Morphology successfully separates third instar larvae of *Muscina*

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Abstract. Three representatives of the muscid genus *Muscina* Robineau-Desvoidy (Diptera: Muscidae), *Muscina levida* (Harris), *Muscina prolapsa* (Harris) and *Muscina stabulans* (Fallén), are well known for their medical, veterinary and forensic importance. However, data in the literature provide contradictory information for the identification of third instar larvae of these species. This hinders easy species differentiation because it requires the rearing of material to adult stages in order to facilitate reliable identification. Third instar larvae of these three *Muscina* species were studied in detail using light and scanning electron microscopy to provide thorough redescriptions of their morphology. Existing information concerning third instar morphology, with particular reference to its value for taxonomy and identification, is revised and discussed. Emden’s spiracular distance factor (SDF) is considered here as inappropriate for identification purposes in third instar larvae of *Muscina* spp. because its values are not constant ratios but increase during larval maturation and overlap in the examined species. These species were, instead, discriminated here by differences in the spinulation patterns of their abdominal segments.

Key words. Muscidae, *Muscina levida*, *Muscina prolapsa*, *Muscina stabulans*, forensic entomology, scanning electron microscopy, spiracular distance factor, third instar larva.

Introduction

*Muscina* is a small genus of the family Muscidae and currently comprises 14 valid species (Pape & Evenhuis, 2014). Three representatives of the genus, *Muscina levida*, *Muscina prolapsa* and *Muscina stabulans*, are well known for their medical and veterinary importance. Immature stages of *Muscina* spp. have been reported from insects’ and birds’ nests and mostly from decomposing organic matter (Skidmore, 1985). Larvae of *Muscina* are facultative carnivores. Thus, despite their potential as vectors of pathogens in the adult stage, in the larval stage they can effectively limit the abundance of other dipteran species by preying on their immatures (Duarte et al., 2013). The medical and veterinary importance of *Muscina* spp. is also demonstrated by the occurrence of myiasis in humans and animals (Zumpt, 1965). In addition to their medical and veterinary significance, larvae of *Muscina* spp. are known for their forensic relevance because they are able to colonize both exposed and buried decomposing human bodies (Greenberg & Kunich, 2002; Gaudry, 2010). Under certain circumstances, *M. stabulans* may exclusively colonize a dead body, or may even colonize the body before death in cases of myiasis (Smith, 1986). *Muscina levida* and *M. prolapsa* have both been reported from the Holarctic region and the latter is also known to occur on St Helena Island (Pont, 1986). By contrast, *M. stabulans* is a cosmopolitan insect spread by commerce and is currently known to occur in all biogeographic regions. It is worth mentioning that some authors still use species names recognized as junior synonyms (Pont, 1986), particularly in medical and veterinary entomology textbooks (Gaudry, 2010; Gestmann et al., 2012). Hence *Muscina assimilis* (Fallén) and *Muscina pabulum* (Fallén) refer to *M. levida* and *M. prolapsa*, respectively.

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Because of their occurrence in a broad range of breeding media (Skidmore, 1985), wide distribution and close association with human settlements, these representatives of *Muscina* are of great medical and veterinary importance and their proper species identification is a vital first step in any analysis of entomological material in medical, veterinary or forensic contexts. The literature concerning the larval morphology of *Muscina* is large. Not surprisingly, the quality of data published varies from superficial to exhaustive, but unfortunately the former predominates. Earlier students of larval morphology often described only a small number of characters, such as general body shape, or the arrangement of anterior and posterior spiracles or the outline of the cephaloskeleton (Bouché, 1834; Portchinsky, 1910; Engel, 1915; Séguy, 1923), but this tendency is also found in more recent studies (Liu & Greenberg, 1989; Iancu & Pârvu, 2013).

