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Larval muscle attachment site (MAS) patterns are a conserved character among Piophilini flies (Diptera, Piophilidae)

Daniel Martín-Vega[1,2], Senta Niederegger[3]

1 *Departamento de Ciencias de la Vida, Universidad de Alcalá, E-28871 Alcalá de Henares, Madrid, Spain*

2 *Department of Life Sciences, Natural History Museum, SW7 5BD London, UK*

3 *Institut für Rechtsmedizin, Universitätsklinikum Jena, D-07743 Jena, Germany*

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Corresponding author: *Daniel Martín-Vega* (daniel.martinve@uah.es)

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Abstract

The dorsoventral muscle attachment sites (MAS) patterns are described 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). Comparison between the MAS patterns of Piophilini and previous descriptions for 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 *C. furcata* were found. Nevertheless, these differences in MAS patterns were subtle and some intraspecific variability was observed; hence, the MAS patterns do not appear to be suitable as diagnostic characters allowing for species determination of Piophilini larvae.

Key Words

Larval morphology
Forensic entomology
Phylogeny
Identification
Centrophlebomyia furcata
Liopiophila varipes
Piophila casei
Piophila megastigmata
Prochyliza nigrimana
Stearibia nigriceps

Introduction

Lacking legs or prolegs, the larvae of Diptera Cyclorrhapha move by the contraction of longitudinal and dorsoventral muscles, increasing the haemolymph hydrostatic pressure (Roberts 1971). However, in spite of its importance, the anatomy of these muscles has only been described for some cyclorrhaphous species; see for example the works of Hooper (1986), Bate (1990) and Wipfler et al. (2013) on *Drosophila melanogaster* Meigen, Hewitt (1908) on *Musca domestica* Linnaeus, and Crossley (1965) and Hanslik et al. (2010) on *Calliphora vicina* Robineau-Desvoidy. All of those studies described a great number of longitudinal muscles usually extending between two segmental borders, and a small number of dorsoventral muscles in the lateral, ventrolateral and dorsolateral regions of each segment (Wipfler et al. 2013).

Recently, Niederegger and Spieβ (2012), and Niederegger et al. (2013, 2015) studied the larval dorsoventral muscles and their cuticular attachments in forensically important blow fly species (Diptera: Calliphoridae). These muscular attachment sites (MAS) are easily visualized as clusters of dots in the larval cuticle and form distinct and both genus- and species-specific patterns in blow fly larvae, allowing for species identification (Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015). Furthermore, the MAS patterns are constant throughout larval development and the length of the MAS rows is linearly correlated with the larval body length (Niederegger et al. 2013). Reliable identification of the material collected is particularly crucial in forensic

investigations as the immature stages of necrophagous insects are usually the only entomological evidence collected at autopsies and crime scenes (Amendt et al. 2007). However, the identification of immature stages remains a difficult task for some forensically important insect groups like the commonly named 'skipper flies' (Diptera: Piophilidae). This common name is due to the skipping behaviour showed by the third-instar larvae, which is mediated by contraction of the musculature. According to the phylogeny proposed by McAlpine (1977), the family Piophilidae includes two subfamilies: Neottiophilinae, which includes ectoparasite species of nestling birds, and Piophilinae, which includes two tribes: the Mycetaulini, whose larvae develop mainly on rotten fungi, and the Piophilini, whose larvae develop mainly on decaying organic matter and which is divided in two subtribes: Piophilina and Thyreophorina. Both subtribes Piophilina and Thyreophorina are typically associated with cadavers in advanced stages of decay and can be potentially useful as forensic indicators; moreover, some Piophilina species can also be major pests for the food industry and agents of human myiasis (Martín-Vega 2011). Given this forensic, economic and medical importance, methods for the identification of piophilid immature stages are strongly needed. However, barcode sequences for molecular identification are still only available for a few species (e.g. Boehme et al. 2012) and, although several recent studies have yielded new insights into the morphology of the immature stages of the Piophilidae (e.g. Martín-Vega et al. 2012, 2014; Paños et al. 2013; Martín-Vega and Baz 2014), the larval morphology of most piophilid genera and species remains undescribed. As a consequence, some diagnostic characters within existing identification keys must be used with caution (Martín-Vega et al. 2014). It is therefore desirable to explore additional morphological characters which may increase not only the accuracy and reliability of the species identification (which is particularly essential for a correct use of insects as forensic indicators), but also may support the reconstruction of phylogenetic relationships.

