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Ultra structure of second instar larva of *Hemipyrellia ligurriens* (Wiedemann) (Diptera: Calliphoridae), a forensically important blow fly species from India

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ABSTRACT: Ultra structural characters of second instar larvae of *Hemipyrellia ligurriens* are elucidated through micrographs (Scanning Electron Microscope). Morphological details of maxillary palpi, antennae, oral cirri, facial mask, labial lobe, spinulations, and papillae of anal segment are described. Oral cirri are ten in number, arranged bilaterally on each side of the functional mouth opening and gently curved medially. The labial lobes are distinctively demarcated with fleshy projections antero–ventrally and have a characteristic shape. Thoracic spines have a bulbous base, slender sharp tips and are directed backwards. Prominent dorsal and ventral anal papillae with projected tips and broad conical base were present surrounded by microtrichia. The ultrastructure details of *H. ligurriens* would help in the rapid and accurate identification of the species in forensic investigations and to estimate time since death in medico legal cases. This is the first report on the ultra-structural features of *H. ligurriens.*

KEYWORDS: *Hemipyrellia ligurriens,* identification, micrograph, scanning electron microscope

Forensic examinations involving decomposed dead bodies need a careful scrutiny of the entomological evidence as the latter being very significant in calculating time of death when the natural postmortem signs of body hold no significance beyond certain level of putrefaction. Studies on insects of forensic significance is very much rudimentary in India except for a few reports on selective species (Bala and Singh, 2015; Bharti and Singh, 2003; Kulshrestha and Chandra, 1987; Rao *et al.,* 1984). *Hemipyrellia ligurriens* (Calliphoridae: Luciliinae) seems to be a synanthrope found in close association with human habitats, garbage dumps, decaying animal bodies and cadavers. The adult flies are generally considered as the vectors of many enteric pathogens (Sinha and Nandi, 2007). Kano and Sato (1952) reared this species on raw fish in Japan and described the larval stages. Ishijima (1967) described the third instar larvae of *H. ligurriens* while Bunchu *et al.* (2012) studied the morphological characters of larval stages of the species using light and stereo microscopy.

The oldest descriptions about all the three larval instars of *H. ligurriens* were provided by Tao (1927) and Knipling (1939). The keys provided in these works were of limited application owing to lack of species specific details. In recent works, blowfly species were identified based on the

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structure of antennae, maxillary palpi, cirri, spines and anal papillae/tubercles (Bunchu *et al.,* 2012; Sukontason *et al.,* 2008). The need for identification of larvae becomes significant especially during forensic investigations when larval specimens are presented. Studies suggest that rapid and more accurate identification of the species is possible through detailed examination of the ultra structural features of the larvae (Liu and Greenberg, 1989; Mendonca *et al.,* 2013).

Blowflies are normally the first insects to infest dead bodies (Smith, 1986). Their larval stages can be aged using knowledge of their developmental rates, and this can then be used to calculate time of death (Erzinclioglu, 1989). Morphological studies of larvae using light microscopy do not provide species specific details and hence larvae should be reared till adult fly emerges. Therefore scanning electron microscopic (SEM) examination of ultra structural details of larvae is very important and would help to identify the species rapidly and accurately (Liu and Greenberg, 1989; Mendonca *et al.,* 2013). SEM studies of larvae of few species of *Luciliinae* have been done by different workers in different parts of the world (Sukontason *et al.,* 2008; Sandeman *et al.,* 1987; Klongklaew *et al.,* 2012; Szpila *et al.,* 2013). However similar studies are lacking in India. Hence the ultra-structural features of *H. ligurriens* in the region was studied for the rapid and accurate identification of this species in forensic investigations to estimate time since death

Second instar larvae of *H. ligurriens* were collected from the outdoor rearing facility for blow flies in Kolangattukara, Thrissur, Kerala (10.5808°N, 76.1875°E). Adult females of *H.ligurriens* were trapped and isolated in the rearing cabinet with decomposing bovine meat as bait. The adult flies were identified using the morphological keys provided in standard literature (Senior-White *et al.,* 1940). Molecular diagnosis of the species was done based on Cytochrome oxidase Subunit I (COI) gene. The DNA sequencing was done at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. Sequence similarity was searched using NCBI BLAST. The sequence was submitted in GenBank, NCBI (GenBank Accession No: MN831480). The insects were reared in the rearing cabinets positioned in the outdoor facility during September 2019. Relative humidity, rain fall and temperature were monitored. The insects were provided with honey and water as food and liquid sources. The flies arrived near the cabinet were trapped with fly net and female of the species was kept in the cabinet. The bovine meat kept in rearing cabinet served as reflex stimuli for the adult female fly to lay eggs. Vermiculite was kept as the bottom layer in the cabinet to assist migration of third instars for pupation.