The third instar larva of *M. levida* was described several times by, respectively, Keilin (1917 (as *M. assimilis*), Séguy, (1923) (as *M. assimilis*), Zimin (1948) (as *M. assimilis*), Skidmore (1985) and Liu & Greenberg (1989) (as *M. assimilis*). That of *M. prolapsa* was studied by Thomson (1937) (as *M. palbularum*), Ishijima (1967) (as *M. palbularum*) and Skidmore (1985). However, reports concerning *M. stabulans* larval morphology are the most numerous (Bouché, 1834; Portchinsky, 1910; Engel, 1915; Séguy, 1923; Zimin, 1948; Matheson, 1950; Zumpt, 1965; Ishijima, 1967; Skidmore, 1985; Queiroz & de Carvalho, 1987; Liu & Greenberg, 1989). Zumpt (1965) suggested that morphological differences among *Muscina* spp. had been only superficially or inadequately studied and recommended the rearing of larvae for identification purposes. Since Zumpt’s (1965) statement, very little new information concerning the morphology of immature stages of *Muscina* has been provided (Ishijima, 1967; Skidmore, 1985; Queiroz & de Carvalho, 1987; Liu & Greenberg, 1989). Moreover, these more recent data still do not enable species discrimination and often provide contradictory information. However, species identification of larvae remains of great importance, especially because rearing to adults or the alternative application of molecular identification tools may be unsuccessful or even impossible in some cases. Furthermore, classical morphological species identification can still be faster and cheaper. Thus, the aim of this paper is to study and document in detail the third instar larva of three *Muscina* species using light microscopy and scanning electron microscopy (SEM).

Materials and methods

Gravid females of *Muscina* were attracted to slightly decomposed chicken liver at a number of locations in Poland (Table 1) and collected using an entomological net. Insects were transported to the laboratory in separate 2-mL Eppendorf tubes covered by perforated caps to allow for aeration. Insects were supplied with sugar, water and liver and kept alive in separate containers until they oviposited. Laboratory rearing and killing of larvae were performed according to Grzywacz et al. (2014), with rearing containers additionally covered with nylon mesh material to avoid contamination by oviposition of flies attracted by the odours of decomposing liver. Both newly moulted and post-feeding third instar larvae were sampled. After oviposition, females were killed with ethyl acetate vapour and were pinned, labelled and identified (Gregor et al., 2002). Voucher specimens are deposited in the collection of the Chair of Ecology and Biogeography, Nicolaus Copernicus University, Toruń, Poland.

Third instar larvae for SEM examination were prepared by dehydration through 80.0%, 90.0% and 99.5% ethanol and critical point drying in carbon dioxide (CO2) using a CPD 030 (Bal-Tec GmbH, Balzers, Lichtenstein) or Autosamdri®-815, Series A (Tousimis Research Corp., Rockville, MD, U.S.A.) critical point dryer. Larvae were then mounted on aluminium stubs with double-sided adhesive tape and sputter-coated with platinum for 140 s (20 nm of coating) or gold for 210 s (30 nm of coating) using a JEOL JFC 2300HR high-resolution fine coater (JEOL Ltd, Tokyo, Japan). Scanning electron microscopy images of *M. prolapsa* and *M. stabulans* were taken with a JEOL scanning microscope (JSM-6335F; JEOL Ltd). Images of *M. levida* were obtained with a variable pressure SEM LEO 1455 (Carl Zeiss Microscopy GmbH, Jena, Germany).

Light microscopy observations were performed with a Stemi 2000 stereomicroscope (Carl Zeiss Microscopy GmbH). Because the larval spines are colourless, examination of the spinulation pattern was preceded by staining with a 1% potassium permanganate solution for a few minutes according to Sukontason et al. (2004). This treatment visualizes spines through their contrast with the background of the adjacent cuticle. For observations of details of the cephaloskeleton, larvae were examined with methyl salicylate. For microscopic slide preparations, larvae were mounted in Hoyer’s medium. Slides were examined with a Nikon Eclipse E200 microscope (Nikon Corp., Tokyo, Japan). Images for light microscopy illustrations were taken with a Nikon 8400 digital camera mounted on either a Nikon Eclipse E200 microscope or a Nikon SMZ 1500 stereomicroscope (Nikon Corp.).

