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Morphology of preimaginal stages of *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera, Calliphoridae): A comparative study

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ABSTRACT

A comparative morphological study of preimaginal stages (larvae and pupae) of *Calliphora vicina* Robineau-Desvoidy, 1830 is presented. The entomological samples came from laboratory colonies bred under controlled environmental conditions (25 °C and 60% relative humidity). In this study, a recently published technique to clear Diptera larvae for light microscopy and a standard protocol for scanning electron microscopy were used. For the morphological comparison of larval instars I, II and III, and pupae of *C. vicina*, different larval regions (cephalic, thoracic and abdominal, including anal division), as well the internal chitinised cephalopharyngeal skeleton, were considered separately. Our results focus on showing the changes observed throughout development for the most important structures in the cephalic region (sensilla of maxillary palpus, antennae and oral ridges), the thoracic region (the first segment and its anterior spinose band) and in the anal division of the abdominal region (posterior spiracles and shape of the papillae). In addition, some morphological structures are described or pictured for the first time, such as the ventral organ and the anterior spiracle of larva I and the antenna sensilla, Keilin's organ and wrinkled area of the anal division of all instars. The cephalopharyngeal skeleton is an important structure for the taxonomy of Diptera larvae in all instars, including Calliphoridae. Our observations in *C. vicina* indicate that an in-depth review of the sclerite composition is needed. Pupae and larvae stages can only be compared by following the segmentary spinose bands and the anal segment, where the morphology of the posterior spiracles and papillae can be observed, in some cases despite the reduced condition of the latter.

Preimaginal stages of sarcosaprophagous Diptera are very interesting in forensic sciences from an applied point of view, because they provide relevant evidence for estimating the postmortem interval (e.g., Refs. [1–4]). Sometimes, the specific identification of preimaginal stages is difficult because some species are very similar morphologically, and they have not been studied in detail. Thus, in many cases, there are no good references to distinguish among them, especially in some geographical areas. Currently, the identification of preimaginal specimens requires breeding in the laboratory in order to obtain the adult stage. These adult stages have been studied in detail, and, for their identification, precise morphological parameters exist [5–9].

There is currently a large interest in studying preimaginal stages, and many articles have provided morphological descriptions of larvae and/or pupae, using mainly techniques of scanning electron microscopy (SEM). However, some of these publications are focussed solely in describing one of the larval stages, usually the first or the third stages, or the pupae [10–23]. Only a few studies have dealt with the morphology of the complete preimaginal life cycle [24–31]. As mentioned above, the study of all the immature stages of sarcosaprophagous Diptera is of great interest, because the characteristic morphology of each one could provide specific features that will become essential for a proper identification.

Historically, the studies of compared larval morphology of Diptera have been performed using light microscopy, especially when cephalopharyngeal skeleton was the focus of the study [12,32–34]. Hence, the main keys to distinguish Calliphorids, as well other Dipteran, have been performed based on this type of data [12,18,32,35,36]. Unfortunately, light microscopy is still the only technique available for many researchers in this kind of studies. We considered that morphological studies based on SEM techniques should be supplemented with light microscopy observations, because these techniques are complementary (e.g., Refs. [17,18,23,28,29,36,37]). While SEM provides accurate high-resolution images of structures, light microscopy allows discrimination between structures with the same density to electrons, as cirri [22,23], or to observe internal structures, such as the cephalopharyngeal skeleton.

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Calliphora vicina Robineau-Desvoidy, 1830 (Calliphoridae) is a cosmopolitan species, occurring in urban and natural environments. It is highly associated with forensic cases involving dead humans and has also been related to different types of myiasis [38–41]. Because of the above, many aspects of its biology have been considered and studied [10,32,42–44]. The morphology of its preimaginal stages has been thoroughly studied and described using mainly light microscopy techniques (e.g., Refs. [12,32]), but SEM studies are rare, and only deal with individual instar [10,12,23]. Besides this, larva II are practically undescribed from a morphological point of view, not only by SEM, but also by light microscopy, and some features of other instars are either not described, such as the wrinkled area of the anal division, or are not pictured, such as the ventral organ and anterior spiracle in larva I. Our work attempts to extend previous studies, not only by describing the morphological characteristics of every preimaginal stage of the life cycle of *C. vicina* using SEM and light microscopy techniques, but also by comparing the changes undergone by the main structures of the body. This article attempts to facilitate the specific identification of *C. vicina* in all preimaginal instars found in carrion, without the necessity to rear them in a laboratory. It also offers precise descriptions of morphological features already known, poorly characterised or not yet described, for systematic purposes.