The second instar larvae were collected and washed many times in distilled water. To kill the larvae and to prevent deformation changes, the larvae were kept in boiling water (96-99°C) for two minutes and finally preserved in 70% alcohol (Adams and Hall, 2003). Sample preparation for SEM included dehydration using 99.5% alcohol followed by ultra sonification and air drying. After being dried at room temperature, larval specimens were gently placed on to stubs fixed with double tape. The specimen was coated with gold using gold sputtering for 10 seconds with 10mA current in the sputter unit (JFC 1600, Japan). Images were taken under JEOL Model JSM-6390 LV, Scanning electron microscope (SEM), JEOL Ltd. Japan, in Sophisticated Analytical Instrumentation Facility (SAIF), Cochin University of Science and Technology, Kochi, Kerala. Larval terminology follows Courtney *et al.* (2000) with a few additional terminology prepared by Szpila and Villet (2011).

The second instar larvae of *H. ligurriens* were 5.27±0.43 mm in length, muscoid, vermiform, pointed anteriorly and blunt posteriorly. Average relative humidity, rain fall and temperature during September 2019 were 88.16 ± 4.38 %, 20.33 ± 10.65 mm and 27.01 ± 1.15°C respectively.

Cephalic region: The antero-dorsal side of both pair of pseudocephalon are occupied by an antenna in the shape of a dome which has a superior cleft and is placed on a ring like base (Fig.1a) and a circular disc shaped maxillary palpus (Fig.1b).The

