Revista Brasileira de Entomologia 60 (2016) 109–113

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution
www.rbentomologia.com

Short Communication

Clearing and dissecting insects for internal skeletal morphological research with particular reference to bees

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ARTICLE INFO

Article history:
Received 20 July 2015
Accepted 4 November 2015
Available online 1 December 2015
Associate Editor: Rodrigo B. Gonçalves

Keywords:
Apidae
Apoidea
Comparative morphology
Hymenoptera
Internal morphology

ABSTRACT

A detailed protocol for chemical clearing of bee specimens is presented. Dry specimens as well as those preserved in liquid media can be cleared using this protocol. The procedure consists of a combined use of alkaline solution (KOH or NaOH) and hydrogen peroxide (H_2O_2), followed by the boiling of the cleared specimens in 60–70% EtOH. Clearing is particularly useful for internal skeletal morphological research. This procedure allows for efficient study of internal projections of the exoskeleton (e.g., apodemes, furcae, phragmata, tentoria, internal ridges and sulci), but this process makes external features of the integument, as some sutures and sulci, readily available for observation as well. Upon completion of the chemical clearing process the specimens can be stored in glycerin. This procedure was developed and evaluated for the preparation of bees and other Apoidea, but modifications for use with other insect taxa should be straightforward after some experimentation on variations of timing of steps, concentration of solutions, temperatures, and the necessity of a given step. Comments on the long-term storage, morphological examination, and photodocumentation of cleared specimens are also provided.

The study of the morphology of different kinds of organisms, especially animals, certainly is one of the easiest, cheapest, and most readily accessible ways of obtaining large amounts of information and understanding of the planet's biodiversity. Morphological comparative studies have had a long life, as exemplified by the classic treatise *De Partibus Animalium* by Aristotle more than 2300 years ago. In the case of Entomology, external and internal morphological studies have always had great appeal because of the enormous diversity of insects, but also because of large amount of variation observable in their exoskeleton. Despite the emergence of new techniques for imaging complex insect anatomy such as μCT-Scan, MRI, CLSM (e.g., Beutel and Friedrich, 2008; Deans et al., 2012), traditional light optical microscopes remain as the most common instruments of assessing morphological diversity. Specimen dissection and preparations for morphological research using optical microscopy is benefited by clearing the cuticle using alkaline solutions, such as NaOH or KOH. Clearing is particularly useful when internal projections of the exoskeleton (e.g., apodemes, furcae, phragmata, tentoria, internal ridges and sulci) are investigated, but this process makes external features of the integument, as some sutures and sulci, more readily available for observation. Furthermore, cleared material is appropriate for line drawings and photography, in addition to remain available for posterior preparation for SEM execution after being critical pointed dried if desired (Porto et al., 2015).

Although alkaline solutions (e.g., NaOH or KOH) have been commonly used in entomological clearing procedures by researchers working with various insect taxa, protocols combining treatments with these kinds of bases and hydrogen peroxide (H_2O_2) are not usually done. Melo (1999) reported the combined application of an alkaline compound (KOH) and a solution intended to make the insect integument more translucent (H_2O_2). The clearing procedure presented by Melo (1999) contained the general steps but a protocol comprising detailed approximate times and alternative routes for these steps into a more readily applicable protocol appears to be lacking. A modified version of the Melo's (1999) clearing method is provided in Table 1. Our protocol was developed and evaluated for the preparation of bees (and other Apoidea, as done by Melo (1999)), but modifications for other insect taxa should be straightforward after some experimentation on variations of timing of steps, concentration of solutions, temperatures, and the necessity of a given step. The understanding of the relationships between the main lineages of bees has been greatly improved during the XX century by many morphological works, particularly those by

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http://dx.doi.org/10.1016/j.rbe.2015.11.007

Table 1
Protocol containing the suggested steps for clearing and dissecting specimens of bees (and other relatively large and well sclerotized hymenopteran representatives) for the study of external and internal skeletal morphology (modified from Melo, 1999).