1. Materials and methods

Adults of *C. vicina* were collected in the Campus of Murcia University (Southeastern Spain) using a modified Schoenly trap [45]. Selected specimens were bred in the laboratory under controlled environmental conditions (25 °C and 60% relative humidity), using pig liver as substrates for egg-laying and larval food. Pupation substrate was sand surrounding the liver. All the immature specimens studied came from these sister colonies. All studied specimens, including adults, have been retained in the collections of the Área de Zoología in the Departamento de Zoología y Antropología Física of Universidad de Murcia.

A total of 120 specimens were studied: 40 specimens of larvae I and II, 20 specimens of larvae III and 20 specimens of pupae. They were selected, rinsed, euthanised in water near to boil (except pupae), and fixed in McDowell fixative solution (4% formalin, 1% glutaraldehyde mixture in cacodylate buffer solution, pH 7.4) [46], at 4 °C for 24 h. Specimens were rinsed again twice in sodium cacodylate + sucrose solution 0.1 M, dehydrated until absolute acetone in a gradient of increasing concentration of ethanol and ethanol–acetone solution (50% of each reagent) and dried using critical point method or by air-drying after hexamethyldisilizane treatment [47]. Dried specimens were mounted on SEM stubs with conductive adhesive tape, mounting them in different positions (dorsal, lateral and ventral views). For third instar larvae and pupae, larger than other instars, it was necessary to cut the specimens to study efficiently the fore and hind parts of the body. Specimens were coated with Au–Pd in a Polaron Bio Rad Sputter Coat and observed in a Jeol 6100 SEM. Pictures were directly obtained and digitised from the SEM.

For light microscopy study, 10 specimens of larvae I and II and 5 specimens of larva III were chosen, euthanised in water near to boiling, fixed in ethanol 70% for 2 h, dehydrated until ethanol absolute and cleared in methyl salicylate (wintergreen oil) [48] for 1–3 h depending on the larval instar. Specimens were mounted in concave microscope slides using Canada Balsam as mounting medium. Microscope slides were observed using a Leica MZ 9.5 stereomicroscope with episcopic and diascopic illumination devices and a Nikon Eclipse i80 microscope with bright field and Nomarski interference contrast. Pictures from both microscopes were taken using a Nikon DS-Fi1 digital camera of 5-megapixel CCD and Nis-Elements software.

To describe the morphological characteristics of larvae, cephalopharyngeal skeleton and pupae, the body divisions and the terminology given by Erzinçlioglu [32], Courtney et al. [49], Sukontason et al. [15] and Szpila et al. [23] were followed.

1.1. Abbreviations used in text and figures

aI-VII, abdominal segments; *ad*, anal division; *ae*, additional spiracle structure; *al*, anterior labial sclerite; *an*, antenna; *ans*, antennal sensilla; *a|ms*, antennal or mandibular sensilla; *ao*, anal opening; *ap*, anal protuberance; *as*, anterior spiracle; *asb-2*, ventrolateral secondary anterior spinose band of tI; *asb*, anterior spinose band; *b*, buttom; *bm*, bubble membrane; *br*, basal ring; *cd*, cephalic depression; *ci*, cirri; *cl*, dephalic lobe; *cf*, cleft; *cp*, circular plate; *cs*, cephalopharyngeal skeleton; *cw*, creeping welt; *db*, dorsal bridge; *dc*; dorsal cornu; *dm*, antennal dome; *es*, ectostomal sclerite; *fa*, fan-shaped structure; *fl*, folding tegument; *fm*, facial mask; *ig*, intersegmental groove; *is*, intermediate sclerite; *lb*, labrum; *la*, labial arch; *ll*, labial lobe; *lo*, labial organ; *ls*, labrum supporting sclerite; *mk*, mouthhook; *mk-3*, infant mouthhook of instar III; *mo*, functional mouth opening; *mp*, maxillary palpus; *mps*, maxillary palpus sensilla; *ms*, medial spine; *no*, nose-like structure; *os*, oral sclerite; *or*, oral ridges; *p1-7*, posterior papillae; *pap*, papillae; *pb*, parastomal bar; *pd*, anal pads; *pe*, posterior spiracle; *pl*, posterior labial sclerite; *pp*, posterior projection; *pr*, peritreme; *ps*, pseudocephalon; *ps-c*, pseudocephalon collapsed; *psb*, posterior spinose band; *pt*, peristigmatic tuft; *rh*, respiratory horn; *rp*, rounded protuberance; *rs*, respiratory slit; *sb1-3*, basiconic sensilla of maxillary palpus; *sc1-3*, coeloconic sensilla of maxillary palpus; *sd*, dental sclerite; *sp*, spines; *tI-III*, thoracic segments; *td*, tiny digitations; *vc*, ventral cornua; *vo*, ventral organ; *vop*, ventral organ prominence; *vos*, ventral organ sensilla; *vp*, ventral plate; *w*, cornu window; *wa*, wrinkled area; *?*, unidentified maxillary palpus sensilla.