The aims of the current study were (i) to describe the dorsoventral muscle equipment in a representative set of piophilid species from the tribe Piophilini, comparing them with previous data on the anatomy of Cyclorrhapha larval muscles; and (ii) to determine if those patterns are genus- or species-specific and can thus be used as an additional tool for Piophilidae species determination.

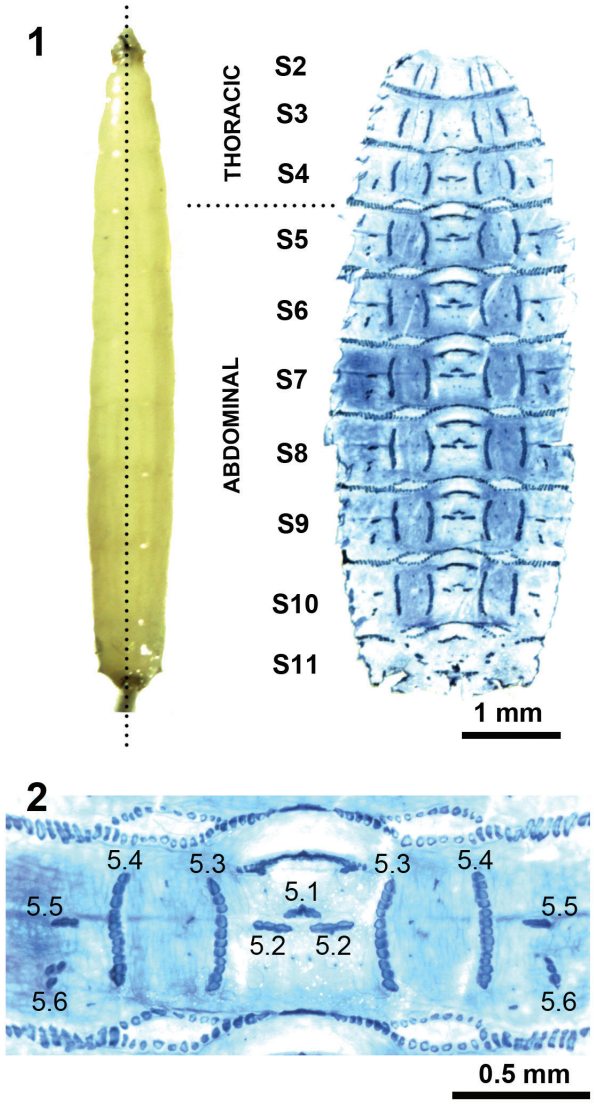
Material and methods

Six target species were selected for the current study: five species belonging to subtribe Piophilina—*Liopiophila varipes* (Meigen), *Piophila casei* (Linnaeus), *Piophila megastigmata* McAlpine, *Prochyliza nigrimana* (Meigen) and *Stearibia nigriceps* (Meigen); and one species belonging to subtribe Thyreophorina—*Centrophlebomyia furcata* (Fabricius). These six species are among the most common piophilid species occurring on carrion and show wide geographical distributions (Martín-Vega 2011).