Terminology follows that of Courtney et al. (2000) for general larval morphology, with a few modifications proposed by Szpila & Pape (2005). For family-specific structures Skidmore’s (1985) terminology was applied with a few modifications proposed by Grzywacz (2013). The following measurements were recorded in both young and mature larvae: maximum body length and

| Species          | Females | Larvae per female, range | Locality and year (May–July) of capture |
|------------------|---------|--------------------------|----------------------------------------|
| *Muscina levida* | 5       | 15–60                    | Las Piwnicki Nature Reserve, near Toruń (2010–2011) and Plawin (2011) |
| *Muscina prolapsa* | 3     | 7–19                     | Airport area in Toruń (2010) and Plawin (2009) |
| *Muscina stabulans* | 4     | 19–60                    | Plawin (2009–2011) |
width; distance between posterior spiracles (dps); width of posterior spiracle in horizontal plane (wps), and spiracular distance factor (SDF) defined after van Emden (1965) as the ratio of the distance between the posterior spiracles to the width of the posterior spiracle. However, the latter ratio was modified after Grzywacz (2013) and expressed as the maximum horizontal diameter of the spiracle rather than its greatest width. Measurements were taken with a Nikon Eclipse E200 microscope or Nikon SMZ 1500 stereomicroscope, both equipped with a micrometer eyepiece.

Results

Details of the larval morphology of third instars of *M. levida*, *M. prolapsa* and *M. stabulans* are jointly described below to avoid repetition because the species examined were identical in most aspects. Exceptions are emphasized.

*Muscinia levida* (Harris), syn. *M. assimilis* (Fallén)

Length: 7.12–14.62 mm; width: 1.08–2.46 mm; wps: 0.14–0.17 mm; dps: 0.10–0.20 mm; SDF: 0.62–1.31 (examined larvae, \( n = 154 \); measured larvae, \( n = 40 \)) (Figs 1A, B, E; 2A, B; 3–5).

*Muscinia prolapsa* (Harris), syn. *M. pabulorum* (Fallén)

Length: 7.42–11.92 mm; width: 1.12–2.62 mm; wps: 0.13–0.16 mm; dps: 0.07–0.16 mm; SDF: 0.46–1.08 (examined larvae, \( n = 38 \); measured larvae, \( n = 10 \)) (Figs 1C, F; 2C, D; 6; 7).

*Muscinia stabulans* (Fallén)

Length: 6.62–13.46 mm; width: 1.04–2.50 mm; wps: 0.13–0.19 mm; dps: 0.08–0.19 mm; SDF: 0.44–1.33 (examined larvae, \( n = 165 \); measured larvae, \( n = 30 \)) (Figs 1D, G; 2E, F; 8; 9).

The third instar larva in all three species has a long, slender body that tapers gradually anteriorly and shows an obliquely truncated anal division when viewed laterally (Fig. 1E–G). Twelve visible body segments include a bilobate pseudocephalon (ps), three thoracic segments (\( tI–tIII \)), seven abdominal segments (\( aI–aVII \)) and an anal division (\( ad \)) (Fig. 1E). The first thoracic segment and the anal division carry a pair of anterior (\( as \)) and posterior (\( ps \)) spiracles, respectively. Minute open apertures of probably non-functional spiracles are present in each of the anterolateral margins of \( tIII–aVII \) (Fig. 9A). The body surface is smooth.

