



RESEARCH ARTICLE - TERMITES

Step-by-step Dissection Protocol of Apicotermitinae Worker (Blattaria: Isoptera)

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Abstract

Apicotermitinae are soldierless termites highly abundant in tropical forests. The taxonomy of this subfamily is based on characters of worker caste and winged forms when present. However, the procedures necessary to dissect termite workers to observe their external and internal morphological characteristics are not well-detailed in any study. Here, we describe a step-by-step protocol for worker dissection of soldierless termite species. We suggest the use of Polyvinyl Alcohol (PVA) for cleaning and visualization of the gizzard and enteric valve, and glycerin to remove tergites and sternites and describe in detail the dissection of the gizzard and enteric valve, and how to visualize the insertion of Malpighian tubules.

Introduction

Termite taxonomy is mainly based on the external morphology of the soldier caste, as it has the most significant interspecific variation (Constantino, 1999). However, this caste is absent in all neotropical Apicotermitinae (Inward et al., 2007; Bourguignon et al., 2016). Although workers and alates are used in the descriptions of Apicotermitinae taxa, most identifications are made with the worker caste, as it is the only one available in most samples. Only a few genera, such as *Tetimatermes*, *Ruptitermes*, and *Tonsuritermes*, and a few species can be identified only by external characters (Fontes, 1986; Acioli & Constantino, 2015; Constantini et al., 2018). However, for most of these taxa, observing characters in the mandibles and internal characters in the digestive tract is necessary.

The observation of the intestine segments and the morphology of the mandibles of termite workers can be done through the dissection of the specimens, performed with the aid of fine-tipped tweezers, entomological pins, and micro-pins

(Constantino, 1999). Conducting studies on genera and species through dissection can be especially challenging due to certain species' diminutive size and characters (Hanley & Ashe, 2003). In addition, patience and training are necessary to dissect these specimens, as a misstep can ruin the specimen (Constantino, 1999). Despite the range of studies on termite workers' morphology regarding mandibles (Deligne, 1999), enteric valve (Donovan, 2002), and gut morphology (Noirot, 2001; Rocha & Constantini, 2015; Rocha et al., 2019), those studies do not summarize the specimen preparation and dissection procedures. Thus, one of the main obstacles to speeding up the taxonomic knowledge spreading of Apicotermitinae is the need for detailed protocols of techniques for visualizing the morphological structures. Therefore, a general protocol for the dissection of termite workers becomes necessary to observe the structures of the mandibles and digestive tract.

We aim: 1) to produce a step-by-step protocol to dissect a termite worker to observe the structures commonly used in descriptions and for species identification, such as the shape



of the digestive tract, enteric valve, gizzard, and insertions of Malpighian tubules, and 2) illustrate the instruments used for the dissection, as well as the reagents used. This protocol was developed based on the author's experience with the group and aimed to facilitate the training of new taxonomists and make the group identification more accessible to taxonomists who need to identify or morphotype Apicotermitinae specimens.

Material and Methods

The mandibles and digestive tract terminology followed Fontes (1987) and Noiro (1995; 2001). The specimens were photographed with a Leica DFC295 camera coupled to an M205 stereoscopic microscope. The Malpighi tubules and enteric valve were photographed with a Leica DFC295 camera attached to a CTR5000 microscope. Inkscape software was used to make the drawings, and an ocular micrometer was used to measure the structures. To dissect specimens, we utilized stylets made with insulin needles, entomological micropins, brush bristles, or human hair (Fig 1A, D). To handle smaller specimens and dissected structures, we suggest a featherweight tweezer. A Petri dish or shallow crucible is used to hold the specimen. It is filled with alcohol and sterilized filtered sand at the bottom to visualize external structures (Fig 1G) and glycerin for specimen dissection (Fig 1F).

