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RESEARCH ARTICLE

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A new method using Scanning Electron Microscopy (SEM) for preparation of anisopterous odonates

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Abstract

Anisopterous odonate male's secondary genitalia is a complex of several structures, among them the vesica spermalis is the most informative with important specific characters. The observation of those characters, mostly of membranous nature, is difficult in the Scanning Electron Microscope due to dehydration and metallization processes. In this contribution, we discuss a new and low cost procedure for the observation of these characters in the SEM, compatible with the most common agents used for preserving specimens.

KEYWORDS

anisopterous, critical point drying, *Erythrodiplax*, Odonata, scanning electron microscope, vesica spermalis

1 | INTRODUCTION

The scanning electron microscope (SEM) has been a preferred instrument for the study of ultrastructures of insects for more than 40 years and the critical point drying (CPD) is the most commonly used method for preparation of biological specimens for examination (Bray, Bagu, & Koegler, 1993).

The preparation (fixation, dehydration, and drying) and mounting procedures of the biological material to be examined must be adequate to the taxa being studied (Ronderos, Spinelli, & Sarmiento, 2000). The improvement of those methods is essential for the successful observation of the characters of interest in each group.

In many odonate taxa, genus *Erythrodiplax* Brauer (Anisoptera, Libellulidae) has no unique characters, and as for all the suborder Anisoptera, reliable specific characters mostly stem from the secondary genitalia, which are located in the venter of the second and third abdominal segments (Garrison, von Ellenrieder, & Louton, 2006). The secondary genitalia of male Anisoptera is a complex of several structures (Figure 1); one of the most informative is the last (fourth) segment of the vesica spermalis (Fraser & Asahina, 1970; Miller, 1991). In *Eryhrodiplax* species, the vesica spermalis is a long and tubular structure, usually dilated in the posterior end with a group of lobes and spines developing which defines a unique specific set of structures (Figure 1).

The observation of these structures poses a great difficulty when working with the SEM because of, due to the membranous nature of the lobes, many artifacts are caused by the acetone or alcohol dehydration of the specimens.

The dehydration by acetone is the most commonly method for preservation of adults odonates (Barlow, Jurzitza, & Cook, 1991; Cook, 1990; Garrison et al., 2006) and is also, widely considered good fixatives for small samples, and for preserving proteins (e.g., enzymes), glycogen, and pigments. Because of this, it can also be used as a conservator of the samples. These procedures have some drawbacks such as producing hardening and retraction of the tissues; according to Bray et al. (1993), air drying from acetone was found to be an unacceptable for preparing animal tissue for SEM, as it produces severe drying artifacts.

Conversely, the membranous nature of the lobes requires, in most cases, the clearing and softening of the cuticle for their eversion, which can be accomplished with a variety of methods (Borror, 1942; Pinto, José, & Lamas, 2010), all of them converging in the storage in glycerin

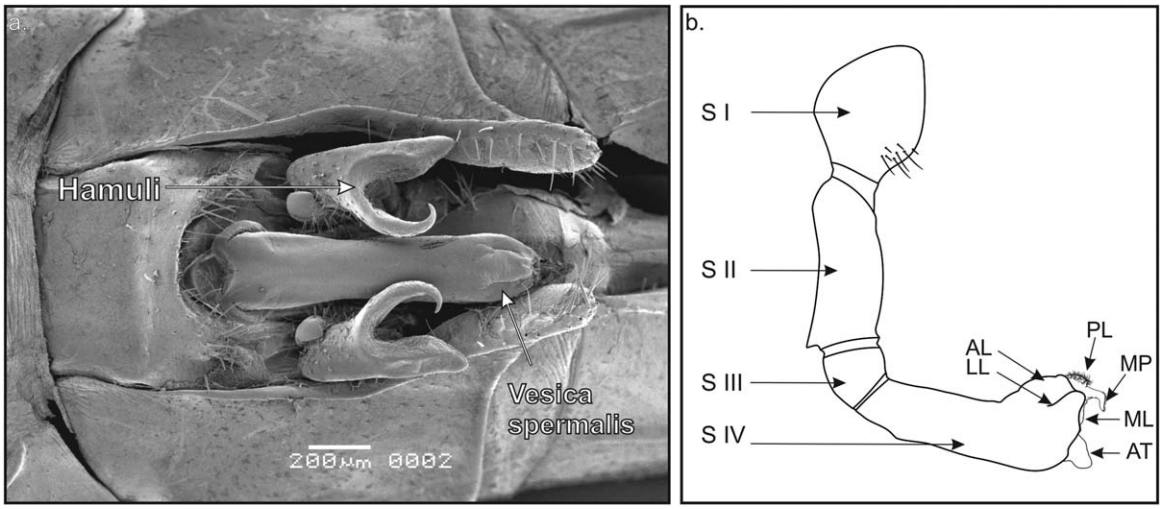

FIGURE 1 *Erythrodiplax chromoptera*'s vesica spermalis photography in SEM using the new methodology proposed

or 70% alcohol. For the use of SEM, the material must be dry; thus, a re-dehydration has to be done on the now soft vesica spermalis, which produces an even wider arrange of artifacts. As a result of the setup and metallization procedures, the membranes of the lobes generally collapse.

There are two procedures commonly used to avoid those artifacts arising during the air drying process: the use of hexamethyldisilazane (HDMS) and the CPD. HDMS is one of the most widely use alternatives to CPD (Braet, De Zanger, & Wisse, 1997; Nation, 1983; Shively & Miller, 2009), but it is a dangerous compound that reacts with moisture to generate ammonia (a strong base), which can be harmful to tissues of the skin, eyes, and mucous membranes. Conversely, the CPD involves, after dehydration in acetone of the sample, its replacement by successive steps with liquid CO_2 at 10°C, then the temperature rises to 31°C and the pressure reaches 73.8 Bar at which point it vaporizes (critical point of the CO_2) and is released thereby avoiding any deformation related to the surface tension of the sample surface

The aim of this work is to provide a new procedure for the use of CPD for the observation of the vesica spermalis in anisopterous Odonata.

