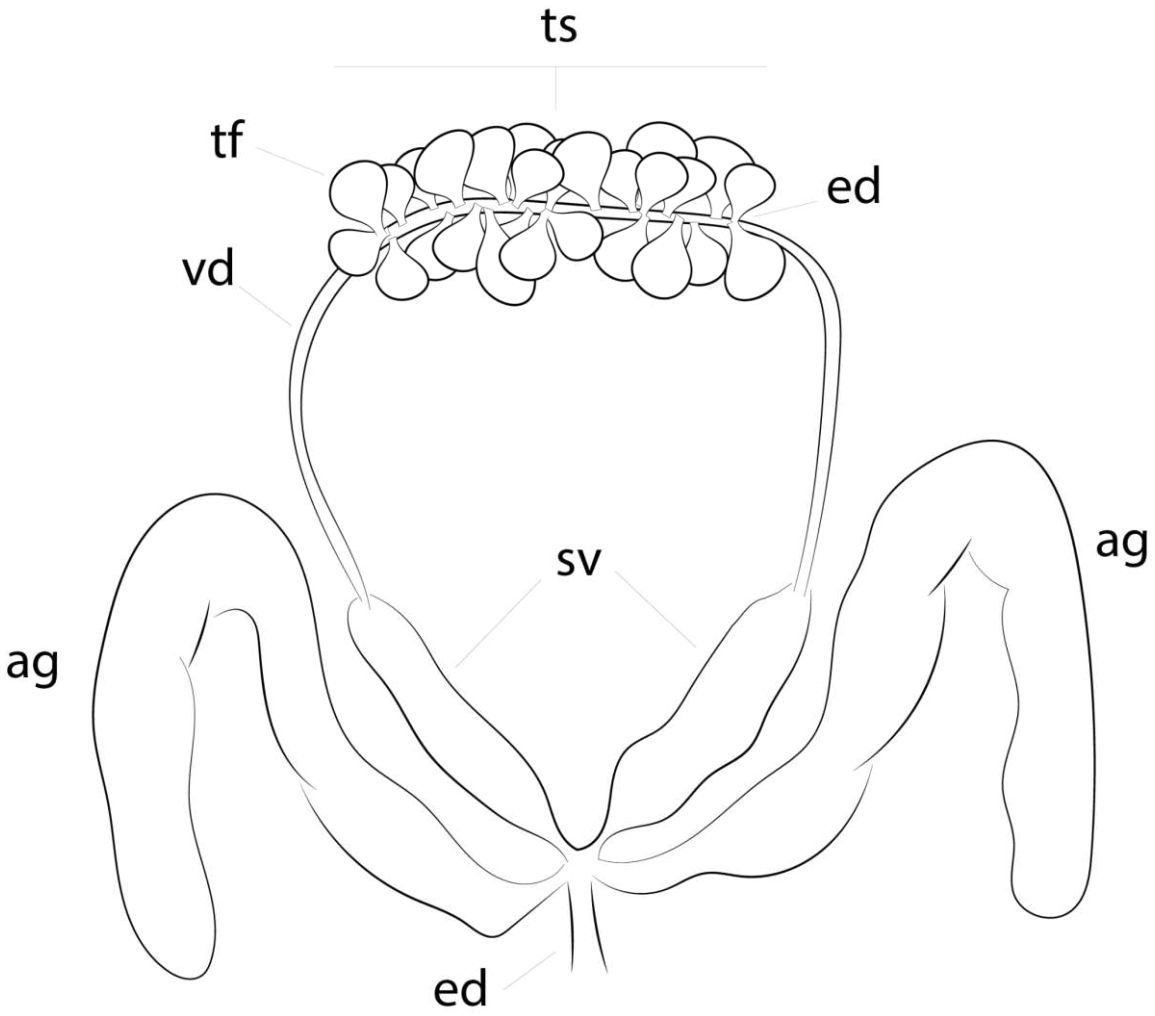


FIGURE 4. Illustration of the male reproductive system of *Kempnyia obtusa* showing a pair of testes fused medially (ts) with numerous testicular follicles (tf), connected to the vas deferens (vd) by the efferent ducts (ed). The arc-shaped vas deferens extend to the seminal vesicles (sv). The ejaculatory duct (ed) originates from the meeting of the ducts of the seminal vesicles (sv) and the accessory gland (ag).

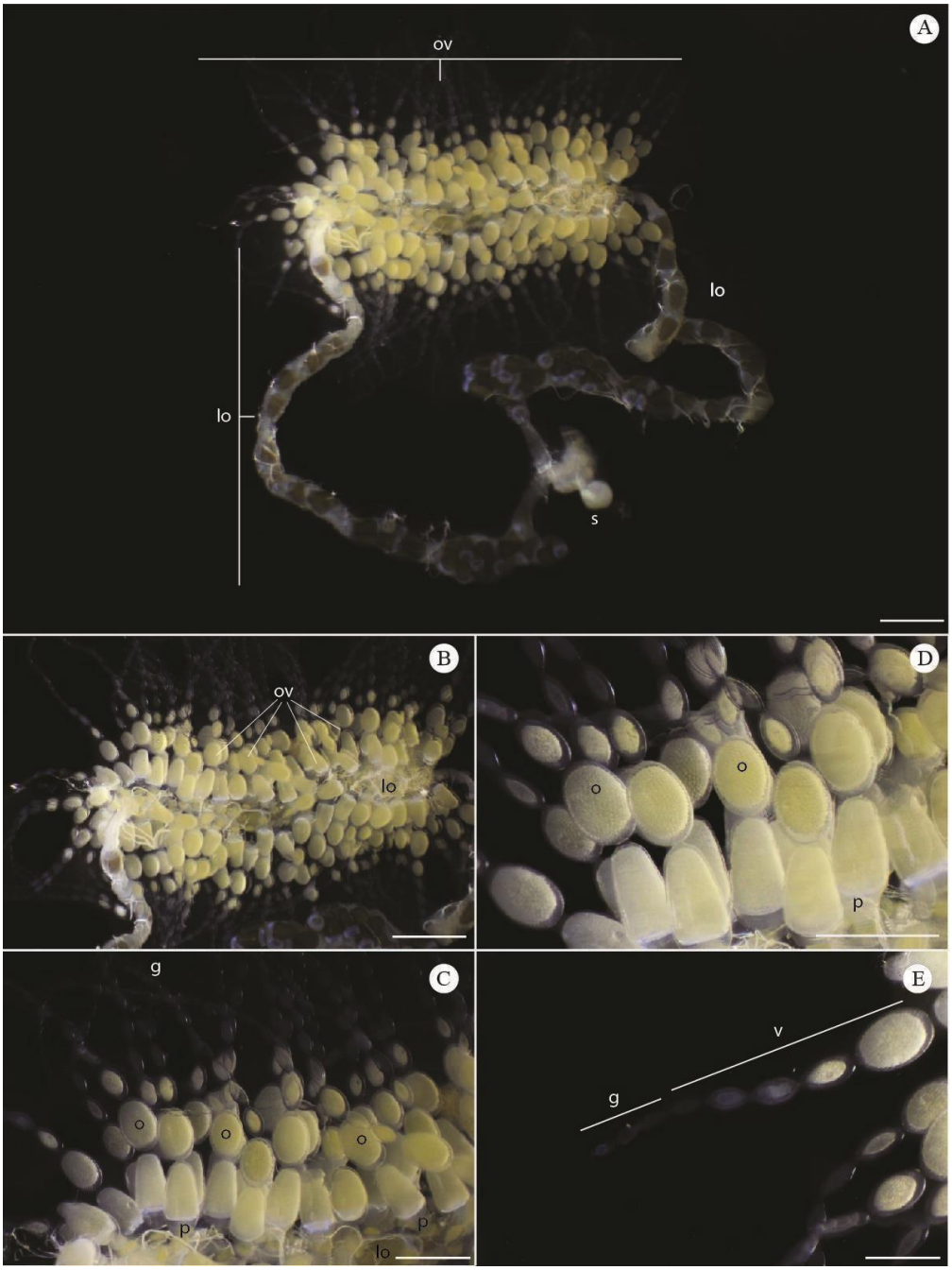
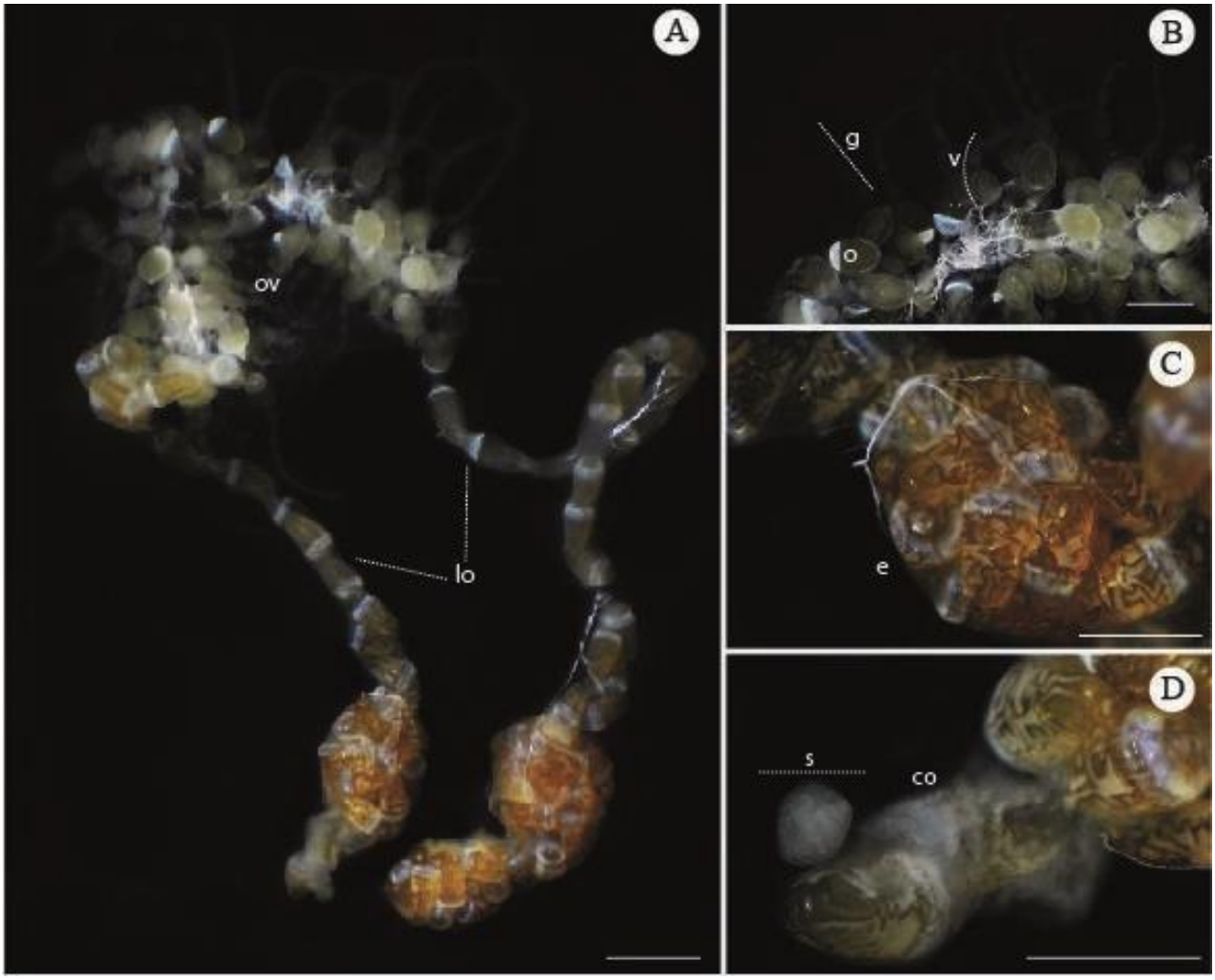


FIGURE 5. The female reproductive system of *Anacroneuria sp*. A - The female reproductive system of *Anacroneuria* sp. is composed of multiple ovarioles (ov), and lateral oviducts (lo) that hold the eggs until oviposition and converge into a common oviduct. Notice the oval-shaped spermatheca (s) located beside the common oviduct. B - Detailed view of the ovarioles (ov). C - Details of the oocytes (o) and pedicels (p). D - Note the connection of the pedicels (p) to the lateral oviducts (lo). E - Detailed view of the germarium (g) and vitellarium (v) region. Scale bars: A and B - 1.0mm; C, D, and E - 0.5mm.

FIGURE 6. The female reproductive system of *Anacroneuria* sp. A - Ovarioles (ov) at the top, and lateral oviducts (lo) on the sides, joining at the base to form the common oviduct (co). Note the presence of the spermatheca (s) and bursa copulatrix (bc) in the region of the common oviduct. B - Detail of the oocytes (o), germarium (g), and vitellarium (v) in the ovariole region. C - Detail of the distal portion of the female reproductive system highlighting the spermatheca (s). D - Notice that the bursa copulatrix (bc) has a more translucent appearance. E - Detail of the eggs (e) inside the lateral oviducts. Scale bars: A - 1.0mm; B, C, D, E - 0.5mm.

FIGURE 7. The female reproductive system of *Kempnyia* sp. A - Organization of the ovarioles



(ov), with the lateral oviducts (lo) located just below. B - Ovariole region, highlighting the germarium (g), and below each germarium, the vitellarium (v) is composed of oocytes in the regions close to the oviduct. C - Detail of the egg mass (e). D - Detail of the common oviduct (co) and laterally the spermatheca (s). Scale bars: A - 1.0mm; B, C, D - 0.5mm.

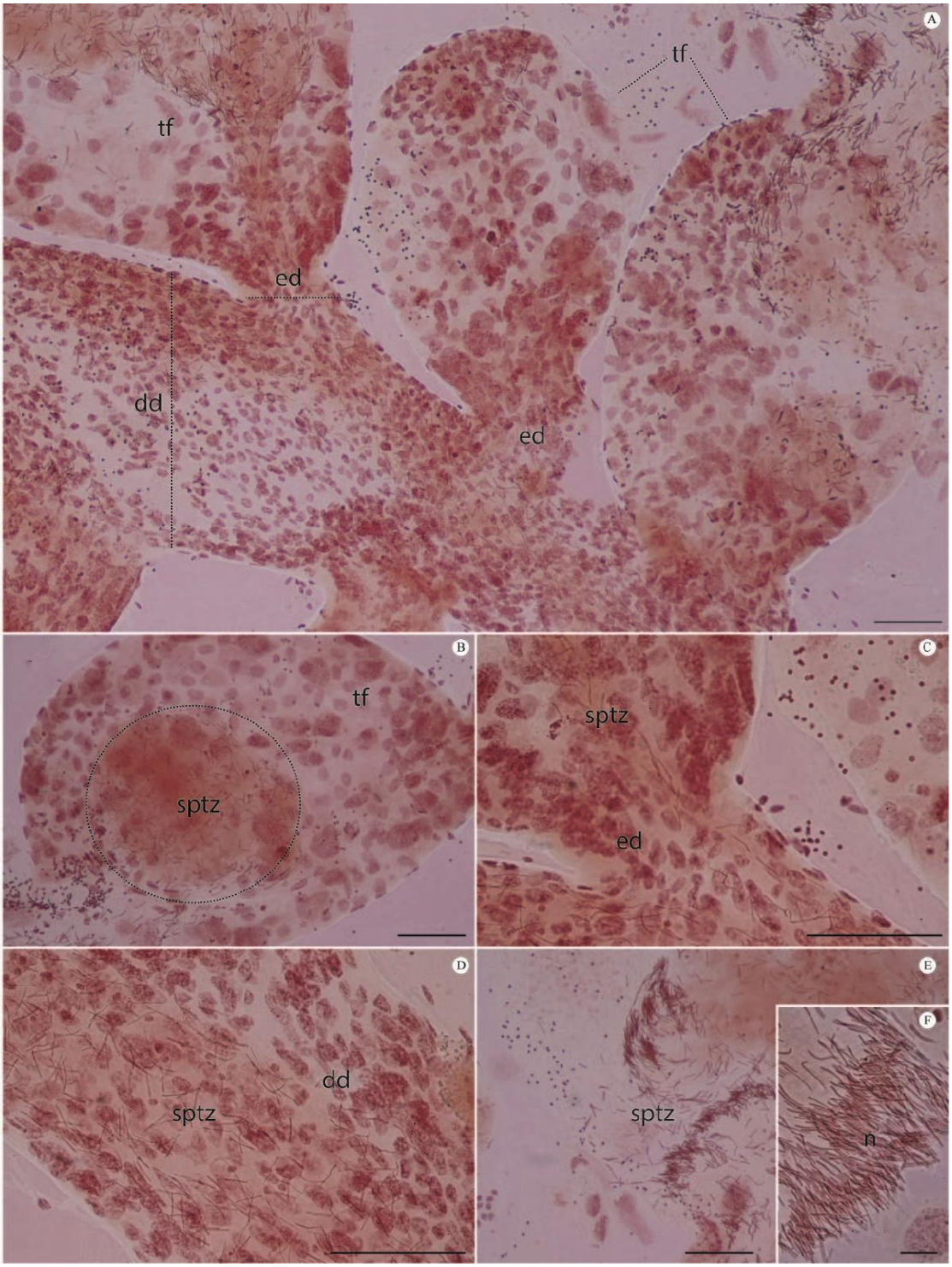


FIGURE 8. Testes of *K. obtusa* stained with orcein acetic acid. A - General structure of the testes of *K. obtusa*. Note the testicular follicles (tf) on the sides. B - Detail of a mass of mature spermatozoa (sptz) inside the testicular follicle (tf). C - Passage of free sperm (sptz) through the efferent duct (ed). D - Arrangement of sperm passing through the vasa deferens (vd). E and F - Spermatozoa (sptz) grouping in the vas deferens (vd). Scale bars: A, B, C, D - 50 µm; E - 10 µm.

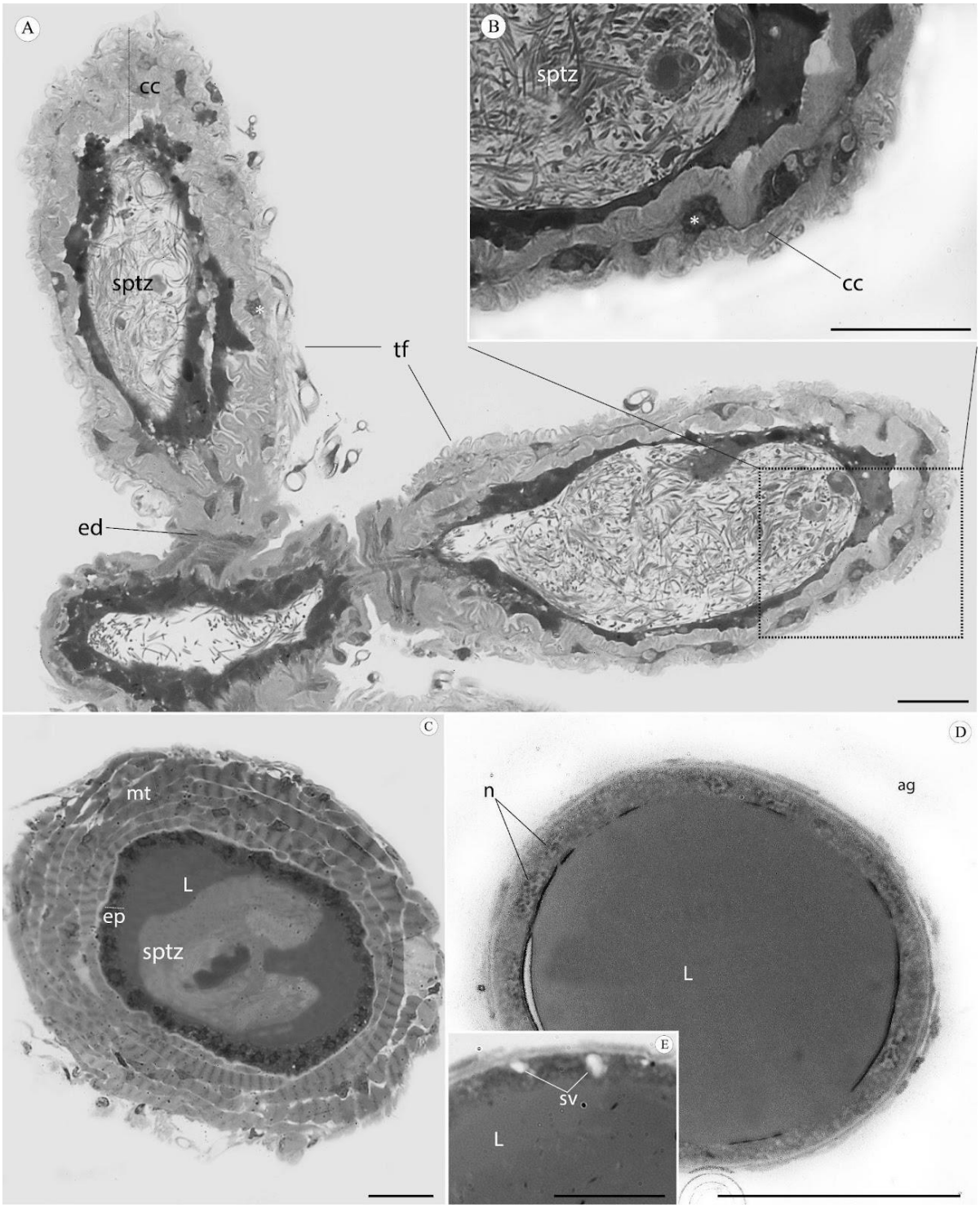


FIGURE 9. Histology of the male reproductive system of *Kempnyia obtusa*. A – Longitudinal section of testicular follicles (tf) containing internal spermatozoa (sptz) and covered by conjunctival capsule (cc). Note the small efferent ducts (ed). B - Detail of a testicular follicle highlighting the arrangement of nuclei (n) in the epithelial cells of the external testicular capsule (tc). C - Transverse section of the ejaculatory duct. Observe the dense layer of muscular tissue (mt), a thin layer of cuboidal epithelial cells (ep), and an extensive lumen (L) just below. D -

Transverse section of an accessory gland. Note the centrally positioned nuclei (n) in each cell, and the lumen (L) containing homogeneous content. E - Inset of the longitudinal section of an accessory gland (ag), where it is possible to observe secretory vesicles (sv) in its epithelium and the uniform content within the lumen (L). Scale bars - 20µm.