Adult males and females of *C. furcata*, *L. varipes*, *P. casei*, *P. megastigmata* and *P. nigrimana* were collected on animal carcasses and carrion baits in different habitats of central Spain, identified using published taxonomical keys (McAlpine 1977, 1978) and subsequently used to start laboratory cultures. Details on collection sites and on the conditions and maintenance of the laboratory colonies for each species can be found in Martín-Vega et al. (2012, 2014) and Martín-Vega and Baz (2014). For each species, larvae were reared to the third-instar, killed in near-boiling water and then preserved in 70% ethanol for more than 24 hours to allow the muscles to detach from the cuticle (see Niederegger and Spieβ 2012). This fixation and storage method is also recommended to achieve best preservation of larval samples (Amendt et al. 2007). Furthermore, larvae of *S. nigriceps* were obtained from a human corpse being object of a current investigation in the Institute of Legal Medicine of the Friedrich-Schiller-University of Jena (Germany). Additional larvae of *S. nigriceps* were kindly supplied by Dr Krzysztof Szpila (Nicolaus Copernicus University, Poland). Several third-instar larvae were killed and preserved following the aforementioned method, while the remaining larvae were placed in a plastic container containing minced meat and reared to adulthood. Ten third-instar larvae of each target species were randomly collected and dissected for the study. Before dissection, the length and diameter of the larvae were measured (accuracy ± 0.1 mm) using a calibrated ocular micrometre.

Preparation of larvae and evaluation of the MAS patterns followed the methodology described in Niederegger and Spieβ (2012) and Niederegger et al. (2013, 2015). The dorsally dissected cuticle was cleaned and stained with Coomassie brilliant blue solution (Sigma, 1% in tap water), cutting off the first segment (Fig. 1). The stained cuticles were flattened and covered with a glass slide cover and mounted onto the dissecting microscope. After study, the cuticles were stored in 70% ethanol at the Institute of Legal Medicine of the Friedrich-Schiller-University of Jena.

Each MAS is visible on the cuticle as a 'dot'. The dots are grouped in distinct clusters which are arranged symmetrically along the ventral midline (Figs 1, 2). Following Niederegger and Spieβ (2012), a cluster of dots is called 'row' and the term 'pattern' refers to the shape of a row. All rows in segments 2–11 were documented and labelled following Niederegger et al. (2015): rows were numbered according to the segment and the position within the segment, starting at the centre (Fig. 2). The individual rows were photographed for each segment using a digital camera (BeyTec, Moticam 1000) attached to the dissecting microscope, using identical magnification in every preparation. Then, on the computer, each MAS dot was covered with a semitransparent coloured circle using graphic software (Adobe Photoshop CS). The resulting rows of circles were stacked, using the most anterior circle (in longitudinal rows) or the left circle (in transverse rows) as reference point. The resulting areas with a high degree of overlap were marked as 'condensed pattern' to allow the direct comparison between species. Moreover,



Figures 1–2. *Liopiophila varipes* (Meigen), third-instar larva. **1.** Dorsal view of a pinned larva before dissection (left) and stained larval cuticle (right), showing the symmetrical muscular attachment sites on segments S2 to S11; **2.** Detail of the abdominal segment S5 showing the label for each muscular attachment site pattern.

the number of MAS per row was documented for each specimen, and the average number of MAS per row and the standard deviation were computed for each species (see Suppl. material 1).

