THE IMPORTANCE OF SCANNING ELECTRON MICROSCOPY (SEM) IN TAXONOMY AND MORPHOLOGY OF CHIRONOMIDAE (DIPTERA)

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ABSTRACT

The paper reports on the value of scanning electron microscopy (SEM) in the taxonomy and morphology of Chironomidae. This method has been relatively rarely used in Chironomidae studies. Our studies suggest that the SEM method provides a lot of new information. For example, the plastron plate of the thoracic horn of *Macropelopia nebulosa* (Meigen) under light microscopy is visible as points, while under SEM we have found that it consists of a reticular structure with holes. By using SEM a more precise picture of the body structure of Chironomidae can be revealed. It allows researchers to explain inconsistencies in the existing descriptions of species. Another advantage of the SEM method is obtaining spatial images of the body and organs of Chironomidae. However, the SEM method also has some limitations. The main problem is dirt or debris (e.g. algae, mud, secretions, mucus, bacteria, etc.), which often settles on the external surface of structures, especially those which are uneven or covered with hair. The dirt should be removed after collection of chironomid material because if left in place it can become chemically fixed to various surfaces. It unnecessarily remains at the surface and final microscopic images may contain artifacts that obscure chironomid structures being investigated. In this way many details of the surface are thus unreadable. The results reported here indicate that SEM examination helps us to identify new morphological features and details that will facilitate the identification of species of Chironomidae and may help to clarify the function of various parts of the body. Fast development of electron microscope technique allows us to learn more about structure of different organisms.

Keywords: Chironomidae, pupae, scanning electon microscopy, SEM

Introduction

A serious problem in Chironomidae systematics is the difficulty in identifying related species. For the determination of particular species of Chironomidae new methods, such as cytogenetics (Michailova 1989), can also be used in addition to the classical techniques. It is clear that scanning electron microscopy (SEM) can also be useful in these studies.

The use of scanning electron microscopy in biological research dates back to the 1970s. In some biological disciplines SEM is the standard method, for example in studies of diatoms (Bacillariophyceae) (Henderson and Reimer 2003; Houk et al. 2010). However, in studies of Chironomidae, despite the large number of publications appearing every year (Hoffrichter 2009), SEM has been applied in relatively few papers, in some papers only in a fragmentary way (Michailova 1980; Kobayashi 1995; Hughes and Murray 2001; Michailova et al. 2005). SEM was used extensively in the description of the morphology of *Chironomus tentans* Fabricius (Sublette and Martin 1995), differentiation of some larvae of Chironomini on the basis of morphology of the head capsule and ventromental plates (Webb 1980) and in determination of aberration amongst larvae of *Chironomus samoensis* Edwards whose eggs were affected by irradiating with ultraviolet light (UV) (Percy et al. 1986).

Therefore, the aim of our investigation was to determine the importance and limitations of scanning electron microscopy in taxonomy and morphology of Chironomidae.

Methods

General Methods for Using SEM

In our studies we compared the pictures obtained from scanning electron microscopy (SEM) with those obtained using light microscopy.

The successive steps for preparing the material for observation using an scanning electron microscope are shown in Fig. 1.

The first stage causes the fixation of the material and increases its mechanical and thermal stability through cross binding proteins. Usually, for fixation chemicals are used: aldehydes, osmium tetroxide and FAA – 10% formalin, 85% ethanol, 5% glacial acetic acid. An appropriate concentration of fixatives, pH, buffer type, temperature and time for good fixation must be taken into account. However, this method is not as demanding as compared to Transmission Electron Microscopy (TEM) (Maunsbach and Afzelius 1999). With SEM the outer layers of the preparation are observed so that the fixative makes immediate contact and ensures stability very quickly.

