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DEVELOPING AND EASY AND EFFICIENT PROTOCOL FOR THE STUDY OF DIFFERENT BLOWFLY INSTARS THROUGH SCANNING ELECTRON MICROSCOPY

Estibaliz Etxeberria-Rekalde[1]
Maite GilArriortua[1]
Marta I. Saloña-Bordas[1]
María Luisa Nó[2]

Key words: *Calliphora vicina*, SEM, egg, maggot, pupa, puparium, adult, ultrastructural microscopy optimization.

Abstract: Forensic entomology studies the arthropods to provide useful information in judicial proceedings, being the *postmortem* interval (PMI) estimation one of its most important contribution. Scanning electron microscopy (SEM) has been broadly used for the accurate identification of blowflies of forensic interest, but usually the sample preparation lasts too long and/or can produce the introduction of artefacts to the image. In that sense, the development of a reliable protocol for insect sample examinations through SEM is needed. The blue bottle fly *Calliphora vicina* Robineau-Desvoidy, 1830 is a blowfly related to decomposing remains, commonly reported worldwide in forensic caseworks and easy to be identified with basic blowfly knowledge. Therefore, the present work uses *C. vicina* as a model to design and develop an adequate, fast and simple protocol for the proper observation of blowflies through SEM. During the optimization of the protocol, the perfect combination of good image contrast, not too much artefact introduction and quick sample preparation were obtained using a mean time of glutaraldehyde treatment and no osmium tetroxide.

[1] Departamento de Zoología y Biología Celular Animal, Facultad de Ciencia y Tecnología, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Spain.

[2] Departamento de Física Aplicada II, Facultad de Ciencia y Tecnología, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Spain.

It was also observed that the use of critical point drying or hexamethyldisilazane to dry samples is not necessary, as air drying at room temperature on a blotting paper is safe, faster, cheaper and gives good results. In this way, and focused on *C. vicina*, the most adequate protocol to each developmental stage of blowflies (eggs, larvae, pupae, puparia and adult genitalia) was achieved.

Resumen: La Entomología forense estudia los artrópodos que aportan información útil en procesos judiciales, siendo la estimación del intervalo *postmortem* (IPM) una de sus contribuciones más importantes. La microscopia electrónica de barrido (MEB) ha sido ampliamente utilizada para una identificación precisa de moscardas de interés forense pero, frecuentemente, el procedimiento seguido es muy largo o puede introducir artefactos en la imagen. Por ello, se precisa desarrollar una técnica fiable para el estudio de insectos por medio del MEB. El moscardón azul *Calliphora vicina* Robineau-Desvoidy, 1830 es una especie asociada a restos en descomposición ampliamente citada en casos forenses y fácil de identificar con unos conocimientos básicos sobre las moscardas. Por ello, el presente trabajo ha empleado *C. vicina* como modelo para diseñar y desarrollar un protocolo sencillo, rápido y adecuado para la observación de moscardas por MEB. Durante la optimización del protocolo se logró la mejor combinación de eficiencia en el tiempo empleado para obtener un buen contraste de imagen sin introducir demasiados artefactos con el empleo de glutaraldehido y sin el uso de tetraóxido de osmio. También se observó que no es preciso el secado de las muestras por punto crítico o por el uso de hexametildisilazano dado que el secado al aire a temperatura ambiente sobre un papel presenta menos problemas, es más seguro, rápido, barato y da buenos resultados. Así, centradas en el moscardón azul *C. vicina*, se detalla el protocolo más apropiado para cada fase de desarrollo (huevos, larvas, pupas, pupas, pupario y genitalias de los adultos genitalia).

Palabras clave: *Calliphora vicina*, MEB, huevo, larva, pupa, pupario, adulto, optimización microscópica ultraestructural.

INTRODUCTION

Forensic entomology studies the arthropods to provide useful information in police investigations or judicial proceedings, being the *postmortem* interval (PMI) estimation one of its most important contribution (Arnaldos *et al.,* 2005, Gómez-Gómez *et al.,* 2007, Lord 1990, Wells & LaMotte, 2001). Medico legal issues in particular, need accurate identification of insect specimens (immature stages and adults) as a critical prerequisite to conduct correct analyses, since the developmental rate of each species differ with the temperature.

Many entomological studies (Chaiwong *et al.,* 2008, Martins Mendonça *et al.,* 2008, 2010, Sukontason *et al.,* 2004, 2006a, 2006b, 2006c, 2006d, 2008a, 2008b, Szpila & Villet, 2011, Ubero-Pascual, 2005, 2012) have used scanning electron microscopy (SEM) through different techniques, to describe fine morphological details of different structures in different phases of the blowfly cycle (eggs, larvae, puparia, adults), which may be useful in forensic investigations. The procedure for preparing biological samples for SEM analysis involves several progressive stages such as cleaning, fixation, dehydration or drying. To obtain good results, careful manipulation of specimens from the moment of the collection is required. A deficient treatment in any of these stages may produce distortions in the shape, general appearance or specific features of samples, as well as introduction of artefacts to the image or produce the shrinking of samples. Such deformations can lead to incorrect interpretations.

In that sense, the development of an appropriate and simple protocol for insect sample examination through SEM is the first step to provide correct diagnostic characterizations. Taking into account that in forensic laboratories the quickness in giving a result is maybe as important as the result itself, the development of an easy, fast and reliable protocol would be of a great interest.

Calliphora vicina (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae), is a synanthropic fly often associated with decomposing remains (Bonacci *et al.,* 2009, García-Rojo, 2004, Reibe & Madea, 2010). Due to its cosmopolitan distribution, this blowfly is reported worldwide in forensic investigations. Besides, having a preference for urban environments it is associated to public health problems, as carrier of pathogenic microorganisms (Greenberg, 1971). Furthermore, it has medical and veterinary importance as it can cause accidental myiasis (Soler Cruz, 2000). Its life cycle is holometabolous and this necrophagous species feeds on dead bodies, being extremely common on human corpses in temperate regions throughout the United States and Europe (Byrd & Castner, 2010).