*Pseudocephalon.* Lobes of the bilobate pseudocephalon carry an antennal complex (\( an \)), maxillary palpus (\( mp \)) and ventral organ (\( vo \)) (Fig. 3A, B, D). The antennal complex consists of an antennal dome (\( and \)), encircled with seven basal pores, situated on a basal ring (\( abr \)) which carries dorsally a lateral pore equipped with a sensillum (Figs 6C, 8B). The former is conical and its length is similar to the height of the latter. The maxillary palp is surrounded by three circular folds and consists of three sensilla coeloconica (\( sc \)), three sensilla basiconica (\( sb \)) and up to five small additional sensilla all arranged in a tight cluster with two sensilla coeloconica of non-maxillary origin (\( ns \)) (Courtney et al., 2000) located laterodorsally (Figs 3C, 6D, 8C). The functional mouth opening is surrounded by a facial mask composed of numerous oral ridges (\( or \)) and posteriorly is closed by a pair of longitudinal labial lobes (\( ll \)) equipped apically with two sensilla of the labial organ (\( lo \)), one of which is a sensillum coeloconicum (Figs 3D, 6E, 8D). Internally, the basal part of the labial lobes is covered by slightly sclerotized spines directed anteriorly. A ventral organ (\( vo \)) is located on each side of the mouth opening at the anterolateral margin of the oral ridges (Figs 3D, 6E, 8D). The ventral organ is bulge-shaped and equipped with three sensilla ampullacea and one sensillum resembling a sensillum placodeum (Figs 3E, 6F, 8E). Distinctly sclerotized suprabuccal teeth (\( subt \)) are present laterally to the anterior end of the functional mouth opening; there are two rows of minute cutaneous teeth (\( cut \)) on its sides (Figs 3D, 6E, 8D).

*Cephaloskeleton.* Mouthhooks (\( mh \)) are well separated and are basally robust with slender, symmetrical distal parts (Fig. 1B). An unpaired longitudinal sclerite (\( us \)) in the form of a spine lies freely between the \( mh \), in the dorsal area close to the border between their basal and apical parts (Fig. 1B). The basal part of the \( mh \) joins with an oral bar (\( ob \)) through a small accessory rectangular process (\( rp \)) (Fig. 1A, C, D). An anterior rod (\( aro \)) articulates with the anterodorsal margin of the anteriorly extended and serrated \( ob \). Both \( aro \) curve inwards around the front of the tips of the \( mh \) but do not join anteriorly. Suprabuccal teeth and two rows of minute \( cut \) are present below the \( ob \) and \( aro \) (Fig. 1A, C, D). Paired dental sclerites (\( ds \)), accessory stomal sclerites (\( acc \)) and supplementary accessory stomal sclerites (\( accs \)) are placed ventrally to the basal part of each mouthhook (Fig. 1A, C, D). The latter sclerites develop during larval maturation. Dental sclerites are fused ventrally, thus forming an inverted arch (Fig. 1B); however, in newly moulted specimens these sclerites are not fused. The intermediate sclerite (\( is \)) is H-shaped (Fig. 1B). An epistomal sclerite (\( es \)) and a pair of labial sclerites (\( lb \)) lie freely between the anterior part of the arms of the \( is \). The long basal sclerite consists of paired vertical plates (\( vp \)) with dorsal (\( dc \)) and broad ventral cornua (\( vc \)), connected anterodorsally by a perforated dorsal bridge (\( db \)) and anteroventrally by a narrow ventral bridge (\( sb \)) (Fig. 1A, C, D). The \( dc \) is shorter than the \( vc \) and the latter bears a well-developed dorsal expansion in the posterdorsal part and in the lower posterior part carries a sensory organ X (\( x \)) equipped with paired sensilla (Fig. 1A, C, D). An optic depression (\( od \)) is present anteriorly to both \( vp \) and below the dorsal bridge. In *M. levida*, the \( dc \) and \( vc \) (Figs 1A, 2A), and in *M. prolapsa*, the \( dc \) (Figs 1C, 2C) may be equipped with additional sclerites [e.g. dorsally forming a bar-like structure (\( bl \)) as in *M. levida*]. The degree of sclerotization differs intraspecifically and depends on the stage of larval maturity.