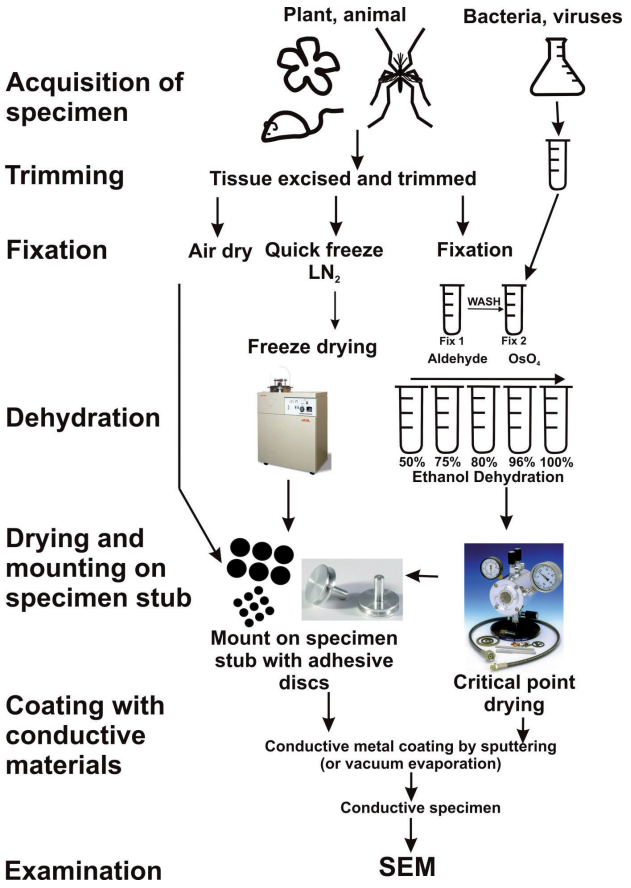

Fig. 1 Diagram showing the steps in the preparation of biological material for the SEM (according to Bozzola and De Russel 1999).

After fixation, the material must be washed out several times to remove buffer salt. Then the material is dehydrated in alcohols of increasing concentration (50%, 75%, 80%, 96%, 100%) and dried because samples have to be compatible with the vacuum in the microscope. Air drying is not recommended because of surface tension forces which causes contraction and shrinkage of the material surface. Any material affected by contraction or shrinkage has to be excluded from further investigation. Preparations should therefore be dried under appropriate conditions, which are provided during freezing, critical point drying or in a vacuum coater. Very good results are obtained after critical point drying with carbon dioxide at a temperature of 31 °C and a pressure of 74 bar since it is suitable for biological material and easy to obtain. It must be taken into consideration that carbon dioxide has a serious drawback as an intermediate fluid because it is not miscible with water. Therefore, the water is replaced with ethanol and acetone, which in turn can be mixed with water and liquid carbon dioxide. In this process the acetone is replaced by the intermediate fluid under high pressure to provide a complete exchange of fluids.

In the next step, warming is done until the liquid reaches phase transition in which the liquid phase passes into vapor and the physical characteristics of liquid and gas are not distinguishable. After reaching critical point, the material is still embedded in a dense vapor phase so it prevents the material from damage by surface tension forces of the liquid / air. Heat is continuously supplied to the CPD (Critical Point Drier) device and ensures that the vapors will not go back to a liquid state. The vapors are very gently released from the CPD device until atmospheric pressure is obtained. The dried material can be mounted on holders or stubs, sputter coated with a thin layer of conductive material, and is then ready to be viewed with a scanning electron microscope.

Methods Used in this Study

Permanent slides of pupal exuviae and larvae were prepared with Faure liquid. Pictures were taken using a Nikon-Eclipse 50i light microscope fitted with a Digital sight DS-U1 camera.

Using a scanning electron microscope (SEM) the following procedure was applied. The samples were fixed in 2.5% glutaraldehyde GLU in 0.1 phosphate buffered saline PBS for 2 hours, rinsed with PBS 2 × 10 min and dehydrated in a graded series of alcohols. Finally they were placed in a transitional liquid (100% acetone) and transferred to a Critical Point Drier (CPD E3000/E3100, Quorum Technologies). Specimens were then coated with gold using a JFC – 1100E Ion sputter (JEOL). For coating, the material was placed on a holder with conductive carbon adhesive tabs (Electron Microscopy Sciences). Photographs of morphological structures were taken by means of Scanning Electron Microscope (SEM), a JSM – 5410, operated at an accelerating voltage of 15 kV in the Scanning Microscopy Laboratory of the Jagiellonian University.

Results and Discussion

Our studies show that the SEM method provides a lot of new information on chironomid structures and surfaces. For example, the plastron plate of the thoracic horn of *Macropelopia nebulosa* (Meigen) in the light microscope is covered by points, while in the SEM we can see that it consists of a reticular structure with holes (Fig. 2). This type of structure helps explain part of the process of oxygen uptake by pupa for the developing imago.