2 | MATERIAL AND METHODS

Male adults of several species of *Erythrodiplax* were captured with aerial nets, deposited in envelopes and dried with acetone according with the commonly used method for preservation used in odonates: a syringe was used to inject live specimens with acetone, inserting between the third pair of legs. The specimens were then placed in glassine envelopes and steeped in an acetone bath in a jar for 24 or 48 hr. They were then removed from the acetone and are air dried for about 3 hr.

The vesica spermalis was cleared by introducing it into a test tube with KOH 10%, then placed in a recipient with water at a constant temperature of 80–90°C (to avoid artifacts due to fast heating), between 5 to 15 min. Then, it was kept in vials with acetone or glycerin.

For vesica spermalis kept in acetone, the proposed new procedure is a consecutive rinse in 25%, 50%, and 75% acetone-water solution, each concentration for a 24-hr period. It is recommended to do a change in the solution after the first 12 hr, and finally, it can be storage in 100% acetone before CPD.

After CPD the setup for SEM observation is done on double sided tape and carefully positioning the proximal segment to mount the vesica spermalis (depending on the number of segments remaining on the vesica, but ideally it should have at least segments III-IV), using filaments of the adhesive to keep it on the desire position. The setup must be done with extreme care due to the fragility of the vesica after the CPD.

If the vesica spermalis was stored in glycerin, first it must be washed in alcohol at 70% and then in 10% KOH for 5–10 min) to completely clean it. The same steps as before are followed after the cleaning.

3 | RESULTS AND DISCUSSION

The success of this procedure for the characters observation of the vesica spermalis in *Erythordiplax* species are shown in Figures 2 and 3. Although some variations are perceived, in all cases the vesica shows all the diagnostic characters; fully extended lobes can be appreciated, with very few artifacts (most of them due to the air drying of the specimens during preservation).

There are two important advantages in this procedure:

1. As it involves common solutions used in entomological laboratories, it is safer and more cost effective than other techniques, particularly HDMS.

FIGURE 2 Secondary genitalia in shown in *Erythrodiplax atroterminata*. (a) second abdominal segmen, ventral view; (b) vesica spermalis, lateral view. AL: Apical Lobe; AT: Apical Tubercle; LL: Lateral Lobe; ML: Median Lobe; MP: Median Process; PL: Posterior Lobe; S I-IV vesica spermalis segments

2. It allows utilization of specimens deposited in scientific collections, either dried with acetone, silica or pinned, thus making this procedure ideal for use in almost all the collections worldwide.
3. As the vesica spermalis of *Erythrodiplax* is similar in general structure and morphology to the rest of the Anisoptera taxa, this procedure can be used throughout the entire suborder.

As HDMS is widely used in several orders of Arthropods, this procedure could be utilized also for other orders which rely on it (e.g., eggs and male's pedipalps from the order Araneae, male reproductive system in Branchiura, etc). In addition, the low cost and time saving, this procedure's easy handling allows for its use by students for educational purposes in classrooms.

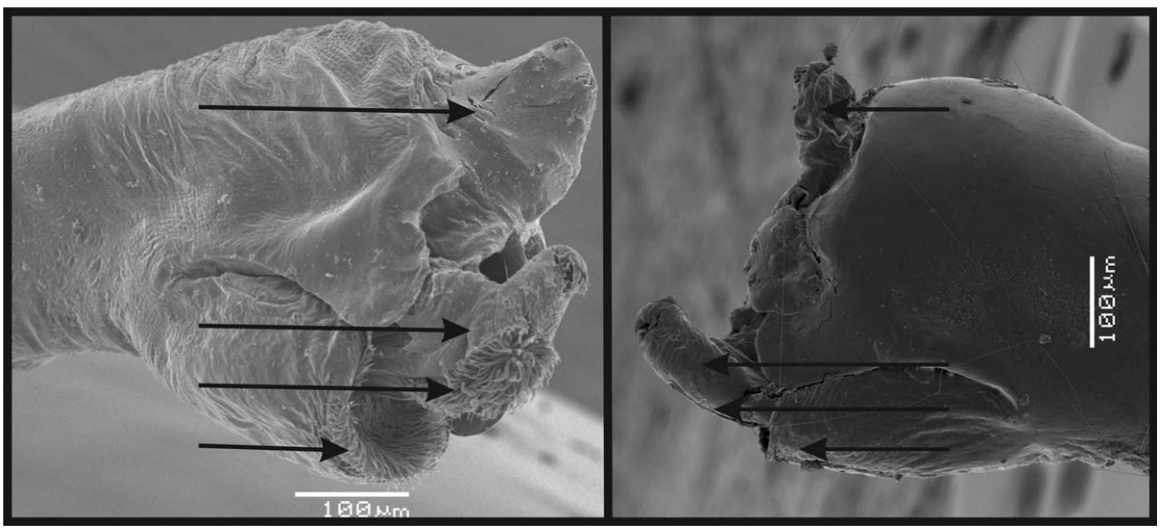

FIGURE 3 Vesica spermalis' median processes of *Erythrodiplax chromoptera*, frontal view. Specimen air dryed (left) and prepared according to the present procedure (right). Arrows showing major changes. Vesica spermalis' median process of *Erythrodiplax fusca*, lateral view. Specimen air dryed (right) and prepared according to the present procedure (left). Arrows showing major changes

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