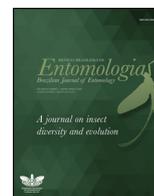




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The coffee leaf miner, *Leucoptera coffeella* (Lepidoptera: Lyonetiidae): identification of the larval instars and description of male and female genitalia

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ABSTRACT

The coffee leaf miner *Leucoptera coffeella* (Guérin-Mèneville & Perrottet) is a key pest in coffee producing countries. During their development, the larvae feed on the palisade parenchyma of the leaves, forming mines and necrotic areas. As a result, the photosynthetic area of the plant decreases, affecting coffee production. Despite the severity of the damage caused by coffee leaf miner (CLM), morphological aspects of the larval development and the adult genitalia remain unknown. This work presents the first morphological description of the four larval instars and the adult genitalia of *L. coffeella*. In each larval instar, we measured the Mean \pm SD (mm) of the cephalic capsules (1st 0,14 \pm 0,03; 2nd 0,25 \pm 0,04; 3rd 0,32 \pm 0,03; 4th 0,42 \pm 0,03) and observed the following morphological details: primary setae, prolegs, crochets and ecdysial line of the cephalic capsule. In the adults, we show how to differentiate adult sexing and observed the sexual structures present in both genitalia: male - bulbus ejaculatorius, valva, gnathos and aedeagus, and female - ovipositor, sclerite and corpus bursae.

Introduction

Annually, more than 160 million coffee bags are consumed on all continents (Conselho Nacional do Café, 2019). Coffee is produced in the countries of the neotropical region in America, where the coffee leaf miner *Leucoptera coffeella* (Guérin-Mèneville & Perrottet, 1842) occurs (Mey, 1994; Pereira et al., 2007). Even though Brazil is the world's largest coffee producer, the coffee leaf miner (CLM) is a key pest causing damages every year (Medina Filho et al., 1977; Vieira et al., 2006; Consórcio de Pesquisa do Café, 2020). The CLM infestation impacts severely the coffee yield and bean quality in all Brazilian producing regions (Parra et al., 1981; Pantoja-Gomez et al., 2019) (Figure 1).

The CLM is a monophagous pest (Reis and Souza, 1986) which feeds exclusively on coffee leaves, seriously compromising the health of the plant. *L. coffeella* has been reported as responsible for losses that can reach up to 87% drop in productivity (Neves, 2006). Depending on the season, the defoliation of the coffee plant can reach up to 75% (Reis and Souza, 1996; Neves, 2006).

The life cycle of *L. coffeella* is holometabolic, including the stages of egg, larva, pupa, and adult in its winged version (Box, 1923) (Figure 2).

After being described by Guérin-Mèneville and Perrottet in 1842, the immature forms were only described in 1923 by Harold Box. The larval phase penetrates the palisade parenchyma of the leaves and feeds there forming mines, which gives rise to the common name of this pest. The lesions caused by the mines cause necrosis, thus reducing the photosynthetic area, which can cause severe defoliation in more sensitive coffee genotypes, such as *Coffea arabica* (Medina Filho et al., 1977; Ramiro et al., 2004). The symptoms of this pest attack consist of mines that progress from light green to brown, as the larva develops and moves to new feeding sites.

The knowledge of life cycle phases is critical to allow the development of pest control methodologies, e.g., oviposition assays, infestation challenge to test pesticide products or resistant cultivars. Studies aimed at chemical control, biological control, transcriptome, and plant resistance to Lepidoptera species pests reported the use of specific larval instars. Wheatley and Crowe (1964) conducted a study to control of mining larvae, *Leucoptera meyricki* Ghesquiere, 1940, in the field with the application of insecticides sprayed on mined leaves in order to kill larvae of the 2nd, 3rd and 4th instar, decreasing the development of the infestation. Frago et al. (2002) conducted a study, in the laboratory, with the use of organophosphate insecticides in larvae of the 3rd instar

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of *L. coffeella*. Biological control agents of mining larvae have been studied by Draganova and Tomov (1998), in which the fungus species *Beauveria bassiana* (Bals.) Vuill., is a pathogen of last instar larvae of the species *Leucoptera malifoliella* (Costa, 1836). Camargo et al. (2015) conducted a transcriptome study with the larval stages of the tomato leaf miner *Tuta absoluta* (Meyrick, 1917). Perthuis et al. (2005) tested the resistance of transformed *C. canephora* 126 using fourth instar larvae of CLM and Meriño-Cabrera et al. (2018) conducted a study with plant defense as an alternative to control CLM, using larvae of the fourth instar.