2. Results and discussion

The actual number of segments constituting the larval body of Cyclorrapha has been discussed, mainly those forming the cephalic region [49], but externally 12 can be identified (Figs. 4A and 5A) [11,16,19–21,23,25–27,29,32]. In Calliphoridae, the noticeable segments of the larvae could be grouped as pseudocephalon or cephalic region (first membranous segment, probably evolved from three embryonic segments), thorax (from second to fourth segments; they will be called *tI*, *tII* and *tIII*) and abdomen (from fifth to twelfth segments; they will be called *aI* to *aVIII*) [23,32,49], although the 12th segment is also known as the anal division (e.g., Refs. [32,49]: Fig. 7). The 12 segments can also be identified in Calliphoridae pupae, although the first and several parts of the anal division are collapsed [10,15,49]. Therefore, our results are presented regarding these different regions, except for those relating to the cephalopharyngeal skeleton and pupae.

2.1. Pseudocephalon morphological description and comparison

Pseudocephalon is a highly specialised region of larval body; it contains several sensorial structures and, ventrally, the functional mouth opening. Morphologically, this region is very unique and its structures develop in a more complex way than in other segments. Pseudocephalon is bilobate anteriorly (Figs. 1A, 5C and 6B) or shows two latero-dorsal cephalic lobes. This feature is very clear in instar III because the lobes show a round shape and are separated from the rest of the pseudocephalon by a neck (Fig. 3A). In instars I and II the cephalic lobes are less conspicuous (Figs. 1A and 2A) and the bilobate characteristic is noticeable only dorsally; laterally and ventrally the cephalic lobes are delimited by the facial mask (Figs. 1A and 2A). The number of sense organs or, simply, cephalic structures used to describe this region, varies in the literature and, generally, depends on the larval instar. For instance, while Erzinçlioglu [32] notes five types of papillae to describe the cephalic region of Calliphoridae (antenna, oral papilla, maxillary palpus and two supramaxillary papillae), Courtney et al. [49] propose several other structures, such as labial lobe, cirri and oral ridges, some of which are described in detail by Szpila et al. [23] for *C. vicina* larvae I. Sometimes, the terms and structures considered in different articles do not agree completely, making their use difficult. Therefore, we attempt to describe the cephalic region in the three larval instars integrating and, where possible, discussing the different terminologies.

Cephalic lobes are very interesting since each harbours two of the main sensorial structures, the antenna and the maxillary palpus. Their homologies to adult structures are not currently understood [32,49]. Antenna is disposed dorsally to maxillary palpus, which is placed in an antero-lateral position (Figs. 1A, 2A and 3A), and the relative distance between them is constant in all instars. Antenna is composed of two structures, a basal ring or socket and a distal dome (Fig. 1C). The relative size between dome and basal ring decreases from instar I to III (Figs. 1C, 2C and 3C). Antenna of instar I have a dome like a bullet and almost twice as long as the length of the basal ring (Fig. 1C), while in instars II and III the dome is conic-shaped and has a length equal to or less,