VOLUME 10 ISSUE 2 2024

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 10, No. 2, 410-417 (2024)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Ultrastructure of Cuticular Muscle Attachment Sites (MAS) of Third Instar of *Chrysomya megacephala*: A Unique Technique for Blowfly Larval Identification

Anika Sharma and Madhu Bala*

Department of Zoology and Environmental Sciences, Punjabi University, Patiala, Punjab, India

**Corresponding Author*

Received: 11th March, 2024; **Accepted:** 6th June, 2024; **Published online:** 18th August, 2024

https://doi.org/10.33745/ijzi.2024.v10i02.038

Abstract: Post-mortem interval estimation is one of the most imperative applications of forensic entomology. During PMI estimation most advanced immature stage of forensically significant insects play vital role in estimation of time elapsed death. Since blowflies are the first visitor on corpse, so their larval stages are forensically substantial in crime investigation. Vital step while utilising larvae in PMI estimation is quick and authentic identification of larval species. To ensure this task the present study was designed. In the present study muscle attachment sites of transversal muscles of third instar larvae of *Chrysomya megacephala* (Fabricius, 1794) which appears as dots are utilized in larval identification. Muscle attachment sites are formed at the place where the muscle strands were attached to the cuticle. These dots were visible when muscles are removed and arranged in a row. Muscle attachment site is a species-specific character and provide reliable larval species identification. SEM micrographs of MAS are obtained during present study to enhance the resolution of these species-specific character.

Keywords: Post-mortem interval, Muscle attachment sites, SEM, Blowfly species identification, Larvae

Citation: Anika Sharma and Madhu Bala: Ultrastructure of cuticular Muscle Attachment Sites (MAS) of third instar of *Chrysomya megacephala*: A unique technique for blowfly larval identification. Intern. J. Zool. Invest. 10(2): 410-417, 2024.

https://doi.org/10.33745/ijzi.2024.v10i02.038

Introduction

To establish the Post-mortem interval (PMI), the forensic entomologist aims to determine, the age of the oldest colonizing insects' species. However, for the age to be determined the insect species firstly needs to be identified. Species identification is more challenging in immature stage than in adult form (Smith, 1986; Byrd and Castner, 2009). For species identification and PMI estimation, forensic entomologist rear the immature insect's stage till adult flies. Insect rearing at the same temperature and humidity of the surroundings from where the insects were collected is important as these factors have been shown to affect the rate of the development (Marchenko,

2001; Tarone *et al*., 2011). Also, previous studies have shown that the type of tissue on which the insect species were fed during the developmental process could impact on the size (length and weight) and developmental rate (Kaneshrajah and Turner, 2004; Clark *et al*., 2006).

Muscle attachment sites (MAS) found to be a good tool for species identification in case of larval stage. The aim of this study was to simplify species identification for forensic purposes. A crucial issue in the application of forensic insects is adequate species identification of the entomological evidence collected from the crime scene (Amendt *et al*., 2011). An expeditious and unchallenging method for the determination of species of the forensically important blowflies' larvae is required; and is important in forensic entomology for the minimal PMI calculation. A trailblazing approach for fast and easy species determination in the third instar blowfly larvae based on visualization of species-specific muscle attachment sites is complemented with SEM. Even the species identification of poorly preserved and damaged sample also becomes possible with SEM studies. The advantage of incorporation of SEM studies along with initial technique provides high-resolution results. In the present study muscle attachment sites of transversal muscles of third instar larvae of *Chrysomya megacephala* (Fabricius, 1794) which appears as dots are utilized in larval identification.

Materials and Methods

Sample collection to study muscle attachment sites:

Third instar larvae of *Chrysomya megacephala* were obtained from a laboratory colony established at Department of Zoology and Environmental Sciences, Punjabi University Patiala, India. Larvae were killed by hot water (90ºC) treatment and then stored in 70% ethanol until the muscles got detached from the cuticle (usually it took 5 days to degrade the muscle). These samples were stored at 4 ºC until required for dissection.