Adult sexing is used in experiments aimed at the mortality rate of females and males, laboratory rearing, sexual behavior, and pheromone employment. Information about the male and female mortality rate of *L. coffeella* was observed by Notley (1948). Katiyar and Ferrer (1968) conducted a study with CLM adult rearing techniques, presenting a cage model and suggesting feeding to keep specimens in laboratory conditions. The sexual behavior of CLM adults has been described in

detail by Michereff et al. (2007). Malo et al. (2009) conducted a study on the chemical composition of pheromones released by females of *L. coffeella*. Although the sexual dimorphism in adults of *L. coffeella* is mentioned in the literature (Parra, 1985), there is no information available about the male and female genitalia of this species or the external aspects to differentiate them.

Despite the economic importance and geographical scope of the CLM, further studies on the larval morphological characteristics and sexual dimorphism of *L. coffeella* are lacking. In this work, we present a detailed study on the morphology of larvae for the identification of the four larval instars and description of male and female genitalia of *L. coffeella*.

Material and methods

Obtaining insects and rearing: *L. coffeella* collections were carried out at Embrapa Cerrados (Federal District, Brazil, 15°36'15.1" S, 47°42'44.4" W) and Embrapa Genetic Resources & Biotechnology (Federal District, Brazil, 15°43'52.8" S, 47°54'10.2" W) between May and August 2020. Leaves with mines and pupae were collected directly from the coffee plant or leaves fallen on the ground around the plant. The leaves with larvae and pupae were taken to the laboratory where they were placed in rearing containers until adult emergence. A cotton soaked with a sugary solution of water with industrialized sugar was offered *ad libitum* for adult feeding.

Larvae: To examine the larvae and determine the number of instars, the infested leaf mines were opened using tweezers and scalpels and observed with a Leica EZ-4 © stereomicroscope. Cephalic capsule width and body length measurements were made using a Leica M205C © stereomicroscope and processed with the Leica LAS © V3.8 to morphometry and image acquisition software. The width of the cephalic capsule and the body length of 50 larvae were measured in each of the four larval instars. Larval morphological characteristics, associated with cephalic capsule measurements, follow Nielsen and Common (1991). To identify morphological characteristics to distinguish the larval stages, we used (1) presence of primary body setae in the first instar, (2) presence or absence of prolegs in the third instar, (3) presence or absence of crochets in the fourth instar, (4) presence or absence of ecdysial line in the frontal suture of the fourth instar. To observe these structures, the cephalic capsules were processed through diaphanization with 10% KOH heated for about 2 min, and larvae were processed through diaphanization with 10% KOH heated for about 6 minutes and then washed in water. The images were taken with a Leica DM-2000 © microscope coupled to a photographic camera and analyzed with Leica LAS © V3.8 software.

Sexing: To identify the sex of the adults, the specimens were individualized in Eppendorfs and observed in a ventral view, to analyze the last abdominal segment, with Leica EZ4 © stereomicroscope.

Genitalia: Male and female genitalia were processed through diaphanization of the abdomen with 10% KOH heated to 70°C, for about five minutes. Subsequently, they were washed in water and dissected in 70% alcohol. The photographs were taken with light microscopy (LM) using a Leica DFC295 camera mounted on a Leica M205C © stereomicroscope and Leica DM2000 © microscope. The specimens were measured (mm) with the Leica LAS © V3.8 software. The terminology of Nielsen and Common (1991) was used for the external morphology of the immature; Box (1923) for external adult morphology; Bradley and Carter (1982); Mey (1994) and Solis and Metz (2016) for male genitalia; Schmitt et al. (1996) and Matthews and Miller (2010) for female genitalia.