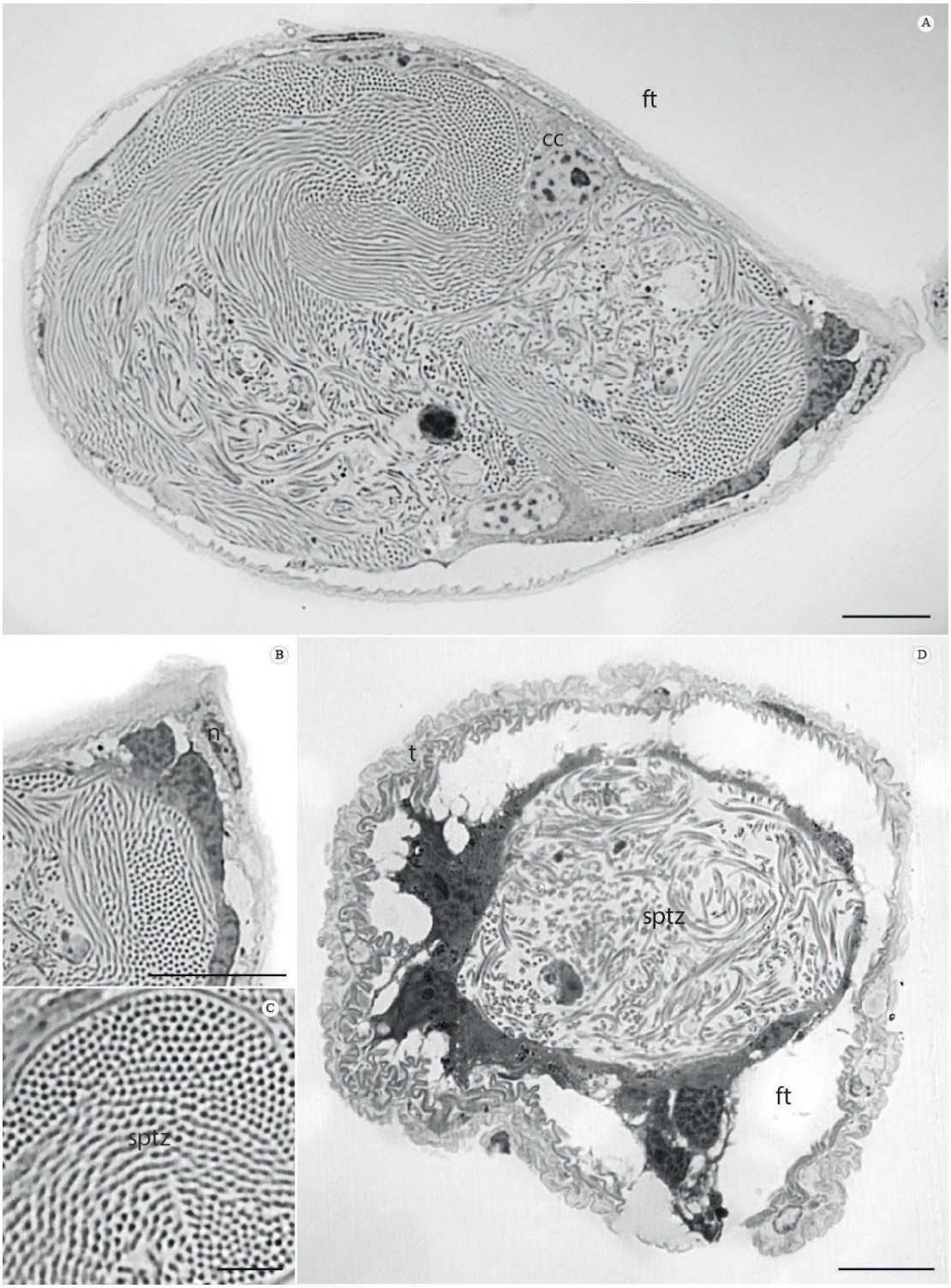


FIGURE 10. Histology of the male reproductive system of *Anacroneuria itatiaiensis*. A - Testicular follicle (tf) containing mature spermatozoa and cystic cells (cc). B - Detail of the simple squamous epithelial tissue of the conjunctival capsule covering the follicle. Nucleus (n). C - Spermatozoa (sptz). D - Detail of another testicular follicle, but with a dense layer of peritoneal tunic (t). Scale bars: A, B, and D - 20µm; C - 5µm.

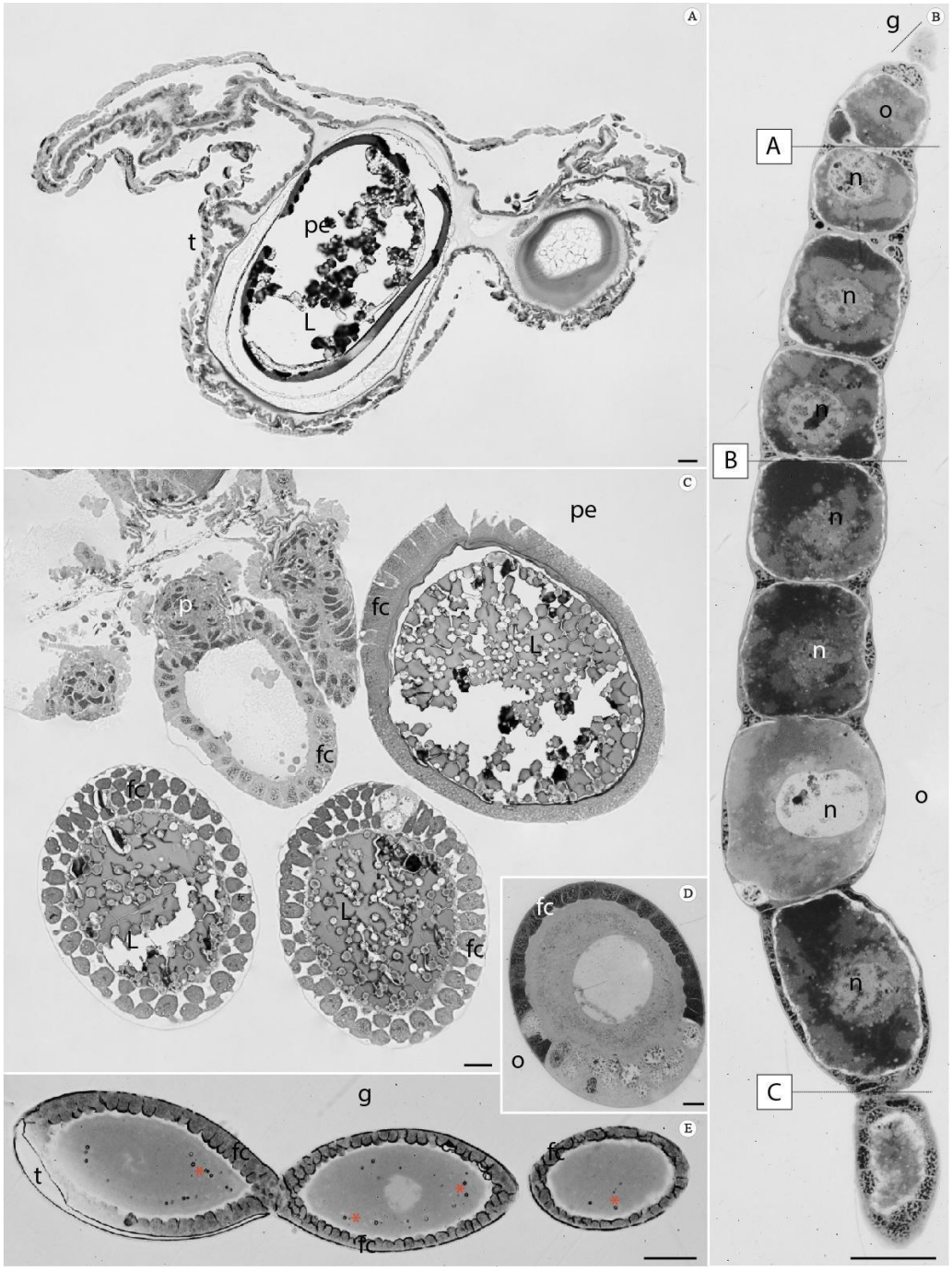


FIGURE 11. Histology of the ovariole of *Anacroneuria sp*. A - Detail of an oocyte at the permeability stage (pe). B - Consecutive developmental stages of the oocyte (o) in the

vitellarium (A-D). Note the germarium (g) at the apical region and the oocytes at various developmental stages below, highlighting the centrally located nucleus (n). C - Oocytes at the permeability stage. Internally, lipid droplets (L) are observed, surrounded by follicular cells (fc), and a small pedicel (p). D - Detail of an oocyte in the developmental stage. E - Detail of a germarium from an ovariole, with each oocyte containing lipid droplets (asterisk - *). Scale bars - 20µm.

CHAPTER 3 - SPERM MORPHOLOGY OF PERLIDAE (PLECOPTERA, INSECTA): A COMPARATIVE DESCRIPTION

5. CHAPTER 3 - SPERM MORPHOLOGY OF PERLIDAE (PLECOPTERA, INSECTA): A COMPARATIVE DESCRIPTION

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Abstract

In this study, we investigate the sperm morphology of Plecoptera genera *Anacroneuria* and *Kempnyia*. Insects exhibit remarkable variations in sperm morphology, which can provide insights into their reproductive strategies and evolutionary dynamics. Despite species identification challenges within Plecoptera, this research presents detailed observations of spermatozoa from various species. The study covers sperm polymorphism, size variations, and nucleus characteristics among male and female specimens. Our findings highlight significant differences in sperm morphology, providing valuable insights into potential genetic diversity and adaptive variations within these species. These variations affect reproductive success, mating behavior, and species relationships. Our study underscores the potential of sperm morphology analysis as a cost-effective and rapid method for understanding the reproductive biology, taxonomy, and evolutionary history of Plecoptera.

Keywords: species identification, spermatozoa, reproductive strategies, evolutionary dynamics.

Highlights:

- This study delves into the sperm morphology of Plecoptera genera *Anacroneuria* and *Kempnyia*, contributing to our understanding of their reproductive biology and potential taxonomic applications.
- The sperm morphometry reveals significant variations among species, suggesting genetic diversity and adaptive differences.
- Variability in sperm morphology within Plecoptera species reflects adaptations to ecological contexts.
- Complex sperm polymorphism in Plecoptera highlights its role in enhancing reproductive success in competitive environments.
- Sperm morphology analysis proves valuable for species identification, understanding mating behavior, and deciphering species relationships.
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5.1. INTRODUCTION

Insect sperm cells exhibit significant morphological diversity (Phillips, 1970; Jamieson, 1987; Simmons, 2002; Swallow & Wilkinson, 2002; Sasakawa, 2007). This diversity extends to various groups, with variations in size and morphology even among closely related species (Pitnick *et al*., 1999; Immler & Birkhead, 2007; Sasakawa, 2009). Insects developed a wide variety of reproductive strategies, and the structure of their sperm cells can offer valuable information on their biology, ecological relationships, and evolutive patterns (Jamieson *et al*., 1999; García-González *et al*., 2005; Birkhead *et al*., 2008; Fisher *et al*., 2023). The structural and ultrastructural characters of these cells have been extensively used in taxonomic and phylogenetic studies of different animal groups, especially insects (Dallai & Afzelius, 1991, 1995; Jamieson *et al*., 1999; Lino-Neto & Dolder, 2001; Alves *et al*., 2006; Mancini *et al*., 2009; Sasakawa, 2009; Dallai, 2014; Dallai *et al*., 2016).

Like some other insect orders, our understanding of Plecoptera's sperm structure remains limited. Baccetti *et al*. (1970) used only one Plecoptera species, *Nemoura cinerea* (Retzius, 1783) (Nemouridae), to investigate the ultrastructure of the spermatozoon. Fausto *et al*. (2001) conducted a study on comparative spermatology analyzing the sperm ultrastructure of four Plecoptera species, *Leuctra fusca* (Linnaeus, 1758) (Leuctridae Klapálek, 1905); *Brachyptera risi* (Morton, 1896); *Taeniopteryx stankovitchi* Ikonomov, 1978 and *T. kuehtreiberi* Aubert, 1950 (Taeniopterygidae Klapálek, 1905), indicating that the spermatozoa of Plecoptera species have a complex structure and discussing the phylogenetic affinity between Plecoptera and other Polyneoptera orders. Soon after, Fausto *et al*. (2002) increased the study with four additional species, *Dinocras cephalotes* (Curtis, 1827); *Perla grandis* (Rambur, 1841) (Perlidae); *Siphonoperla torrentium* (Pictet, 1841) (Chloroperlidae) and *Isoperla grammatica* (Poda, 1761) (Perlodidae) to investigate the systematics and phylogenetic relationships within the order and in comparison, with other insect orders. The results demonstrated that in Systellognatha, sperm are heterogeneous, and this infraorder is monophyletic with shared sperm features.

Of the 3,900 extant species of Plecoptera, 1,000 belong to Perlidae (Arctoperlaria: Systellognatha), which is the most diverse family in the World (Fochetti & Tierno De Figueroa, 2008; DeWalt *et al*., 2023). Perlidae comprises two subfamilies, Perlinae and Acroneuriinae (Stark *et al*., 2009). The latter is divided into three tribes, of which Anacroneuriini is restricted to the Neotropics and the southern portion of the Nearctic (Murányi & Weihai Li, 2016).

Anacroneuriini comprises four genera, *Anacroneuria* Klapálek, 1909, with about 380 species; *Enderleina* Jewett, 1960, with ten species; *Kempnyia* Klapálek, 1914, with 36 species and *Macrogynoplax* Enderlein, 1909, with 16 species (Pessacq *et al*., 2019; DeWalt *et al*., 2023). Recent studies have increased our knowledge about the diversity of this tribe (Stark, 2001; Fochetti & Tierno de Figueroa, 2008; Froehlich, 2010; Pessacq *et al*., 2019), but information on species-level identification, the association of males and females, and phylogenetic relationships among genera is lacking (Gamboa & Monaghan, 2014; Avelino-Capistrano *et al*., 2018; Almeida & Bispo, 2020).

In general, identification at the species level in Plecoptera is difficult, as the morphology of stoneflies can be quite variable and difficult to discriminate from closely related species (Avelino-Capistrano *et al*., 2011; Almeida & Bispo, 2020). Recently, in addition to morphological characters, genetic analysis using DNA sequencing techniques is also being applied to identify varied species within the order (Avelino-Capistrano *et al*., 2016; Elwess *et al*., 2018; Almeida & Bispo, 2020; Chen *et al*., 2020).

Despite the extensive research (Nelson, 1984; Zwick, 2000; South *et al*., 2021), there are still unresolved aspects concerning the phylogenetic relationships of Plecoptera, as highlighted by Nelson (1984). One such aspect is the need for additional characters to elucidate these relationships better. In this regard, comparative spermatology can significantly contribute to phylogenetic and taxonomic issues that traditional taxonomy could not solve (Burrini *et al*., 1988).

This information might be able to help in the inference of phylogenetic relationships between the families and the relationships among Plecoptera and other insect orders; can be used to identify and classify new species, and to resolve taxonomic problems; also can provide important information about mating behavior, such as the presence of sperm competition.

Given the importance of the study of sperm morphology through comparative spermatology with the potential to contribute significantly to our understanding of the evolutionary history, taxonomy, and reproductive biology and the absence of studies on Neotropical components of Plecoptera, the objectives are to describe the sperm morphology of two genera of Anacroneuriini. We compare in this study the spermatozoa of varied species within the genera *Anacroneuria* and *Kempnyia*.

5.2. MATERIAL AND METHODS

5.2.1. Collection and deposition

We sampled 17 specimens of *Anacroneuria* (eight males, nine females) and nine of *Kempnyia* (seven males, two females) using light traps equipped with white or black light fluorescent tube lamps, following the protocol described by Upton & Mantle (1991). The collection localities included the following sites in Southeastern Brazil: Serra do Brigadeiro - Araponga; Cachoeira Grande - Canaã; Parque Nacional da Serra do Cipó - Jaboticatubas, Minas Gerais (MG), and Parque Nacional da Serra dos Órgãos - Teresópolis, Rio de Janeiro (RJ). Detailed information regarding the species, locality, date of collection, and assigned codes to the specimens are shown in Table 1. We deposited all specimens in the Museu de Entomologia, Universidade Federal de Viçosa (UFV), Viçosa (MG) - Brazil.

5.2.2. Identification

For identification purposes, we subjected the terminalia of male adults to a clearing process, adapted from Blahnik & Holzenthal (2004), involving a 10% KOH solution and exposure to incandescent light for 10 minutes. Subsequently, they were rinsed for 1 minute sequentially in solutions of distilled water, 80% ethanol, 90% ethanol, and an acidified ethanol solution (absolute ethanol mixed with acetic acid in a ratio of 1:9). We identified the male specimens at the species level using the descriptions by Zwick (1971), Froehlich (2002), Bispo & Froehlich (2004, 2008), Baldin *et al*. (2013), Bispo *et al*. (2014), Novaes & Bispo (2014), Almeida *et al*. (2018), and Almeida & Bispo (2020). We did not identify the females due to the difficulty of identifying them. We photographed the specimens using a Leica M205A stereomicroscope with an MC170 HD digital camera.

5.2.3. Sperm morphometry

We dissected the individuals, and the spermatozoa from the seminal vesicles of males and spermatheca of females and spread them on histological slides. We stained the histological slides for 20 min with 0.2 mg/ml DAPI (4.6-diamino-2-phenylindole), washed in distilled water, and mounted with 50% sucrose. We photographed the slides stained with DAPI under epifluorescent illumination in this same microscope equipped with a BP360-370 nm excitation filter. We stained part of these preparations with Giemsa, washed them in running water, and dried them at room temperature. We made the observations and light photographs using an Olympus BX-60 microscope with an Olympus Q-Color3 digital camera attached. To obtain the total length of spermatozoa and their respective flagella and nuclei, we measured ten

spermatozoa of each individual. We made all measurements using the free software ImageJ (https://imagej.nih.gov/ij/).

5.2.4. Statistical analysis

We built linear models with Gaussian error distribution to compare flagellum and nucleus size (response variables) across individuals (explanatory variable) for each genus – *Anacroneuria* and *Kempnyia* (i.e., four models in total). In each analysis, we did pairwise comparisons using the package emmeans v1.8.6 (Lenth, 2021), with a Tukey adjustment for multiple comparisons. We analyzed the residuals to check for distribution suitability and fit in all models using the DHARMa package v0.4.6 (Hartig, 2022). We performed all statistical analyses in the software R (R Core Team, 2022).

5.3. RESULTS

In both genera, the spermatozoa were long, filiform, and composed of a distinctive head (with acrosome and nucleus) and a flagellum. The spermatozoa were organized into bundles in the seminal vesicles.

The spermatozoa from males and females of *Anacroneuria* exhibited reduced or absent acrosomes. These cells from four males of *A. debilis* [AdM1 (Figure 1), AdM2 (Figure 2), AdM3 (Figure 3), and AdM4 Figure 4)] had total lengths averages of 139.9 µm, 109.212 µm, 115.983 µm, and 122.454 µm, respectively. The sperm nuclei of AdM1 had an average length of 10.053 µm, while of AdM2 was 11.006 µm, AdM3 of 11.802 µm, and AdM4 of 13.118 µm.

The two male specimens of *A. flintorum* collected were coded AfM1 (Figure 5) and AfM2 (Figure 6). The male AfM1 exhibited individualized sperm with an average length of 150.350 µm, the sperm nuclei measured an average of 11.969 µm. In comparison, the male AfM2 showed sperm bundles measuring an average of 199.204 µm in length, and sperm with an average of 141.103 µm, and their nuclei 10.904 µm. In the only male specimen of *Anacroneuria itatiaiensis* collected [AiM1 (Figure 7)], the spermatozoa showed a length of 135.513 µm and the nuclei of 13.3128 µm. The single sperm bundle found measured 185 µm in length.

In an unidentified male specimen of *Anacroneuria*, possibly a new species [AMsp (Figure 8)], based on the lengths of the sperm, it was possible to classify these cells into three groups (a, b, and c Figure 8-G), therefore exhibiting sperm polymorphism. The average lengths were 268.255 µm (the largest observed in the genus), 150.401 µm, and 104.017 µm,

respectively. The nuclei could also be classified into three groups, although the differences in the mean lengths of this region between the groups were more discrete: 13.478 µm, 12.539 µm, and 11.859 µm. In the seminal vesicles, we observed spermatozoa individually rather than in bundles.