SEM observations also allow us to explain inconsistencies in the existing descriptions of species. For instance, in the description of the pupa of *Glyptotendipes glaucus* (Meigen) by Michailova and Contreras-Lichtenberg (1995) they state that the "anal comb on segment VIII consisting of 5–7 short teeth". In the light microscope only the ends of teeth on the anal comb are visible (Fig. 3A), while SEM observations indicate that the teeth are only part of a very large anal comb structure viewed from the ventral side (Figs 3B–C). In the light microscope shagreen on tergite II of *G. glaucus* is composed of minute spinules (Fig. 4A), whereas under the SEM differentiation of the shagreen is distinctly visible

(Fig. 4B). Shagreen on the anterior part of tergite II has smaller spinules (Fig. 4C) than the shagreen on the posterior part (Fig. 4D). Under SEM the structures of hooklets (Fig. 4E) and epaulettes (Fig. 4F) are more visible. The SEM method allows us also to see the spatial structure of the object, while in the light microscope a flattened picture can be observed. For example, the spatial structure of the epaulette on segment III of *G. glaucus*, viewed under light microscope is flat and shows little differentiation (Figs 5A–B), while under SEM an almost three-di mensional picture can be seen (Figs 5C–D). The spatial

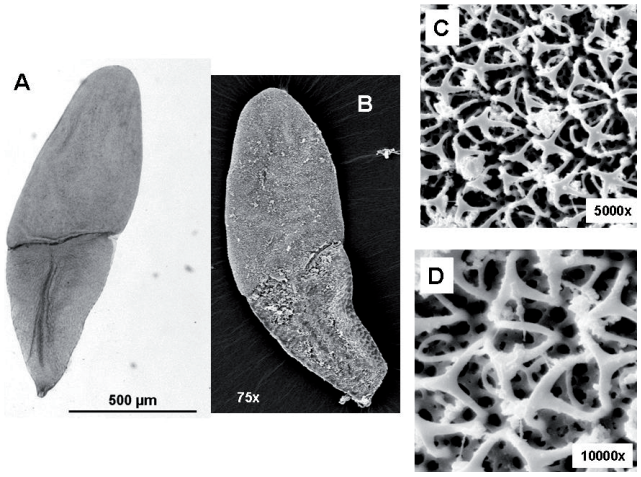

Fig. 2 *Macropelopia nebulosa* pupa; A – thoracic horn (light microscope); B – thoracic horn (SEM); C, D – structure of the plastron plate (SEM) (according to Michailova et al. 2014).

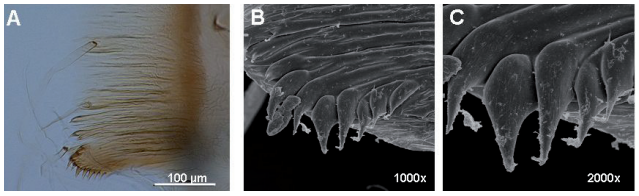

Fig. 3 *Glyptotendipes glaucus* pupa; A – anal comb, dorsal view (light microscope); B, C – anal comb, ventral view (SEM).

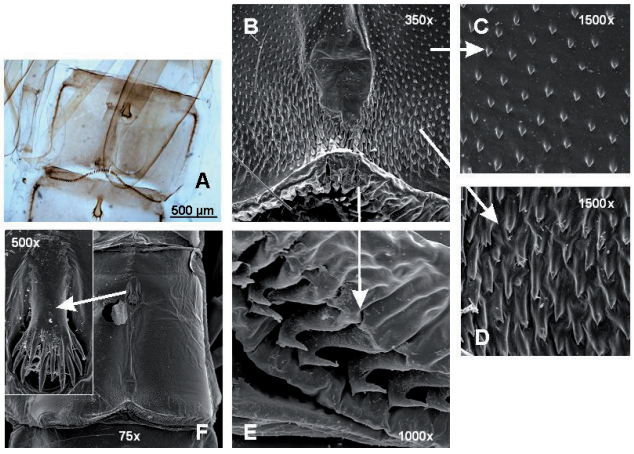

Fig. 4 *Glyptotendipes glaucus* pupa; A – tergite II (light microscope); B – tergite II (SEM); C – shagreen on the anterior part of tergite II (SEM); D – shagreen on the posterior part of tergite II (SEM); E – hooklets on tergite II (SEM); *f* – epaulette (SEM).