Map: The incidence map of the coffee leaf mining in Brazil was built using the SimpleMappr © program.

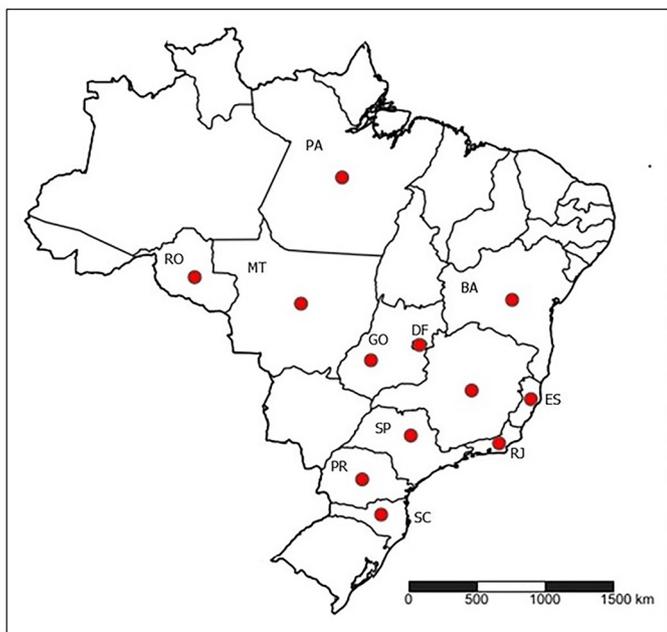


Figure 1 States of Brazil with the incidence of *Leucoptera coffeella* (red dots). Rondônia (RO), Mato Grosso (MT), Pará (PA), Goiás (GO), Distrito Federal (DF), Bahia (BA), Minas Gerais (MG), Espírito Santo (ES), São Paulo (SP), Rio de Janeiro (RJ), Paraná (PR), Santa Catarina (SC).

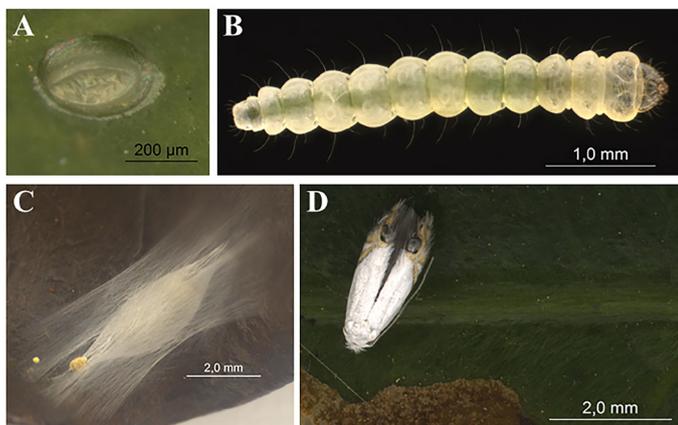


Figure 2 *Leucoptera coffeella* life cycle phases. (A) Egg Stage. (B) Larval stage. (C) Pupa stage. (D) Adult.

Results

Morphological characteristics and chaetotaxy of the head and thorax were used to identify the four larval instars of *L. coffeella*. Also, we present a detailed study of the genitalia of males and females to better characterize both sexes.

