Transactions of the American Entomological Society Volume 134, Number 3+4: 471-476, 2008

# A new method using acid to clean and a technique for preparation of eggs of biting midges (Diptera: Ceratopogonidae) for Scanning Electron Microscope

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## ABSTRACT

A new procedure for clearing eggs belonging to genera of Ceratopogonidae is described. In the new procedure eggs in different states, with different ultra structures of the exochorion, were immersed in 10% glycolic acid (CH2OH-COOH) and 20 % mandellic acid (C6H5.CHOH.COOH) for 1-4 minutes. Eggs, prepared by both acids and subsequently neutralized by distilled water, were placed in a saturated solution of phenol, and then passed through a battery of alcohol solutions. Finally, critical point was performed and gold metallized. After preparation the eggs of this group retained their natural form under vacuum long enough to yield good microphotographs of the outer layer of the chorion.

## INTRODUCTION

The Scanning Electron Microscope (SEM) is a valuable instrument in biological research. Hard structures such as the exoskeleton of many Diptera usually present no problems and can be cleaned with distilled water and solution of Na (OH) 0.1 N, they may not be effective or they may damage cells. Important advances have been made in the technical improvement of the SEM. Preparation and posterior mounting procedures of the different biological material to be examined, suitable for the different states of the ontogeny cycle being studied, have been greatly improved as well.

The structures to be protected by fixation vary according to the chemical nature of the fixative and the image obtained, and will be more complete if several procedures are used together.

Ronderos *et al.*, 2000, described techniques for the preservation of the original morphology, especially in larvae and adults. Some methods of fixation, dehydration and drying, were used by Murphree & Mullen (1991), Day *et al.* (1997), Breidenbaugh & Mullen (1999), with reference to treatment in the different gerenra of diptera Ceratopogonidae. Forattini & Marucci (1993) using a method for SEM for observation eggs of *Anopheles sp* (Kerteszia); Bosworth *et al.*, 1998, studied methods for use on the exocorion and endochorion of *Psorophora columbiae* eggs.

In previous studies, we used the SEM to observe larvae, pupae and adults of the Ceratopogonidae.

In this study we report on the results obtained by clearing procedures, along

with the results of different treatments, and posterior preparation and mounting of the eggs of Ceratopogonidae. These include, partly, the already proved techniques considered by Ronderos (1998, 1999) in adults of *Culicoides insignis* Lutz, as well as Ronderos *et al.*, 2000 in larvae and adult of Ceratopogonidae.

## MATERIAL AND METHODS

This paper concerns the eggs of certain Ceratopogonidae. The eggs were taken from gravid females from different environments. Therefore, exochorionic quality varies, not only according to its mature state but also in different generic and specific ultra cells. The particular cellular exochorionic characteristics need adapted treatment based on tissue type.

**Group a**. Eggs taken from the abdomen of gravid females, and exochorion labile.

**Group b**. Eggs captured in a plastic container, 13.50 cm diameter, containing sand, organic matter and rain water. These eggs were taken to the laboratory in their original medium (immersed in water rich in nutrients). A second sample was sucked with pipette from a micro-capsule, 3 cm diameter, containing rainwater and different organic matter in tubes at the laboratory.

**a.** We obtained eggs from gravid females, and eggs holding exochorionic characteristics were placed in mandellic acid (C6H5.CHOH.COOH) in 20 % for about 1-2 minutes. Later they were brushed to remove the remains. These were in line and joined to one another. Once separated, they were placed in glassed micro capsules with distilled water to neutralize them. Subsequently, they were fixed in phenol (C6H5OH) saturated in absolute alcohol, for a period of 24-48 hours, and then, dehydrated in increasing concentrations of alcohols (90%, 100%). For the drying procedure, the critical point in drying system was used (Balzers equipment, CP-30), the material was immersed in amyl acetate which was replaced by liquid carbon dioxide at 10C and aired at 32C during 30 minutes. As a final step, eggs underwent gold metallized in a Jeol Fine Coat Ion Sputter, JFC 1.100.

**b.** Approximately 60 eggs were captured in organic matter and rain water. In the laboratory, masses of eggs were placed in Petri capsules. The temperature oscillated between 20 and 25°C and the humidity between 60 and 70%. Previous to fixation, a fraction of eggs was treated with 10% glycolic acid (CH2OH-COOH) for 3-4 minutes. Later they were brushed to remove the cementing remains from the union of the egg mass; then ultrasound was applied for three 30" periods. Once they were cleaned, they were placed in a saturated solution of phenol and then passed through a battery of alcohols. Finally, critical point was performed and gold metallized.

All the material, group (a) and group (b), were finally mounted in a double side carbon tape for observations and photomicrographs. Observations were carried out with Scanning Electron Microscope, whose brand is JEOL JSM-6360LV, and JEOL JSM-TS100.

## RESULTS AND DISCUSSION
Figs 1-8

The different techniques for preparation (group a and group b) clearly indicate the best conditions in which eggs type should be treated.