Results

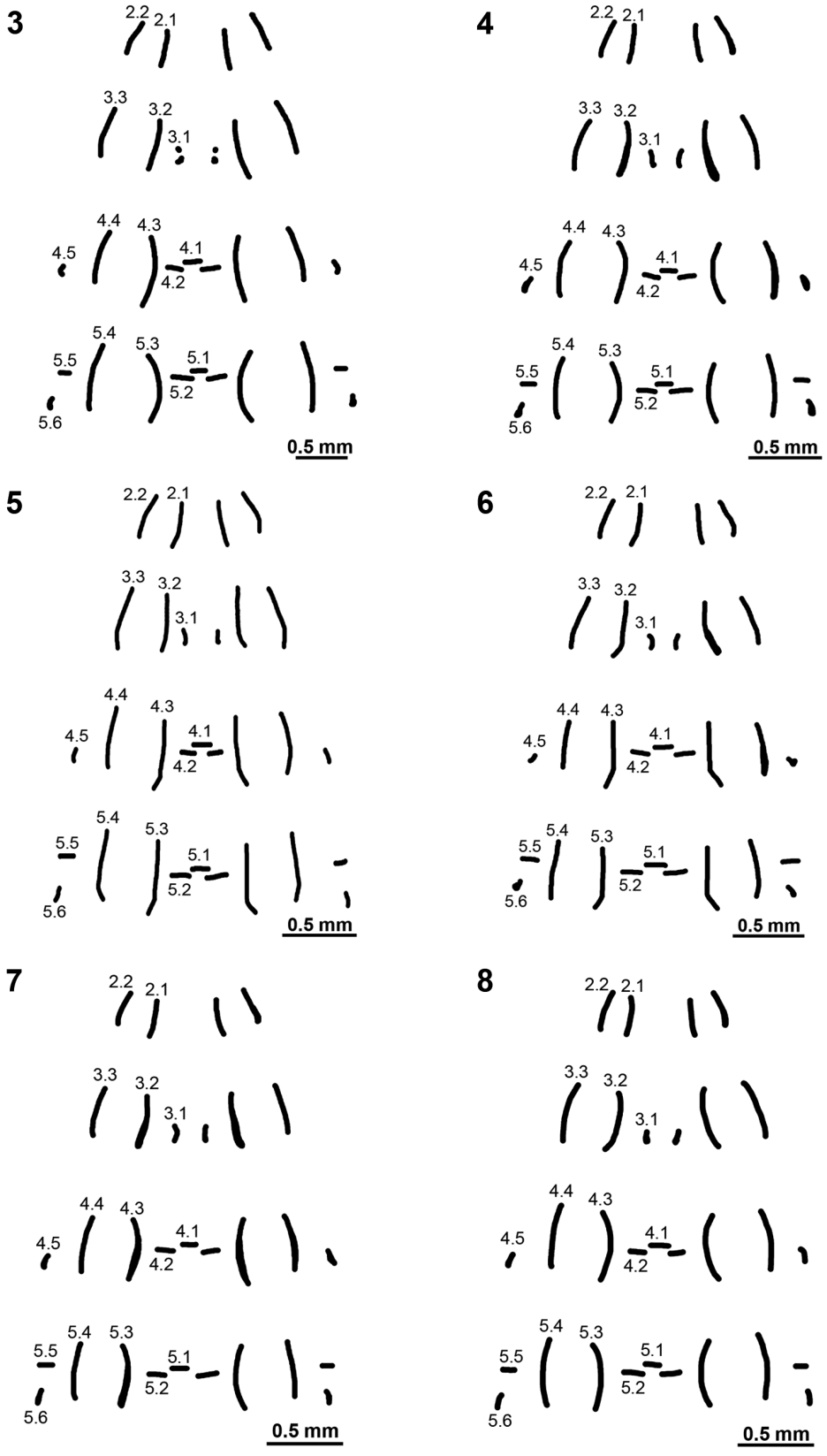
Larval muscle equipment

The MAS patterns followed the same general model in the six target species (Figs 3–8). The three thoracic segments (S2 to S4) and the last abdominal segment (S11) showed different muscle equipment (i.e. number of MAS rows) than abdominal segments S5 to S10, where the muscle equipment was identical (Fig 1). Thoracic segment S2 was equipped with only two pairs of symmetrical, longitudinal rows, labelled 2.1 and 2.2 (Figs 3–8). Thoracic segment S3 showed two small, usually symmetrical, oblique rows, labelled 3.1; and two pairs of symmetrical, longitudinal rows, labelled 3.2 and 3.3 (Figs 3–8). Thoracic segment S4 showed a single transverse row, perpendicular to the ventral midline, labelled as 4.1; two symmetrical, transverse rows placed under row 4.1, labelled 4.2; two pairs of symmetrical, longitudinal rows 4.3 and 4.4; and two small, distal symmetrical oblique rows, labelled 4.5 (Figs 3–8). Abdominal segments S5 to S10 showed the same muscle equipment than thoracic segment S4, plus two additional symmetrical, transverse rows, labelled 5.5–10.5, depending on the segment number. No significant differences were found between abdominal segments S5–S10 within the same individual (Fig. 1), so only the MAS patterns from abdominal segment S5 are shown (Figs 3–8). Finally, the last abdominal segment S11 showed only two irregular, asymmetrical clusters with a varying number of dots (Fig. 1). These clusters of dots did not follow any distinct pattern between individuals within any species. Moreover, because of the pinning for dissection (see Material and Methods), the abdominal segment S11 was usually broken and the sample size was below ten for every species. Therefore, no stacked or condensed patterns for segment S11 are shown.