Immature forms: 1st larval instar (Figure 3A, B). Cephalic capsule variation 0,09–0,21 mm and body length variation 0,43–1,16 mm. Body with translucent whitish coloration, pale yellow cephalic capsule with a defined chewing mouthpiece type (Figure 3B), primary setae on the side of the body (Figure 4A), and without prolegs. **2nd larval instar** (Figure 3C, D). Variation of the cephalic capsule 0,19–0,32 mm and variation in body length 0,71–1,70 mm. Body with pale yellow coloration, first body segment wider than the cephalic capsule, body setae longer than in the first instar and without prolegs. Mouthpiece with small spines (Figure 4B). **3rd larval instar** (Figure 3E, F). Variation of the cephalic capsule 0,27–0,44 mm and variation in body length 1,39–2,38 mm. Body and cephalic capsule with yellowish coloration, long body setae, presence of prolegs without defined crochets (Figure 4C). **4th larval instar** (Figure 3G, H). Cephalic capsule variation 0,35–0,54 mm and body length variation 2,54–4,71 mm. Yellowish body, prolegs with crochets in the shape of a uniordinal circle (Figure 4D, E), and cephalic capsule with ecdysial line above the adfrontal suture (Figure 4F).

The morphometry of the immature forms was made based on the width of the cephalic capsule. The head grows in geometric progression and the width increases in a constant ratio, showing that there is variation in the size of the head in changing the larval instars as shown

by Dyar (1890), Bigger (1969), Ecole et al. (1999), Delbac et al. (2010). The average cephalic capsule width and body length of the different instars are shown in Table 1.

Imago. Male (Figure 5A). Average body length of 1,98±0,03 mm. **Head.** White scales on the dorsal apex. Base of the antenna white, with brownish length. **Thorax.** Covered with white scales. **Legs.** Long white scales. **Wings.** Long and thin, with two rounded black dots surrounded by yellowish scales. Apex of the wings with a row of black scales, and with a “V” shape facing the posterior region of the body (Figure 5B). **Abdomen.** White colored tergites. In ventral view, the last bipartite segment (Figure 6A). **Genitalia.** Tergite 8 with coremata, forming a pair of elongated limbs with setae at the apex (Figure 7A). Bilobate sternite 8, with an oval bulbus ejaculatorius (Figure 7A). Bipartite valva, setose, and with small teeth in the internal region of the apex (Figure 7B). Gnathos elongated, forming a thin pair of arms (Figure 7C). Short, cylindrical, and slender aedeagus at the apex (Figure 7D). **Female** (Figure 8A): Similar to the male except by the average body length of 2,11±0,10 mm. Apex of the wings with a “C” shape facing

Table 1

Mean (±SD) of variation in cephalic capsule measurements and body length in the 4 instars of *Leucoptera coffeella*.

Larval Instar	no.	Cephalic capsule width (mm)		Body length (mm)	
		Range	Mean ±SD	Range	Mean ±SD
1 st	50	0,09 - 0,21	0,14±0,03	0,43 - 1,16	0,64±0,15
2 nd	50	0,19 - 0,32	0,25±0,04	0,71 - 1,70	1,22±0,23
3 rd	50	0,27 - 0,44	0,32±0,03	1,39 - 2,83	2,06±0,37
4 th	50	0,35 - 0,54	0,42±0,03	2,54 - 4,71	3,60±0,63