Due to the difficulty of identifying them, the nine female collected specimens of Anacroneuria remained unidentified. We assigned each specimen with a unique code for differentiation: AF1, AF2, AF3, AF4, AF5, AF6, AF7, AF8, and AF9 (Figures. 9-17). The female designated as AF1 exhibited bundles with an average length of 177.152 µm, a spermatozoa mean total length of 124.157 µm, and a nucleus of 12.403 µm. In AF2, AF3, and AF4 females, the sperm were individualized rather than in bundles. The average lengths of these cells were 146.765 µm, 121.928 µm, and 149.130 µm, respectively, and the nuclei had average lengths of 12.386 µm, 12.766 µm, and 12.226 µm, respectively (Table 2). In the AF5 female, sperm were in bundles with an average length of 168.416 µm. The average sperm length was 124.560 µm, and the nuclei 11.565 µm. In the AF6 female sperm were in individualized rather than in bundles. The average length of these cells was of 151.579 µm and of the nuclei 12.073 µm. In the AF7 female, the sperm were in bundles with a mean length of 174.445 µm, and the average sperm length was 145.199 µm with nuclei measuring an average of 11.459 µm. In both AF8 and AF9 females, the sperm were individualized, with an average length of 132.974 µm and 144.671 µm, respectively. The average lengths of their nuclei were 10.784 µm and 11.355 µm, respectively.

The acrosome was evident in the spermatozoa from both males and females of *Kempnyia*, but the nuclei were commonly detached from the flagella. Three males of *K. neotropica* collected were coded KnM1 (Figure 18), KnM2 (Figure 19), and KnM3 (Figure 20). None of them showed spermatozoa into bundles. In these individuals, their sperm had average lengths of 907.150 µm, 862.117µm, and 897.570 µm, respectively. In comparison, the nuclei had an average of 21.540 µm, 20.512 µm, and 20.620 µm long, respectively while the nuclei had an average of 21.540 µm, 20.512 µm, and 20.620 µm long, respectively.

Four specimens of *K. obtusa* collected were designated as KoM1, KoM2, KoM3, and KoM4 (Figures 21-24). The mean sperm lengths of these individuals were 567.826 µm, 551.092 µm, 586.329 µm, and 591.085 µm, respectively, and the average nuclei lengths were 21.554 µm, 21.631 µm, 22.152 µm, and 19.716 µm, respectively.

The two female specimens of unknown Kempnyia species collected we coded as KF1 (Figure 25) and KF2 (Figure 26). The average sperm length of specimen KF1 was 825.068 µm with a nuclear length of 19.957 µm, while for specimen KF2, the sperm had an average length of 578.614m with a nuclear length of 18.107 µm.

The sperm morphometry was unique for each species, as most could be distinguished by flagellum length (Graphs 1 and 2). The pairwise analysis comparing the groups using the emmeans package is available in Tables 2 and 3 (supplementary material).

We observed the shorter sperm in *A. debilis* (AdM2) (around 109 µm; Figure 4G), while in *K. neotropica* (KnM1) they were the longest (around 907 µm, Figure 22F). Regarding the unidentified male of *Anacroneuria* (Amsp), it exhibited sperm polymorphism, with three distinct lengths, including one that represented the largest found within this genus in the conducted analyses (Figure 19G).

5.4. DISCUSSION

The variations in total sperm length and respective nuclei between the four specimens of *A. debilis* showed significant differences. These differences can also be observed in the external morphological characteristics of the individuals, such as the size and coloration of the individual and the genital shape. These differences can be attributed to various factors, including genetic diversity, environmental conditions and potential sexual dimorphism, as found in other studies (Dallai & Afzelius, 1980; Yoshimura & Oishi, 2003; Dallai *et al*., 2016; Rezende *et al*., 2021). These differences may also be an indication that *A. debilis* may be a cryptic species. However, a larger sample of the different populations is needed to confirm this hypothesis.

The long flagellum observed in the unidentified *Anacroneuria* along with its distinctive polymorphism displaying three different sperm sizes, sets it apart from the other specimens. Comparing it with other specimens may help to determine if it represents a new species. A larger sample size would be necessary to accurately describe the sperm morphology of the unidentified male and to further investigate the implications of this polymorphism.

We were unable to establish clear associations between males and females of *Anacroneuria*, a phenomenon partly attributed to the relatively slow evolution of spermatozoa compared to other morphological or genetic characteristics within some organism groups. Sperm cells often maintain highly conserved basic structures and fertilization processes

throughout evolutionary changes (Jamieson *et al*., 2000). As a result, closely related species may possess spermatozoa with significant morphological similarities, rendering it challenging to distinguish them solely based on sperm morphology. This may have contributed to the complexity of associating males with their corresponding females within *Anacroneuria*.

The comparison of spermatozoa measurements in two Kempnyia species revealed exciting differences. Precisely because the spermatozoa observed in KF1 and KF2 females also displayed significant differences, with KF1 exhibiting longer sperm than KF2. The three male specimens of *K. neotropica* exhibited similar total sperm lengths. Likewise, the specimens of *K. obtusa* showed comparable total sperm lengths; however, they were significantly smaller than the sperm lengths observed in *K. neotropica*. Thus, it was possible to infer that the KF1 is closely related to *K. neotropica*, whereas the KF2 female is associated with *K. obtusa*. This inference, pointed out by the difference in sperm lengths, is also supported by the external morphology of adult individuals. These findings provide valuable insights into male-female association based on sperm characteristics and can aid in discriminating between species and understanding relationships among closely related species as found in other studies (Pereira *et al.,* 2008; Barcellos *et al*., 2015; Cursino & Duarte, 2016).

The use of sperm morphology for species differentiation and male-female association varies in its effectiveness across different groups. This discrepancy occurs because spermatozoa exhibit a high degree of structural conservation in some organisms, making it challenging to distinguish between closely related species based solely on sperm characteristics. Nevertheless, in certain cases, variations in sperm morphology can provide valuable insights, allowing for species discrimination. To enhance the utility of sperm morphology in phylogenetic analyses, conducting ultrastructural analyses becomes crucial, especially when dealing with closely related species showing minimal differences in their sperm characteristics. Ultrastructural investigations can reveal subtler differentiating features at the cellular level, allowing for a more comprehensive understanding of the evolutionary relationships among these species. This approach further refines the accuracy and reliability of using sperm morphology as a valuable tool in phylogenetic studies, making it particularly useful in cases where external traits or genetic data may not provide the needed resolution.

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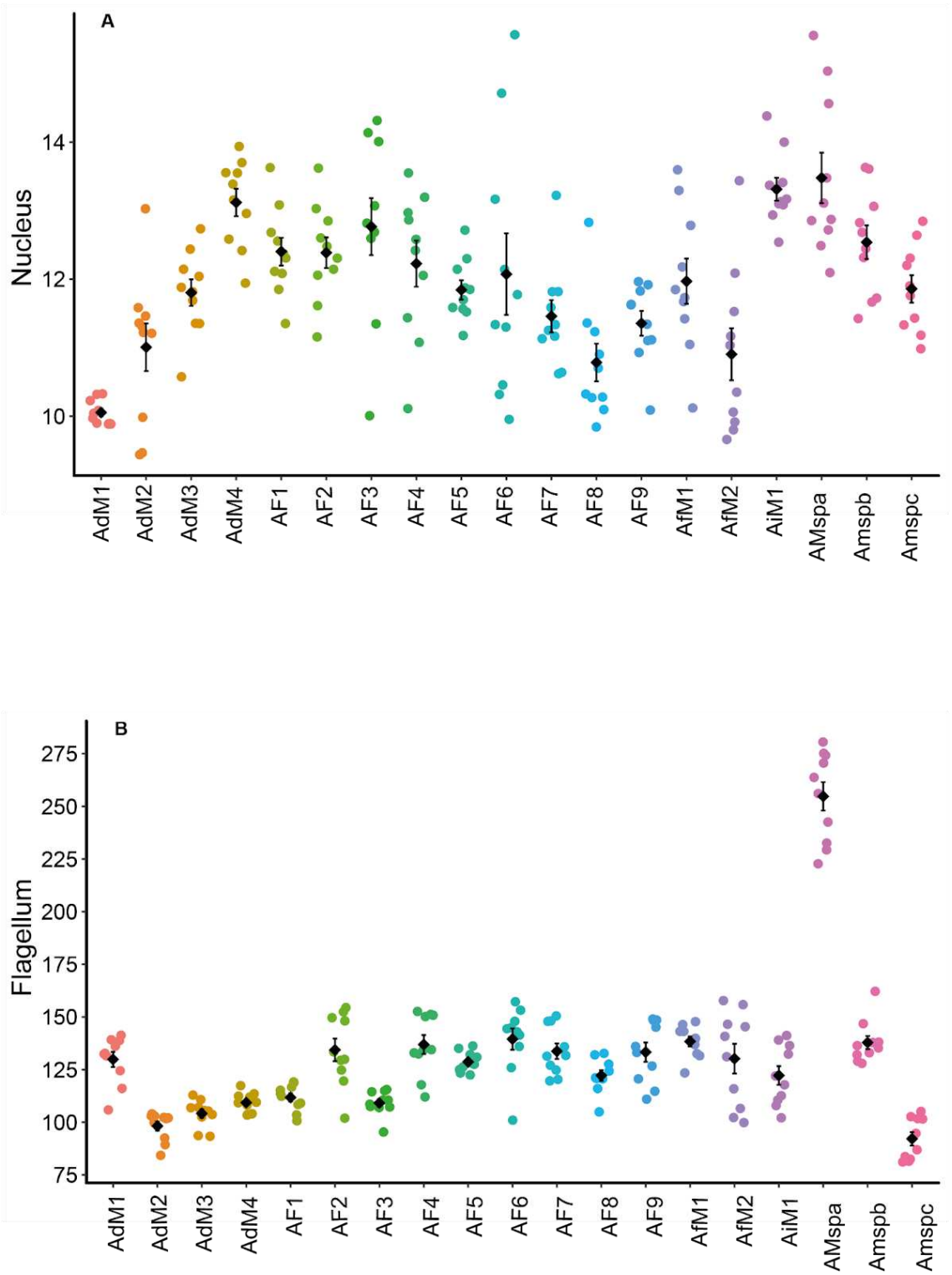
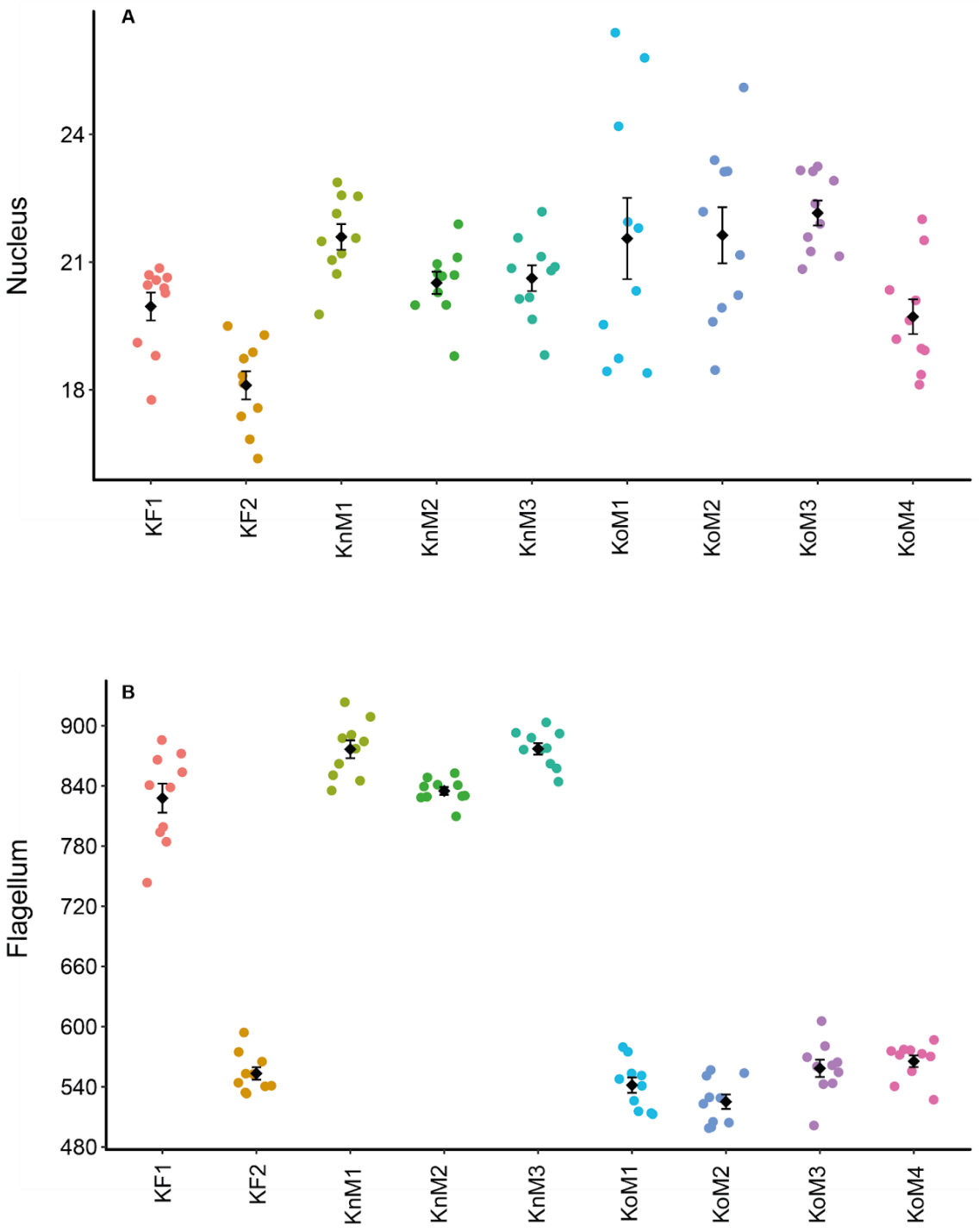
5.6. LIST OF ILLUSTRATIONS



GRAPH 1: Comparison of *Anacroneuria* sperm measurements for each individual. A - nucleus. B - flagellum. Individuals: *A. debilis* (AdM1, AdM2, Adm3, Adm4); *A.*sp females (AF1, AF2, AF3, AF4,AF5, AF6, AF7, AF8, AF9); *A. flintorum* (AfM1, AfM2); *A. itatiaiensis* (AiM1) and *A.* sp male non indentified (AMspa, AMspb, AMspc).



GRAPH 2: Comparison of *Kempnyia* sperm measurements for each individual. A - nucleus. B - flagellum. Individuals: *Kempnyia* sp females (KF1, KF2); *K. neotropica* (KnM1, KnM2, KnM3) and *K. obtusa* (KoM1, KoM2, KoM3, KoM4).

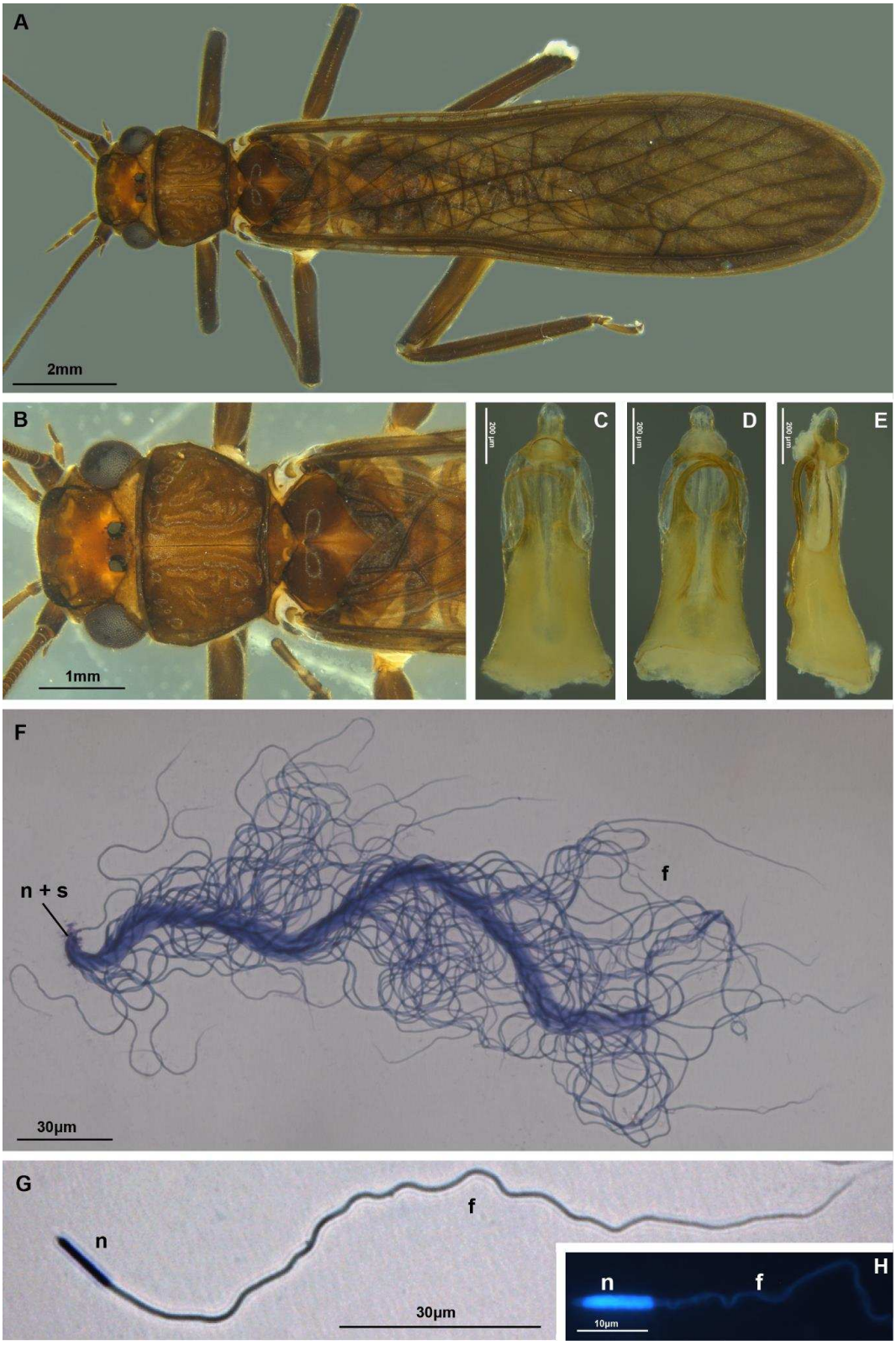


FIGURE 1: *Anacroneuria debilis* (AdM1). (A) Habitus. (B) Head and pronotum. (C,D, E) Male Genitalia. (F) Bundle. (G) Spermatozoa. (H) Nucleus. n+s: nucleus and stem, f: flagellum, n: nucleus.

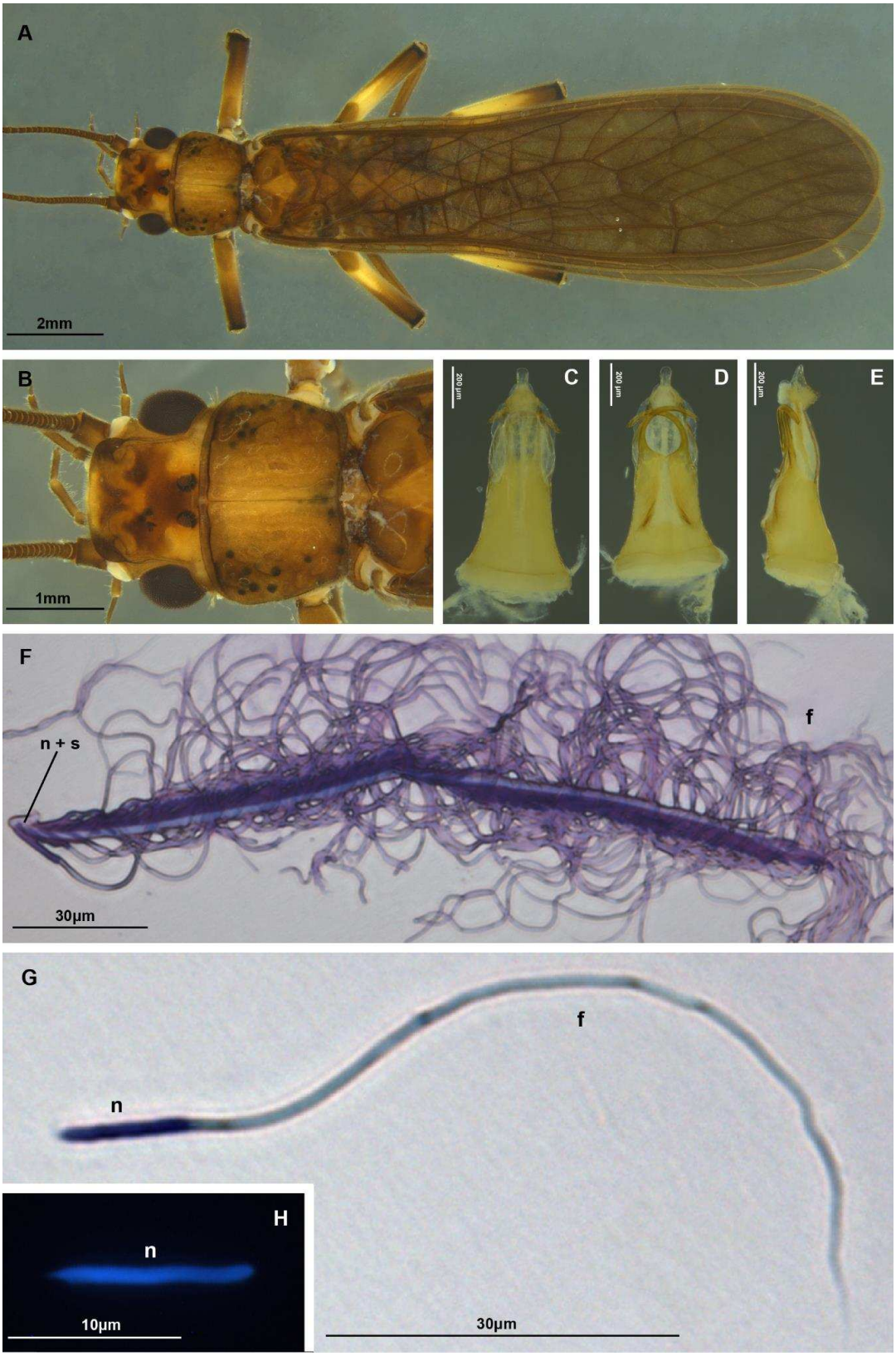


FIGURE 2: *Anacroneuria debilis* (AdM2). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Sperm bundle. (G) Spermatozoa. (H) Nucleus. n+s: nucleus and stem, f: flagellum, n: nucleus.

