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Ultrastructure analysis of the immature stages of *Ravinia belforti* (Diptera: Sarcophagidae), a species of medical-veterinary and forensic importance, by scanning electron microscopy

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ABSTRACT

The postmortem interval is related to the age of immature species of flies found on corpses and can be estimated using data available in the literature on the biology of the species. The flesh fly *Ravinia belforti* is a carrier of enteric pathogens that can affect human and animal health as well as being of forensic importance. As the morphology of many immature Sarcophagidae is unknown, these immature forms must be collected and characterized after the emergence of the adult male. Here we describe and analyze the morphological characteristics of the larvae stages L1, L2, L3 and the puparium of *R. belforti* by scanning electron microscopy (SEM). Ten specimens of each stage were analyzed. Larvae of *R. belforti* follow the typical muscoid vermiform pattern with 12 segments. The anterior region is pointed, while the posterior region is thicker. The spines of the cephalic collar are flattened and with double, triple or quadruple points, different from the spines along the body that only have a single point. In L2, the anterior spiracle is present with a varying number of papillae (16–22), differing from other species. The posterior spiracles are located within the peritreme. The spiracular cavity is internalized in the posterior region, following the pattern that differs Sarcophagidae from other families. L3 features more visible and developed spines around the cephalic collar, getting thicker and denser near to the first thoracic segment. Puparium is similar to other species of Sarcophagidae. This paper presents important data on this family which has both health and forensic importance. Furthermore, *R. belforti* shows significant differences from other species of Sarcophagidae.

1. Introduction

An important application of forensic entomology is to estimate the postmortem interval (PMI) when a body is already in an advanced stage of decomposition and the traditional methods are not realiable (Liu and Greenberg 1989; Benecke 1998; Greenberg and Kunich 2002; Vairo et al., 2015a). The PMI can be calculated based on the age of immature muscoid flies collected from the corpse using data available in the literature on the biology of the species in question (Salviano et al., 1996; Campobasso and Introna, 2001; Oliveira-Costa and Mello-Patiu, 2004; Amendt et al., 2007; Nassu et al., 2014; da-Silva-Xavier et al., 2015). Various species of flesh flies (Diptera, Sarcophagidae) are attracted to decaying corpses, and therefore may be used as entomological evidence in criminal investigations (Catts and Goff, 1992; Benecke, 1998; Introna et al., 1998; Al-Mesbah et al., 2011).

The flesh fly *Ravinia belforti* Prado & Fonseca, 1932 is distributed throughout Argentina, Brazil, Colombia, Paraguay and Trinidad & Tobago (Mello-Patiu et al., 2009). This species has forensic importance due the fact that both immature and adult flies are found on animal carcasses and human cadavers (Carvalho and Linhares, 2001; Oliveira-Costa et al., 2001; Barros et al., 2008; Barbosa et al., 2009; Oliveira and Vasconcelos, 2010; Rosa et al., 2011; Alves et al., 2014). In addition, d'Almeida and Salviano (1996) reported that *R. belforti* has a preference to deposit their larvae on animal feces, including human feces. Besides being used to estimate the PMI, this species may indicate cases of abuse or neglect of the elderly, children and vulnerable persons (Benecke and Lessing, 2001; Benecke et al., 2004). As *R. belforti* uses feces as substrate to lay larvae and is a species with a high level of synanthropy, it is a potential carrier of enteric pathogens (*e.g.* bacteria, fungi, protozoa, viruses, worms

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and helminthic eggs) that can affect human and animal health (Linhares, 1981; d'Almeida and Mello, 1996).

Accurate identification of the Sarcophagidae species is usually made through the adult male genitalia (Carvalho and Mello-Patiu, 2008). However, it is very common to find immature stages on a corpse as adult females use the carcass to deposit their larvae (Anderson, 1999; Otranto and Stevens, 2002; Cherix et al., 2012). The few keys to identify immature forms of Sarcophagidae refer mainly to the species of the northern hemisphere, most commonly in Europe and Asia (Zimin, 1948; Ishijima, 1967; Smith, 1986; Velásquez et al., 2010; Szpila et al., 2015). Consequently, the morphology of many immature forms of Sarcophagidae species in the New World is poorly understood, which impedes the species identification *via* the larval instars or puparium.

In these cases the immature forms collected from corpses needed to be reared in the laboratory and after the emergence of the adult male the identification can be made (Smith, 1986; Byrd and Castner, 2010). In addition while waiting to complete the life cycle of the fly, other problems may prevent the identification of the species, for example, the small number of specimens collected, the low survival rate of the specimens that reach the laboratory and even the difficulty to breed a particular species under artificial conditions (Sukontason et al., 2003a; Pujol-Luz et al., 2008).

Scanning electron microscopy (SEM) is an extremely important tool for the morphological characterization of immature flies. SEM is able to reveal structures that cannot be viewed through the optical microscope and thus enrich the taxonomic data of the morphology of these understudied immature forms (Leite and Lopes, 1987; Liu and Greenberg, 1989; Dahlem, 1991; Sukontason et al., 2003b; Ubero-Pascal et al., 2010, 2015; Singh et al., 2012; Samerjai et al., 2014).