Popularly known as blue bottle fly, *C. vicina* adults can easily be recognized by the metallic blue colour of the abdomen, the yellow basicosta in the upper border of the wing and the yellowish anterior spiracle (Rognes, 1991). These flies lay a large amount of white elongated egg masses. The eggs contain a furrow that runs almost the entire length of the egg, called median area (Mendonça *et al.,* 2008). The larvae that hatch from these eggs are soft-bodied and are commonly known as maggots due to the wormlike appearance/aspect of the body, they can be recognized due to the presence of an additional sclerite pigmented between the mouthparts. The posterior spiracles are surrounded by a complete peritreme and at a distance between each other similar to their diameter. The openings of the anterior spiracles are disposed in a single row of 5-7-openings. The body segments are delimited by few rows of thin spines arranged in groups (Szpila, 2010). *C. vicina* moves into three instars before hardening the cuticle during pupariation. Pupae are

cylindrical in shape and composed of the hardened larval integument of the last larval instar (Sukontason *et al.*, 2006d). Empty puparia have a smooth surface and preserve the remains of the mouthparts in the anterior part, allowing us to a quick confirmation of its identity.

As *C. vicina* is a common species, easy to be identified with a basic knowledge on blowfly diversity, we decided to use it as model to find an adequate protocol to be applied to future studies of blowflies through SEM. Therefore, the present work analyzes previous research published on scanning electron microscopy applied to insects, to develop an appropriate and simple protocol for the observation of the developmental stages of blowflies through SEM, focused on *C. vicina*.

MATERIALS AND METHODS

All the instars of *C. vicina* examined in this study were obtained from a colony maintained at the Forensic Entomology laboratory of the Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Biscay, Spain. Adults were captured using a selective attraction trap placed in a window of the laboratory, with animal viscera as bait, following a double funnel model (Hwang & Turner, 2005). They were then morphologically identified using taxonomic keys (Carles-Tolrá Hjorth-Andersen, 2004, González-Mora, 1989, Peris, 2004) before being transferred to breeding cages. Breeding conditions of laboratory colonies were room temperature and natural light/darkness photoperiod. Maggots were supplied with pork kidney and adults with water and sugar as culture substrates (Ireland & Turner, 2006, Kaneshrajah & Turner, 2004).

After reviewing literature sources, different protocols to prepare samples for SEM were designed, all of them summarised on Table 1. The first two protocols are mainly based on previous studies by Sukontason *et al.*, 2003 and Szpila & Pape, 2008, with little changes (Andrade, per. com). Remaining protocols were designed after observing the results from first assays. Changes consisted on the variation in treatment time with glutaraldehyde/ethanol and the use of different ways of drying, such as air drying on blotting paper. In the tested protocols, samples were boiled (in the case of eggs and maggots only) for a minute and washed by ultrasounds and saline solution (not protocol version 1) to prevent artefacts and dirty surfaces. They were later fixed (not protocol version 1) with a 2,5% glutaraldehyde mixture in phosphate buffer solution (PBS) with a pH of 7,4 at 4ºC, for different time periods depending on the protocol version. After fixation, samples were rinsed twice with PBS (not protocol version 1) and dehydrated through increasing ethanol concentrations. Some protocols also included treatment in acetone, the time lengths of both steps being

different depending on the protocol. Finally, samples were dried with hexamethyldisilazane or left to air dry on a blotting paper at room temperature and protected against any contamination, depending on the protocol version.

The samples, once treated, were stuck using double-sided conductor tape in cylindrical brass structures. They were then metalized by gold-palladium sputtering (Aspoas, 1991) in a BalTec SCD 004 coater. The metalization was done at about 50 mm in height, to avoid overheating of the sample (Goldstein *et al.,* 2003). The gas used to ionize the gold-palladium tablet was Argon, which was kept at a pressure of $5 \bullet 10^{-2}$ mbar. Samples were coated with a layer of about 10 nm thick with a current of 15 mA acting for 120 seconds. The micrographs were taken in a SEM JEOL 6400 actually at the UPV/EHU «Electronic Microscopy and Material Microanalysis service» installed in the Faculty of Science and Technology. Working conditions were: 15 keV beam energy, a beam current of less than 10^{-10} A, high vacuum and a working distance between 5 and 38 mm.

Complementarily, one sample was examined through low vacuum at UPV/EHU «Analytical and High resolution Microscopy in Biomedicine service», installed in the Faculty of Medicine. This sample did not undergo any prior treatment or metalization, and was seen by low vacuum SEM HITACHI S-3400N. Micrographs were taken under working conditions of 15 keV beam energy, 60 and 200 Pa pressure depending on the case, and at a working distance between 9.6 and 10 mm.

The terminology used in describing the morphology of the eggs in this paper followed Martins Mendonça *et al.,* 2008. Larval terminology follows Szpila, 2010. Pupae and empty puparia terminology follows Sukontason *et al.,* 2006d. Terminology used for adult genitalia follows Rognes, 1991 and Chaiwong *et al.,* 2008.

RESULTS

As cuticular hardness varies from one instar to another, it was unnecessary to test all protocols at all stages, because the information obtained from micrographs of a specific protocol for one stage allowed us to discard this protocol for other instars. In first assays, samples were treated in 1.5ml vials, but it was found that samples were usually deformed and it was not easy to manipulate them properly when changing reactive. Therefore, it was decided to use flat surface containers in following procedures.

Eggs

When trying to differentiate blowfly eggs, the aspect of the median area, how this ends in the anterior and posterior pole or the appearance of the

micropyle are details to focus on. The appearance of the islands inside this median area are also important as diagnostic characteristics, and an appropriate protocol for the observation of this samples through SEM should allow us to see them indubitably.