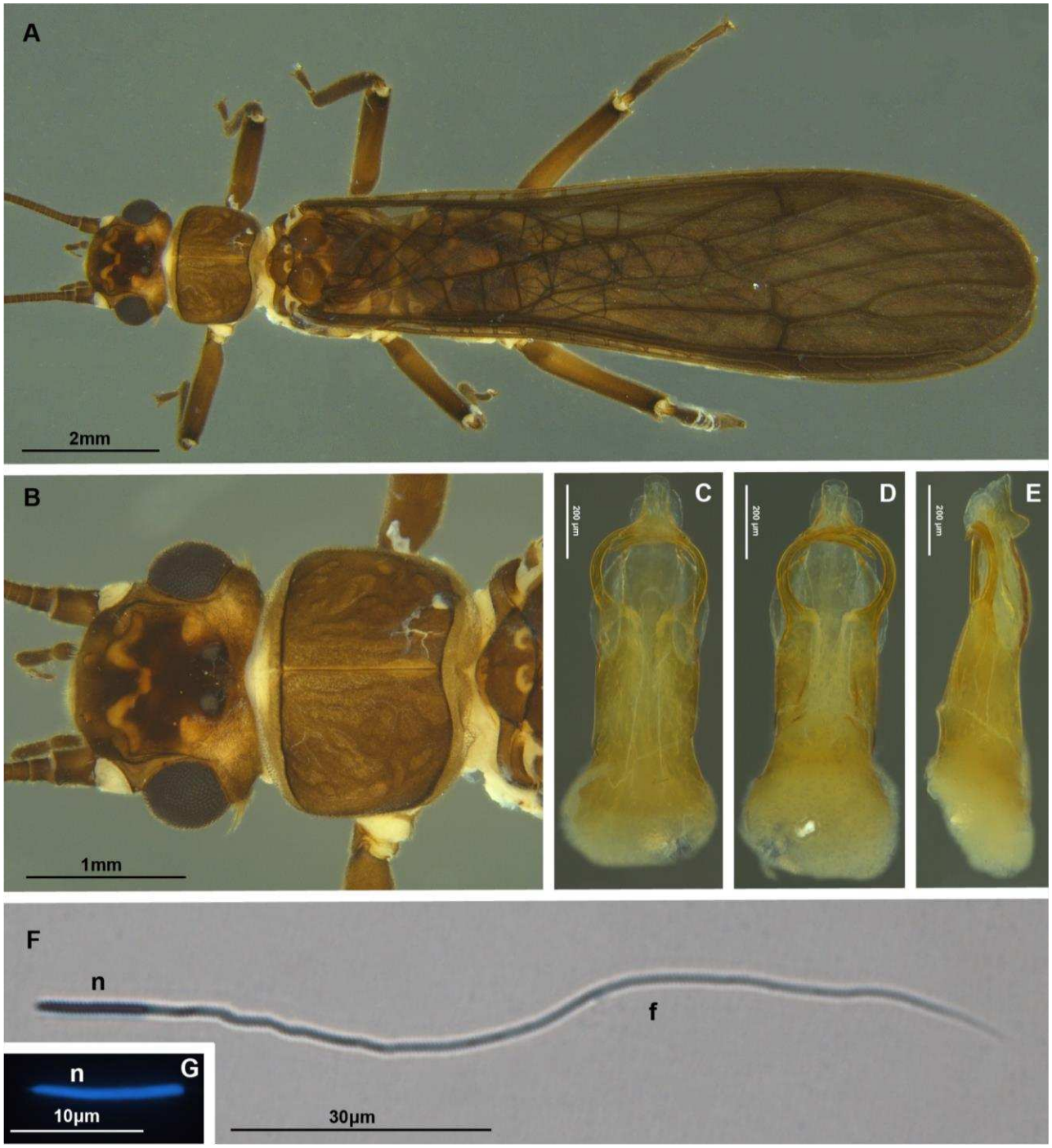


FIGURE 3: *Anacroneuria debilis* (AdM3). (A) Habitus. (B) Head and pronotum. (C,D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

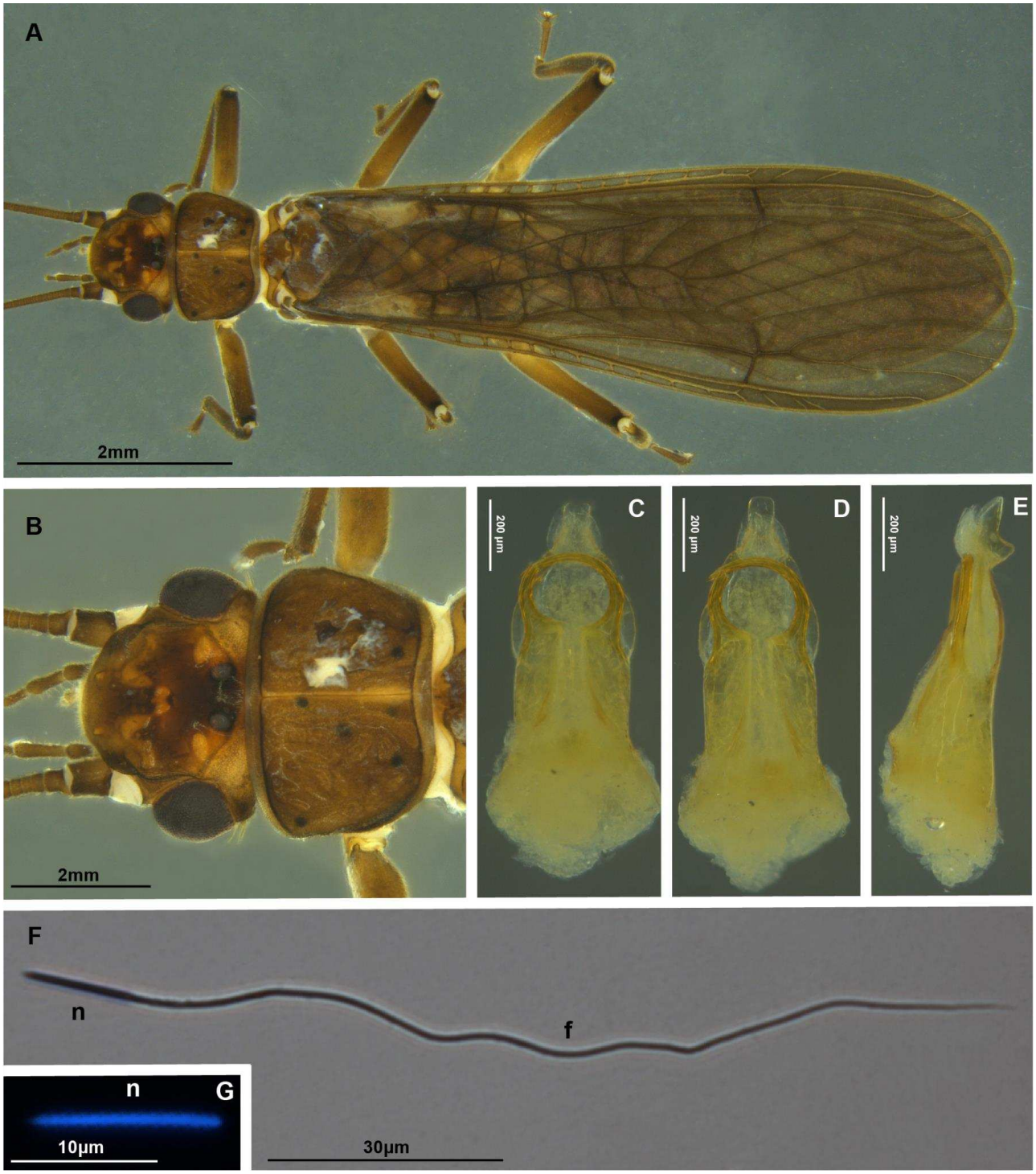


FIGURE 4: *Anacroneuria debilis* (AdM4). (A) Habitus. (B) Head and pronotum. (C,D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

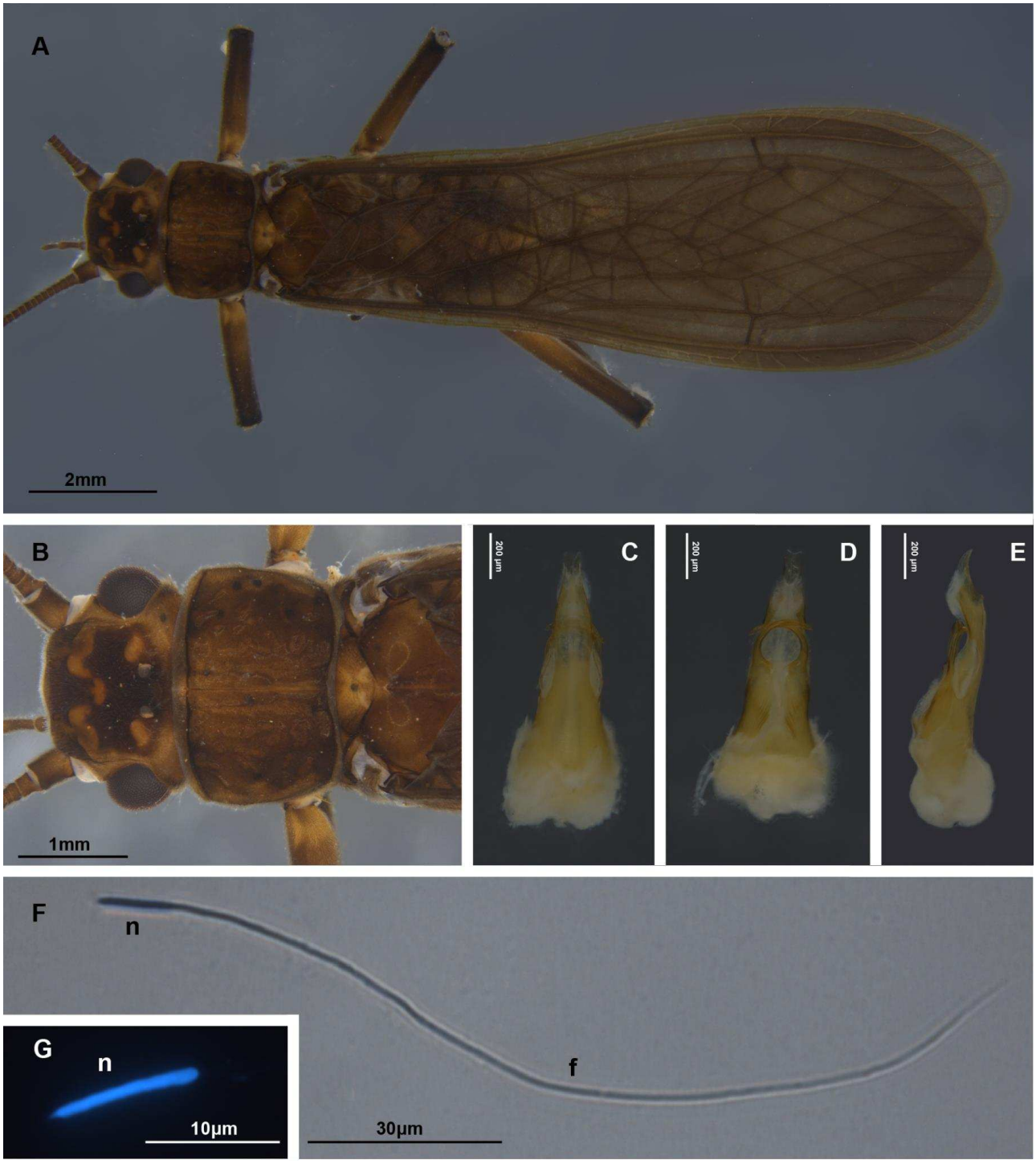


FIGURE 5: *Anacroneuria flintorum* (AfM1). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

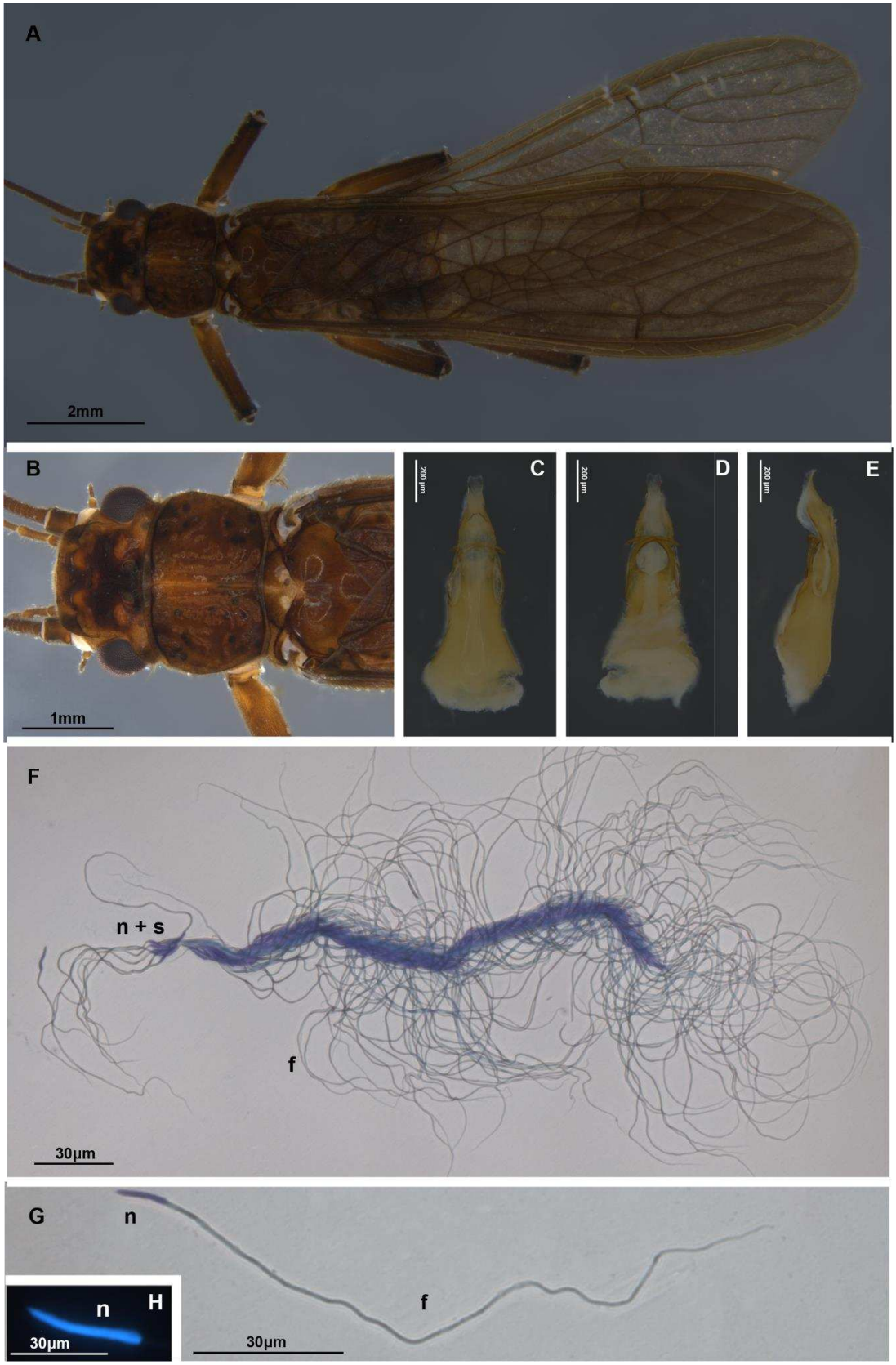


FIGURE 6: *Anacroneuria flintorum* (AfM2). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Sperm bundle. (G) Spermatozoa. (H) Nucleus. n+s: nucleus and stem, f: flagellum, n: nucleus.

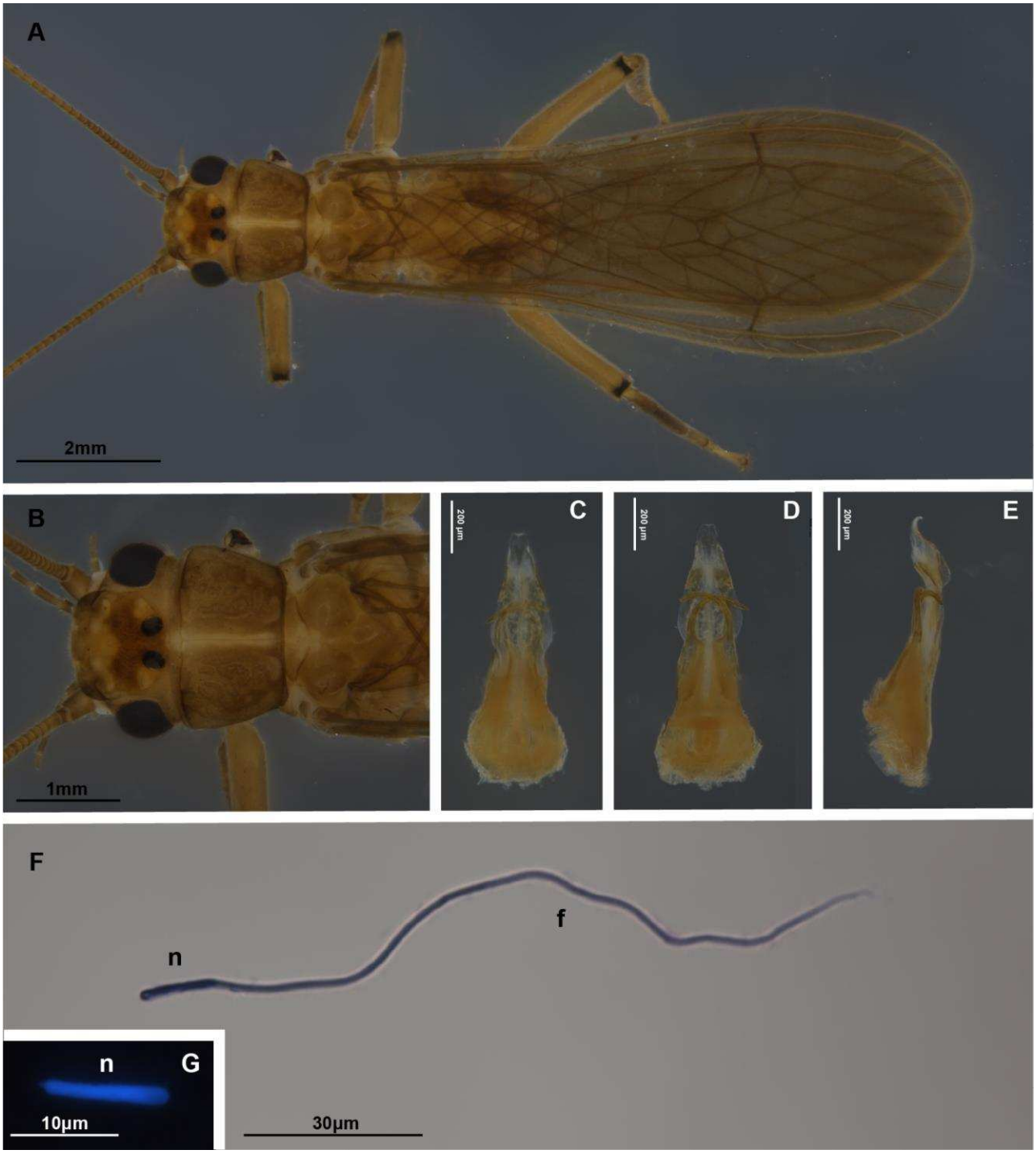


FIGURE 7: *Anacroneuria itatiaiensis* (AiM1). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

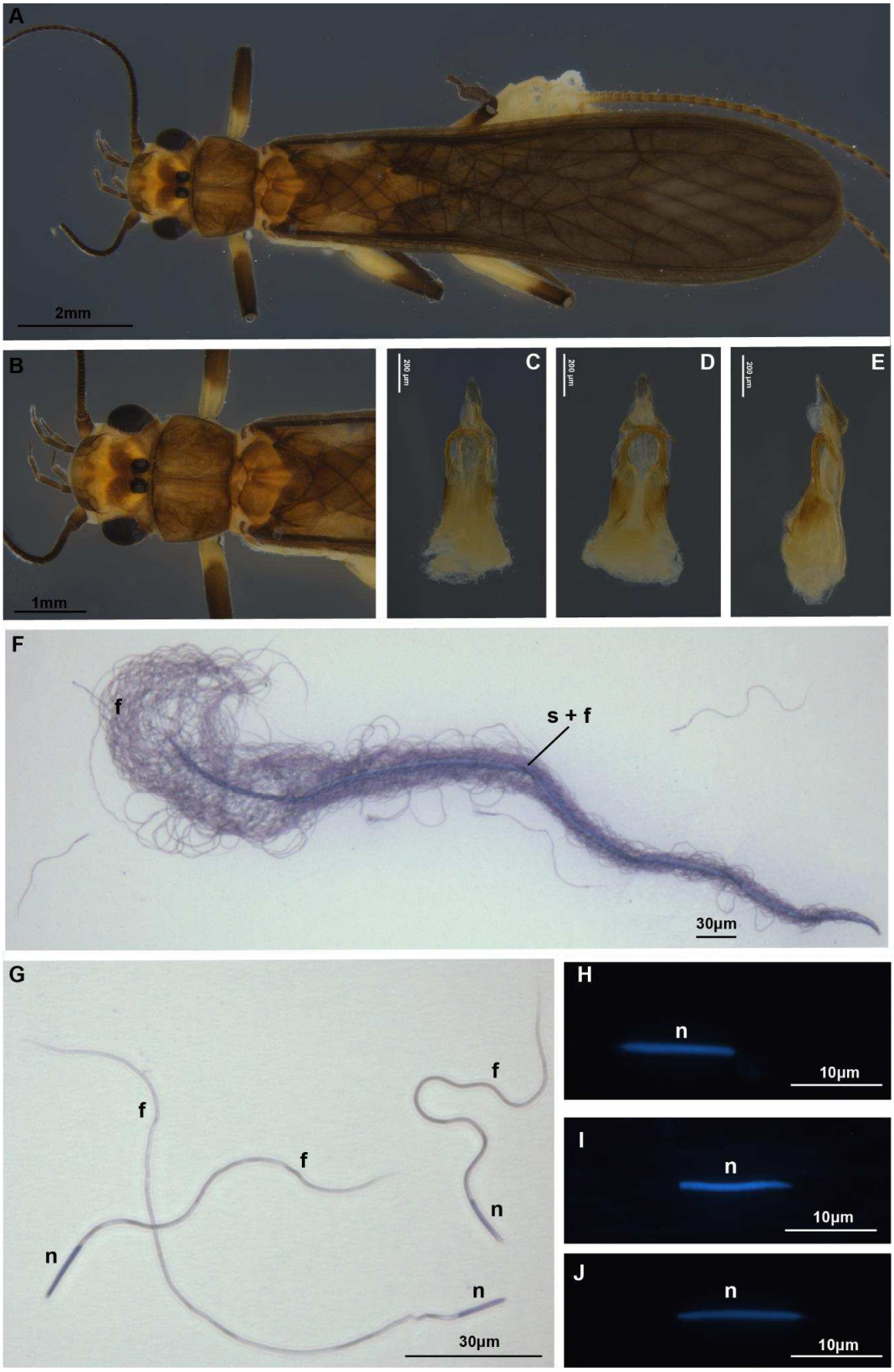


FIGURE 8: *Anacroneuria sp* Male (AMsp). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Sperm bundle. (G) Spermatozoa. (H, I, J) Nucleus. s+f: stem and flagellum, f: flagellum, n: nucleus.