Preparation for Dissection:

The larvae (n=10) were dried on a filter paper, after which their length was measured by using a calibrated microscope. Average body length of third instar larvae was 17.2 ± 0.5 mm. Individual larva was pinned down with dorsal side to upward direction in petri dish coated with wax and covered with distilled water to clear out the muscle remnant. 200 micrometre tungsten needles were used to fix the larvae cuticle as flat as possible. Under the stereo zoom microscope, a slit was made with the help of surgical blade to dissect larval body segments (2, 3 and 4) to investigate the attachment sites for transversal body wall muscles. Cuticle was peeled off from the muscle layer vigilantly and muscles dregs were removed by using fine brush and forceps.

SEM Preparation:

Dissected cuticle of 2nd, 3rd and 4th segment was first immersed in 3% glutaraldehyde buffered with 1.0 M phosphate buffer for 24 h at 4 °C. Samples were rinsed with 0.1 M phosphate buffer (pH =7.2, 3 x 10 min) and post-fixed with 2% osmium tetroxide solution at room temperature for 2 h. Dehydration was done by placing the samples in graded ethanol; 30%, 50%, 70%, 80%, 90%, 100% for 5-15 min each. The samples were then placed in absolute ethanol for another 24 h to complete the dehydration process. Larval cuticle was attached to double-stick tape on aluminium stubs and coated with gold in sputter-coating apparatus, and viewed under SEM. All individual rows (2A, 3A, 3B, 4A and 4B) of the segments 2-4 were photographed to observe if there were any specific cluster pattern made by the dots of attachment sites.

Results

MAS of the transversal muscles were visible on the cuticle as 'dots. These dots were grouped in distinct clusters which are arranged symmetrically along the ventral midline (Figs. 1, 2, 3, 4). After removing the muscles, the attachment sites become visible as laterally symmetrical segmental clusters of dark dots. The pattern of these clusters, located in the second, third and fourth segments,

Table 1: Comparative account of number of dots in meso, meta thoracic and first abdominal segment of third instar larvae of *C. megacephala.* with other blowfly species (Niederegger and Spieß, 2012)

Species (n=10)	Larval Stage	Length of larvae	Body Segment (Number of dots)				
			2A	3A	3B	4A	4B
Chrysomya megacephala	3rd instar	17.5± 0.5 mm	8±1.2	9±1.4	3±1	10±1.6	4±0.9
Lucilia illustris	3rd instar	14.1± 0.9 mm	10±0.8	11±1.2	5.9±1.4	11±1.8	7±1.6
Lucilia sericata	3rd instar	14.9± 1 mm	10±1.7	13±1	6±0.9	13.5±2	6±1.2
Calliphora vomitoria	3rd instar	17.9± 1.5 mm	12±1.6	12.5±1.6	5±1.2	14±1.5	6.5±1.4
Calliphora vacina	3rd instar	16.6± 1 mm	12.5±1.5	14±2	4±1	14±1.2	6.5±1