In the first protocol (Pv1), it was tested what happens when there is no glutaraldehyde fixation. Egg samples were just dehydrated in an ascending series of ethanol, starting from their ethanol concentration of conservation, 70% (Adams & Hall, 2003, Amendt *et al.*, 2007). Structures do not crystallize (Fig. 1A), but due to the lack of glutaraldehyde a loss of contrast in the image was observed, which makes more difficult a proper focus of the sample and the observation on detail of the islands.

A massive crystallization of the structure of the egg samples is produced when fixing them through 24 hours (Pv2) in glutaraldehyde (Fig. 1B). These artefacts produce distortions on the general appearance of the egg. However, this fixation period gives to the sample good contrast and focuses easily.

Three protocols (Pv3, Pv4 and Pv5) with mean times of fixation with glutaraldehyde/ dehydration through ethanol were designed, to test if fixation was unnecessary or glutaraldehyde treatment time should be near 24 hours, as previous protocols produced a lack of contrast (Pv1) or an excessive crystallization (Pv2). When immersing egg samples for 12 hours in glutaraldehyde and 6 hours of dehydrations, (Pv3) a good contrast was obtained but crystallization is still produced, some crystals still appear on the surface of the egg, not allowing us to see details in its surface structure (Fig. 1C). 6 hours/3 hours in glutaraldehyde/ethanol (Pv4) produced even less crystallization, while an acceptable contrast was maintained (Fig. 1D). The immersion in only 10 minutes of glutaraldehyde, followed by 10 minute dehydration lots in ethanol (Pv5), produced no crystallization of the structure but there was a high loss of contrast (Fig. 1E).

When lowering the glutaraldehyde treatment time down to 4 hours, and ethanol dehydration steps to 2 hours (Pv6) it was observed nearly no crystallization of the egg structures maintaining a good image contrast (Fig. 1F). But the appearance of the islands in the median area was not good enough. As in previous protocols the use of hexamethyldisilazane produced a strong drying of the samples and this wrinkle too much, it was decided to let them dry at room temperature on a blotting paper and protected against contamination. Thus, it was seen that samples wrinkle less, although an optimal result was still not achieved.

In the last proposed protocol (Pv7) the glutaraldehyde treatment lasted 2 hours, ethanol dehydrations 1 hour and the drying process was done on a blotting paper, at room temperature and protected against contamination. With this protocol (Figs. 2A-C) egg samples wrinkle less and enough image contrast is achieved without excessive crystallization of the structures, allowing us to see the appearance of the median area (Figs. 2A-B) or even the holes in the islands (Fig. 2C).

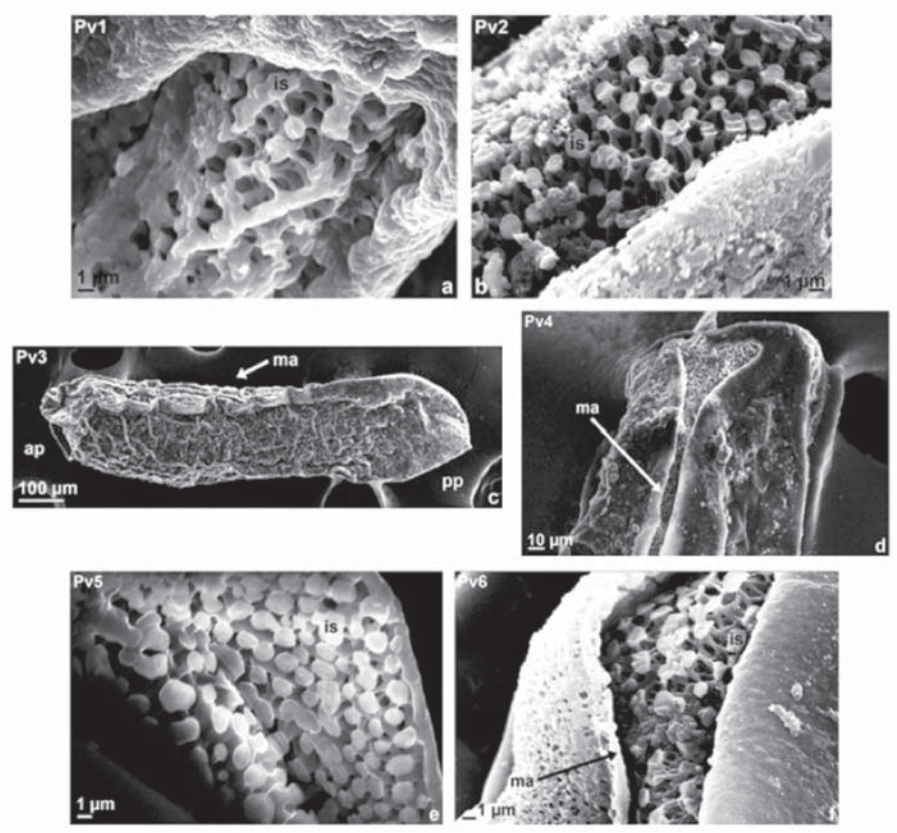

Figure 1. SEM micrographs of *C. vicina* eggs. (a) detail of the anterior pole of egg treated with Pv1. (b) detail of the anterior pole of egg treated with Pv2. (c) general lateral view of egg treated with Pv3. (d) dorsal view of the anterior pole of egg treated with Pv4. (e) detail of the anterior pole of egg treated with Pv5. (f) detail of the anterior pole of egg treated with Pv6. Abbreviations: (ap) anterior pole, (is) islands, (ma) median area and (pp) posterior pole

As eggs where one of the most problematic samples to prepare, for obtaining good results a complementary sample was tested in low vacuum microscope with no chemical treatment. The structure of the egg did not wrinkle, allowing us to see the general aspect and the appearance of the median area, but the lack of contrast when working under these pressures is very high, and it is very difficult to focus the image or see specific details