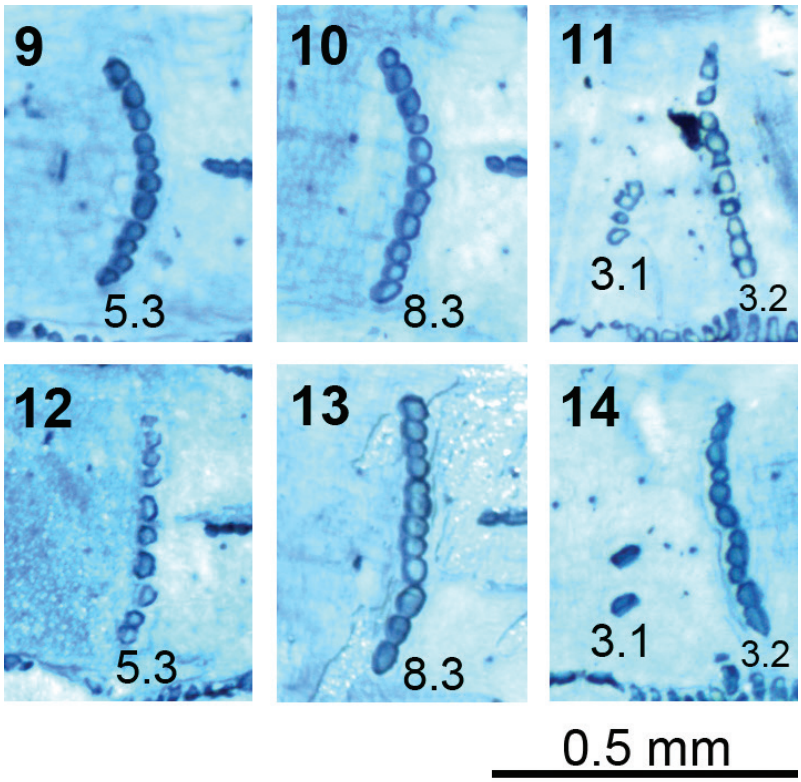
MAS patterns as an identification tool in the Piophilini

Only subtle differences were found in the MAS patterns between some species. The condensed patterns for the longer, longitudinal rows 4.3 and 5.3–10.3 were generally curved in their middle, parentheses-shaped in *C. furcata*, *L. varipes*, *P. nigrimana* and *S. nigriceps* (Figs 3, 4, 7–10). However, the same longitudinal rows 4.3 and 5.3–10.3 were straight but markedly bended on its final third, J-shaped in both *P. casei* and *P. megastigmata* (Figs 4, 5, 12, 13). The longitudinal row 3.2 also showed this pattern, although not so markedly in every individual (Figs 3–8). It must be highlighted that, in some specimens, some individual rows did not show a clear parentheses-shape (in *C. furcata*, *L. varipes*, *P. nigrimana* and *S. nigriceps*) or J-shape (in *P. casei* and *P. megastigmata*), but the correspondent pattern could be clearly observed in other longitudinal rows of the same individual. On the other hand, the oblique row 3.1 showed a clear disruption in every *C. furcata* individual (Fig. 3, 14), but no disruptions were observed in the other species (Figs 4–8, 11). No clear differences were observed in the other rows between species (Figs 3–8). Moreover, the oblique rows 4.5 and 5.6–10.6 showed variable angles within the same segment and within the same species, so no clear condensed patterns can be determined (Figs 3–8).