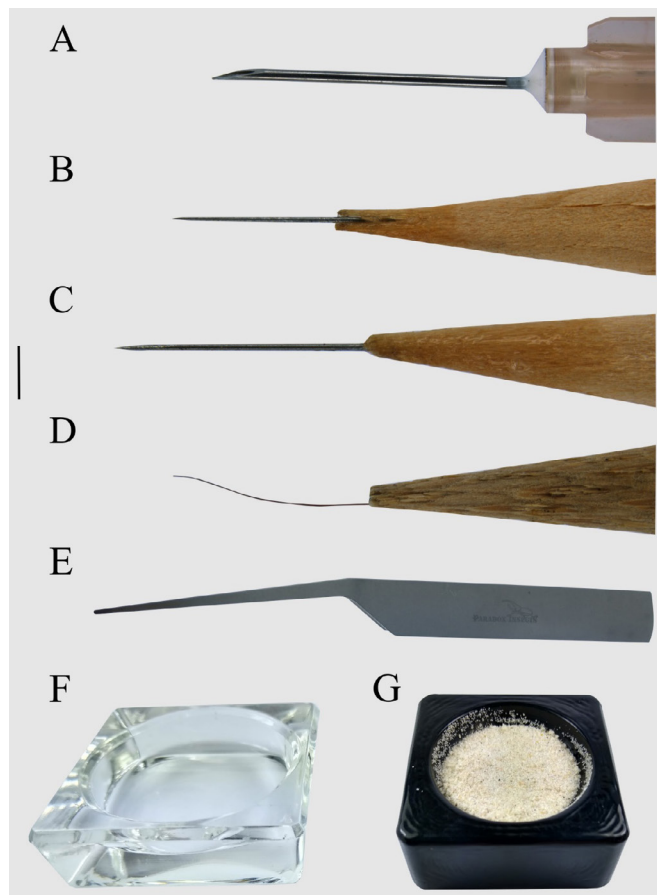


Fig 1. Materials for dissection of termite workers. A: needle pin. B: entomological micro-pin. C: pin with a single bristle. D: Pine-tipped malleable tweezer. E: transparent crucible with glycerin. F: black crucible with washed sand. G: scale bar. A–D = 0.2 mm.

We used PVA as a mounting medium (Downs, 1943) to mount the slides of the enteric valve and gizzard.

Preparation of specimens and tools

To observe the external morphology of the specimens, we recommend using a shallow crucible or Petri dishes with 80% alcohol, preferably with a 5 mm layer of washed sand to facilitate positioning (Constantino, 1999) (Fig 1F, G). We suggest using glycerin P.A. ($C_3H_8O_3$) for specimen dissection without sand (Fig 1F). Glycerin, as it is more viscous than alcohol, improves specimen handling and facilitates the removal of its structures, in addition to hydrating and improving visualization. Dissection can be performed with stylets made with one or zero pins and entomological micropins (Fig 1A, B) (Constantino, 1999). The tools to be utilized depend upon the size of the specimen or the structure to be dissected. While tools with broader tips may be better for the bigger specimens, smaller species, such as those belonging to the *Anoplotermes* genus, require stylets made with micropins, especially for the enteric valve dissection. To position more delicate structures and remove dirt, we suggest using a pin with a single bristle (Fig 1D). This pin can be made from a paintbrush, removing all the bristles until only one is left or gluing a segment of human hair to the end of a rod. The styles can be made using wooden skewers. A transversal slice must be made at the tip of the wooden skewer. After, the pin is inserted deep at the slit. Superglue can be used to fix the pin at the wooden skewer tip. We recommend a set of paired styles of different pin sizes, including a set of micro-pins styles for different situations. In addition, it is necessary to use fine-tipped malleable tweezers to handle the specimens and their morphological structures (Fig 1E).

Results

Step-by-step Dissection Protocol

External morphology

Some external structures are relevant to identify some Apicotermitinae genera and species (Rocha et al., 2019). Some examples are the dilated tibiae of *Tetimatermes* and the bristles pattern of the coxae of *Ruptitermes*. As those structures need no dissection to be visualized, they will not be addressed.

Mandibles (Fig 2A–E)

The specimen is positioned in dorsal view in the Petri dish to visualize the mandible, and then the labrum and clypeus are removed. For this procedure, a pin is inserted in the cephalic capsule's dorsal part and another in the labrum and clypeus. Then, a gentle movement is performed in the proximal-distal direction, starting from the base of the structures (Fig 2A). The teeth and mandibular condyles can be visualized after total removal of the labrum and clypeus (Fig 2B). This technique can be helpful to avoid the total destruction of the individual's head during dissection. However, because most workers die

with their mandibles closed, some structures cannot be fully visualized, requiring the total removal of the mandible. Thus, the musculature that connects the condyles to the head must be cut to fully visualize the mandibular structures. Next, a second stylet must be inserted into the head of the specimen to prevent the structure from moving during the cutting (Fig 2C, D). An alternative procedure is to dissect the mandible from the ventral position, first removing the maxillae and labium and later cutting the mandibular abductor muscles and pressing the labrum between the mandibles. This allows us to visualize the mandibles without removing them from the head.

Digestive tube (Fig 3A-J, supplementary material S1)

For the dissection, it is necessary to use two micro-pin stylets to start the dissection and a bristle or human hair stylet to clean the intestine. The total removal of tergites and sternites must be done to visualize the digestive tract better. First, the individual is positioned in the crucible or the Petri dish with glycerin in a dorsal view. Next, with the aid of a stylet, a longitudinal cut must be made in the abdominal pleura. A second stylet is inserted into the individual's chest to prevent it from moving during the cutting process (Fig 3A, B).