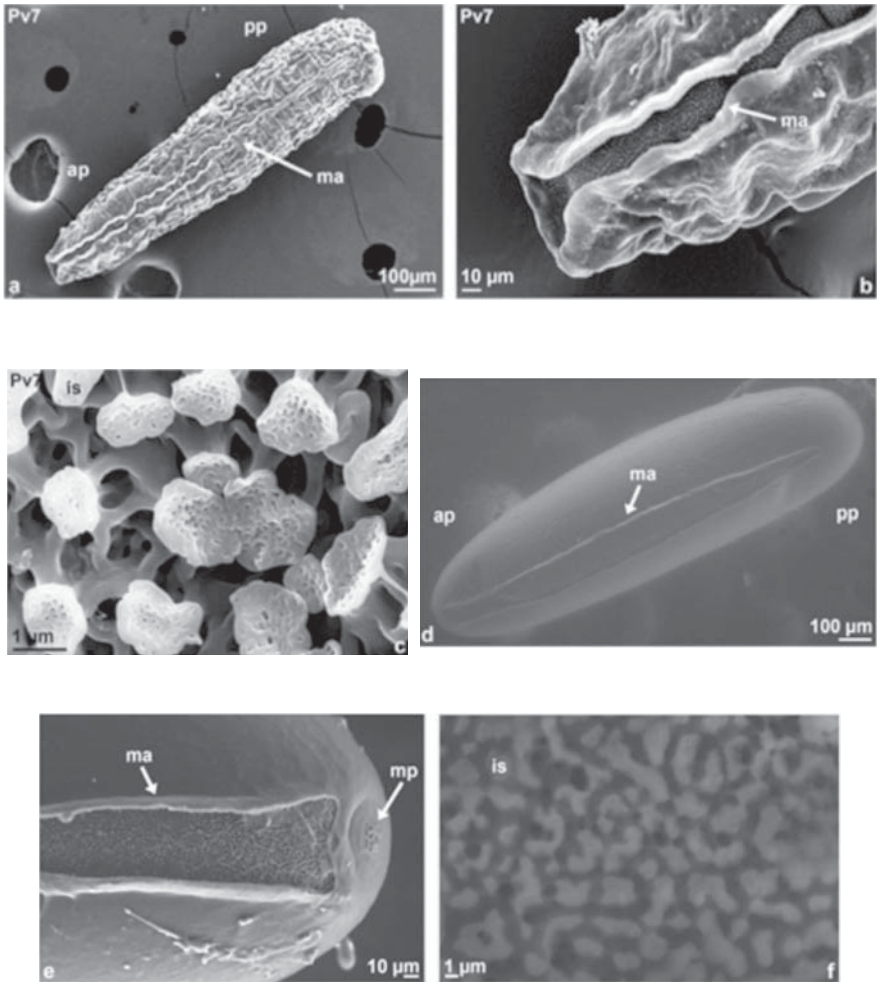

Figure 2. SEM micrographs of *C. vicina* eggs. (a) general dorsal view of egg treated with Pv7, with the median area extending almost through the entire length of the egg. (b) dorsal view of the anterior pole of egg treated with Pv7. (c) detail of the islands with holes in the median area of egg treated with Pv7. (d) general dorsal view of egg under low vacuum microscope, with the median area extending almost through the entire length of the egg. (e) dorsal view of the anterior pole of egg under low vacuum microscope with the micropyle differentiated near to the median area. (f) detail of the islands in the median area of egg under low vacuum microscope. Abbreviations: (ap) anterior pole, (is) islands, (ma) median area, (mp) micropyle and (pp) posterior pole.

of the islands in the median area (Figs. 2D-F). Furthermore, after some time the samples began to deform as a result of the near environmental vacuum low pressure.

Maggots

Although maggots look very similar to untrained eyes, they have many structures of diagnostic value. On the anterior spiracles, it is important to observe how many papillae there are. On posterior spiracles we should check if the peritreme is complete, focus on the shape of the bottom, how the spiracular discs are located one to each other, how the spiracular hairs

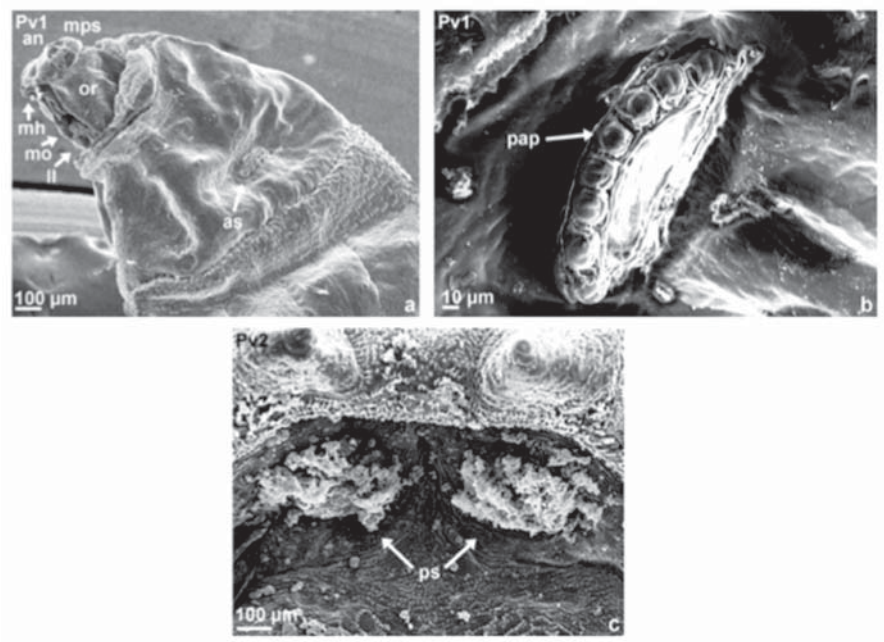

Figure 3. SEM micrographs of *C. vicina* maggots. (a) lateral view of the cephalic segment of a third instar maggot treated with Pv1. (b) anterior spiracle of a third instar maggot treated with Pv1, showing a single row of 8 papillae. (c) posterior spiracles of a third instar maggot treated with Pv2, completely crystallized. Abbreviations: (an) antenna, (as) anterior spiracle, (ll) labial lobe, (mh) mouthhook, (mo) mouth opening, (mps) maxillary palpus, (or) oral ridges, (pap) papillae and (ps) posterior spiracles.