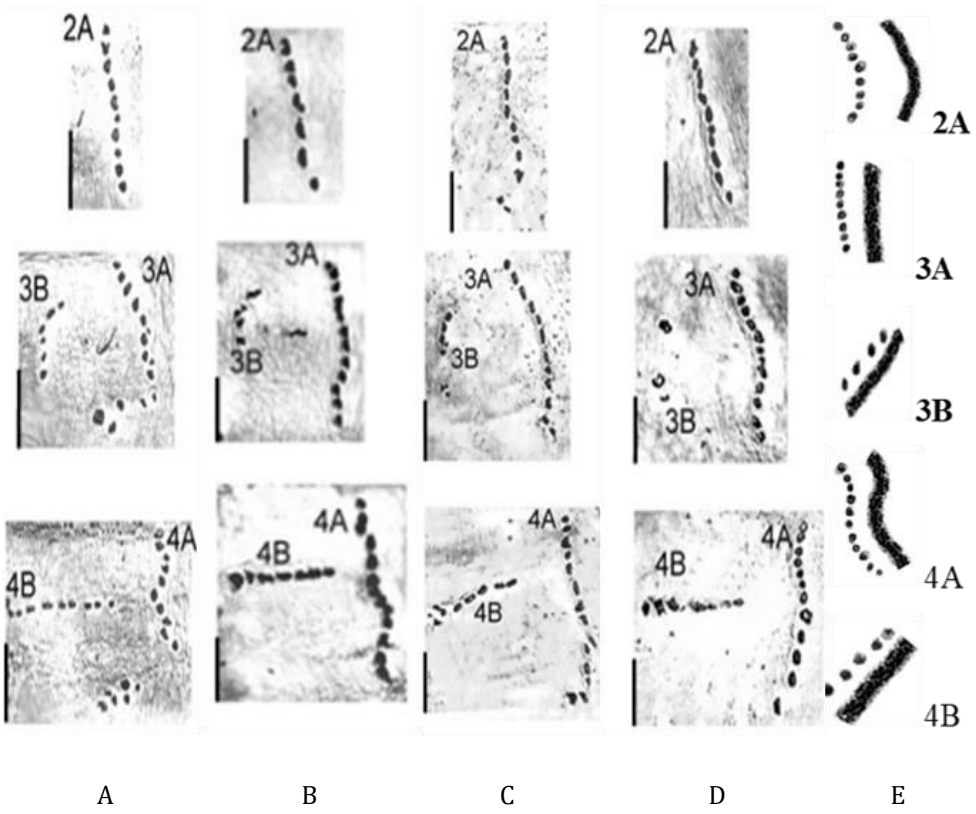


Fig. 1: Comparative account of pattrens of row 2A, 3A, 3B, 4A, 4B in *L. illustris, L. sericata, C. vomitoria, C. vacina* and *C. megacephala* (Niederegger and Spieß, 2012).

showed enough distinction to enable the identification of *C. megacephala* larvae, the most significant calliphorid blowfly species with great medical, veterinary, and forensic importance. The patterns, assembled by cuticular muscle attachment site (MAS) of anterior segments, 2-4 (meso and metathorax and first abdominal segment) were found to be species-specific in blowfly larvae, therefore could be used as an effective tool for species identification purpose (Niederegger *et al*., 2013). MAS of the larval body segment are affected by the growth of the larvae; thus, the dot numbers increase as the larval body length increases (Niederegger *et al*., 2013). Cuticular attachment sites on the body wall were arranged in clusters that form a perceptible pattern. The third instar larvae of this species had an average body length of 17.5± 0.5 mm (Table 1). Dot was formed at the place where the muscle strand was attached to the cuticle (Fig. 1).

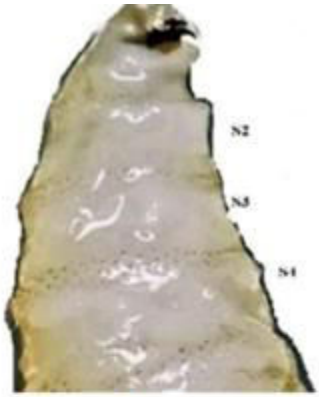
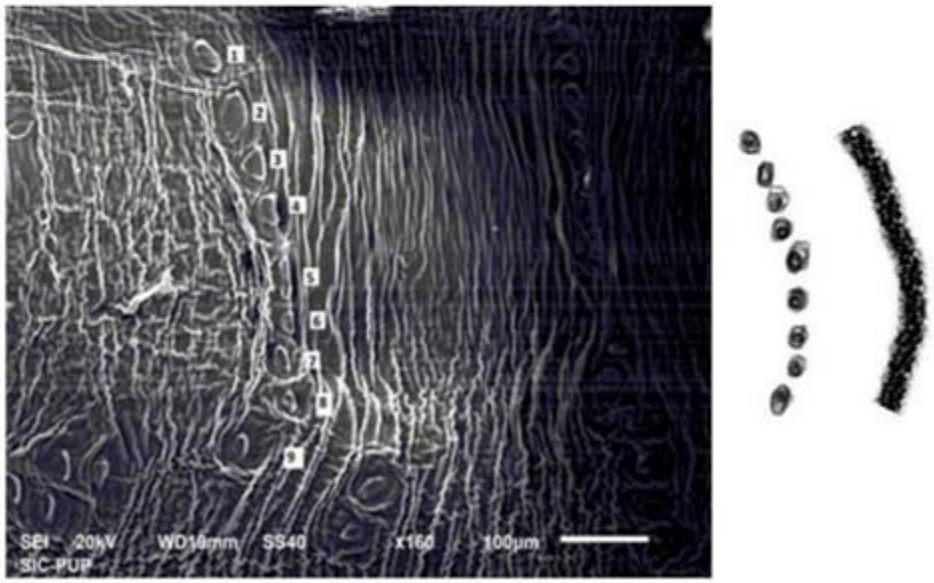