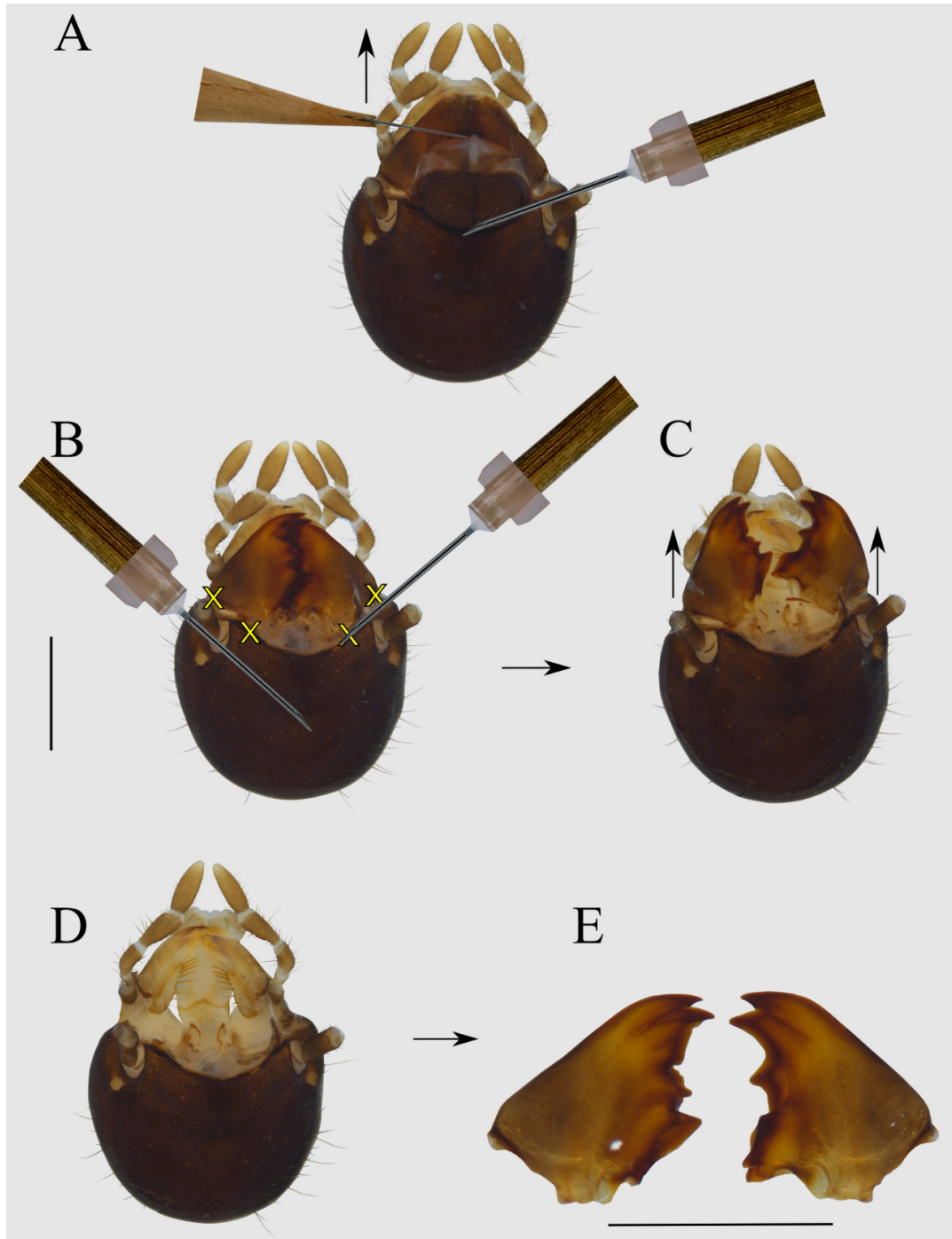


Fig 2. Dissection of worker mandibles of the *Ruptitermes arboreus*. A: Removal of the clypeus and labrum. B: cut into condyles of the mandibles. C: mandibles removal. D: Termite worker head without mandibles. E: termite worker mandibles. Scale bar: A–E = 0.5 mm.

The tergites should be carefully pushed towards the dorsal region of the abdomen, leaving this region fully exposed (Fig 3B). An insulin needle stylet can be used for this procedure if it is a larger individual (Supplementary Material video 1).