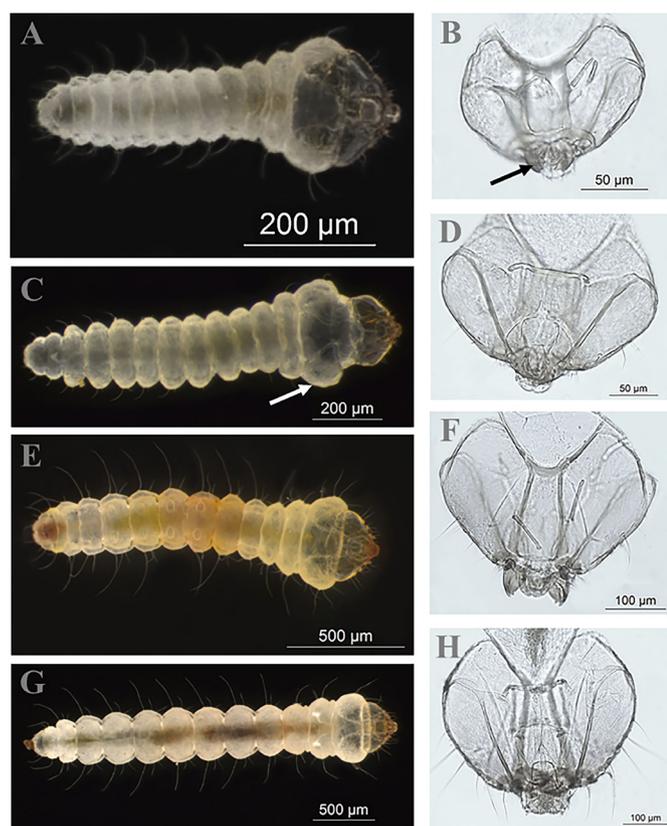


Figure 3 Immatures of *Leucoptera coffeella*. (A) First instar larva. (B) Cephalic capsule of the first larval instar. Arrow indicates chewing mouthpiece. (C) Larva of the second instar. Arrow indicates first body segment. (D) Cephalic capsule of the second larval instar. (E) Larva of the third instar. (F) Cephalic capsule of the third larval instar. (G) Larva of the fourth instar. (H) Cephalic capsule of the fourth larval instar.

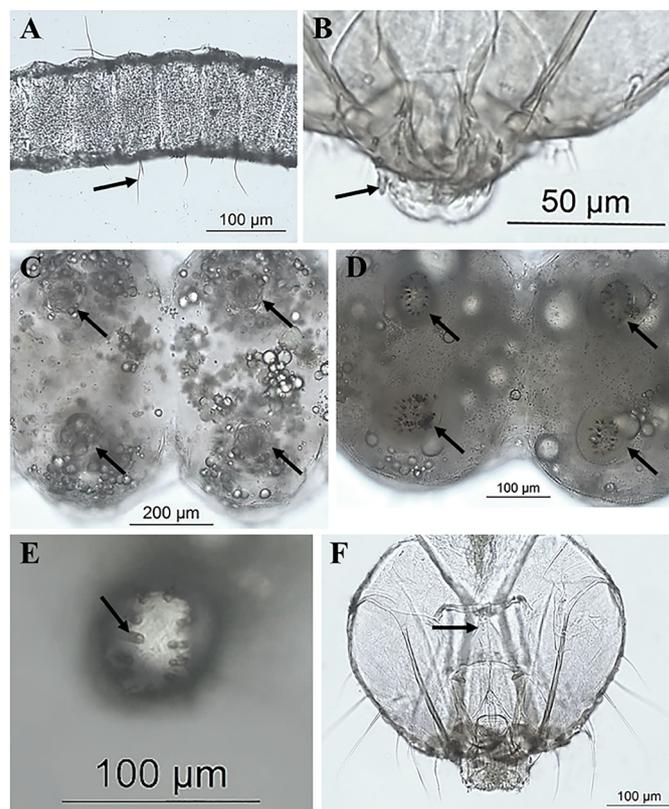


Figure 4 External morphological characteristics of coffee leaf miner *Leucoptera coffeella* larvae. (A) Dorsal view of the larva body of the first larval instar. Arrow indicates primary seta. (B) Chewing mouthpiece of the second larval instar. Arrow indicates spine. (C) Ventral view of the third larval instar. Arrows indicate prolegs without crochets. (D) Ventral view of the fourth larval instar. Arrows indicate prolegs with crochets. (E) Ventral view of crochets in the shape of uniordinal circle in a fourth instar larva. Arrow indicates crochets. (F) Cephalic capsule of the fourth larval instar. Arrow indicates ecdysial line.