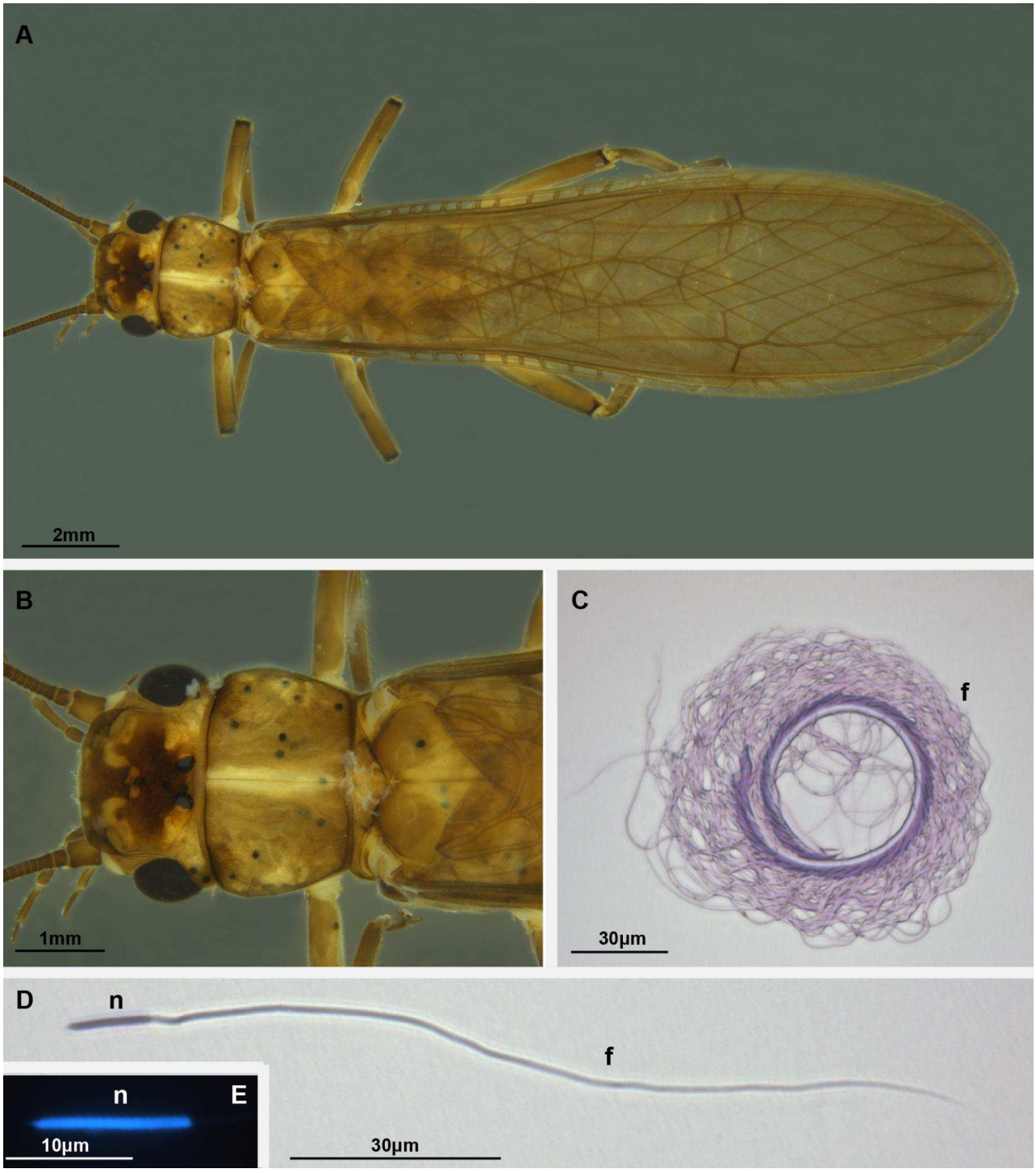


FIGURE 9: *Anacroneuria* - Female (AF1). (A) Habitus. (B) Head and pronotum. (C) Sperm bundle. (D) Spermatozoa. (E) Nucleus. f: flagellum, n: nucleus.

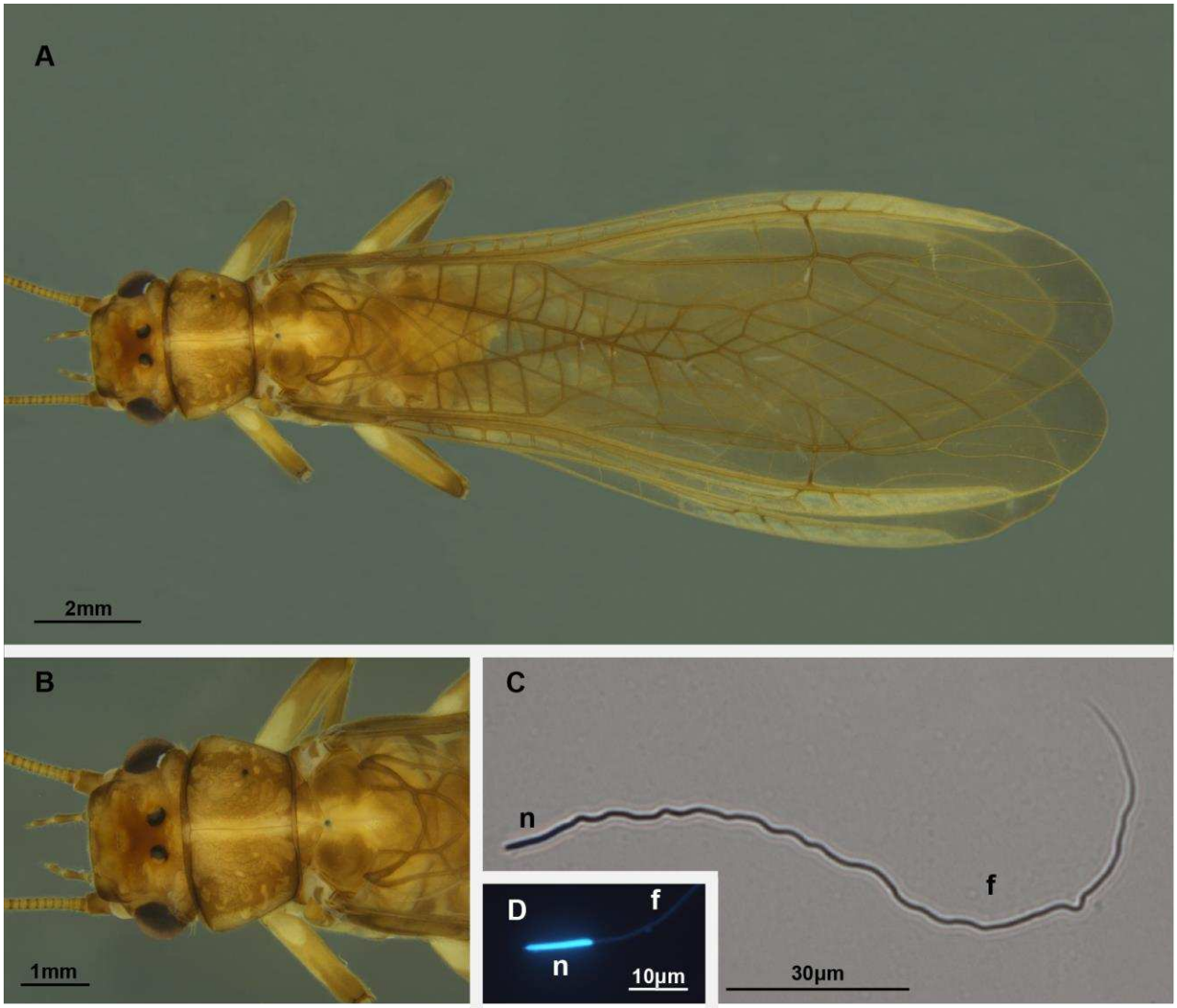


FIGURE 10: *Anacroneuria* - Female (AF2). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

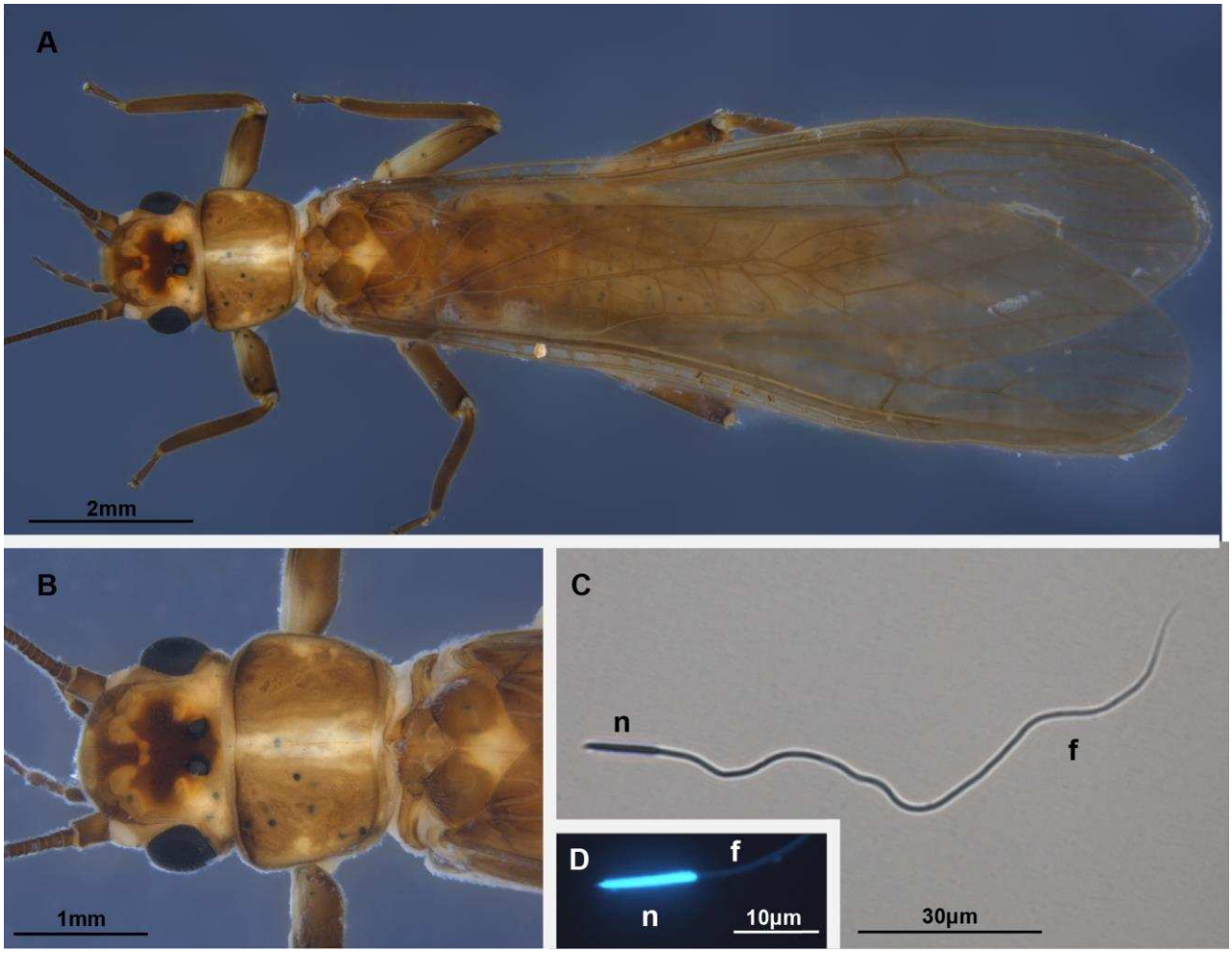


FIGURE 11: *Anacroneuria* - Female (AF3). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

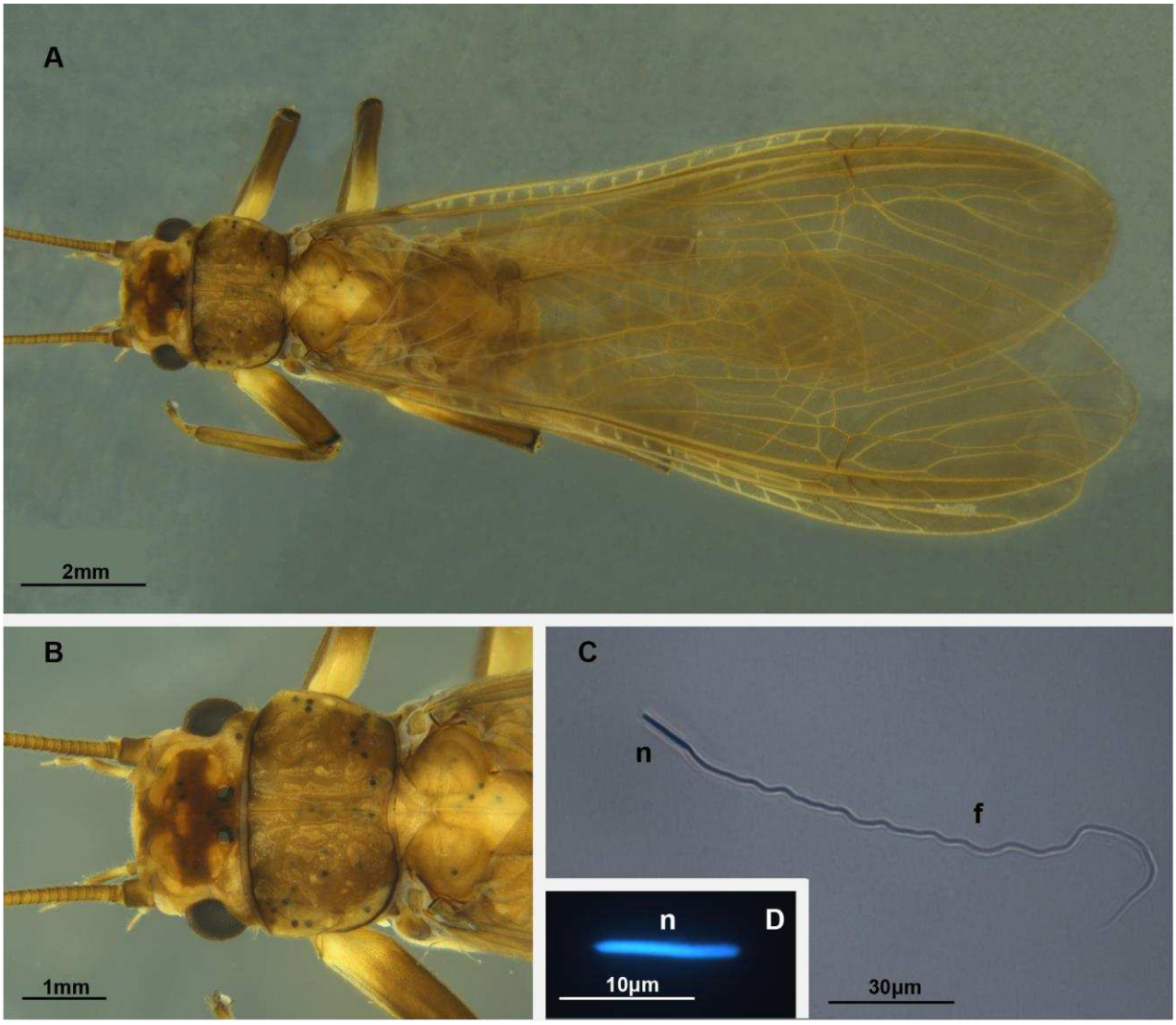


FIGURE 12: *Anacroneuria* - Female (AF4). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

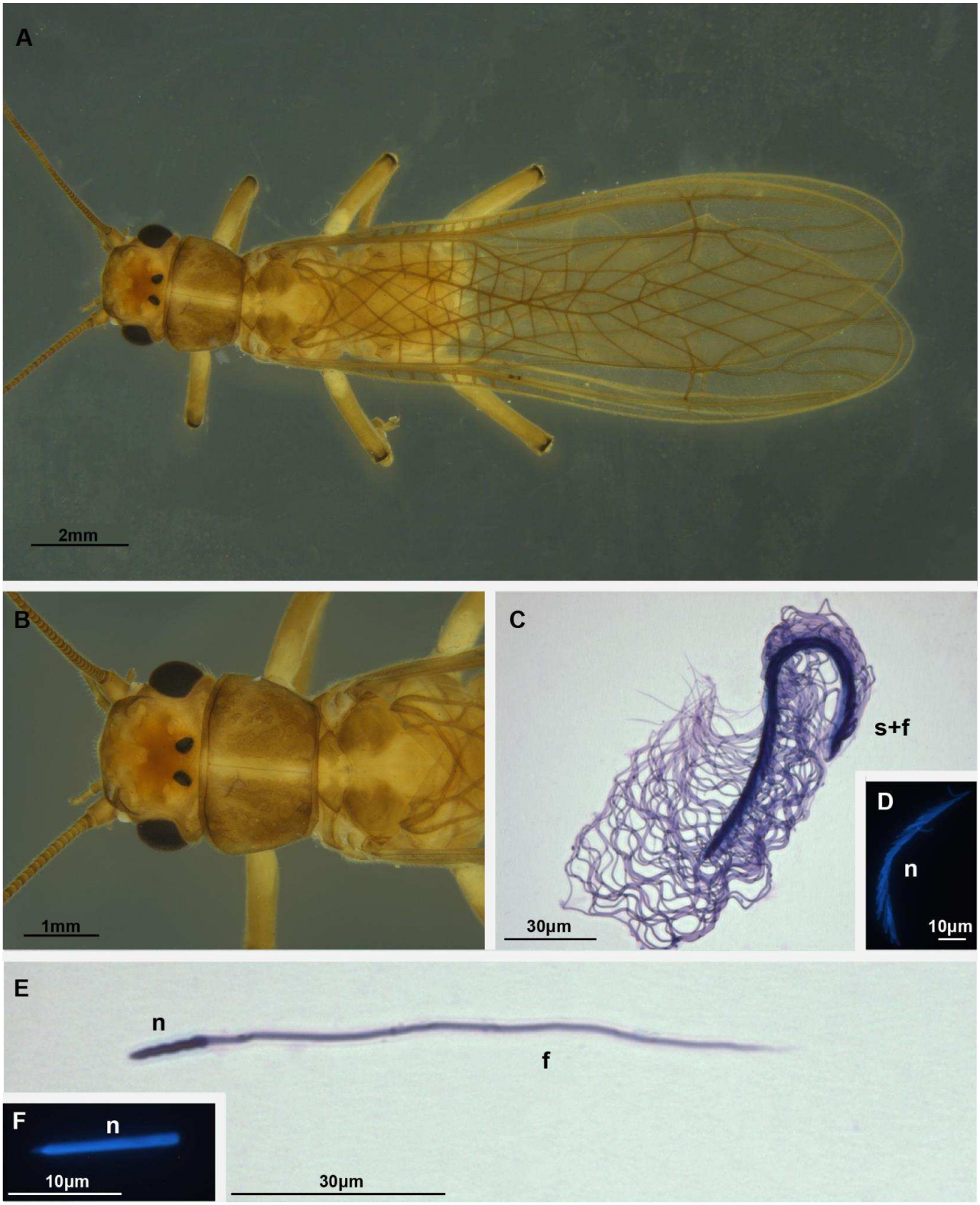


FIGURE 13: *Anacroneuria* - Female (AF5). (A) Habitus. (B) Head and pronotum. (C) Sperm bundle. (D) Nucleus in the bundle. (E) Spermatozoa. (F) Nucleus. s+f: stem and flagellum, f: flagellum, n: nucleus.