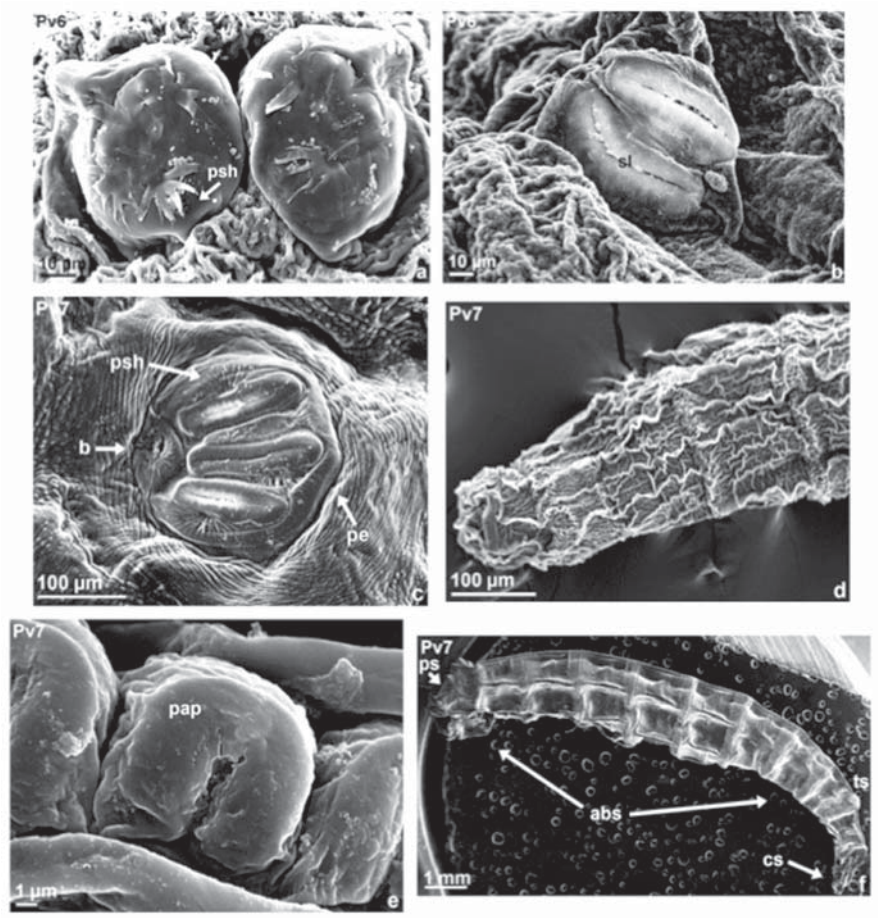

Figure 4: SEM micrographs of *C. vicina* maggots. (a) posterior spiracles of a first instar maggot treated with Pv6, bearing 1 spiracular slit. (b) posterior spiracle of a second instar maggot treated with Pv6, with the characteristic 2 separated and practically straight spiracular slits. (c) posterior spiracle of a third instar maggot treated with Pv7, with the characteristic 3 linear and separated spiracular slits. (d) detail of the cephalic and thoracic segments of a first instar maggot treated with Pv7 seen laterally, with the absence of the anterior spiracle. (e) detail of an anterior spiracle of a second instar maggot treated with Pv7. (f) lateral view of the entire body of a third instar maggot treated with Pv7. Abbreviations: (abs) abdominal segments, (b) button, (cs) cephalic segment, (pe) peritreme, (pap) papillae, (ps) posterior spiracles, (psh) posterior spiracular hairs (sl) spiracular slits and (ts) thoracic segments.

look like in each disc, or how many slits have the spiracular discs, together with their shape and position. The shape and disposition of the intersegmental spines have also taxonomic value.

Based on previous appreciations done on egg micrographs, maggots were not observed with protocols Pv3, Pv4, and Pv5. It was noticed that larvae reacted similarly to eggs to the use of different protocols, but they wrinkle less. When not using glutaraldehyde for the fixation (Pv1), a lack of contrast is observed but the structures are not crystallized at all (Figs. 3A-B). When using 24 hours of glutaraldehyde immersion and hexamethyldisilazane drying (Pv2), structures get completely crystallized and wrinkle a lot (Fig. 3C), not allowing us the observation of taxonomic details as the slits in the spiracular discs.

Using either glutaraldehyde immersions of 4 hours (Pv6) or 2 hours (Pv7), almost the same crystallization of structures is produced (Fig. 4). The contrast is good in both cases, but 2 hours of glutaraldehyde immersion give the right combination of little crystallization and enough contrast with shorter preparation time, useful in forensic investigations. The shape of the spiracular slits, or the details of the spiracular hairs in all the tree instars (Figs. 4A-C), the absence of anterior spiracle in the first instar (Fig. 4D), details of the papillae of anterior spiracles (Fig. 4E) or the general appearance of the maggot (Fig. 4F) are clearly seen with these protocols.

Pupae and empty puparia

When trying to identify a pupa, we have to look for the same characteristics as in maggots. Pupae and empty puparia have a hardened skeleton, and air drying is enough for SEM after cleaning the surface by ultrasounds (Pv1). This was the first and only protocol tested for these instars and as expected, being quite hard structures, enough image contrast is achieved with neither fixation in glutaraldehyde nor ethanol dehydrations, with the absence of artefact introductions (Figs. 5A-D). We can perfectly see the form of the intersegmental spines and how they are arranged in groups (Fig. 5A), the papillae in the anterior spiracle (Fig. 5B), how the distance between the spiracular discs is similar to their diameter (Fig. 5C) as well as the remains of the mouthhook inside the empty puparia (Fig. 5D), a very useful tool for specific identification.