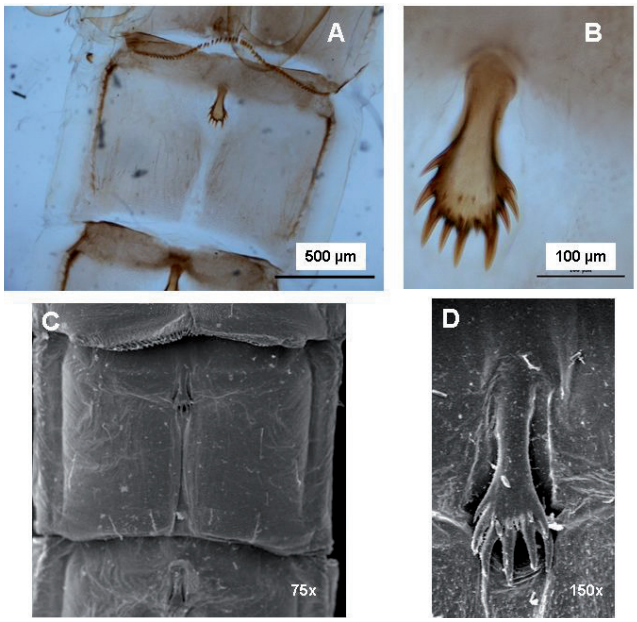

Fig. 5 *Glyptotendipes glaucus* pupa; A – tergite III (light microscope); B – tergite III, epaulette (light microscope); C – tergite III (SEM); D – tergite III, epaulette (SEM).

structure of the epaulette on segment III is relatively simple and in the SEM image is clearly visible (Fig. 5D).

However, the SEM method also has some limitations. The main problem is dirt (e.g. algae, mud, debris, etc.), which often settles on the surface of external structures, especially those covered with hair or which are uneven. Attempts to remove these contaminants do not always give satisfactory results, particularly on surfaces on which there are structures with high spatial diversity. For example, the epaulette on segment VI of *G. glaucus* has a lot of spines which collect dirt (Fig 6C–D). These impurities, after being coated with gold, are more pronounced under SEM than the real structure of the epaulette. In the light microscope, the impurities are overexposed and are not seen on the picture (Figs 6A–B).

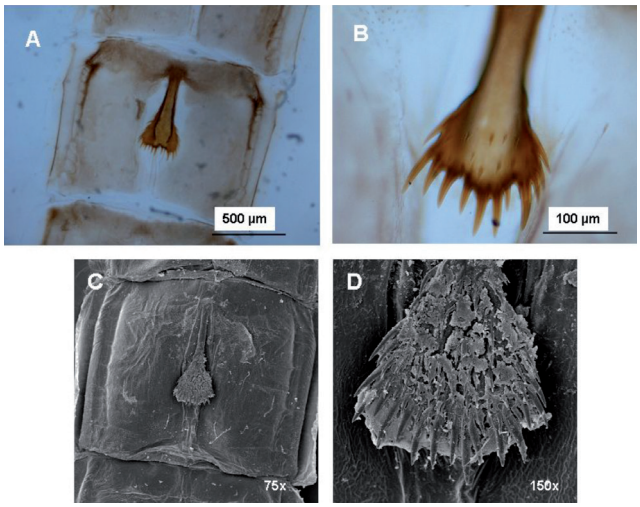

Fig. 6 *Glyptotendipes glaucus* pupa; A – tergite VI (light microscope); B – tergite VI, epaulette (light microscope); C – tergite VI (SEM); D – tergite VI, epaulette (SEM).

It is worth mentioning that sometimes good results are obtained by using simple methods. One way of cleaning the surfaces can be gentle, repeated washing of the sample by using a pipette.

Conclusions

Our results indicate that the SEM examination helps us to get to discover, investigate and understand new morphological details which will greatly facilitate the identification of Chironomidae species and may help to clarify the functions of various body parts. Fast development of the electron microscope technique allows us to find new features for differentiating particular species from one another and even to distinguish Chironomidae species new for science. However, the SEM method also has some limitations if the specimens are not carefully cleaned to ensure that important structures are not obscured by dirt and other debris.

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