Care should be taken with P5 as it is often easily removed during this process. Next, the sternites are pushed with the specimen in the dorsal position, always holding the specimen with another stylet. After removing the tergites and sternites, the abdomen should be entirely exposed (Fig 3C and Supplementary Material video 1). Few Apicotermite species have dehiscent organs so large. For this protocol to comprehend the most species possible, we opt to maintain the dehiscent organs in the images. This technique is helpful

for a photograph of the specimen with the abdomen exposed. Then, holding the specimen in dorsal view, the abdomen is removed from the rest of the body. For that, a stylet is inserted into the metanotum, and, using a second stylet, the abdomen must be pushed away from the rest of the body, starting from the crop. Subsequently, the individual must be placed in a crucible or Petri dish containing alcohol to clean the remaining musculature trapped in the intestine. At that moment, the individual is ready to be photographed in the four views or drawn using a camera lucida (Supplementary Material video 1 and Supplementary Material Fig 1A–D). Glycerin can dry out the contents of some parts of the intestine, requiring its placement in alcohol.

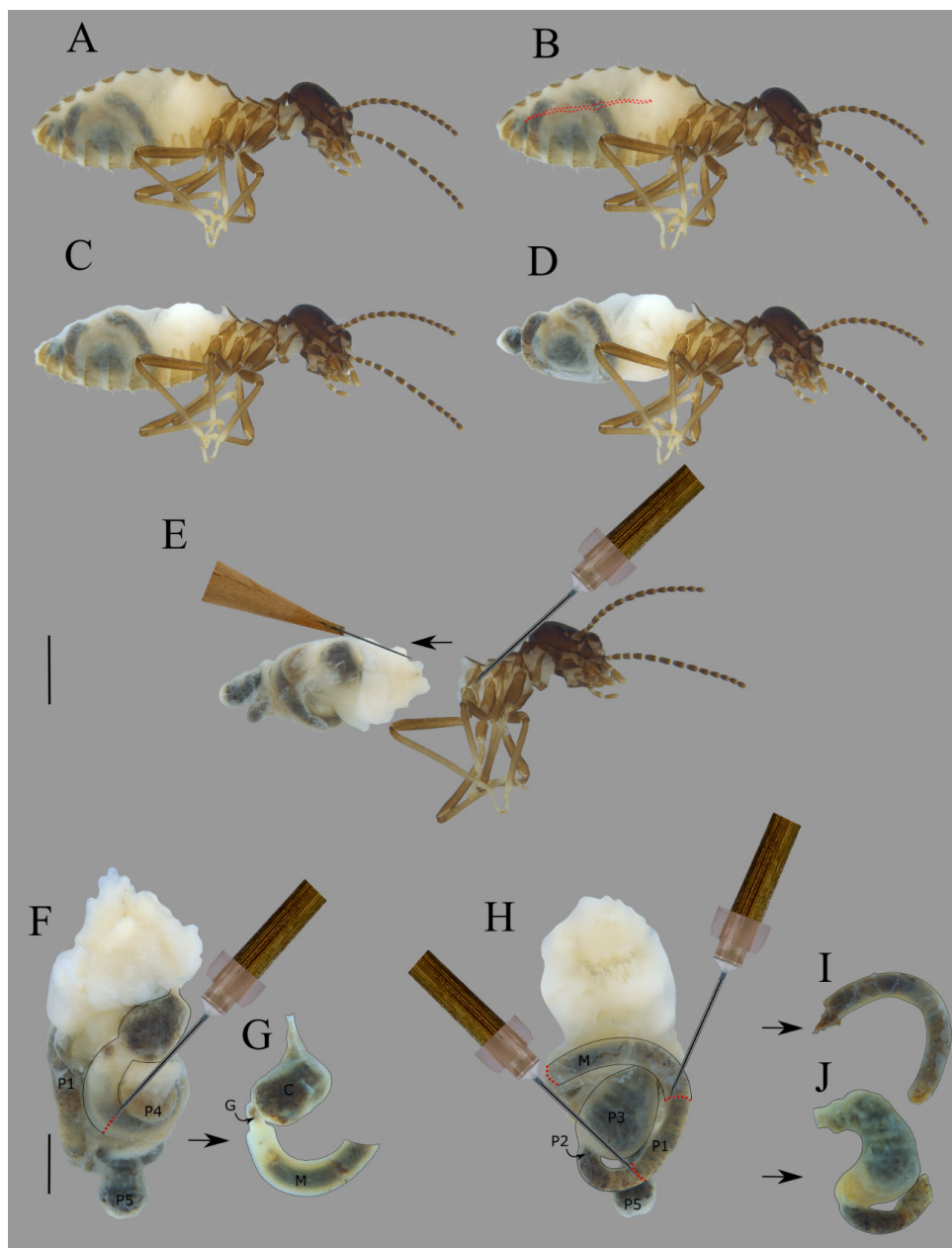


Fig 3. Dissection of worker gut of *Ruptitermes arboreus*. A: whole worker. B: cut in the pleura. C: Tergites removed. D: tergites and sternites removed. E: Movement against the chest to remove the gut. F: cut in the mesenteron and crop. G: crop and mesenteron removed for gizzard visualization. H: mesenteric cuts and P1 and P1 and P3. I: mesenteron and P1 removed for visualization of Malpighian tubules. J: P3 and P1 were removed to view the enteric valve. C = crop, G = gizzard, M = mesenteron, P=first proctodeal segment, P2=enteric valve, P3 = paunch, P4=colon, P5 = rectum. Scale bar: A–E = 1 mm; F–J = 0.5 mm.