Fig. 2



2A

Fig. 2: Larval Body Segment 2, 3 and 4. Fig. 3: (2A) Muscle attachment site pattern of mesothorax (2nd body segment) of third instar larvae of *C. megacephala.*

These dots were visible when we removed the muscles and found to be arranged in a row. 8± 1 dots of row 2A (mesothorax) formed an inverted C– shaped line (Fig. 3). Row 3A (metathorax) consisted of 9± 1 dot (Fig. 4). This row formed I shaped pattern. Row 3B (short row of metathorax closer to midline) consisted of 3± 1 dots forming a diagonal line (Fig. 4). In the fourth segment row 4A (first abdominal segment) had 10± 1 dots forming inverted S shaped pattern (Fig. 5). The four (± 1) dot of row 4B (Short row of first abdominal segment closer to midline) formed a diagonal straight-line (Fig. 5). The differences in dot number of rows 2A, 3A and 4A were evinced in the pattern. These patterns should be considered a key feature for the identification of this species. The characteristic feature of this species is "I" shape of row 3A and row 4A forms an inverted "S" shape.

Discussion

When a forensic entomologist attends a crime scene it is crucial, they can identify the species of the insect collected from corpse. The reason that

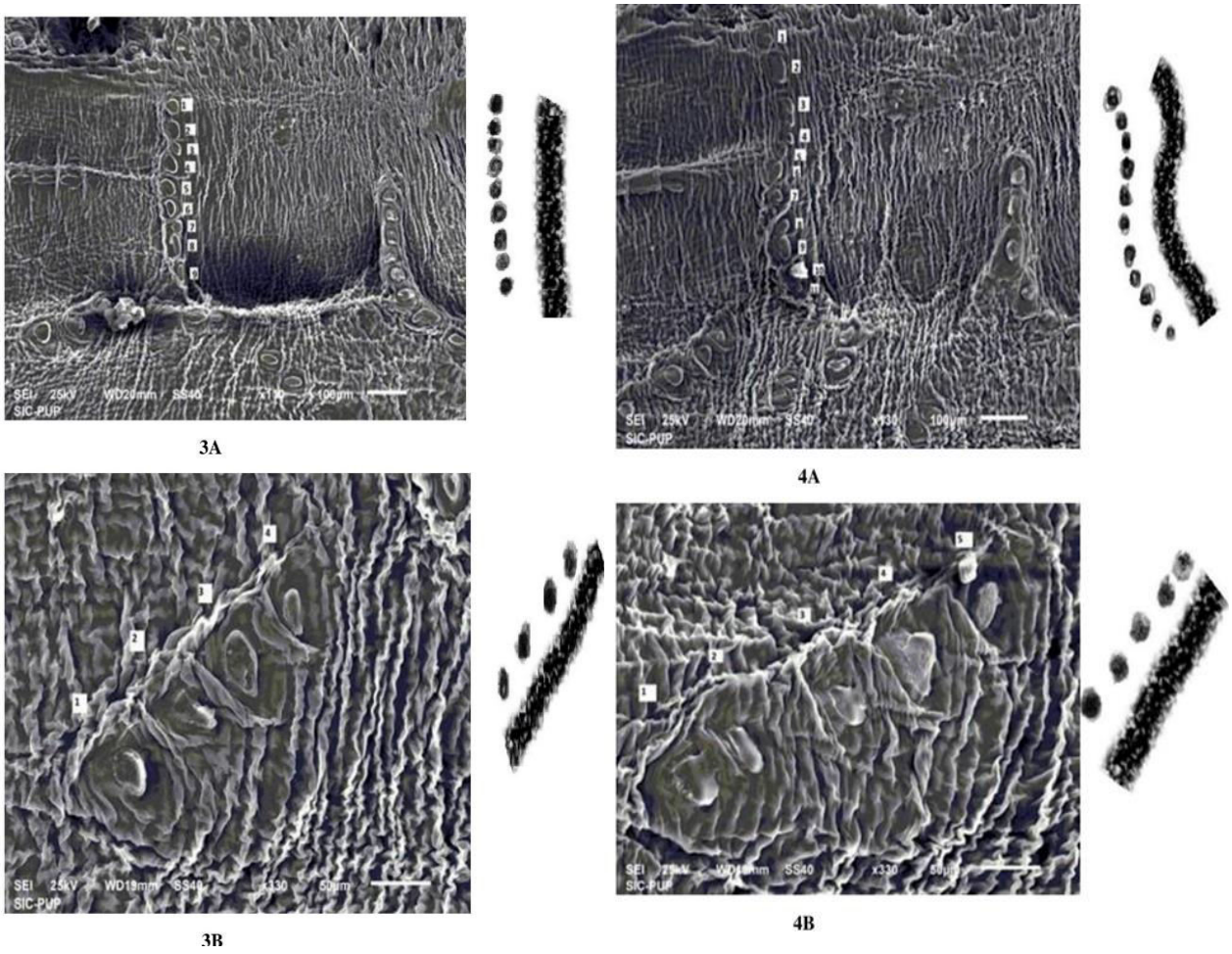


Fig. 4: (3A & 3B) Muscle attachment site pattern of metathorax (3rd body segment) of third instar larvae of *C. megacephala.* Fig. 5: (4A & 4B) Muscle attachment site pattern of 1st abdominal (4th body segment) segment of third instar of *C. megacephala.*

identification is so important is because different species of insects have different life stage timings therefore to utilize the correct developmental information, correct identification is required, and misidentifications can lead to inaccurate PMI estimations. The identification of larvae is possible using morphological techniques, but it can be challenging, and an experienced entomologist is required to carry out the procedure. The analysis of muscle attachment sites compliments the current identification techniques used for larval stage and has the potential to be applied to unknown specimens or to confirm the species identification if it is uncertain.