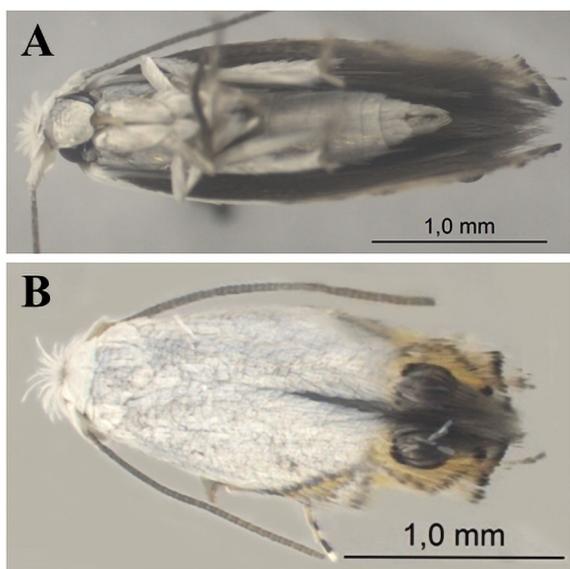


Figure 5 *Leucoptera coffeella*, adult male. (A) Male ventral view. White-colored body scales. (B) Male dorsal view. Apex of the wings with black dots surrounded by yellow scales, with a "V" shape facing the posterior region of the body.

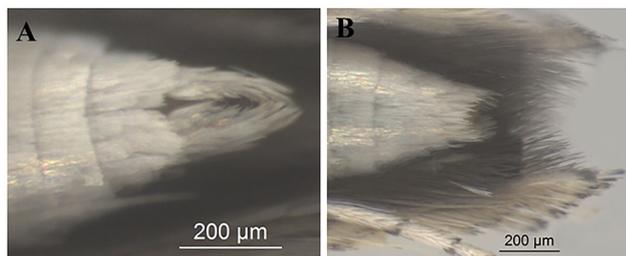


Figure 6 *Leucoptera coffeella* last abdominal segments. (A) Male last abdominal segment in ventral view, with a white bipartite segment. (B) Female last abdominal segment in ventral view, with a white tubular shape.

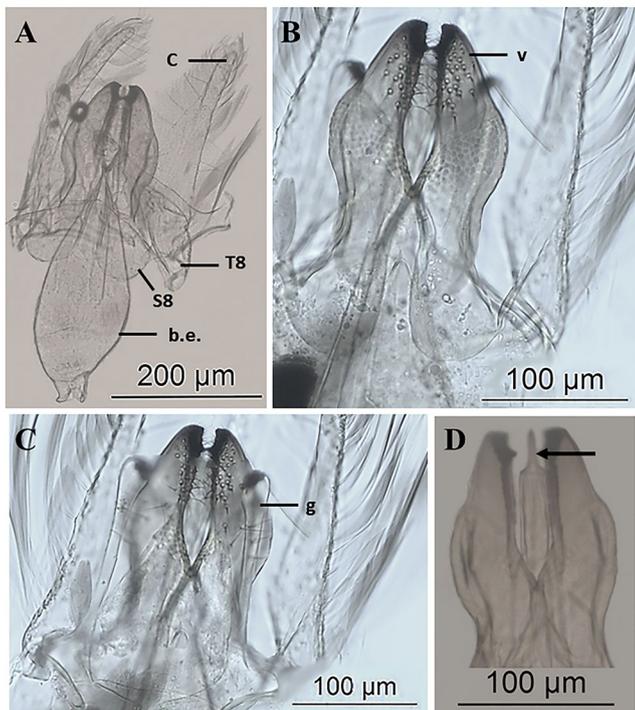


Figure 7 *Leucoptera coffeella*, male genitalia. (A) Tergite 8, coremata, sternite 8 and ejaculatory bulb ventral view. (B) Valva ventral view. (C) Gnathos ventral view. (D) Aedeagus dorsal view. Arrow indicates apex of aedeagus. C = coremata, T8 = tergite 8, S8 = sternite 8, b.e. = bulbus ejaculatorius, v = valva, g = gnathos.

the anterior region of the body (Figure 8B). In ventral view, the last segment of the abdomen with a tubular shape (Figure 6B). **Genitalia.** Bilobed ovipositor with long apical setae (Figure 9A). Scleritis of the ductus bursae apically tapered and enlarged in the anterior region; extremities of the anterior region are tapered and have a concave shape facing the apex (Figure 9B). Oval membranous corpus bursae with small spines (Figure 9C).