Gizzard (Fig 3 and 4)

To visualize this structure, a longitudinal cut in the median region of the mesenteron is made to remove the crop and gizzard (Supplementary Material video 2). A second stylet is inserted at the apex of the crop to prevent the structure from moving during cutting (Fig 3F). Then, a horizontal cut is made at the base of the crop, pressing the structure against the crucible (Fig 3G). Next, with the aid of a stylus with bristles, the interior of the gizzard begins to be cleaned. Cleaning should be done by pressing the structure very lightly against the crucible. A stylus with a brush bristle or human hair prevents the structure from being damaged. After cleaning the structure, a longitudinal cut is made by pressing the gizzard wall against the crucible so that it is fully open (Fig 4A).

Finally, the structure is placed in Polyvinyl Alcohol (PVA) for consumption by the musculature (Fig 4B).

Malpighian tubules (Fig 3 and 4)

Malpighian tubules are usually located at the connection of the mesenteron and first proctodeal segment. However, for *Apicotermitinae*, the Malpighian tubules are inserted at the mesenteron. To find the Malpighian tubule insertion, it is necessary to find its connection, which is often sinuous or has a mesenteric tongue. Then, a longitudinal cut is made in the middle part of the mesentery and another right at the beginning of the first proctodeal segment (Supplementary Material video 2 and Fig 3H, I). Next, a second stylet must be inserted in the mesentery or first proctodeal segment to prevent