Muscle attachment site (MAS) patterns on the inside of the cuticula of fly larvae are species specific and grow proportionally with the animal. The patterns can therefore be used for species identification in forensically important dipteran larvae. The previous literature present MAS as a species determination tool (Niederegger and Spieß, 2012; Niederegger *et al*., 2013, 2015, 2019; Martín-Vega and Niederegger 2015) and indicated the general viability of the method. The results presented for *C. megacephala* vindicate the concept. For *Lucilia illustris* and *Lucilia Caesar*- where species determination of larvae was reported to be difficult (Smith, 1986; Szpila, 2010; Szpila *et al*., 2013) with genetic methods (Sonet *et al*., 2012), MAS pattern was found as a distinctive feature (Niederegger *et al*., 2015). These clusters of dots represent both genus and species-specific pattern in the larval stages, allowing for accurate species identification (Niederegger and Spieβ, 2012; Niederegger *et al*., 2013, 2015). The length

of the row increases as the larvae grows; therefore, this method can be used for age determination as well (Niederegger *et al*., 2013).

Only five attachment site clusters in the mesothorax, metathoracic and first abdominal segments are highlighted in this study. Yet, there are seven more abdominal segments (Wallman, 2001), each bearing at least three more clusters per hemi segment. In future project, a complete map could be drawn of all clusters in all accessible developmental stages of forensically relevant larvae. For a fruitful progress, however, the analysis of many more larvae from a broader range of species and genera will be imperative.

Niederegger and Spieß (2012) presented the first result of a modern method of species identification in third instar larvae of saprophagous blowflies. The light microscope was used to visualize the transversal muscle attachment sites. These sites are present in clusters of dark dots. The combined pattern of such clusters was investigated in the second, third, and fourth larval segments. Results revealed that the pattern of these clusters shows enough differences to allow reliable separation of *L. sericata* and *Phormia regina* as well as externally very similar *Calliphora vomitoria* and *Calliphora vicina*, the most common saprophagous blow fly species in the Europe. This method can also determine the species in case of poorly conserved, discoloured, and fragmented larvae. After detaching the muscles from the cuticle, it takes about six minutes to visualize the attachment sites under a binocular microscope.