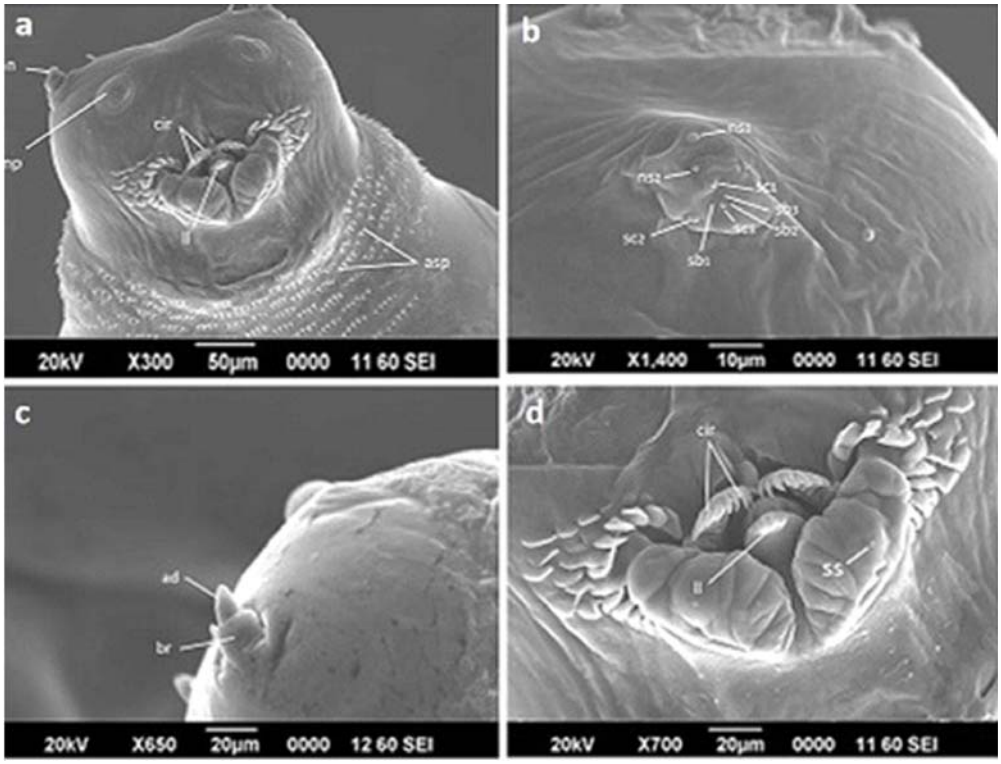

Fig. 1 Micrographs of second instar of *H.ligurriens.* a) pseudocephalon showing antennal complex (an), maxillary palpus (mp), cirri (cr), labial lobe (ll) and anterior spinous process of the first thoracic segment (asp), b) Maxillary palpus showing eight sensillae (sc1-3; sb1-3; ns1-2), c) Antennal complex showing antennal dome, d) functional mouth opening showing cirri (cr) and labial lobe (ll), spines (sp), sensory structures (SS)

height of dome is shorter than that of the height of basal ring (Fig.1c) in contrast to some European *Lucilia* species (*sericata, ampullacea, caesar, cuprina, richardsi, silvarum*) where it is greater (Szpila and Villet, 2011). The diameter of the maxillary palpus is more than the antennal length (Fig.1c). Groups of many sensilla were present in the maxillary palpus (Fig.1b). Sensilla coeloconica (sc1- 3) are three in number and are arranged in a single row with some space in between them. Sensillae basiconicum (sb1-3) are also three in number, highly reduced and not visible prominently and positioned adjacent to the sensilla coeloconicum. Two more sensillae known as 'first and second additional sensillum coeloconicum' are seen dorsal to sensilla coeloconicum and basiconicum cluster. These arrangements are similar to the observations made by earlier workers (Sukontason *et al.,* 2008; Klongklaew *et al.,* 2012; Szpila *et al.,* 2013).

Facial mask is very prominent on the ventral aspect of the pseudocephalon (Fig.1a). Numerous well-structured cirri are dominating in the facial mask and are ten in number. They are characteristically arranged bilaterally on each side of the mouth opening and are gently curved medially. This kind of arrangement of spinulous cirri was not reported in the earlier studies (Sukontason *et al.,* 2008). Three rows of spine clusters were present dorso-medial to the functional mouth opening. The first and second anterolateral rows of spines were with shapes of elongated pyramids of different sizes having broad bases and flat blunt ends .Third postero-medial row of spines were with broad bases and thin concavo-convex apex. Oral ridges were not prominent. Labial lobe was well developed with fleshy lateral lobes constituting a very distinctively demarcated ventral arch area and a medial small lobe which is different from the observations made