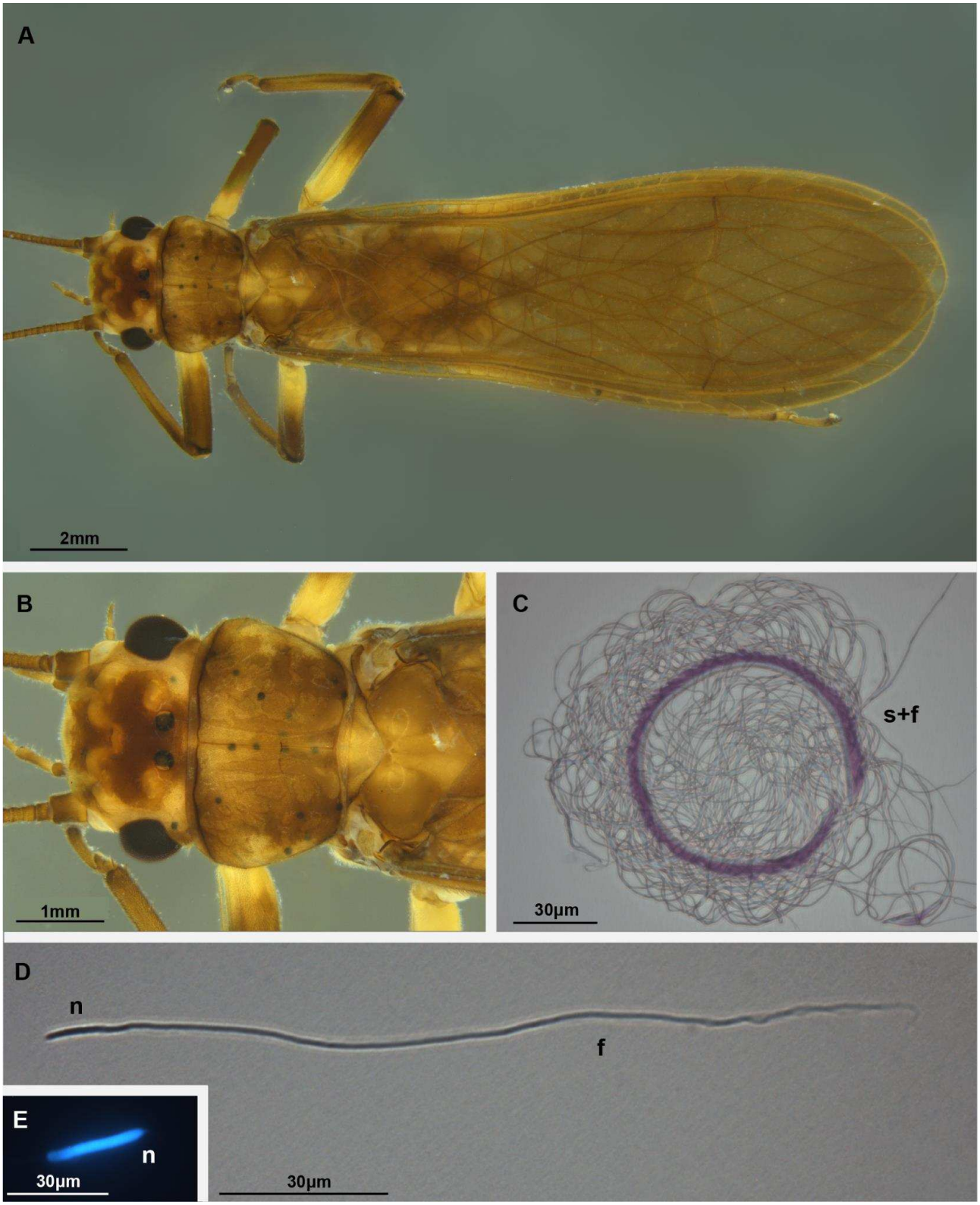


FIGURE 14: *Anacroneuria* - Female (AF6). (A) Habitus. (B) Head and pronotum. (C) Sperm bundle. (D) Spermatozoa. (E) Nucleus. s+f: stem and flagellum, f: flagellum, n: nucleus.

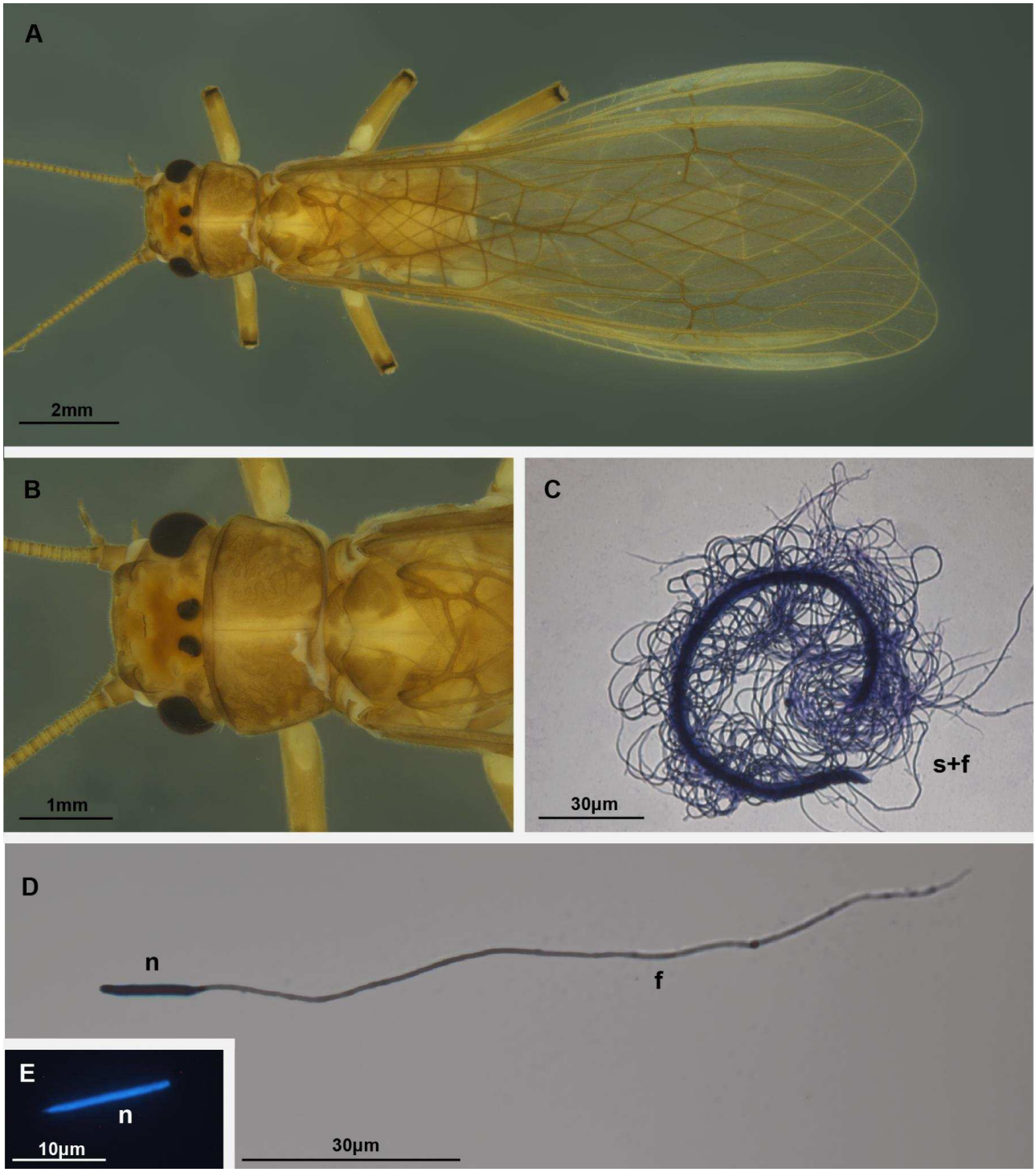


FIGURE 15: *Anacroneuria* - Female (AF7). (A) Habitus. (B) Head and pronotum. (C) Sperm bundle. (D) Spermatozoa. (E) Nucleus. s+f: stem and flagellum, f: flagellum, n: nucleus.

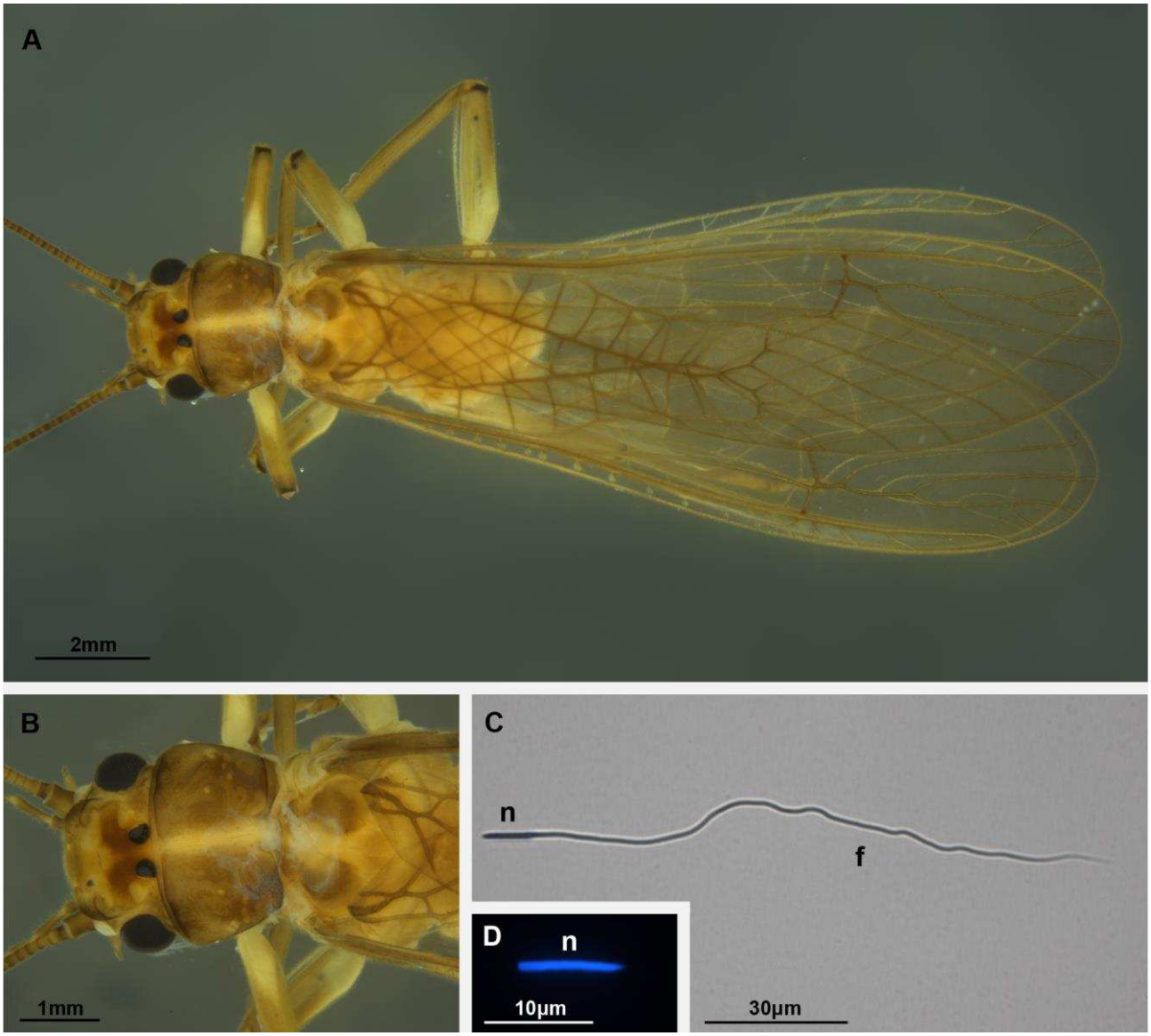


FIGURE 16: *Anacroneuria* - Female (AF8). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

FIGURE 17: *Anacroneuria* - Female (AF9). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

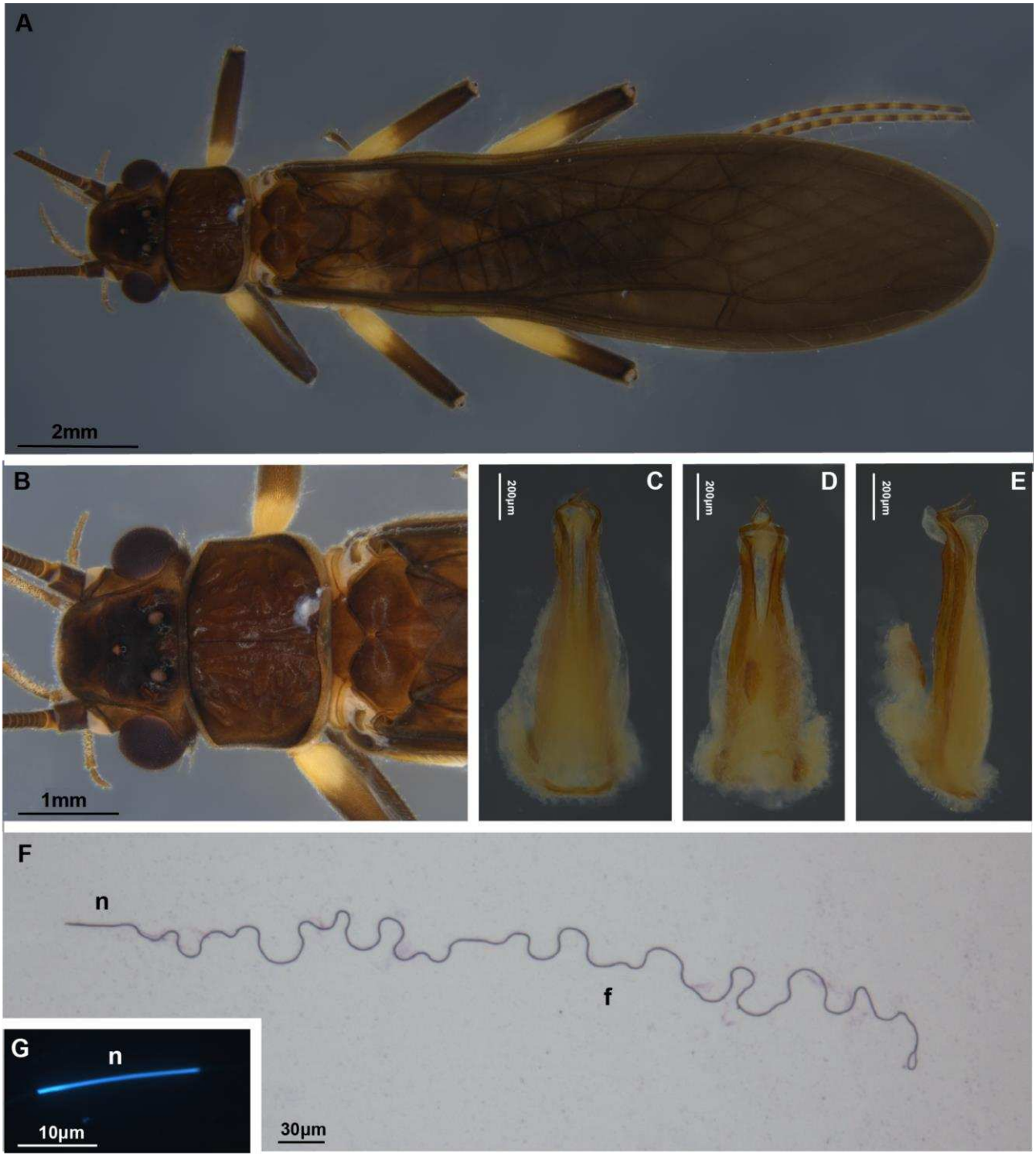


FIGURE 18: *Kempnyia neotropica* (KnM1) sperm under light microscopy. (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

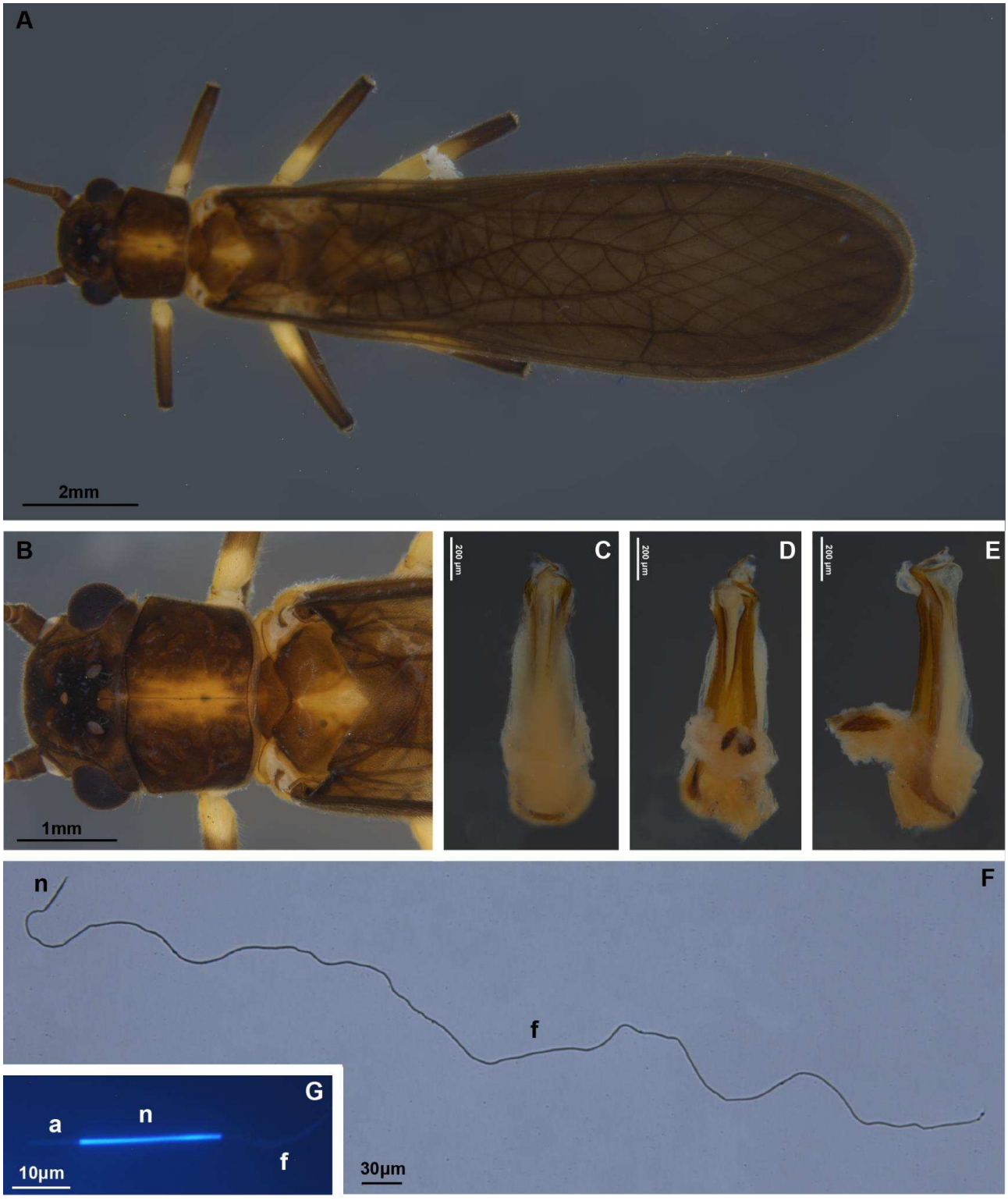


FIGURE 19: *Kempnyia neotropica* (KnM2). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum, a: acrossome.