Suppl. material 1 shows the average number of MAS per row within each segment. More dots were generally found in the longitudinal rows 4.3–10.3 in *C. furcata*, *L. varipes* and *S. nigriceps*, but the observed range overlapped in any case in the six target species.



Figures 3–8. Condensed larval muscular attachment site patterns for six species of Piophilini. **3.** *Centrophlebomyia furcata* (Fabricius). Larval length = 11.91±1.03 mm; diameter = 1.45±0.12 mm; n = 10; **4.** *Liopiophila varipes* (Meigen). Larval length = 7.34±0.3 mm; diameter = 0.75±0.03 mm; n = 10; **5.** *Piophila casei* (Linnaeus). Larval length = 7.43±0.42 mm; diameter = 0.87±0.05 mm; n = 10; **6.** *Piophila megastigmata* McAlpine. Larval length = 7.69±0.28 mm; diameter = 0.82±0.03 mm; n = 10; **7.** *Prochyliza nigrimana* (Meigen). Larval length = 6.61±0.24 mm; diameter = 0.72±0.02 mm; n = 10; **8.** *Stearibia nigriceps* (Meigen). Larval length = 7.28±0.37 mm; diameter = 0.76±0.06 mm; n = 10.



Figures 9–14. Muscular attachment sites in Piophilini larvae. **9.** *Liopiophila varipes* (Meigen), third-instar larva, detail of abdominal row 5.3; **10.** *Prochyliza nigrimana* (Meigen), third-instar larva, detail of abdominal row 8.3; **11.** *Stearibia nigriceps* (Meigen), third-instar larva, detail of thoracic row 3.1; **12.** *Piophila casei* (Linnaeus), third-instar larva, detail of abdominal row 5.3; **13.** *Piophila megastigmata* McAlpine, third-instar larva, detail of abdominal row 8.3; **14.** *Centrophlebomyia furcata* (Fabricius), third-instar larva, detail of thoracic row 3.1.

Discussion

Larval muscle equipment

Previous studies had described a variation in the muscle equipment (i.e. variation in the number of MAS rows) between the larval segments of the same individual in different cyclorrhaphous species (Hewitt 1908; Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015; Wipfler et al. 2013). As described by Wipfler et al. (2013) for *D. melanogaster*, the thoracic segments and the first and last abdominal segments of the Piophilini show a varying muscle equipment, whereas the abdominal segments S5–S10 appear uniform (Fig. 1). There is a progressive increase in the muscle equipment from segment S2 to segments S5–S10, but the number of muscles decreases drastically in the last abdominal segment (Fig. 1); this is also in accordance with the observations of Wipfler et al. (2013) on *D. melanogaster*.

On the other hand, the current study of the larval MAS patterns of the Piophilini shows that their muscle equipment is clearly different from the Calliphoridae (Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015). The thoracic segments of Calliphoridae larvae show more MAS rows and contain a higher number of MAS (Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015; MAS patterns of the abdominal segments were not described). The higher number of muscles in blowfly larvae in comparison to the smaller larvae of Piophilini is very likely due to the difference in larval size between both families, but it also suggest variation in the muscle equipment among different Diptera families. The preparation of larval specimens for the study of the MAS patterns is fast and simple (Niederegger and Spieβ 2012), so it may provide a potential useful tool for comparative anatomy studies on cyclorrhaphous Diptera. In the current study, the similar MAS patterns observed between both closely related Piophilina genera (Figs 4–8) and a more distantly related genus of Thyreophorina (Fig. 3) suggest that the larval MAS patterns may be highly conserved among the Piophilini. Hence, it would be desirable to describe the larval MAS patterns in species of the piophilid tribe Mycetaulini and subfamily Neottiophilinae, as well as in species of related families (see McAlpine 1977, for a phylogeny of the Piophilidae and related families), in order to know either if the general pattern described here is apomorphous in the Piophilini or if it is a conserved character among more taxa.