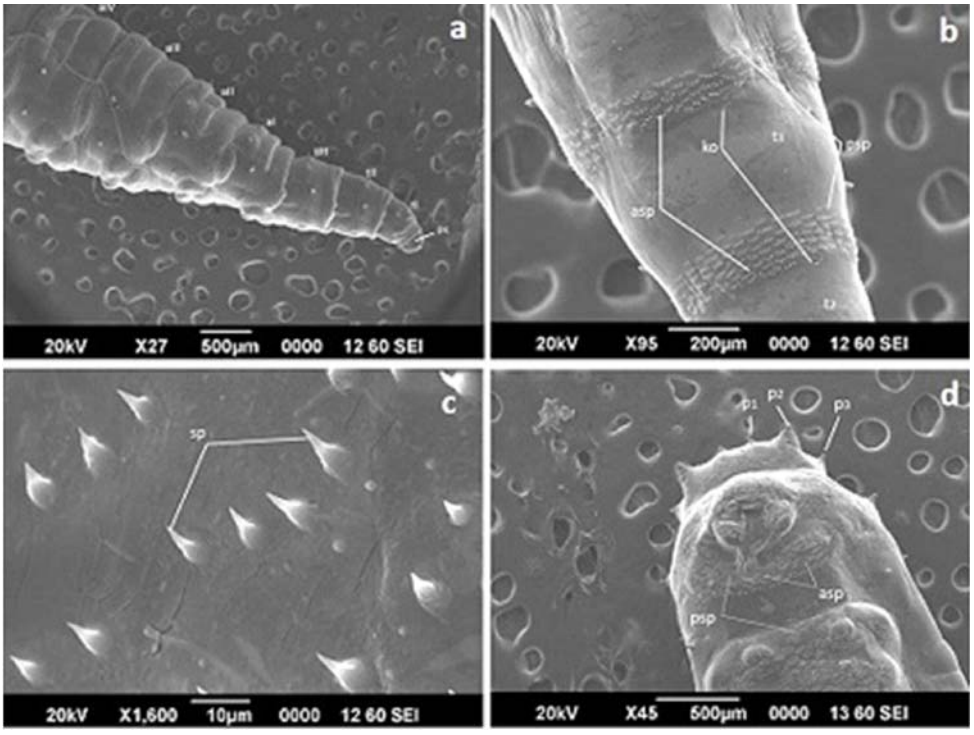

Fig. 2 Micrographs of second instar of H. *ligurriens.* a) second instar showing body segments till fourth abdominal segment, b) second and third thoracic segments showing anterior spinous processes (asp), posterior spinous process (psp), Keilin's organ (ko), c) SEM images of spines with bulbous base and sharp tips between first and second thoracic segments d anal segment displaying dorsal papillae (p1-p3), d) anal segment displaying dorsal papillae (p1-p3), anterior and pesterior spinous processed asp & psp)

by Sukontason *et al.* (2008). Rounded sensory structures were seen on lateral lobes (Fig. 1d) and it is similar to the earlier observation by Sukontason *et al.* (2008) on the same species. In *L. cuprina* and in *L. sericata*, the cirri were seven and eight in number (Szpila *et al.,* 2013). In *L. sinensis* oral cirri were seen with flattened bases and constricted tips (Sanit *et al.,* 2017).

Thorax: Characteristic acuminate spines were present on the anterior and posterior margins of the ventral and lateral surfaces of all the three thoracic segments (Fig. 2a). Spines have a bulbous base, slender sharp tips and are directed backwards (Fig. 2c). These characters were different from the observations made by Sukontason *et al.* (2008) on *H. ligurriens*, where spines were acuminate with flat broad bases. Special sense organs known as Keilin's organs which are sensitive to humidity are present on the ventral side of all thoracic segments (Fig. 2b). In Lucilinae, the thoracic spines show several variations in their shape and structure. Szpila *et al*. (2013) described the spinulations on the thoracic segments of *L. sericata, L. cuprina and L. ampullacea* which are flattened with triangular bases and curved hook like tips. In *L. sinensis,* the thoracic spines are flattened with pigmented sharp tips (Sanit *et al.,* 2017) in *L. porphyrina* and the spines are triangular with dark tips (Klongklaew *et al.,* 2012).

Abdomen: In all abdominal segments, spines were present on the ventral and lateral surfaces. The shape of spines in all abdominal segments were similar to thoracic segments except the last anal segment which have filiform spines in contrast to the verrucate and echinate spines observed by Sukontason *et al*. (2008). Anal papillae are prominent (Fig. 2d) with a broad conical base especially in outer dorsal and outer ventral papillae. These papillae were surrounded by microtrichia (Fig. 2d). Anterior spinous bands are 4-5 in number

and posterior spinous bands are narrow and 2-3 in number. The spinulation pattern was similar to that of the thoracic segments. In *L. sinensis*, the outer ventral papillae were extremely elongated (Sanit *et al.,* 2017) whereas in *L. porphyrina*, all six pairs of inner, middle, outer dorsal and ventral tubercles are prominent but not very large in size like that of *L. sinensis* (Klongklaew *et al.,* 2012). In *L. cuprina*, the dorsal and ventral tubercles were prominent but not so elongated like that of *L. sinensis* (Mendonca *et al.,* 2013)

In most of the studies conducted across the world (in Europe, Africa, Australia and in Asian countries like Thailand, Malaysia) the larval morphology of Luciliinae species were discussed with emphasis on the specific keys to identify the species in those specific geographical locations. This is to address the wide variety of variations in the larval morphology observed in this subfamily. An attempt was made in this study to identify *H. ligurriens* through the unique ultra structural details of second instar larvae. The diagnostic features which helped to differentiate *H. ligurriens* from other species were the spinulous oral cirri which consist of three rows of spine clusters present dorso-medial to the mouth opening .The postero-medial rows were with broad bases and thin concavo-convex apex. The well-developed labial lobes, characteristic thoracic and abdominal spines and prominent outer dorsal and ventral anal papillae were also diagnostic. The ultra structural details of *H. ligurriens* provided in this study would definitely help in the rapid and accurate identification of this species in forensic investigations to estimate time since death.

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