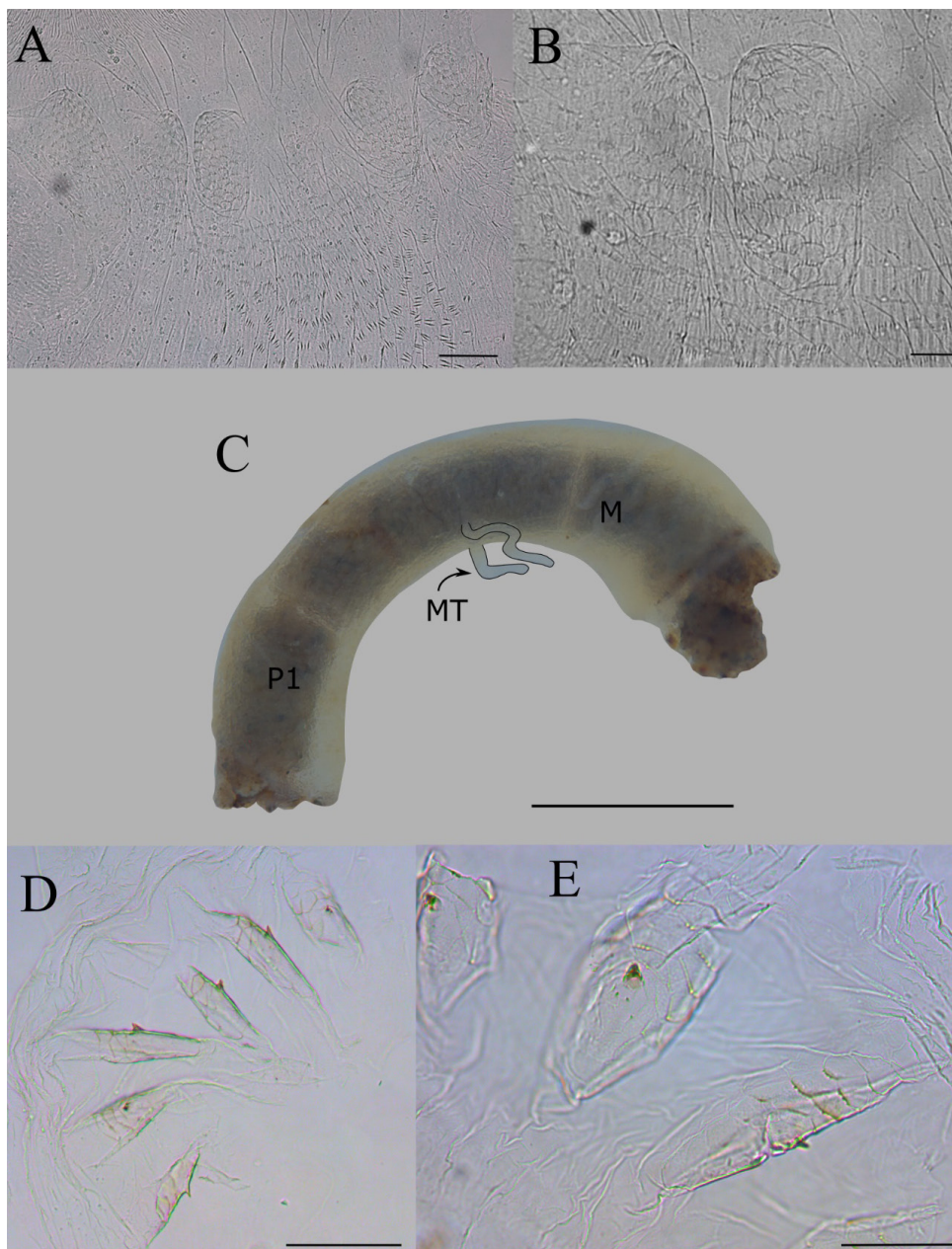


Fig 4. Dissection of worker gut of *Ruptitermes arboreus*. A: dissected gizzard. B: gizzard details. C: Malpighian tubules. D: enteric valve. E: Enteric valve details. Scale bar: A–B = 200.00 μ m; C = 0.5 mm; D–E = 50 μ m.

the intestine from moving during the cut. After removing the connection, the mesentery and the first proctodeal segment must be cleaned with the stylet with a brush bristle. Cleaning must be done sequentially and by lightly pressing the structure against the crucible. This structure is very sensitive, and cleanliness is essential for visualizing the insertion of Malpighian tubules (Fig 4C).

Enteric valve (Fig 3 and 4)

For enteric valve dissection, a cut in the paunch and a cut in the first proctodeal segment are often necessary (Supplementary Material video 2). Some valves easily separate from the paunch, leaving them exposed. However, some are more complicated, and we will follow the same steps for both. After removing the paunch and the first proctodeal segment (Figure 3H, J), it is necessary an internal cleaning of these structures (Supplementary Material video 3). Cleaning with the stylet with a brush bristle makes it easy to separate the valve from the belly. Then, a longitudinal cut is made in the valve, pressing its wall slightly against the bottom of the crucible (Constantino, 1999) (Figure 4D); later, the valve must be placed in PVA to degrade the musculature. After opening and without musculature, it is possible to observe the valve structures more efficiently (Figure 4E). It is essential to notice that some genera and species have taxonomic characters that can be observed in loco before opening the enteric valve, such as *Dissimulitermes* reduced plate.

Enteric valve seating

To visualize the enteric valve seating, isolating part of P3 and P1 from the gut is necessary. This step is essential, especially for identifying some genera, such as *Compositermes*.

Mixed segment

The mixed segment also possesses relevant characters for Apicotermatinae species identification. Noirot (2001) describes the possible states of the mixed segment, which can be absent, present, or vestigial, in which there is no mesenteric tongue but an oblique portion of the midgut. For the observation of this structure, it is only needed to remove the sternites, as shown for the dissection of the digestive tube (Fig 3A-J).

Storage of termite parts and dissected specimen

The dissected parts and the dissected specimen should not be discarded but stored in a separate microvial attached to the vial of the original sample. The slides must be stored separately in a proper slide box to avoid the need to dissect other specimens in the future.

Discussion

This protocol details the step-by-step procedure for dissecting an Apicotermatinae specimen, which also serves workers from other termite subfamilies. We have shown that

the process of dissecting and treating the internal structures of the intestine (gizzard, Malpighian tubule connections, and enteric valve) is important.

Taxonomists have almost ignored the gizzard because of its generally regressed state in soil-feeding termites (Noirot, 2001; Arias et al., 2020). However, this structure has variations that can be used as phylogenetic characters and to discriminate species (Arias et al., 2020). The enteric valve is particularly variable in the Apicotermatinae (Donovan et al., 2002), making it one of the main structures to separate and identify species and is always described (Constantino, 1999). Both the gizzard and the enteric valve need to undergo a cleaning, which removes the musculature surrounding them. However, it is difficult to perform this cleaning mechanically without the aid of an acid that serves to dissolve the musculature and facilitate its removal. In this study, we suggest using Polyvinyl Alcohol (PVA) to clean and visualize the gizzard and enteric valve. PVA is a synthetic vinyl alcohol polymer used to assemble permanent slides of biological and botanical specimens, as it does not dehydrate the tissue. In addition, its primary laboratory function is the assembly and cleaning of specimens and biological tissues (Downs, 1943). In this study, we suggest that PVA can be used to assemble semi-permanent blades of the gizzard and enteric valve. Because these structures are delicate, PVA can continue acting on and damaging the tissue. Some classic studies of termite taxonomy use other methods for mounting the blades, such as Swan's Berlese (Sands, 1972), the Hoyle Medium (Constantino, 1999), and potassium hydroxide (KOH), which can be used to remove musculature. Visualizing the insertion of Malpighian tubules requires a specialized technique. However, dissecting and visualizing this structure without applying a clarifying agent is possible. The location of this insertion is always located between the junction of the midgut (mesentery) and the hindgut (first proctodeal segment) (Noirot, 2001). This visualization requires practice and trained eyes, especially in small specimens, such as an *Anoplotermes* worker.