MAS patterns as an identification tool in the Piophilini

Both genus- and species-specific MAS patterns have been described in Calliphoridae larvae (Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015). It provides a

simple identification method which may be particularly useful in the analysis of blow fly larvae collected in a forensic case. However, the current results show that larval MAS patterns show no significant differences among a set of different Piophilini genera, and therefore cannot provide a reliable identification at a species level. Indeed, the current results suggest that the larval MAS patterns may be also conserved among other species of the subtribe Piophilini, as mentioned.

At a genus level, the two *Piophila* species showed a distinctive, J-shaped pattern in longitudinal rows 4.3 and 5.3–10.3 (Figs 5, 6, 12, 13), in comparison to the parentheses-shaped pattern of the other species (Figs 3, 4, 7–10). This genus-specific character should be taken with caution as some intraspecific variability was observed, although the analysis of several segments in the same individual may provide additional support in order to determine the pattern of the longitudinal rows of muscle attachments in a particular specimen. Nevertheless, it is recommended to use alternative morphological characters for the identification of *Piophila* larvae, like the shape of the cephalopharyngeal skeleton or the arrangement of the spines of ventral creeping welts (Martín-Vega et al. 2012; Paños et al. 2013). Similarly, the distinctive disruption observed in the oblique row 3.1 of *C. furcata* larvae (Figs 3, 14) may also be present in the larvae of the other *Centrophlebomyia* species or in other Thyreophorina genera, so it is not possible to confirm if it is a species-, genus- or subtribe-specific pattern. The larvae of *C. furcata* are highly distinctive and easily distinguishable from the larvae of Piophilini (Martín-Vega and Baz 2014); however, the larval morphology of most Thyreophorina genera remains undescribed.

Even though it is true that the larval MAS patterns have not been shown to be a valid tool for species identification in the Piophilini, the conserved pattern among species of this tribe and the observed differences in comparison to Calliphoridae larvae raise interesting questions on the larval muscle anatomy and functioning. How do these muscles operate in the typical skipping behaviour of piophilid larvae? Is the conservation of the MAS patterns related to the performance of that kind of movement? Further studies on the mechanics of the piophilid skipping behaviour may answer these questions. Moreover, given the paucity of anatomical descriptions of the muscular system of Diptera larvae, the current study also suggests the potential use of this simple method in comparative studies. As MAS patterns have shown to be highly conserved among the Piophilini but significantly different from those described for a relatively distant family (Niederegger and Spieβ 2012; Niederegger et al. 2013, 2015), they might represent a valuable tool in the reconstruction of phylogenetic relationships.

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References

Amendt J, Campobasso CP, Gaudry E, Reiter C, LeBLanc HN, Hall MJR (2007) Best practice in forensic entomology—standards and guidelines. International Journal of Legal Medicine 12: 90–104. doi: 10.1007/s00414-006-0086-x

Bate M (1990) The embryonic development of larval muscles in *Drosophila*. Development 110: 791–804.

Boehme P, Amendt J, Zehner R (2012) The use of COI barcodes for molecular identification of forensically important fly species in Germany. Parasitology Research 110: 2325–2332. doi: 10.1007/s00436-011-2767-8

Crossley AC (1965) Transformations in the abdominal muscles of the blow-fly, *Calliphora erythrocephala* (Meig.), during metamorphosis. Journal of Embryology and Experimental Morphology 14: 89–110.