Adults

In the case of identifying adults, observing some details of the genitalia as the shape of the supraanal plate, cerci, phallum or epandrium is usually helpful and basic in some other species of the family. Adult genitalia, as pupae and empty puparia, were just cleaned by ultrasounds and air dried (Pv1). It is a fast and simple protocol which, when used in samples with hard structures adequately cleaned, results in images with a good quality,

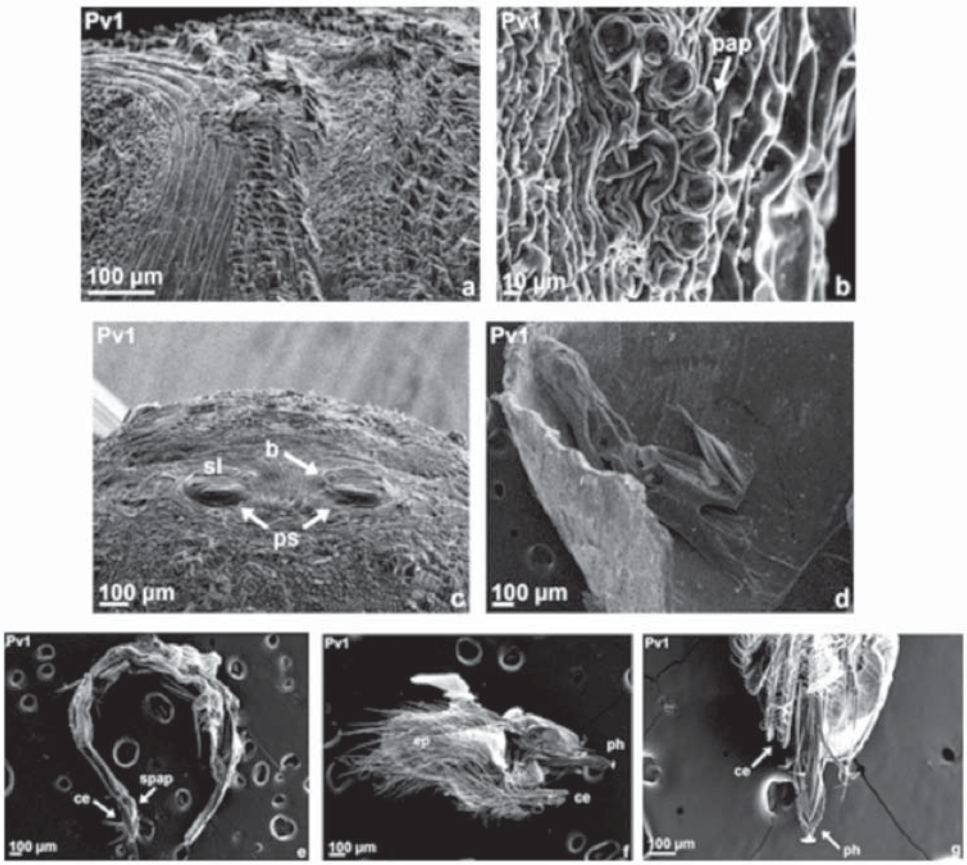

Figure 5: SEM micrographs of *C. vicina* pupae, empty puparia and genitalia treated with Pv1. (a) detail of the intersegmental spines. (b) anterior spiracle of the pupa displaying 8 papillae arranged in a single row. (c) posterior spiracles of the pupa having three spiracular slits on each spiracular disc. (d) detail of an empty puparium with remains of the larval mouthhook. (e) lateral view of female genitalia. (f) lateral view of male genitalia. (g) detail of the cerci and phallus of male genitalia. Abbreviations: (b) button, (ce) cerci, (ep) epandrium, (pap) papillae, (ph) phallus, (ps) posterior spiracles, (sl) spiracular slits and (spap) supraanal plate.

enough contrast and a lack of artefact introductions (Figs. 5E-G). This protocol allows us to see the details of the female (Fig. 5E) and male genitalia (Figs. 5F-G), as well as the long and thin form of male cerci, an important diagnostic character.

DISCUSSION

The procedure for preparing biological samples for SEM analysis involves several progressive stages and, a deficient treatment in any of these stages may produce distortions, introduction of artefacts to the image or produce the shrinking of the samples that lead to the risk of a misidentification. As such deformations can lead to misinterpretations, they should be minimized as much as possible, keeping at the same time the best contrast of what we want to observe. Between two protocols having similar results we will choose the easier and faster one, as in forensic laboratories the quickness in giving a result is maybe as important as the result itself.

To prepare eggs and maggots for SEM, it is advisable to boil them (1 minute in 100ºC water), so that they elongate adequately and putrefaction of the samples is prevented. It is also important to clean them by ultrasounds to prevent artefacts and dirty surfaces, and to wash them several times in a saline solution. During the treatment applied to the samples, it is important to prevent deformations or inadequate manipulations when changing reactive, so it was decided to use containers having a flat surface.

It was confirmed that when samples are not treated with glutaraldehyde (Pv1, Figs. 1A and 3A-B), the structures do not crystallize, an advantage when analyzing features of diagnostic value. But it is also observed a loss of contrast in the image, making difficult a proper focus of the sample and consequently having a loss of information. When fixing samples with glutaraldehyde, a good image contrast is obtained, but it also produces crystallization of the structures introducing artefacts to the sample. This was attributed to the exposed time, as for example with 24 hours immersion in glutaraldehyde (Pv 2, Figs. 1B and 3C), structures suffer a massive crystallization, not allowing us to see characteristics of diagnostic value such as the aspect of posterior spiracles (Fig. 3C).