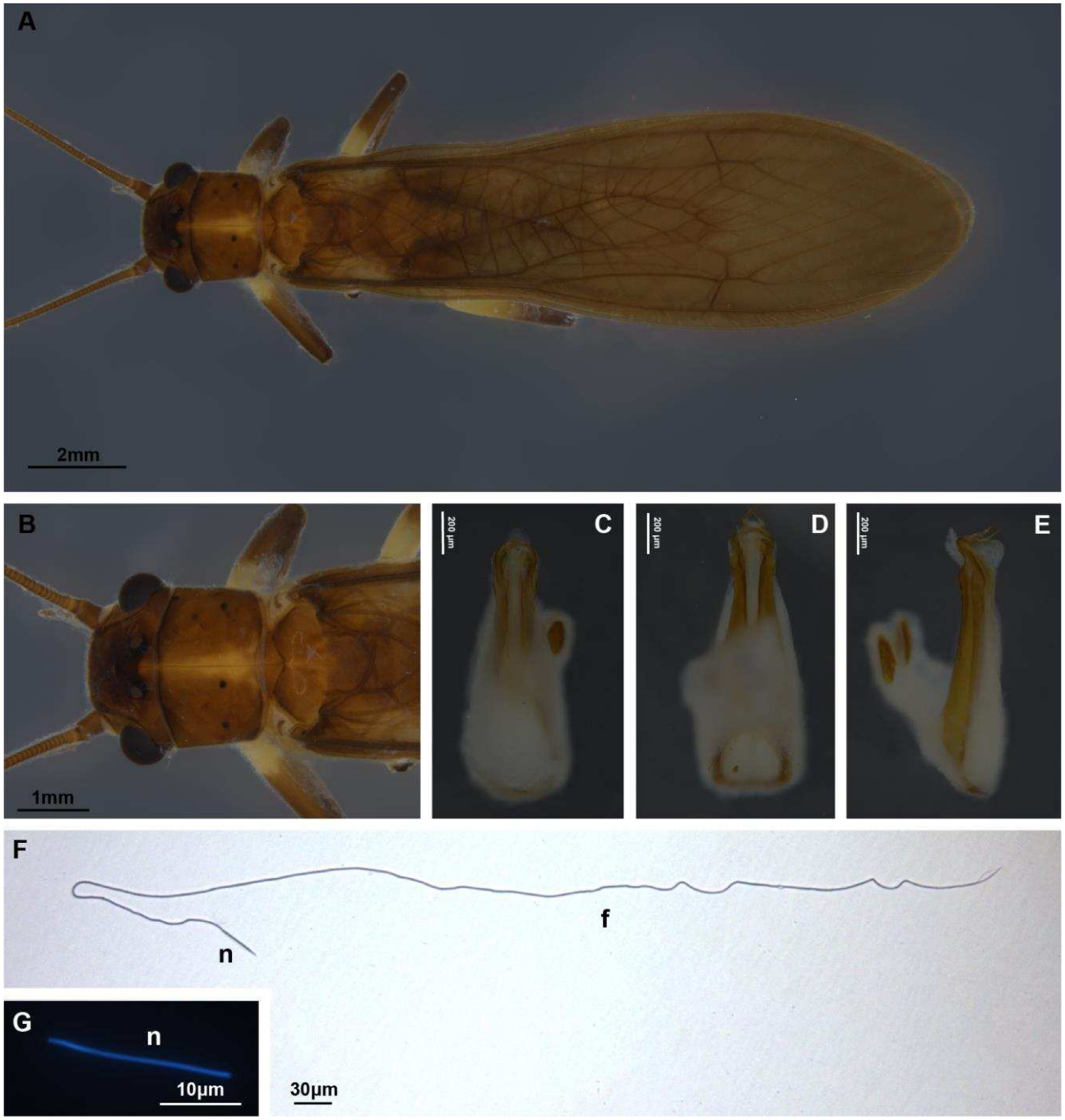


FIGURE 20: *Kempnyia neotropica* (KnM3). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

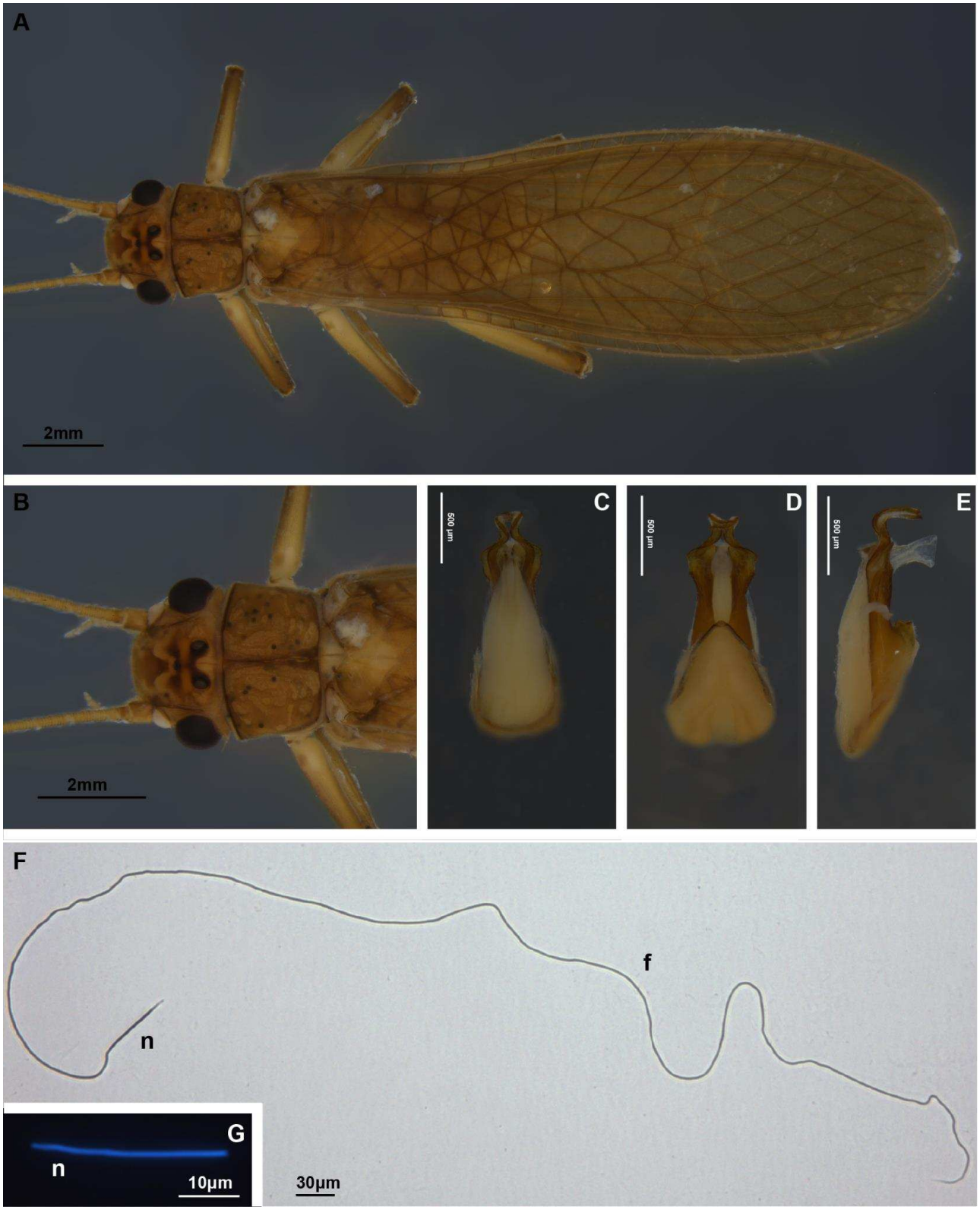


FIGURE 21: *Kempnyia obtusa* (KoM1). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

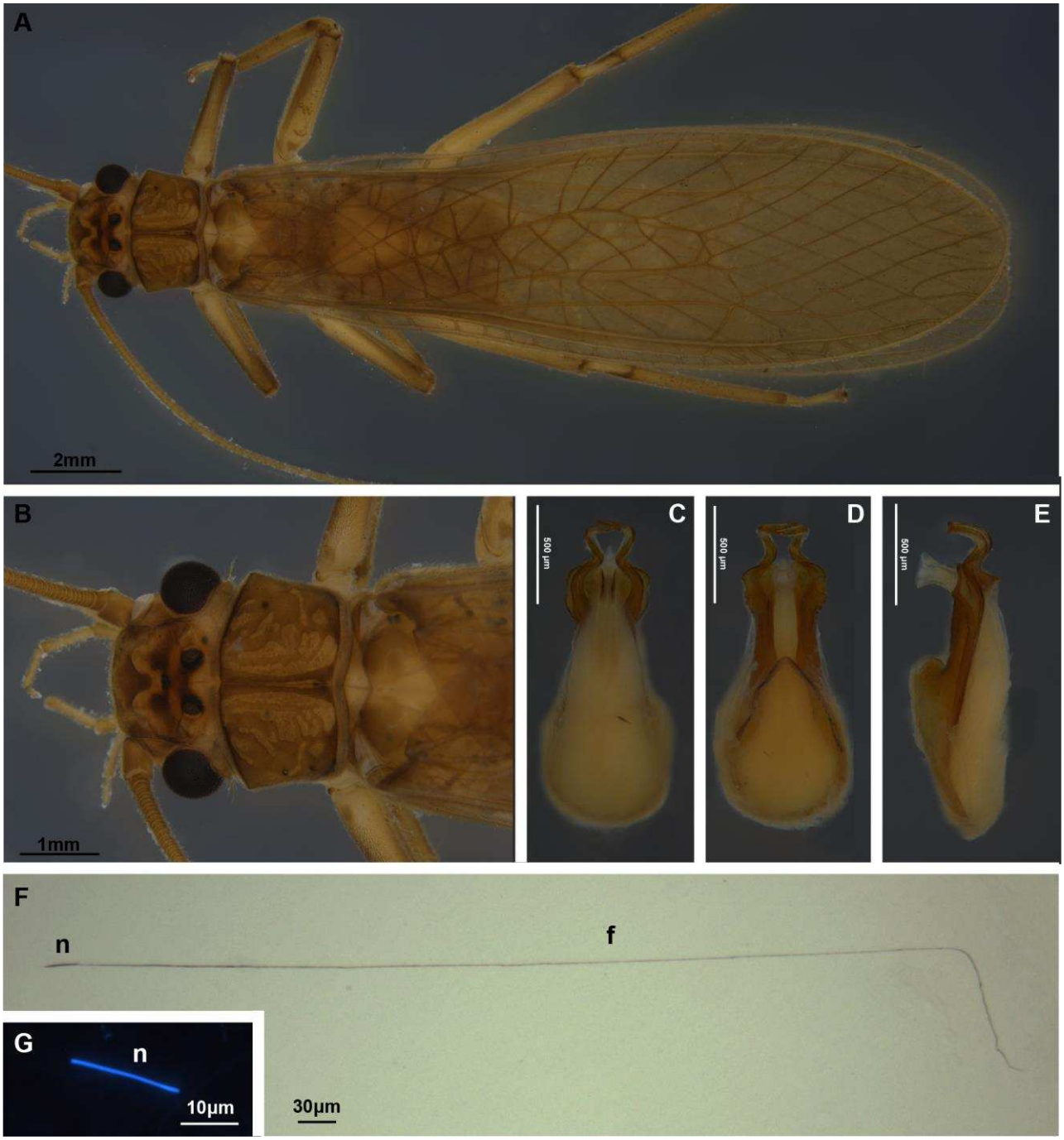


FIGURE 22: *Kempnyia obtusa* (KoM2). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

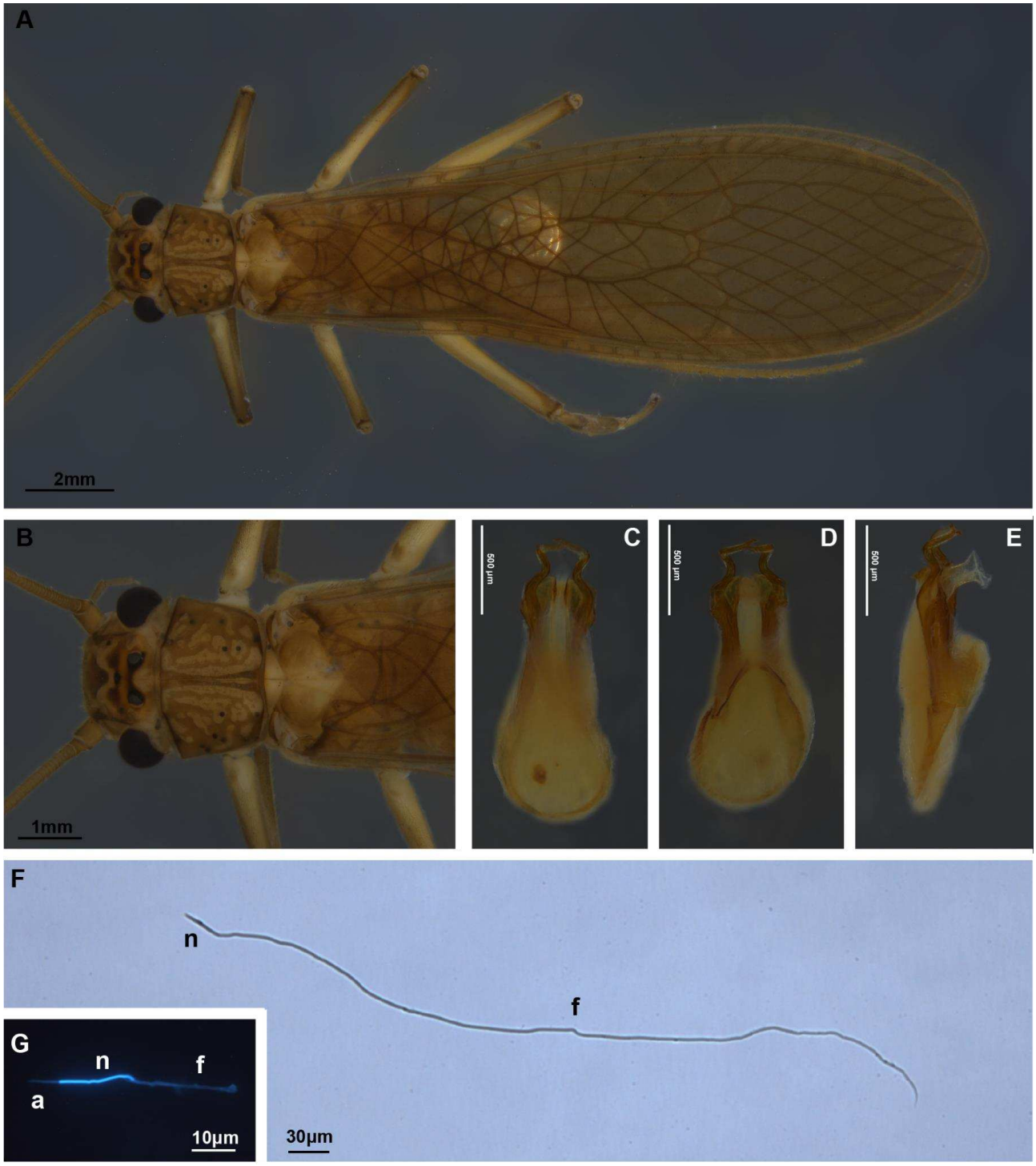


FIGURE 23: *Kempnyia obtusa* (KoM3). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum.

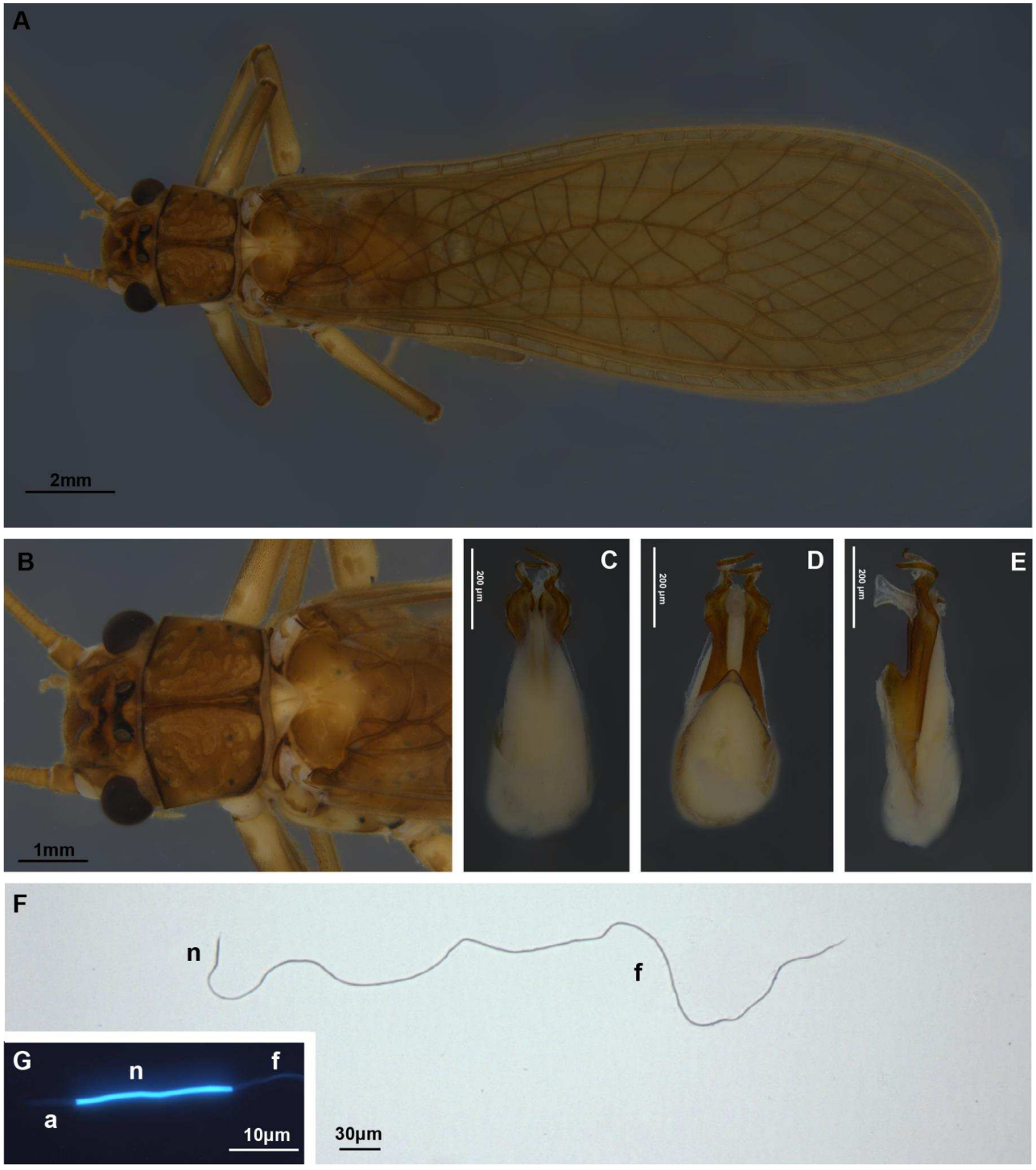


FIGURE 24: *Kempnyia obtusa* (KoM4). (A) Habitus. (B) Head and pronotum. (C, D, E) Male Genitalia. (F) Spermatozoa. (G) Nucleus. n: nucleus, f: flagellum, a: acrossome.

FIGURE 25: *Kempnyia sp* Female (KF1). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

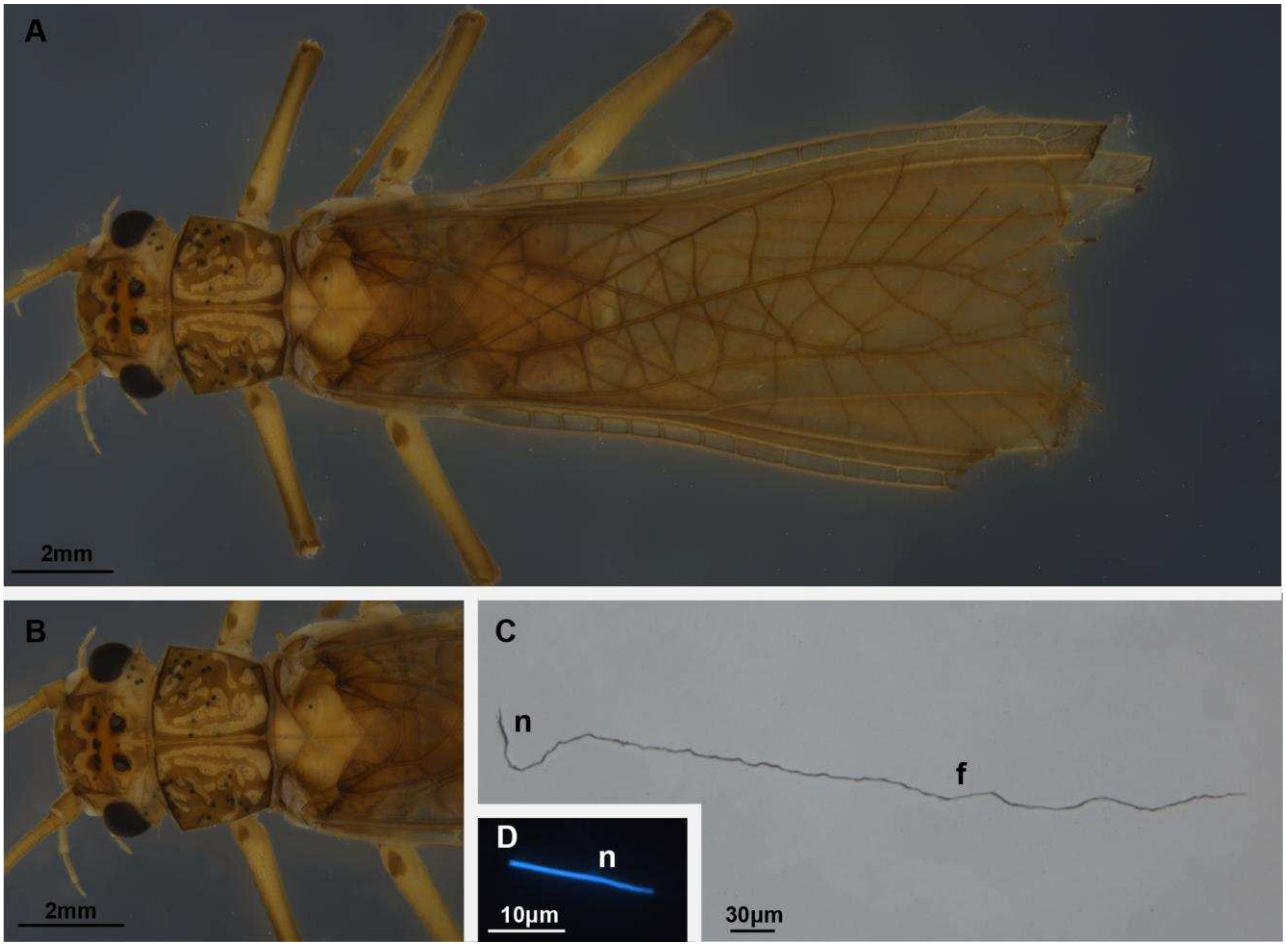


FIGURE 26: *Kempnyia sp* Female (KF2). (A) Habitus. (B) Head and pronotum. (C) Spermatozoa. (D) Nucleus. n: nucleus, f: flagellum.

5.7 SUPPLEMENTARY MATERIAL - LIST OF TABLES

TABLE 1. Detailed information regarding the species, locality, date of collection, and assigned codes.