Despite being central to ecosystem functioning, termites still need to be studied compared to other taxa (Jouquet et al., 2016). We believe that this is in part due to the difficulty of collecting and identifying them, mainly because Apicotermatinae can compose about a third of termites of a sampled area (Ackerman et al., 2009), and only in the last decades, the taxonomy of this group is being regularly worked on. We hope this protocol will enable researchers to begin working with this group independently of being close to a termite research laboratory or specialist. We reiterate that it requires a long time of practice and experience dealing with multiple species before being able to dissect important samples or with a few individuals. Moreover, this protocol may not apply to some samples, such as specimens from type series, which need experience to be handled safely and preferably kept intact. For this reason, we advise that for those who want to work with Apicotermatinae, especially with a taxonomic focus, to seek the aid of a specialist.

The Apicotermittinae taxonomy can benefit from this protocol and, thus, advance and consolidate the technique of recognizing and efficiently dissecting the main structures used for identifying the species. Furthermore, this study can help taxonomists by showing the step-by-step procedures.

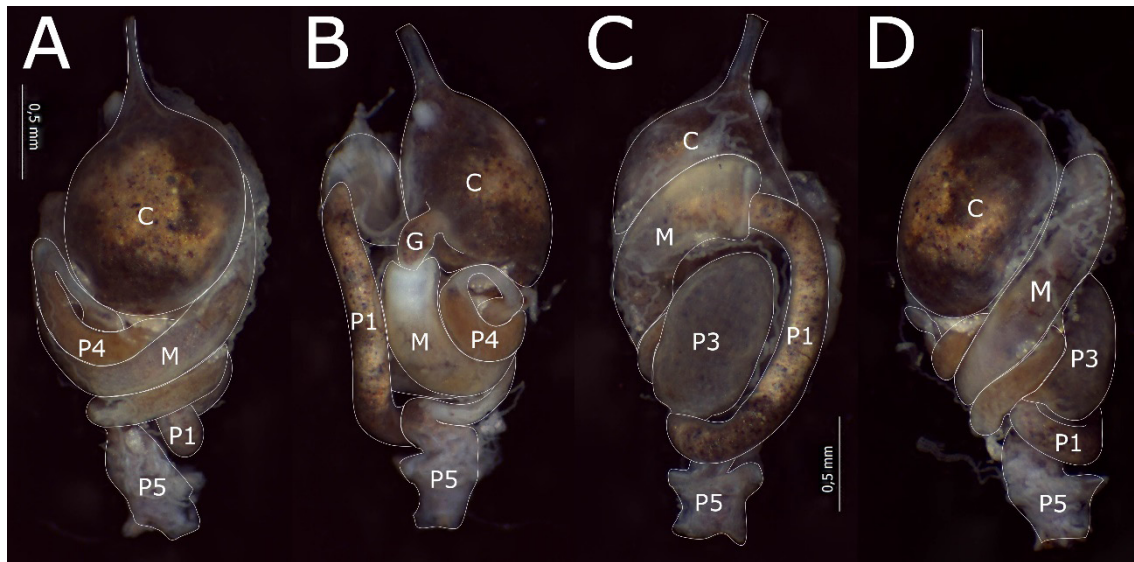
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References