Although many previous studies have used prolonged times of glutaraldehyde fixation (Chaiwong *et al.,* 2008, Chen & Fadamiro, 2008, Pellegrini *et al.,* 2011, Radhakrishnan *et al.,* 2009, Sukontason *et al.,* 2003, 2007a, 2008a, 2008b, Žďárek *et al.,* 1996), analyzing the images of the different protocols, we can conclude that glutaraldehyde is necessary to achieve good contrast, but produces strong artefacts that alter the surface appearance. Because of that reason, the best option is to use a mean time of glutaraldehyde treatment, enough to have a good image contrast at SEM

and not too much crystallization with the introduction of artefacts to the image. In addition, this makes the protocol faster. In the specific case of crystallization by glutaraldehyde resulting in artefacts that avoid the recognition of specific structures, one could choose to sacrifice image quality and try to see the structure without glutaraldehyde fixation (Pv1: Figs. 1A and 3A-B), a less contrasted protocol but without crystallization.

Osmium tetroxide has been broadly used in previous similar research (Chaiwong *et al.*, 2008, Chen & Fadamiro, 2008, Martins Mendonça *et al.*, 2008, 2010, Pellegrini *et al.*, 2011, Radhakrishnan *et al.*, 2009, Ruiz-Martinez *et al.*, 1989, Sukontason *et al.*, 2003, 2007a, 2008a, 2008b) as postfixation after glutaraldehyde, as it gives better fixation and contrast to the samples. But it can also introduce new artefacts, has a complicate manipulation because of its toxicity, and makes the protocol last longer. Taking all of this into account, we recommend not using osmium tetroxide, as glutaraldehyde gives enough image contrast in our assays.

When using dehydration phases for just 1 or 2 hours, and stopping them at the concentration of conservation of each kind of sample, EtOH 70% for eggs and EtOH 80% for maggots (Adams & Hall, 2003, Amendt *et al.*, 2007), better results were found (Pv6 and Pv7, Figs. 1F, 2A-C and 4), samples dry in a better form and wrinkle less. This makes the protocol faster than some of those previously described (Chaiwong *et al.*, 2008, Chen & Fadamiro, 2008, Sukontason *et al.*, 2003, 2008a, 2008b).

Most of similar SEM studies (Aspoas, 1991, Chaiwong *et al.*, 2008, Chen & Fadamiro, 2008, Martins Mendonça *et al.*, 2008, 2010, Pellegrini *et al.*, 2011, Radhakrishnan *et al.*, 2009, Ruiz-Martinez *et al.*, 1989, Sukontason *et al.*, 2003, 2007a, 2008a, 2008b, Szpila & Pape, 2008, Szpila & Villet, 2011, Ubero-Pascal & Puig, 2009, Ubero-Pascal *et al.*, 2005, 2012, Žďárek *et al.*, 1996) use the critical point drying technique or hexamethyldisilazane in the drying process. As hexamethyldisilazane achieves a faster and cheaper dryness than the critical point drying (Andrade, per.com.), this last one was not tested. In fact, we found that hexamethyldisilazane produces a strong drying of the samples, and these wrinkle too much (eggs and maggots in Pv1, Pv2, Pv3, Pv4 and Pv5, Figs. 1A-E and 3). Because of that, it was tried to leave samples at room temperature on a blotting paper, protected against any contamination (pupae and empty puparia in Pv1, Pv6 and Pv7, Figs. 1F, 2A-C, 4 and 5). As it can be seen when comparing the images through the different methods of drying, this is the most appropriate drying option, as samples wrinkle less, it is faster and it is also cheaper.

Apart from these general assessments, they were also some specific appreciations done for each specific stages of development of *C. vicina*.

Eggs

Because of its small size (2 mm in length), the diagnostic structures of the eggs are very difficult to assess through optical microscopy. This makes

SEM techniques particularly useful in describing features of diagnostic value in fly eggs (Martins Mendonça *et al.,* 2008) and it is therefore interesting to find an easy preparation protocol that allows us to appreciate them.

The egg samples wrinkle in excess during the drying process, because during the dehydration steps the embryo may shrink and the chorion get separated from it. Therefore, to see the egg form it is preferable to see them under a low vacuum microscope in which dehydration steps are not necessary. (Figs. 2D-F). Also, an advantage of this method is that in the early stages of observation the different parts of the egg preserve its shape and size, being possible to make real measurements that can't be done on eggs that have been fixed and/or dehydrated.

However, although under the low vacuum scanning electron microscope structures wrinkled less, the lack of contrast was very high and it was very difficult to focus the image at high magnification and to appreciate the details of the islands in the median area (Fig. 2F), important diagnostic characteristics. In addition, after some time, the samples began to deform as a result of the near environmental vacuum pressure and probably due to an overheating under the beam.

If a low vacuum microscope is not available or specific details are needed to be seen, the best analyzed option is the high vacuum SEM using 2 hours of glutaraldehyde fixation and air drying on a blotting paper (Pv7, Figs. 2A-C). This protocol gives enough image contrast without excessive crystallization of the structures, allowing us to see even the holes in the islands (Fig. 2C).

For instance, the use of an environmental microscope in which the samples will not suffer as they would be at atmospheric pressure is not advised, because the eggs could be heated under the beam even if a thermo-ionic gun is used. A good implementation to the low vacuum microscope would be a cryogenic plate, which should maintain the egg's structure without an increase of the temperature.

Maggots

The immature stages of most Dipteran families remain poorly understood. Although previous research report the presence of 5-7 papillae in the anterior spiracle (Szpila, 2010), we observe specimens with 8 natural openings surrounded by the papillae in these spiracles (Fig. 3B). Scavengers are very significant in forensic entomology, being calliphorids the most important in terms of utility (Pérez-Moreno *et al.,* 2006). Therefore, more detailed research involving other populations of these worldwide distributed species are already needed. This makes of special interest the development of an appropriate protocol for the preparation of these samples for SEM, being also able to extrapolate the results to other species.

When observing maggots, there is not much difference between the crystallization occurring after 4 hours of glutaraldehyde (Pv 6, Figs. 4A-B) and 2 hours of glutaraldehyde (Pv7, Figs. 4C-F). Both protocols use air drying on blotting paper and as the last one is faster, we consider it the appropriate protocol for the forensic study of such samples.