Niederegger *et al.* (2013) utilized a fast and easy method for larval species determination by analyzing cuticular attachment sites of body wall muscles. Muscle attachment site (MAS) pattern was investigated for *Protophormia terraenovae* and throughout larval development of *C. vicina* and *C. vomitoria*. After removing the muscles and staining the cuticle, the attachment sites become visible as clusters of distinct patterns. Attachment sites of *P. terraenovae* showed different patterns ("M" shaped pattern in the first abdominal segment) than the *Calliphora* species, thus providing further evidence for the viability of this method as a tool for species identification. It was observed that the MAS patterns are constant throughout the larval development and the length of the MAS rows is linearly correlated with the larval body length.

Martin-Vega and Niederegger (2015) described the dorsoventral muscle attachment sites (MAS) patterns for six species of the tribe Piophilini (Diptera: Piophilidae): *Centrophlebomyia furcata* (Fabricius), *Liopiophila varipes* (Meigen), *Piophila casei* (Linnaeus), *Piophila megastigmata* McAlpine, *Prochyliza nigrimana* (Meigen) and *Stearibia nigriceps* (Meigen). MAS patterns of Piophilini and Calliphoridae (Diptera) revealed differences in the muscle equipment between the larvae of both taxa. Among the Piophilini, the MAS patterns were highly conserved, and only a genus-specific pattern for *Piophila* species and a species-specific pattern for *Centrophlebomyia furcata* (Fabricius) were found. Niederegger *et al.* (2015) also presented a distinctive Muscle Attachment Site (MAS) pattern for European *L. ampullacea*, *L. caesar*, *L. illustris*, *L. richardsi*, *L. sericata*, and *L. silvarum*. The attachment sites of the transversal muscles form distinguishable, bilaterally symmetrical rows in each segment. The thoracic segments of Calliphoridae larvae show more MAS rows and contain a higher number of MAS. The segments in all analysed species showed an average number of 36 (±8) MAS per hemi segment and an average total of 108 (±14) MAS for all three hemi segments.

Niederegger *et al.* (2016) used Muscle Attachment Site (MAS) pattern method for species identification in case of third instar larvae of five forensically significant species of *Sarcophaga* Meigen: *Sarcophaga argyrostoma* (Robineau Desvoidy), *Sarcophaga caerulescens* (Zetterstedt), *Sarcophaga melanura* (Meigen), *Sarcophaga albiceps* (Meigen) and *Sarcophaga similis* (Meade). Attachment sites of transversal muscles were easy to distinguish after staining with Coomassie-

Brilliant blue solution and showed species-specific pattern variations. With the augmentation of the MAS method for more species and genera, it becomes apparent that it is a useful and applicable tool for species determination in brachyceran larvae. Niederegger *et al*. (2017) assessed the utility of the MAS method for applications in forensic entomology. This method excludes the requirement of rearing of immatures to adult flies and facilitates species determination of forensically important immatures without too much time and money investment. MAS patterns can also be used for the age estimation of larval species collected from a cadaver. This method suited to all the larval instars and can also be used to a poorly preserved or discolored specimen.

Niederegger *et al.* (2019) analyzed MAS patterns in third instar larvae of six common West Palearctic species of *Wohlfahrtia* -- *Wohlfahrtia bella* (Macquart), *Wohlfahrtia indigens* (Villeneuve), *Wohlfahrtia nuba* (Wiedemann), *Wohlfahrtia trina* (Wiedemann) *Wohlfahrtia villeneuvi* (Salem) and *Wohlfahrtia magnifca* (Schiner). In *Wohlfahrtia,* rows appear shorter and narrower, but cluster patterns are composed of a significantly higher number of attachment sites than the patterns found in *Sarcophaga*. The number of MAS for the investigated body segment (second and third thoracic and first abdominal segment) ranged from 171-234. The least number of attachment sites belonged to *Wohlfahrtia indigens* and the maximum to *Wohlfahrtia bella*.