In general, cleaning with distilled water is not enough. Joining materials, once they are dried, form a fibrous or amorphous layer that obscures the surface to observation (Pickett-Heaps, 1980). That is why the surface must be thoroughly cleaned before any fixation. Na (OH) 0.1 N solutions have shown great utility for removing the glue, but they also can damage the cells. In some instances polysaccharide oxidized and dissolved in an alkaline medium. In order to obtain the desired results, proponed acids have been used. For eggs extracted from the females (group a) (Figs. 1-3) which have a thin and inconspicuous chorion, the treatment determined that the mandellic acid was the most effective on the exochorion, because this acid is weaker and was used in more sensitive tissues. When eggs are removed from females, or in the case of soft structures in immature eggs, ultrasound is not advisable.

On the other hand, eggs loose in the environment and embryonic eggs have a strong chorion (group b) (Figs. 4-8) and treatment with glycolic acid shows better results, and allows the subsequent use ultrasound for cleaning. In these eggs the optimum results were obtained in eggs treated with phenol saturated in absolute alcohol, because of its compound action as fixate and dehydrator. They join the action with absolute alcohol favor the hardening of tissues.

Embryonic eggs and removed eggs were chemically fixed and dried coated with carbon and gold. After preparation, the eggs of this group retained their natural form under vacuum long enough to yield good microphotographs of the outer layer of the chorion.

## ACKNOWLEDGMENTS

We greatefully acknowledge Dr. Gustavo Spinelli for critical review of the manuscript. Lic. Ms. Nelida Caligaris for able technical assistance, and Ms. Maria Cristina Boffo for disinterested assistance.

## REFERENCES

Bosworth, A. B. S., M. Meola, M. Thompson & J. K. Olson. 1998. Chorionic morphology of eggs of the *Psorophora confinnis* complex in the United States. II. Pre-and postdeposition studies of *Psorophora columbiae* (Dyar & Knab) eggs. Journal of American Mosquito Control Association, 14(1): 46-57.

Breidenbaugh, M. S. & B. A. Mullen. (1999). Two new western nearctic *Culicoides* Latreille (Diptera: Ceratopogonidae) described from all stages. Proc. Entomol. Soc. Wash., 101: 149-163.

Day, J. D., D. Duzak, Y. Braverman & A. Chizov-Ginzburg .1997. Ultraestructure of the eggs of *Culicoides circunscriptus*, *Culicoides gejgelensis*, and *Culicoides imicola* (Diptera: Ceratopogonidae) Journal of American Mosquito Control Association, 13: 76-83.

Forattini O. P. & D. Marucci. 1993. Scanning electron microscopy of the eggs of two species of *Anopheles* (Kerteszia) (Diptera: Culicidae). Memórias do Instituto. Oswaldo Cruz, 88 (3): 349-352.

Forattini, O. P., M. A. Mureb Sallum, E. S. Bergo & D. Corugedo Flores. 1998. Ultraestructure of eggs of *Anopheles rondoni*, *Anopheles lutzii*, and *Anopheles parvus*, three species of the subgenus *Nyssorhynchus*. Journal of American Mosquito Control Association, 14(3): 256-265

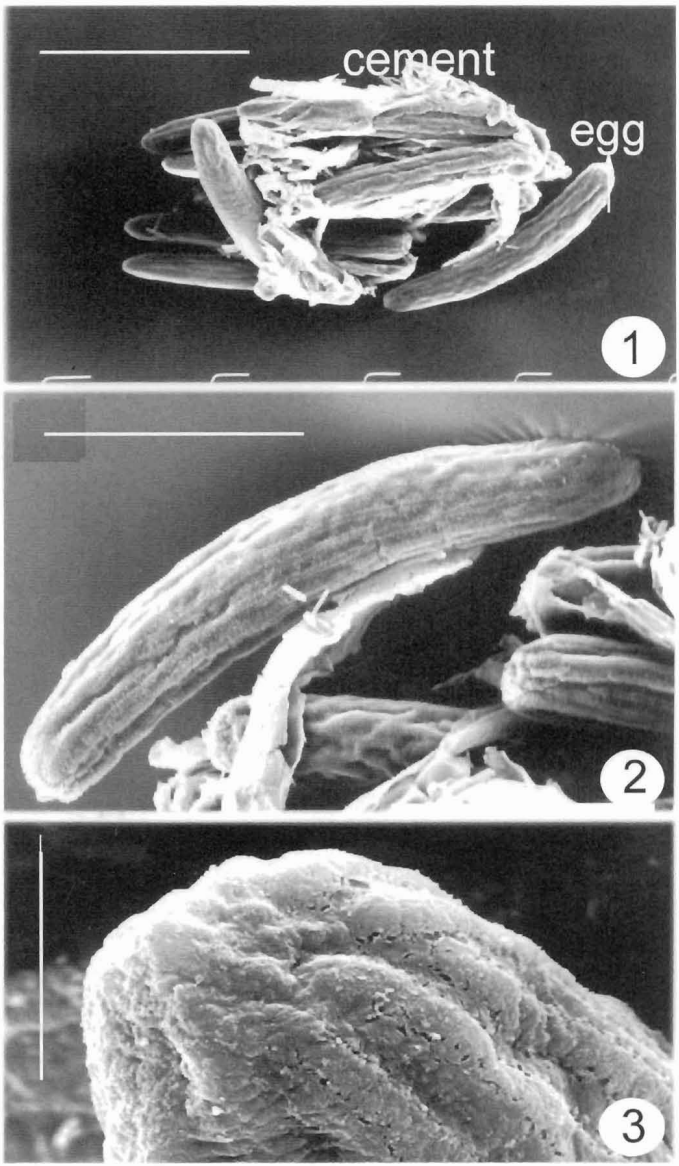
Murphree, C. S. & G. R. Mullen. 1991. Comparative larvae morphology of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) in North America with a key to species. Bulletin of the Society of Vector Ecology, 16(2): 269-399.