Hanslik U, Schoofs A, Niederegger S, Heinzel HG, Spieβ R (2010) The thoracic muscular system and its innervation in third instar *Calliphora vicina* larvae. I. Muscles of the pro- and mesothorax and the pharyngeal complex. Journal of Morphology 271: 960–968. doi: 10.1002/jmor.10848

Hewitt CG (1908) The structure, development, and bionomics of the house-fly, *Musca domestica*, Linn. Part II – The breeding habits, development, and the anatomy of the larva. Quarterly Journal of Microscopical Science 208: 395–448.

Hooper JE (1986) Homeotic gene function in the muscles of *Drosophila* larvae. EMBO Journal 5: 2321–2329.

Martín-Vega D (2011) Skipping clues: Forensic importance of the family Piophilidae (Diptera). Forensic Science International 212: 1–5. doi: 10.1016/j.forsciint.2011.06.016

Martín-Vega D, Baz A (2014) Comparative larval morphology of the European bone-skippers, *Thyreophora cynophila* (Panzer, 1798) and *Centrophlebomyia furcata* (Fabricius, 1794) (Diptera: Piophilidae), with notes on their coexistence and natural history. Journal of Natural History 48: 285–299. doi: 10.1080/00222933.2013.791938

Martín-Vega D, Baz A, Díaz-Aranda LM (2012) The immature stages of the necrophagous fly, *Prochyliza nigrimana*: comparison with *Piophila casei* and medicolegal considerations (Diptera: Piophilidae). Parasitology Research 111: 1127–1135. doi: 10.1007/s00436-012-2943-5

Martín-Vega D, Díaz-Aranda LM, Baz A (2014) The immature stages of the necrophagous fly Liopiophila varipes and considerations on the genus Liopiophila (Diptera: Piophilidae). Deutsche Entomologische Zeitschrift 61: 37–42. doi: 10.3897/dez.61.7534

McAlpine JF (1977) A revised classification of the Piophilidae, including 'Neottiophilidae' and 'Thyreophoridae' (Diptera: Schizophora).

Memoirs of the Entomological Society of Canada 103: 1–66. doi: 10.4039/entm109103fv

McAlpine JF (1978) A new species of *Piophila* from South Africa (Diptera: Piophilidae). Annals of the Natal Museum 23: 455–459.

Niederegger S, Miroschnikow A, Spieβ R (2013) Marked for life: muscle attachment site patterns in blowfly larvae are constant throughout development. Parasitology Research 112: 347–355. doi: 10.1007/s00436-012-3142-0

Niederegger S, Spieβ R (2012) Cuticular muscle attachment sites as a tool for species determination in blowfly larvae. Parasitology Research 110: 1903–1909. doi: 10.1007/s00436-011-2716-6

Niederegger S, Szpila K, Mall G (2015) Muscle attachment site (MAS) patterns for species determination in European species of *Lucilia* (Diptera: Calliphoridae). Parasitology Research 114: 851–859. doi: 10.1007/s00436-014-4248-3

Paños A, Arnaldos MI, García MD, Ubero-Pascual N (2013) Ultrastructure of preimaginal stages of *Piophila megastigmata* McAlpine, 1978 (Diptera, Piophilidae): a fly of forensic importance. Parasitology Research 112: 3771–3788. doi: 10.1007/s00436-013-3567-0

Roberts MJ (1971) On the locomotion of Cyclorrhaphan maggots (Diptera). Journal of Natural History 5: 583–590. doi: 10.1080/00222937100770421

Wipfler B, Schneeberg K, Löffler A, Hünefeld F, Meier R, Beutel RG (2013) The skeletomuscular system of the larvae of *Drosophila melanogaster* (Drosophilidae, Diptera) – A contribution to the morphology of a model organism. Arthropod Structure & Development 42: 47–68. doi: 10.1016/j.asd.2012.09.005

Supplementary material 1

Average number of muscular attachment sites (±STD) per row for six species of subtribe Piophilini

Authors: Daniel Martín-Vega, Senta Niederegger

Data type: dataset