In this paper, we describe and analyze the morphological characteristics of the immature forms of *R. belforti*. The morphology of the first, second and third instar larvae and puparium were analyzed by SEM.

2. Material and methods

Adults of *R. belforti* were collected with the aid of a modified Shannon trap (Barbosa et al., 2009; da-Silva-Xavier et al., 2015) on the campus of Instituto Oswaldo Cruz (IOC, FIOCRUZ) (22°51′06″s 43°14′27″W), in the metropolitan area of Rio de Janeiro, Brazil. The flesh fly *R. belforti* was identified by the adult identification key elaborated by Carvalho and Mello-Patiu (2008) and the captured flies were placed in wooden cages (30 cm × 30 cm × 30 cm) to establish a colony. The colonies were kept at the Laboratório de Entomologia Médica e Forense (LEMEF, Instituto Oswaldo Cruz—IOC, Fundação Oswaldo Cruz—FIOCRUZ) and the rearing followed the methodology previously described by Queiroz and Milward-De-Azevedo (1991).

The second generation larvae from the colony were killed in hot water (75–80 °C) and washed with 2% sodium hydroxide for five minutes. After this process, the larvae were fixed in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The larvae were subsequently washed three times (10 min each) in the same buffer. Then, post-fixation in osmium tetroxide 1% was carried out for 1 h at room temperature and in the dark. After this process, they were washed once more in sodium cacodylate 0.1 M (three times, 10 min each).

The next step was the dehydration of the specimens through increasing ethanol series (7.5, 15, 30, 50, 70, 90 and 100%) for 15 min at each concentration. At the end of this step, the samples were subjected to drying by the critical point method using superdry CO_2 (Hayat, 1970).

The puparium were not subjected to any fixation, post-fixation and drying process due to its rigid cuticle, composed of chitin. The pupae were killed by freezing at −23 °C for approximately 24 h. The larvae and puparium were mounted on specific metal brackets, attached with double-sided tape and covered by a thin layer of white gold (20–30 nm) to be viewed in the scanning electron microscope Jeol JSM 6390LV of the Plataforma de Microscopia Rudolf Barth, IOC, FIOCRUZ.

Ten specimens of each larval and puparia stage were analyzed. The terminology used in the morphology description followed Ishijima (1967), McAlpine et al. (1981) and Courtney et al. (2000).

3. Results

The general morphology of the instars of *R. belforti* follows the typical vermiform pattern of muscoid dipterans. The anterior region is narrower than the posterior region and the cylindrical body of the larvae presents one pseudocephalon, three thoracic segments (T1–T3) and eight abdominal segments (A1–A8) (Figs. 1 A and 2 A).

3.1. First instar—L1

In the first instar the pseudocephalon is divided into two lobes on which there are antennae, sensorial papillae, maxillary palps and oral ridges (Fig. 1B). The presence of maxillary hooks was only observed in the first instar specimens (Fig. 1C). The spines that separate the cephalic region of the first thoracic segment are small, flattened and arranged in groups of double, triple or quadruple tips (Fig. 1D,E). The body of the larva has a wrinkled tegument and the segments are divided by smaller spines, flattened and single tips always pointing to the posterior region. The posterior region is thicker and where the posterior spiracles, with an internalized spiracular cavity, are found. This region presents a large number of spines and the circumspiracular tubercles are not yet well developed in the L1 (Fig. 1F).

3.2. Second instar—L2

The body of L2 is similar to the L1, but is larger (Fig. 2A). The pseudocephalon is more developed. In this region, the dome-shaped antennae, oral ridges and maxillary palps are clearly visible. The spines of the cephalic collar become more dense, flattened and with double, triple or quadruple tips (Fig. 2B,C). The maxillary hooks were retracted in all samples examined. The anterior spiracle has a varying number of papillae (16–22). The papillae are arranged along the anterior spiracle in an irregular row, usually in a pattern of a double row (Fig. 2D). The spines of the abdominal segments have simple tips (Fig. 2E). In the anal segment, the posterior spiracle has a pair of incomplete peritremes with two slits each. The spiracular cavity is internalized in the anal region, which is surrounded by more developed and elongated circumspiracular tubercles. These tubercles are arranged in four groups of three, surrounding the spiracular cavity, making a total of 12 tubercles (Fig. 2F).

3.3. Third instar—L3

The third instar larvae have similar morphologies to the L2 larvae, but are heavier and larger. Furthermore, L3 has fully developed structures and spines in larger quantities (Fig. 3A). The cephalic region shows antennas, oral ridges and maxillary palps all fully-developed (Fig. 3B). The spines of the cephalic collar are more visible and well developed with 1–4 tips, getting thicker and denser as they get closer to the first thoracic segment (Fig. 3C). The anterior spiracles, as well as having more developed and ornate papillae, have undergone a change from the L2 spiracle format. In all the L3