Pickett-Heaps, J. 1980. Preparation of algae for scanning electron microscopy. *In*: E. Gnatt (ed). Handbook of Phycological Methods. Developmental and cytological methods. Cambridge University Press, Cambridge, 367-375.

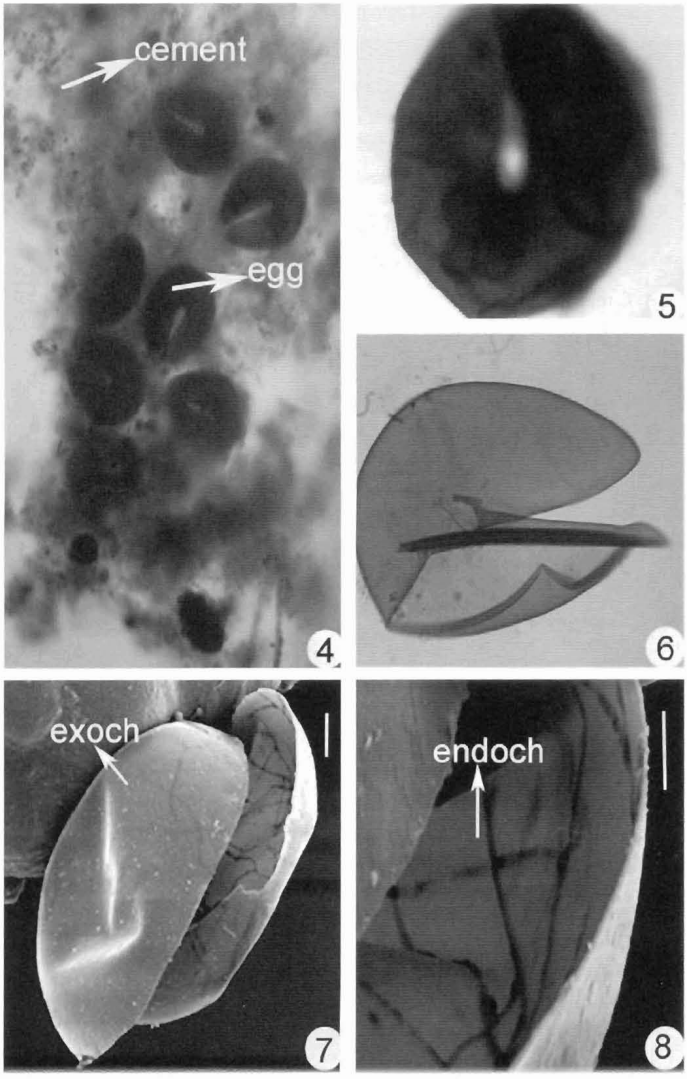
Ronderos, M. M. 1998. Morfología del aparato bucal de *Culicoides insignis* Lutz (Diptera: Ceratopogonidae) bajo Microscopio Electrónico de Barrido. Gayana Zoología, 62: 159-165.

Ronderos, M. M. 1999. Las sensilas antenales y palpales de la hembra de *Culicoides insignis* (Diptera: Ceratopogonidae) a la luz del Microscopio Electrónico de Barrido. Boletín de la Real Sociedad EspaÒola de historia Natural (Sección Biología), 95: 139-146.

Ronderos M. M., G. R. Spinelli & P. Sarmiento. 2000. Preparation and mounting of biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) to be observed with Scanning Electron Microscope. Transactions of the American Entomological Society, 126(1):125-132.




Figs. 1-3. Group a. Eggs *Culicoides venezuelensis* Ortiz & Mirsa. 1, entering egg with adhesive; 2, egg; 3, anterior end showing detail of exochorion. Scale bars: 100 μm.




Figs 4-8. Group b. Eggs *Dasyhelea necrophila* Spinelli & Rodríguez. 4, eggs mass; 5 immature egg; 6, broken egg; 7; ultraestructura off egg exochorion (Scale bars 20 μm) 8, ultraestructura off egg endochorion. (Scale bars: 10 μm).