(1) Place the specimen into a 10% solution of KOH/NaOH to dissolve soft tissues. The specimen may remain soaked in solution for 10–45 h, in room temperature. This step of the procedure can be speed up by heating the KOH/NaOH solution up to ~90–100 °C, preferably in a water bath, in which case the time needed for the initial clearing can be reduced to 10–40 min. The necessary time for heated KOH/NaOH to be effective depends on various traits of the specimen, such as body size, color, thickness of the integument, region of the body. Dry pinned specimens do not need to be relaxed before being placed into the KOH solution, although this might be necessary to safely remove the specimen from the pin. In case the specimen is preserved in ethanol (70–80%), boil it in 100% EtOH for approximately 10 min, then transfer it to heated KOH/NaOH and proceed as indicated in the standard protocol. Overall, no significant differences between ethanol preserved specimens and dry-pinned can be perceived at the end of the clearing process. Delicate parts of the specimen, such as mouthparts and terminalia, may be dissected/disarticulated at this stage to prevent over clearing them. Additionally, the head and propectus (i.e., propleuron and prosternum), mesosoma and metasoma can be separated prior to the next steps. This allows the fine tune of the clearing process for distinct portions of the body, as they normally have different properties (e.g., cuticle thickness, colors, etc.) and, thus, will probably differ in their ideal times in heated solution.
(2) Transfer the specimen to a room-temperature solution of 3–5% H_2O_2, where it shall remain for approximately 10–20 min, when it will be cleared enough. Once again, the necessary time for the clearing to be effective depends on various traits of the specimen, such as size, color, integument thickness, region of the body, etc. Alternatively, a heated (~ 70–90° C) solution of H_2O_2 may be used, but this must me done with caution because the clearing activity might be over accelerated leading to a reduced control over the degree of the overall clearing process. For proper clearing, it is important to emphasize that the specimen needs to be transferred directly from the alkaline solution to the peroxide solution. Do not wash it in water between the two steps. In certain cases, treatment with KOH/NaOH will be effective enough so that it would be advisable to skip the H_2O_2 step (in such cases, the chemical action of KOH/NaOH must be stopped, and this can be accomplished with a bath in diluted acetic acid or lactic acid for a few minutes, followed by washing in pure water. Going to step 3 directly from 1 will also work).
(3) Transfer the specimen and all its previously disarticulated parts (if any) to a 60–70% EtOH boiling solution, leaving them there for 5–10 min. There will be bubbles popping out of the body, but this is normal and necessary to remove small air bubbles from within the exoskeleton and remnants of macerated soft tissues as well as to interrupt the chemical effects of KOH/NaOH and H_2O_2.
(4) Wait for the solution to cool down to naturally reach room temperature.
(5) Transfer the specimen to a glycerol: 60% EtOH mixture (1:1).
(6) After approximately 24 h, remove part of the supernatant EtOH with a pipet and double the amount of glycerol. Ethanol and glycerol do not mix perfectly well because they possess distinct densities. After some time, it is easily noticeable that there will be more EtOH on the upper layer of this solution.
(7) Wait for the specimen to be completely immersed in the glycerol (this may require a few days), and then transfer the specimen to a pure glycerol solution.
(5–7, alternative) The time required for these steps can be significantly shortened using a series of dehydrating alcoholic solutions. After step **4**, the dissected pieces can be soaked into a series of EtOH solutions with increasing concentrations (70–100%) for 20–30 min each. Four baths are sufficient (70%, 80%, 90% and then 100%) for a successful dehydration of the specimens or specimen-parts. After that, store the pieces into pure glycerol.

Roig-Alsina and Michener (1993) and Alexander and Michener (1995), which investigated external and internal skeletal morphology of Apoidea in a comparative manner.

The protocol adopted by Melo (1999) was in current use at the Entomology Division of the University of Kansas Natural History Museum in the 1990s. GARM recalls not learning it from a written document, but mostly from talking to Charles Michener and the late Byron Alexander. He also recalls learning from the late Steve Ashe the step of boiling specimens in diluted ethanol after peroxide clearing to remove the air bubbles from within them. A search in Ashe's publications at that time (e.g., Ashe, 1992; Ahn and Ashe, 1996) reveals that use of hydrogen peroxide to clear dark specimens is indicated, but subsequent boiling in ethanol is not mentioned. A detailed clearing and dissecting protocol for aleocharine staphylinid beetles was later published by Hanley and Ashe (2003) in which this step is mentioned and discussed. Differently from Hanley and Ashe (2003), the protocol detailed in the present paper focuses mostly on preparing specimens that will be kept disassembled permanently in glycerin in individual wells of plastic culture plates, instead of being mounted in permanent slides, as it is done in the work of the former authors.

Protocol

Prior to starting the chemical clearing process of a specimen, it is advisable to remove its wings. There is no harm in leaving them attached to the specimen, especially when it is expected that a study of its microscopic structure will be carried out, but they will be completely deformed by the procedure described in Table 1. It is recommended that one pair is permanently mounted in glass slides for study under light microscopy and the other pair is glued in a piece of paper and pinned with the specimen labels, which then can be properly stored for further records.

Useful techniques for preparation of fine dissecting tools are given by Hanley and Ashe (2003). These pin tools are also quite handful when dealing with the whole storage plates (see below) under dissecting microscopes. They allow proper positioning of the insect parts without requiring that the examined part be taken out of the plate wells.

Step 1 (Table 1)—Two alternatives are possible at this step: either the specimen can be soaked into a 10% KOH/NaOH solution in room temperature overnight (Melo, 1999: step 1), or this solution can be heated at 90–100 °C. The immersion of specimens in solution by a period of 12–24 h (generally done overnight), although guarantee more control over the clearing/softening process of the integument, showed inefficient results in many instances. On the one hand, for very small specimens (<3 mm), this time proved to be too long and resulted into excessive softening of the integument, making the specimens structurally very weak to manipulations during dissections. On the other hand, for larger specimens (>15 mm), sclerites and articulations are kept structurally intact or only slightly affected, even after more than 24 h in a solution in room temperature.

Heating accelerates the reaction of the alkaline compounds with the integument, membranes, and soft tissues, therefore facilitating the breaking of chemical bonds between the linear chains of chitin and the proteins that are responsible for the rigidity/pigmentation of exocuticle. For specimens with body size between 10 and 15 mm (with moderately sclerotized cuticle) heating the 10% KOH/NaOH solution for about half an hour at 90–100 °C suffices in most