- Acioli, A.N. & Constantino, R. (2015). A taxonomic revision of the neotropical termite genus *Ruptitermes* (Isoptera, Termitidae, Apicotermittinae). *Zootaxa*, 4032: 451-492. <http://dx.doi.org/10.11646/zootaxa.4032.5.1>
- Ackerman, I.L., Constantino, R., Gauch, Jr., H.G., Lehmann, J., Riha, S.J. & Fernandes, E.C. (2009). Termite (Insecta: Isoptera) species composition in a primary rain forest and agroforests in Central Amazonia. *Biotropica*, 41: 226-233. <https://doi.org/10.1111/j.1744-7429.2008.00479.x>
- Arias, J.R., Chevalier, C. & Y Roisin (2020). Anatomical specializations of the gizzard in soil-feeding termites (Termitidae, Apicotermittinae): Taxonomical and functional implications. *Arthropod Structure and Development*, 57: 100942. doi: <https://doi.org/10.1016/j.asd.2020.100942>
- Bourguignon, T., Šobotník, J., Dahlsjö, C.A.L. & Roisin, Y. (2016). The soldierless Apicotermittinae: insights into a poorly known and ecologically dominant tropical taxon. *Insectes Sociaux*, 63: 39-50. <https://doi.org/10.1007/s00040-015-0446-y>
- Constantino, R. (1999). Chave ilustrada para identificação dos gêneros de cupins (Insecta: Isoptera) que ocorrem no Brasil. *Papéis Avulsos de Zoologia*, 40: 387-448.
- Constantini, J.P., Carrijo, T.F., Palma-Onetto, V., Scheffrahn, R., Carnohan, L.P., Šobotník, J. & Canello, E.M. (2018). *Tonsuritermes*, a new soldierless termite genus and two new species from South America (Blattaria: Isoptera: Termitidae: Apicotermittinae). *Zootaxa*, 4531: 383-394. <https://doi.org/10.11646/zootaxa.4531.3.4>
- Deligne, J. (1999). Functional morphology and evolution of a carpenter's plane-like tool in the mandibles of termite workers (Insecta: Isoptera). *Belgian Journal of Zoology*, 129: 201-218.
- Donovan, S.E. (2002). A morphological study of the enteric valves of the Afrotropical Apicotermittinae (Isoptera: Termitidae). *Journal of Natural History*, 36: 1823-1840. <https://doi.org/10.1080/00222930110062309>
- Downs, W.G. (1943). Polyvinyl alcohol: a medium for mounting and clearing biological specimens. *Science*, 97: 539-540.
- Fontes, L.R. (1986). Two new genera of soldierless Apicotermittinae from the Neotropical region (Isoptera, Termitidae). *Sociobiology*, 12: 285-297.
- Fontes, L.R. (1987). Morphology of the alate and worker mandibles of the soil-feeding nasute termites (Isoptera, Termitidae, Nasutitermittinae) from the Neotropical Region. *Revista Brasileira de Zoologia*, 3: 503-531.
- Hanley, R.S. & Ashe, J.S. (2003). Techniques for dissecting adult aleocharine beetles (Coleoptera: Staphylinidae). *Bulletin of Entomological Research*, 93, 11-18. <https://doi.org/10.1079/BER2002210>
- Inward, D.J., Vogler, A.P. & Eggleton, P. (2007). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Molecular Phylogenetics and Evolution*, 44: 953-967. <https://doi.org/10.1016/j.ympev.2007.05.014>
- Jouquet, P., Bottinelli, N., Shanbhag, R.R., Bourguignon, T., Traoré, S. & Abbasi, S.A. (2016). Termites: the neglected soil engineers of tropical soils. *Soil Science*, 181: 157-165.
- Noirot, C. (1995). The gut of Termites (Isoptera). Comparative anatomy, systematics, phylogeny. I. Lower Termites. *Annales de la Société Entomologique de France*, 31: 197-226.
- Noirot, C. (2001). The gut of termites (Isoptera) comparative anatomy, systematics, phylogeny. II. Higher termites (Termitidae). *Annales de la Société Entomologique de France (Nouvelle Série)*, 37: 431-471.
- Rocha, M.M. & Constantini, J.P. (2015). Internal ornamentation of the first proctodeal segment of the digestive tube of Syntermittinae (Isoptera: Termitidae). *Deutsche Entomologische Zeitschrift*, 62: 2944. <https://doi.org/10.3897/dez.62.8550>
- Rocha, M.M., Cuezco, C., Constantini, J.P., Oliveira, D.E., Santos, R.G., Carrijo, T.F., & Canello, E.M. (2019). Overview of the morphology of Neotropical termite workers: History and practice. *Sociobiology*, 66: 1-32. <https://doi.org/10.13102/sociobiology.v66i1.2067>
- Sands, W.A. (1972). The soldierless termites of Africa (Isoptera, Termitidae). *Bulletin of the British Museum (National History)*, Supplement, 18: 1-244.



Supplementary Material 1

S1. Scheme with the digestive tube segments. A – Dorsal view; B – Left view; C – Ventral view; D – Right view. C, crop; G, gizzard; M, mesenteron; P1, first proctodeal segment; P3, paunch; P4, colon; P5, rectum.

Supplementary Material 2

Video 1: <http://youtu.be/bvLl0Ky6zVo>

Video 2: <http://www.youtube.com/watch?v=ZlGfWd3tsFw>

Video 3: <https://www.youtube.com/watch?v=hxBpEPswhtk>