Indivídual	Label Code	Genera	Species	Gender	Collection Location	Collection Date
AdM1	1.10.05.21M	Anacroneuria	*Anacroneuria debilis*	M	MG, Canaã, Cachoeira Grande (20°36'17"S 42°36'08"W - 586 m)	09.v.2021
AdM2	4.29.10.19M	Anacroneuria	*Anacroneuria debilis*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019
AdM3	05.07.x.2022M	Anacroneuria	*Anacroneuria debilis*	M	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
AdM4	06.07.x.2022M	Anacroneuria	*Anacroneuria debilis*	M	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
AF1	01.07.10.22F	Anacroneuria	*Anacroneuria sp*	F	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
AF2	1.1.07.21F	Anacroneuria	*Anacroneuria sp*	F	MG, Piranga, Rio Piranga, Hotel Fazenda Pirapetinga (20°41'11"S 43°23'09"W - 646 m)	29.vi.2021
AF3	02.07.10.22AF	Anacroneuria	*Anacroneuria sp*	F	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
AF4	02.09.09.21F	Anacroneuria	*Anacroneuria sp*	F	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	08.ix.2021
AF5	2.28.10.20F	Anacroneuria	*Anacroneuria sp*	F	MG, Canaã, Cachoeira Grande (20°36'17"S 42°36'08"W - 586 m)	27.x.2020
AF6	3.27.02.20AF	Anacroneuria	*Anacroneuria sp*	F	MG, Araponga, Serra do Brigadeiro, Cachoeira do Boné (20°38'48"S 42°27'30"W - 944 m)	26.ii.2020
AF7	3.28.10.20F	Anacroneuria	*Anacroneuria sp*	F	MG, Canaã, Cachoeira Grande (20°36'17"S 42°36'08"W - 586 m)	27.x.2020
AF8	04.07.10.22AF	Anacroneuria	*Anacroneuria sp*	F	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
AF9	4.29.10.21FA	Anacroneuria	*Anacroneuria sp*	F	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	28.x.2021
AfM1	01.09.09.21M	Anacroneuria	*Anacroneuria flintorum*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	08.ix.2021
AfM2	5.29.10.19M	Anacroneuria	*Anacroneuria flintorum*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019

AiM1	4.29.10.20B M	Anacroneuria	*Anacroneuria itatiaiensis*	M	MG, Canaã, Cachoeira Grande (20°36'17"S 42°36'08"W - 586 m)	27.x.2020
AMspa AMspb AMspc	07.07.10.22M	Anacroneuria	*Anacroneuria sp*	M	MG, Jaboticatubas, Parque Nacional da Serra do Cipó, Córrego Capão (19°20'22"S 43°37'07"W - 845 m)	05.x.2022
KF1	7.29.10.19F	Kempnyia	*Kempnyia sp*	F	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019
KF2	10.29.10.19F	Kempnyia	*Kempnyia sp*	F	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019
KnM1	01.08.09.22BM	Kempnyia	*Kempnyia neotropica*	M	RJ, Teresópolis, Parque Nacional da Serra dos Órgãos (22°26'54"S 42°59'00"W - 918 m)	07.ix.2022
KnM2	5.29.10.21BM	Kempnyia	*Kempnyia neotropica*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	28.x.2021
KnM3	8.29.10.19M	Kempnyia	*Kempnyia neotropica*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019
KoM1	1.27.02.20M	Kempnyia	*Kempnyia obtusa*	M	MG, Araponga, Serra do Brigadeiro, Cachoeira do Boné (20°38'48"S 42°27'30"W - 944 m)	26.ii.2020
KoM2	2.27.02.20M	Kempnyia	*Kempnyia obtusa*	M	MG, Araponga, Serra do Brigadeiro, Cachoeira do Boné (20°38'48"S 42°27'30"W - 944 m)	26.ii.2020
KoM3	3.29.10.21M	Kempnyia	*Kempnyia obtusa*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	28.x.2021
KoM4	9.29.10.19M	Kempnyia	*Kempnyia obtusa*	M	MG, Araponga, Serra do Brigadeiro, Pousada Remanso (20°39'23"S 42°27'15"W - 1.083 m)	27-28.x.2019

TABLE 2. Pairwise comparisons of nucleus and flagellum size across *Anacroneuria* individuals. No significant values are (p>0.05) marked in gray.

	Nucleus		Flagellum	
contrast	**t.ratio**	**p.value**	**t.ratio**	**p.value**
AdM1 - AdM2	-2.296	0.7106	5.722	<.0001
AdM1 - AdM3	-4.215	0.0056	4.642	0.0010
AdM1 - AdM4	-7.387	<.0001	3.710	0.0325
AdM1 - AF1	-5.662	<.0001	3.273	0.1175
AdM1 - AF2	-5.621	<.0001	-0.818	10.000
AdM1 - AF3	-6.539	<.0001	3.741	0.0294
AdM1 - AF4	-5.236	0.0001	-1.275	0.9989
AdM1 - AF5	-4.314	0.0038	0.212	10.000
AdM1 - AF6	-4.867	0.0004	-1.745	0.9632
AdM1 - AF7	-3.387	0.0860	-0.703	10.000
AdM1 - AF8	-1.760	0.9601	1.386	0.9969
AdM1 - AF9	-3.136	0.1661	-0.626	10.000
AdM1 - AfM1	-4.617	0.0011	-1.542	0.9896
AdM1 - AfM2	-2.050	0.8592	-0.062	10.000
AdM1 - AiM1	-7.854	<.0001	1.384	0.9970
AdM1 - AMspa	-8.252	<.0001	-22.586	<.0001
AdM1 - Amspb	-5.989	<.0001	-1.448	0.9949
AdM1 - Amspc	-4.350	0.0033	6.816	<.0001
AdM2 - AdM3	-1.919	0.9151	-1.080	0.9999
AdM2 - AdM4	-5.090	0.0001	-2.012	0.8772
AdM2 - AF1	-3.366	0.0913	-2.450	0.5985
AdM2 - AF2	-3.325	0.1021	-6.540	<.0001
AdM2 - AF3	-4.243	0.0050	-1.981	0.8911
AdM2 - AF4	-2.940	0.2601	-6.997	<.0001
AdM2 - AF5	-2.018	0.8748	-5.510	<.0001
AdM2 - AF6	-2.571	0.5067	-7.467	<.0001
AdM2 - AF7	-1.091	0.9999	-6.425	<.0001
AdM2 - AF8	0.536	10.000	-4.336	0.0035
AdM2 - AF9	-0.840	10.000	-6.348	<.0001
AdM2 - AfM1	-2.321	0.6929	-7.264	<.0001
AdM2 - AfM2	0.246	10.000	-5.784	<.0001
AdM2 - AiM1	-5.558	<.0001	-4.338	0.0035
AdM2 - AMspa	-5.956	<.0001	-28.308	<.0001
AdM2 - Amspb	-3.693	0.0344	-7.170	<.0001
AdM2 - Amspc	-2.054	0.8576	1.093	0.9999
AdM3 - AdM4	-3.171	0.1524	-0.932	10.000

AdM3 - AF1	-1.447	0.9949	-1.369	0.9973
AdM3 - AF2	-1.406	0.9964	-5.460	<.0001
AdM3 - AF3	-2.323	0.6914	-0.901	10.000
AdM3 - AF4	-1.021	0.9999	-5.917	<.0001
AdM3 - AF5	-0.099	10.000	-4.430	0.0024
AdM3 - AF6	-0.652	10.000	-6.387	<.0001
AdM3 - AF7	0.828	10.000	-5.345	<.0001
AdM3 - AF8	2.455	0.5942	-3.256	0.1226
AdM3 - AF9	1.079	0.9999	-5.268	0.0001
AdM3 - AfM1	-0.402	10.000	-6.184	<.0001
AdM3 - AfM2	2.165	0.7962	-4.704	0.0008
AdM3 - AiM1	-3.639	0.0407	-3.258	0.1221
AdM3 - AMspa	-4.036	0.0107	-27.228	<.0001
AdM3 - Amspb	-1.773	0.9572	-6.090	<.0001
AdM3 - Amspc	-0.134	10.000	2.174	0.7910
AdM4 - AF1	1.725	0.9671	-0.437	10.000
AdM4 - AF2	1.765	0.9589	-4.528	0.0016
AdM4 - AF3	0.848	10.000	0.031	10.000
AdM4 - AF4	2.151	0.8049	-4.984	0.0002
AdM4 - AF5	3.073	0.1934	-3.498	0.0626
AdM4 - AF6	2.519	0.5457	-5.455	<.0001
AdM4 - AF7	3.999	0.0122	-4.412	0.0026
AdM4 - AF8	5.626	<.0001	-2.324	0.6909
AdM4 - AF9	4.250	0.0049	-4.336	0.0035
AdM4 - AfM1	2.769	0.3648	-5.251	0.0001
AdM4 - AfM2	5.336	<.0001	-3.772	0.0266
AdM4 - AiM1	-0.468	10.000	-2.326	0.6896
AdM4 - AMspa	-0.865	10.000	-26.296	<.0001
AdM4 - Amspb	1.398	0.9966	-5.158	0.0001
AdM4 - Amspc	3.037	0.2101	3.106	0.1788
AF1 - AF2	0.041	10.000	-4.091	0.0088
AF1 - AF3	-0.877	10.000	0.469	10.000
AF1 - AF4	0.426	10.000	-4.547	0.0015
AF1 - AF5	1.348	0.9978	-3.061	0.1989
AF1 - AF6	0.795	10.000	-5.018	0.0002
AF1 - AF7	2.275	0.7253	-3.975	0.0133
AF1 - AF8	3.902	0.0172	-1.887	0.9262
AF1 - AF9	2.526	0.5408	-3.899	0.0174
AF1 - AfM1	1.045	0.9999	-4.814	0.0005
AF1 - AfM2	3.612	0.0443	-3.335	0.0994
AF1 - AiM1	-2.192	0.7795	-1.889	0.9256
AF1 - AMspa	-2.590	0.4928	-25.858	<.0001
AF1 - Amspb	-0.327	10.000	-4.720	0.0007

AF1 - Amspc	1.312	0.9984	3.543	0.0547
AF2 - AF3	-0.918	10.000	4.559	0.0014
AF2 - AF4	0.385	10.000	-0.457	10.000
AF2 - AF5	1.307	0.9985	1.030	0.9999
AF2 - AF6	0.754	10.000	-0.927	10.000
AF2 - AF7	2.234	0.7528	0.115	10.000
AF2 - AF8	3.861	0.0198	2.204	0.7724
AF2 - AF9	2.485	0.5718	0.192	10.000
AF2 - AfM1	1.004	10.000	-0.724	10.000
AF2 - AfM2	3.571	0.0503	0.756	10.000
AF2 - AiM1	-2.233	0.7533	2.202	0.7735
AF2 - AMspa	-2.631	0.4624	-21.768	<.0001
AF2 - Amspb	-0.368	10.000	-0.630	10.000
AF2 - Amspc	1.271	0.9990	7.634	<.0001
AF3 - AF4	1.303	0.9986	-5.016	0.0002
AF3 - AF5	2.225	0.7588	-3.529	0.0570
AF3 - AF6	1.671	0.9757	-5.486	<.0001
AF3 - AF7	3.152	0.1600	-4.444	0.0023
AF3 - AF8	4.779	0.0006	-2.356	0.6682
AF3 - AF9	3.402	0.0824	-4.367	0.0031
AF3 - AfM1	1.921	0.9144	-5.283	0.0001
AF3 - AfM2	4.488	0.0019	-3.803	0.0240
AF3 - AiM1	-1.316	0.9984	-2.357	0.6669
AF3 - AMspa	-1.713	0.9691	-26.327	<.0001
AF3 - Amspb	0.550	10.000	-5.189	0.0001
AF3 - Amspc	2.189	0.7816	3.074	0.1926
AF4 - AF5	0.922	10.000	1.487	0.9930
AF4 - AF6	0.369	10.000	-0.470	10.000
AF4 - AF7	1.849	0.9380	0.572	10.000
AF4 - AF8	3.476	0.0667	2.660	0.4408
AF4 - AF9	2.100	0.8336	0.649	10.000
AF4 - AfM1	0.619	10.000	-0.267	10.000
AF4 - AfM2	3.186	0.1470	1.212	0.9994
AF4 - AiM1	-2.618	0.4715	2.658	0.4421
AF4 - AMspa	-3.016	0.2203	-21.311	<.0001
AF4 - Amspb	-0.753	10.000	-0.173	10.000
AF4 - Amspc	0.886	10.000	8.090	<.0001
AF5 - AF6	-0.553	10.000	-1.957	0.9008
AF5 - AF7	0.927	10.000	-0.915	10.000
AF5 - AF8	2.554	0.5197	1.174	0.9996
AF5 - AF9	1.178	0.9996	-0.838	10.000
AF5 - AfM1	-0.303	10.000	-1.754	0.9614
AF5 - AfM2	2.264	0.7330	-0.274	10.000

AF5 - AiM1	-3.540	0.0551	1.172	0.9996
AF5 - AMspa	-3.938	0.0152	-22.798	<.0001
AF5 - Amspb	-1.675	0.9752	-1.660	0.9773
AF5 - Amspc	-0.036	10.000	6.603	<.0001
AF6 - AF7	1.480	0.9934	1.042	0.9999
AF6 - AF8	3.107	0.1782	3.131	0.1684
AF6 - AF9	1.731	0.9659	1.119	0.9998
AF6 - AfM1	0.250	10.000	0.203	10.000
AF6 - AfM2	2.817	0.3335	1.683	0.9741
AF6 - AiM1	-2.987	0.2349	3.129	0.1691
AF6 - AMspa	-3.384	0.0867	-20.841	<.0001
AF6 - Amspb	-1.122	0.9998	0.297	10.000
AF6 - Amspc	0.517	10.000	8.560	<.0001
AF7 - AF8	1.627	0.9815	2.088	0.8398
AF7 - AF9	0.251	10.000	0.077	10.000
AF7 - AfM1	-1.230	0.9993	-0.839	10.000
AF7 - AfM2	1.337	0.9980	0.640	10.000
AF7 - AiM1	-4.467	0.0021	2.086	0.8407
AF7 - AMspa	-4.865	0.0004	-21.883	<.0001
AF7 - Amspb	-2.602	0.4838	-0.745	10.000
AF7 - Amspc	-0.963	10.000	7.518	<.0001
AF8 - AF9	-1.376	0.9972	-2.012	0.8776
AF8 - AfM1	-2.857	0.3083	-2.927	0.2671
AF8 - AfM2	-0.290	10.000	-1.448	0.9948
AF8 - AiM1	-6.094	<.0001	-0.002	10.000
AF8 - AMspa	-6.491	<.0001	-23.972	<.0001
AF8 - Amspb	-4.229	0.0053	-2.833	0.3230
AF8 - Amspc	-2.590	0.4928	5.430	<.0001
AF9 - AfM1	-1.481	0.9933	-0.916	10.000
AF9 - AfM2	1.086	0.9999	0.564	10.000
AF9 - AiM1	-4.718	0.0007	2.010	0.8784
AF9 - AMspa	-5.115	0.0001	-21.960	<.0001
AF9 - Amspb	-2.853	0.3111	-0.822	10.000
AF9 - Amspc	-1.214	0.9994	7.441	<.0001
AfM1 - AfM2	2.567	0.5097	1.479	0.9934
AfM1 - AiM1	-3.237	0.1290	2.925	0.2681
AfM1 - AMspa	-3.634	0.0413	-21.044	<.0001
AfM1 - Amspb	-1.371	0.9973	0.094	10.000
AfM1 - Amspc	0.267	10.000	8.357	<.0001
AfM2 - AiM1	-5.804	<.0001	1.446	0.9949
AfM2 - AMspa	-6.201	<.0001	-22.524	<.0001
AfM2 - Amspb	-3.938	0.0151	-1.386	0.9969
AfM2 - Amspc	-2.300	0.7082	6.878	<.0001

AiM1 - AMspa	-0.397	10.000	-23.970	<.0001

TABLE 3. Pairwise comparisons of nucleus and flagellum size across *Kempnyia* individuals. No significant values are (p>0.05) marked in gray.

	Nucleus		Flagellum	
contrast	**t.ratio**	**p.value**	**t.ratio**	**p.value**
KF1 - KF2	2.733	0.1529	15.905	<.0001
KF1 - KnM1	-2.413	0.2914	-5.125	0.0001
KF1 - KnM2	-0.819	0.9960	-2.422	0.2867
KF1 - KnM3	-0.979	0.9868	-5.152	0.0001
KF1 - KoM1	-2.358	0.3214	16.665	<.0001
KF1 - KoM2	-2.472	0.2611	17.741	<.0001
KF1 - KoM3	-3.241	0.0432	15.572	<.0001
KF1 - KoM4	0.357	10.000	15.115	<.0001
KF2 - KnM1	-5.146	0.0001	-21.030	<.0001
KF2 - KnM2	-3.552	0.0176	-18.326	<.0001
KF2 - KnM3	-3.712	0.0107	-21.056	<.0001
KF2 - KoM1	-5.091	0.0001	0.760	0.9976
KF2 - KoM2	-5.205	<.0001	1.836	0.6584
KF2 - KoM3	-5.974	<.0001	-0.333	10.000
KF2 - KoM4	-2.376	0.3111	-0.790	0.9969
KnM1 - KnM2	1.594	0.8052	2.703	0.1631
KnM1 - KnM3	1.434	0.8814	-0.027	10.000
KnM1 - KoM1	0.055	10.000	21.790	<.0001
KnM1 - KoM2	-0.059	10.000	22.866	<.0001
KnM1 - KoM3	-0.828	0.9956	20.697	<.0001
KnM1 - KoM4	2.769	0.1409	20.240	<.0001
KnM2 - KnM3	-0.161	10.000	-2.730	0.1539
KnM2 - KoM1	-1.539	0.8336	19.087	<.0001
KnM2 - KoM2	-1.653	0.7722	20.163	<.0001
KnM2 - KoM3	-2.423	0.2862	17.994	<.0001
KnM2 - KoM4	1.175	0.9595	17.536	<.0001
KnM3 - KoM1	-1.378	0.9027	21.817	<.0001
KnM3 - KoM2	-1.493	0.8557	22.893	<.0001
KnM3 - KoM3	-2.262	0.3772	20.724	<.0001
KnM3 - KoM4	1.336	0.9173	20.266	<.0001
KoM1 - KoM2	-0.114	10.000	1.076	0.9761
KoM1 - KoM3	-0.884	0.9933	-1.093	0.9737
KoM1 - KoM4	2.714	0.1593	-1.550	0.8280
KoM2 - KoM3	-0.769	0.9974	-2.169	0.4356
KoM2 - KoM4	2.829	0.1231	-2.626	0.1923
KoM3 - KoM4	3.598	0.0153	-0.457	0.9999

6. CONCLUSIONS

The results of the analysis based on cladistic methodologies showed some differences from the historical phylogenetic relationships proposed for Plecoptera. In the first analysis, the suborder Antarctoperlaria was not recovered as monophyletic, because Eusthenioidea did not appear as a sister group only to Gripopterygoidea. Gripopterygoidea appeared in a polytomy with Scopuridae, Nemouroidea, and Systellognatha, which also did not recover Euholognatha. Systellognatha was recovered as monophyletic. The synapomorphies supporting Systellognatha corroborate those listed by Zwick (2000), such as drumming behavior; adult mandible thin, lacking mola, not sclerotized, soft; egg with anchor and collar, and thoracic postalar bridge present. In the second analysis, the results recovered Systellognatha as monophyletic and supported by two synapomorphies: drumming present and egg with anchor and collar. The suborder Antarctoperlaria was also recovered as monophyletic. However, its placement in the order is dissonant from most phylogenies proposed for Plecoptera, in which it appears as a sister group to Arctoperlaria (Systellognatha + Euholognatha). In our analysis, this clade appears as a sister group to Euholognatha, so the monophyletism of Arctoperlaria is no longer confirmed. Euholognatha was recovered as monophyletic and Scopuridae was recovered as a sister group to Nemouroidea, thus confirming the status of this infraorder.

One of the distinctive characteristics proposed by Zwick (2000) for Plecoptera monophyly is the testes fused medially. In the study of the reproductive system, we demonstrated that the morphology of the adult male reproductive system of *A. flintorum* and *K. obtusa* consists of pair of testes fused medially and show differences in the quantity of testicular follicles. Our study supports the potential for anatomical characteristics, such as the testes, testicular follicles and seminal vesicles, to be employed in phylogenetic analysis. These characteristics can provide distinct traits to elucidate the evolutionary relationships between different groups of stoneflies.

Additionally, our research into Plecoptera sperm morphology, although not directly comparable to previous ultrastructural studies, did highlight significant differences between *Anacroneuria* and *Kempnyia*. In both genera, the spermatozoa were long, filiform, distinguishable by size and composed of a distinct head (with acrosome and nucleus) and a flagellum. The spermatozoa of males and females of *Anacroneuria* exhibited reduced or absent acrosomes, while in males and females of *Kempnyia*, the acrosome was evident, but the nuclei

were commonly detached from the flagellum. The challenge of associating males and females within *Anacroneuria* arises from the slow evolution of spermatozoa in comparison to other morphological and genetic traits. Sperm cells tend to retain consistent basic structures and fertilization processes throughout evolutionary changes, resulting in closely related species having similar sperm morphology. This hinders the differentiation of species based solely on sperm characteristics, contributing to the difficulty of matching males with their corresponding females in *Anacroneuria*. In contrast to the challenge faced with *Anacroneuria*, we were able to identify species and associate males and females in the *Kempnyia* species used in this study. This identification was further supported by the external morphology of the individuals.

Thus, this study highlights the potential of internal characteristics, such as reproductive system structures and sperm morphology, as a valuable complementary approach to phylogenetic analyses. This could be crucial in order to bridge gaps in our understanding of Plecoptera phylogeny and improve our knowledge of these fascinating aquatic insects.