Discussion

Lyonetiidae is a family of lepidopterans consisting of 204 species distributed in 32 genera (Zhang, 2011). *Leucoptera* Hübner brings

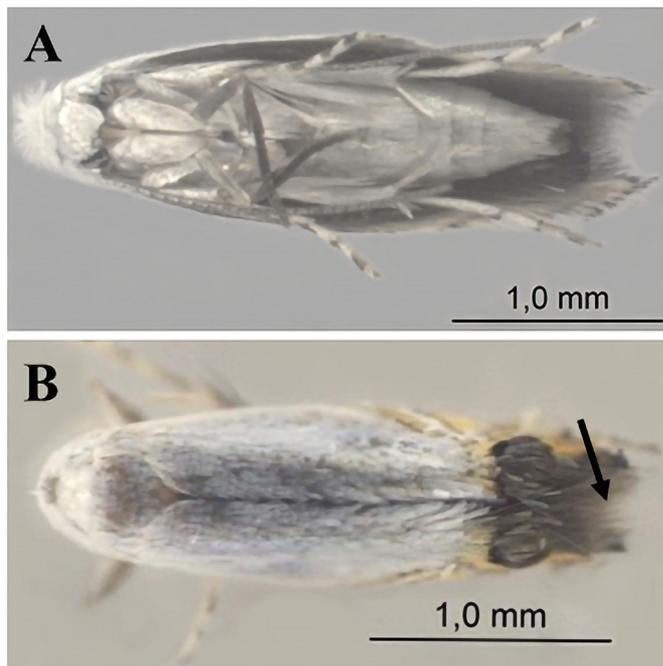


Figure 8 *Leucoptera coffeella*, adult female. (A) Female ventral view. (B) Female dorsal view. Arrow indicates the apex of the abdomen with a "C" shape facing the anterior region of the body.

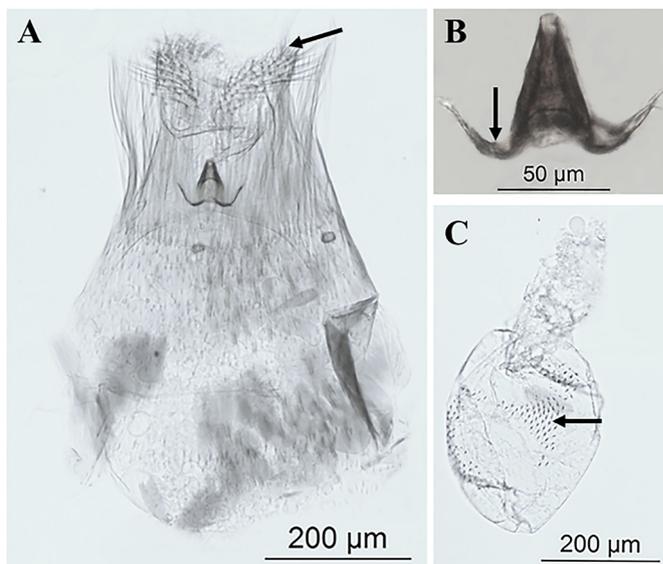


Figure 9 *Leucoptera coffeella*, female genitalia. (A) Ovipositor ventral view. Arrow indicates ovipositor lobe. (B) Sclerite ventral view. Arrow indicates the sclerite's concave end. (C) Corpus bursae ventral view. Arrow indicates spines.

together leaf-mining moths that cause economic damage worldwide (Seven, 2006; Magalhães et al., 2010). Although *L. coffeella* causes serious losses to the coffee crop, knowledge gaps about the biology of the CLM remain to be fulfilled and made publicly available.