Pupae and empty puparia

The use of pupae or empty puparia in forensic entomology is still problematic, since accurate identification of the species is necessary, and the similarities between pupae of different species complicate the process (Sukontason *et al.,* 2007b). Therefore, an adequate protocol to characterize pupae and empty puparia by SEM and to identify them properly, is already needed.

Pupae and empty puparia just need to be cleaned by ultrasounds and air dried (Pv1, Figs. 5A-D) as these kind of samples have hardened structures, and without fixation in glutaraldehyde we can get good contrasted images. Thus, wrinkles on the structure are not formed and we avoid artefacts introduced when using glutaraldehyde. Besides, this protocol is easy and fast.

Adults

In the case of adults, being able to characterize by SEM the genitalia of flies is of interest as it gives indubitable structural information that can be applied in taxonomy (Chaiwong *et al.,* 2008).

Adult genitalia have also hard structures. Because of that reason, the ideal option is an ultrasonic cleaning followed by air drying on a blotting paper. (Pv1, Figs. 5E-G). As mentioned previously, it is a fast and simple protocol and gives images with a good quality, enough contrast, and lack of artefact introduction.

To summarize all the assessments done in this work, table 2 gives easy clues to select the adequate protocol for the examination of each stage of development, focused on *C. vicina,* through SEM, namely, eggs, maggots, pupae and empty puparia, and adults.

In conclusion, the results here obtained may provide a point of reference for future ultramorphological blowfly analysis, as a tool for the insect identification at any stage of development and even from partial remains.

CONCLUSIONS

When preparing samples for SEM, it is advisable to boil them for 1 minute (eggs and maggots), make an ultrasonic cleaning and use containers with flat surfaces.

In prolonged times of glutaraldehyde, it crystallizes under SEM producing unwanted artefacts. In low times we lose contrast, making it impossible to observe certain details. In the specific case of crystallization by glutaraldehyde resulting in artefacts that avoid the recognition of specific structures, one could choose to sacrifice image quality and try to see the structure without glutaraldehyde fixation (Pv1), a less contrasted protocol but without crystallization.

As glutaraldehyde gives enough image contrast we recommend not using osmium tetroxide, taking into account the toxicity, the difficulty of handling osmium tetroxide and the artefacts that could enter the image.

We consider not necessary the use of critical point drying, as hexamethyldisilazane achieves a faster and cheaper dryness. In fact, it was determined that the most appropriate option is to dry the samples at room temperature on a blotting paper and protected against any contamination.

To see the eggs shape, it is preferable to use a low vacuum microscope. If this is not available or specific details are needed to be seen, the best analyzed option is the high vacuum SEM using 2 hours of glutaraldehyde fixation and air drying on a blotting paper (Pv7).

We consider that the proper protocol for observing maggots would be 2 hours of glutaraldehyde immersion (Pv7). Although there is not much difference between the crystallization occurring after 4 hours in glutaraldehyde (Pv6), the first one is faster, making it the appropriate protocol for the forensic study of such samples.

Pupae, empty puparia and adults have the ideal protocol in just an ultrasonic cleaning and the air drying on a blotting paper (Pv1) due to their hard structures.

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TABLES

Table 1. Protocols developed for the study. Abbreviations: **Pv:** protocol version, **Sal. Sol.:** saline solution NaCl (0,9 %), **Glutar.:** glutaraldehyde, **PBS:** phosphate buffer solution pH 7.4, **EtOH:** ethanol, **E:** eggs, **M:** maggots, **PP:** pupae, **P0:** empty puparia, **A:** adults, **h:** hours, **Hexameth.:** hexamethyldisilazane, **AD/BP:** air drying on blotting paper.

	BOIL	CLEANING		FIXATION	CLEANING	DEHYDRATIONS		DRYING
	E and M	**Ultra-sounds**	**Sal. Sol.**	**Glutar.**	**PBS**	**EtOH**	**Acetone 80%**	
Pv1	1'	6-7' in EtOH 70% (M, EtOH 80%)	-	-	-	E, M → 70%, 80%, 90%, 2·100% 12h in each step PP, P0, A → -	E, M 2·12h	E, M → Hexameth. 2·10' PP, P0, A → AD/BP
Pv2	1'	6-7' in PBS	several times	24h	twice	30%, 50%, 70%, 80%, 90%, 2·100% 12h in each step	2·12h	Hexameth. 2·10'
Pv3	1'	6-7' in PBS	several times	12h	twice	30%, 50%, 70%, 80%, 90%, 2·100% 6h in each step	2·6h	Hexameth. 2·10'
Pv4	1'	6-7' in PBS	several times	6h	twice	30%, 50%, 70%, 80%, 90%, 2·100% 3h in each step	2·3h	Hexameth. 2·10'
Pv5	1'	6-7' in PBS	several times	10'	twice	30%, 50%, 70%, 80%, 90%, 2·100% 10' in each step	2·10'	Hexameth. 2·10'
Pv6	1'	6-7' in PBS	several times	4h	twice	30%, 50%, 70%, (M, 80%) 2h in each step	-	AD/BP
Pv7	1'	6-7' in PBS	several times	2h	twice	30%, 50%, 70%, (M, 80%) 1h in each step	-	AD/BP

Table 2. Adequate protocols for the examination of each instar through SEM. Abbreviations: **EtOH:** ethanol, **h:** hour, **AD:** air drying, **BP:** blotting paper.

		EGGS	**MAGGOTS**	**PUPAE AND EMPTY PUPARIA**	**ADULTS**
Boil		1'	1'	-	-
Cleaning	Ultra-sounds	6-7' in PBS	6-7' in PBS	6-7' in EtOH 70%	6-7' in EtOH 70%
	Saline Solution	several times	several times	-	-
Fixation	Glutaral-dehyde	2h	2h	-	-
Cleaning	PBS	twice	twice	-	-
Dehydrations	EtOH	30%, 50%, 70% 1h in each step	30%, 50%, 70%, 80% 1h in each step	-	-
	Acetone 80%	-	-	-	-
Drying		AD/BP	AD/BP	AD/BP	AD/BP