Conclusion

Muscle attachment site is a potential tool for determination of larval stages in case of blowflies as well as flesh flies. During PMI estimation identification of immature stages especially most advance immature stage i.e., third instar larvae is a crucial task. Rearing of larvae to get adults is the only option to get accurate identification of immature stages in the absence of relevant keys, which is a time-consuming task. MAS is a unique technique to get quick identification of immature stages. If it is supplemented with Scanning electron microscopy it enhances the resolution and provide clearer image of species-specific dots on MAS.

Acknowledgements

Authors would like to thank authorities at Department of Zoology and Environmental Sciences for providing laboratory facilities and University Grant Commission for providing financial aid.

References

Amendt J, Richards CS, Campobasso CP, Zehner R, Hall M JR. (2011) Forensic entomology: applications and limitations. Forensic Sci Med Pathol. 7(4): 379-392.

Byrd J and Castner JL. (2010) Forensic Entomology: The Utility of Arthropods in Legal Investigations, 2nd edn., CRC Press, Boca Raton.

Clark K, Evans L and Wall R. (2006) Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. Forensic Sci Int. 156(2-3): 145-149.

Kaneshrajah G and Turner B. (2004) *Calliphora vicina* larvae grow at different rates on different body tissues. Int J Legal Med. 118(4): 242-244.

Marchenko MI. (2001) Medicolegal relevance of cadaver entomofauna for the determination of the time of death. Forensic Sci Int. 120: 89-109.

Martin-Vega D and Niederegger S. (2015) Larval muscle attachment site (MAS) patterns are a conserved character among Piophilini flies (Diptera, Piophilidae). Deutsche Entomologische Zeitschrift 62(2): 239-245.

Niederegger S, Akbarzadeh K and Szpila K. (2019) Muscle attachment site patterns for species determination in West Palaearctic *Wohlfahrtia* (Diptera: Sarcophagidae) of medical and veterinary importance. Sci Rep. 9: 19151.

Niederegger S, Doge KP, Peter M, Eickholter T and Mall G. (2017) Connecting the dots: From an Easy method to computerized species determination. Insects 8(2): 52.

Niederegger S, Miroschnikow A and Spieß R. (2013) Marked for life: muscle attachment site patterns in blowfly larvae are constant throughout development. Parasitol Res. 112(1): 347-355.

Niederegger S and Spieß R. (2012) Cuticular muscle attachment sites as a tool for species determination in blowfly larvae. Parasitol Res. 110(5):1903-1909.

Niederegger S and Spieß R. (2014) Muscle attachment sites of *Phormia regina* (Meigen). Parasiol Res. 113: 4313-4314.

Niederegger S, Szpila K and Mall G. (2015) Muscle attachment site (MAS) patterns for species determination in European species of *Lucilia* (Diptera: Calliphoridae). Parasitol Res. 114: 851-859.

Niederegger S, Szpila K and Mall G. (2016) Muscle attachment site (MAS) patterns for species determination in five species of *Sarcophaga* (Diptera: Sarcophagidae). Parasitol Res. 115(1): 241-247.

Smith KG. (1986) A Manual of Forensic Entomology, The Trustees of British Museum, London.

Sonet G, Jordaens K, Braet Y and Desmyter S. (2012) Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and *L. illustris* (family Calliphoridae) so problematic? Forensic Sci Int. 223(1–3): 153-159.

Szpila K. (2010) Key for the identification of third instars of European blowflies (Diptera: Calliphoridae) of forensic importance. In: Current Concepts in Forensic Entomology, (eds.) Amendt J., Goff M.L., Campobasso C.P. and Grassberger M., Springer, Dordrecht, Netherlands.

Szpila K, Hall MJR, Pape T and Grzywacz A. (2013) Morphology and identification of first instars of the European and Mediterranean blowflies of forensic importance. Part II. Luciliinae. Med Vet Entomol. 27(4): 349-366.

Tarone AM, Picard CJ, Spiegelman C and Foran DR. (2011) Population and temperature effects on *Lucilia sericata* (Diptera: Calliphoridae) body size and minimum development time. J Med Entomol. 48(5): 1062-1068.

Wallman JF. (2001) Third-instar larvae of common carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae) in South Australia. Invertebrate Taxon. 15: 37-51.