The CLM damage causing agents are all the larval stages before pupation (Notley, 1948). Although Notley (1948) declared that *L. coffeella* has four larval instars, there is no description of the morphological characteristics of each instar. The determination of Lepidoptera larval instars can be done by measuring the cephalic capsule width (Dyar, 1890; Ecole et al., 1999). Besides width of the cephalic capsule, lepidopteran instars may also have different morphological characteristics (Nielsen and Common, 1991). We observed that the 1st larval instar of *L. coffeella* has primary arrows and no prolegs; 2nd instar has no prolegs; 3rd instar presents prolegs without defined crochets; and 4th instar presents prolegs with defined crochets and ecdysial line. The larval instars of *L. coffeella* can be determined by the union of the head capsule measurements and the external body morphological characteristics.

To our understanding, the instar determination by the larval age is less precise than the morphological method we described here. In Navarro-Gutiérrez and Gallardo-Covas (2009), a bioassay study that referred the larval instars according to their age, reported that, in laboratory at 27°C, the 1st instar correspond to 3 days, 2nd to 7 days, 3rd to 11 days and 4th to 13 days. However, the CLM life cycle is shorter in dry climates combined with higher temperatures (Wolcott, 1947; Dantas et al., 2020). Hence, it is highly probable that a natural variation is reflected in the life cycle timing due to the climatic conditions in the coffee crop (Costa et al., 2012).

The identification of males and females is essential to understand sexual behavior, sex-related mortality rates, and pheromone production (Notley 1948, Katiyar and Ferrer, 1968, Michereff et al., 2007, Malo et al., 2009). Semiochemicals can be used to alter the natural reproductive behavior of insects, decreasing pest population levels (Cardé and Minks, 1995; Dantas et al., 2020). The CLM sexual pheromone was used to determine the pattern of pheromone production by females, as an indirect measure of the behavior calling for sexual intercourse (Lima et al., 2008). However, although several previous studies have used adult sexing, none has clearly explained how to differentiate males from females, so the methodology of sexing males and females has remained unclear.

Alves et al. (2006) presented for the first time the structure of the male reproductive system of *L. coffeella*, but it did not include information regarding the morphology of the male genitalia. Thus, we present here the morphology of the genitalia of males and females of this species, which was not yet known.

The elements in the male and female genitalia of *L. coffeella* corroborating the structures described for the other species of Lyonetiidae (Bradley and Carter, 1982; Mey, 1994). The *L. coffeella* male exhibits bulbous ejaculatorius and aedeagus. These structures are common with the male genitalia of *L. malifoliella*. In *L. coffeella*, tergite 8 with setose coremata, bilobed sternite 8, bipartite and setose valve, and elongated gnathos were observed, forming two thin arms. In contrast, pleurallobus, vinculum, pedunculus, subanalplatte, tuba analis are observed in *L. malifoliella* and this species does not have a valva (Mey, 1994). In the female genitalia of *L. coffeella*, the structures we observed were bilobed ovipositor, sclerite in a conical shape, and oval corpus bursae with small spines. The corpus bursae was identified by Mey (1994) in the general structure of the *Leucoptera* female genitalia. The sclerite structure was identified by (Schmitt et al., 1996) in another genus belonging to the Lyonetiidae family, where the female genital structure was characterized by a cruciform “T” shape, while in *L. coffeella* the sclerite has a conical shape.

The characterization of larval instars can be important to identify targets that are more susceptible to efficient and narrow control by natural or synthetic approaches. Equally important is the sexing of the adult in experiments to control CLM, such as the study of control methods based on pheromones. The dissection of adult specimens for genitalia studies confirmed that the sexing method described here by observing external morphology is correctly associated with male or female. These results may support innovative and improved control strategies for CLM Integrated Pest Management (IPM).

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution statement

Authors JRP and EVSA conceived and supervised the research.
 Authors IOM, JD, LV and JB conducted the trials.
 Authors JD, JRP and EVSA contributed with the biological materials.
 Authors IOM, JRP and EVSA analyzed data.
 Authors IOM, JD, JRP and EVSA wrote the manuscript.
 Author EVSA secured funding.

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