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**Fig. 1.** Third instar larvae of *Muscina*. (A) *Muscina levida*, cephaloskeleton, lateral view and (B) dorsal view. (C) *Muscina prolapsa*, cephaloskeleton, lateral view. (D) *Muscina stabulans*, cephaloskeleton, lateral view. (E) *M. levida*, habitus. (F) *M. prolapsa*, habitus. (G) *M. stabulans*, habitus. Scale bars: (A–D) 0.1 mm; (E–G) 1.0 mm. al–aVII, abdominal segments I–VII; acc, accessory stomal sclerite; accs, supplementary accessory stomal sclerite; ad, anal division; aro, anterior rod; as, anterior spiracle; bl, bar-like structure; cr, transverse crevice; cut, cutaneous teeth; db, dorsal bridge; dc, dorsal cornu; ds, dental sclerite; es, epistomal sclerite; ls, intermediate sclerite; lw, lateral creeping welt; ls, labial sclerite; mh, mouthhook; ob, oral bar; od, optic depression; pc, pseudocephalon; pre, pre-anal welt; ps, posterior spiracle; rp, accessory rectangular process; sub, suprabuccal teeth; tI–tIII, thoracic segments I–III; us, unpaired sclerite; vc, ventral cornu; vcw, ventral creeping welt; vb, ventral bridge; vp, vertical plate; x, sensory organ X.

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Thorax. Anterior spiracles are equipped with four to six (Fig. 3A, B), four or five (Fig. 6A) and six or seven lobes (Fig. 8A) in *M. levida*, *M. prolapsa* and *M. stabulans*, respectively. The first thoracic segment (*tI*) in all studied species is equipped with a broad and complete band of dark spines, followed by a transverse cleft approximately reaching the middle of the segment (Fig. 3A). This spinose band is further equipped ventrally with an additional patch of spines, present beyond the main broad band and the cleft (Figs 3A, 6A, 8A). Spines are relatively long, single- or double-pointed, arranged individually or in short rows. Anterior spinose bands on the *tII–III* segments are complete and spines are colourless and may be blunt or pointed, fused basally and arranged in short rows (Figs 3F, 6G, 8G). A pair of Keilin’s organs (*ko*), each consisting of three clustered trichoid sensilla (Figs 3G, 8F), are present in the middle ventral part of each of the *tI–III*.

Abdomen. A transverse crevice (*cr*) is present ventrally in the middle part of segments *aI–aVII* (Figs 1E; 4A, B). Elliptical lateral creeping welts (*lcw*) are present between abdominal segments, although they may be barely visible in young third instar larvae and are never covered by spines (Figs 1E, 4A, 9A). A paired structure termed the ‘bubble membrane’ (*bm*) is placed in the posteroventral margin of each of the first abdominal segments (Figs 4A, 7A). The *bm* consists of spherical and conical globules in a cluster, placed at the same level as the adjacent integument (Figs 4C, 7C, 9C). Spines, if present, are generally confined to the anterior spinose bands similarly to the thoracic segments (Fig. 1F–G); however, small groups of spines may also be present in the posterior margin of the ventral surface of some segments (Figs 1E, G; 4D). In *M. levida* and *M. stabulans*, the posteroventral margin of *aI–aVI* (cf. Figs 4D, 9B) are covered by spines randomly arranged in about 10 short rows, whereas in *M. prolapsa* the posteroventral margin of these segments is usually not covered by spines (Fig. 7B), although at most one or two rows consisting of about five spines may be present on one or more segments. First and second abdominal segments are covered with complete anterior bands of colourless, blunt or pointed spines, which are fused basally.
Identification of third instars of *Muscina*

Fig. 3. Third instar larva of *Muscina levida*. (A) Anterior end of body with anterior spinose band further ramified ventrally (arrow), lateral view. (B) Anterior end of body, ventral view. (C) Maxillary palpus. (D) Facial mask, ventral view. (E) Ventral organ. (F) Third thoracic segment, anterior spinose band, lateral view. (G) Trichoid sensilla of Keilin’s organ. (H) Lateral papilla. an, antennal complex; as, anterior spiracle; cl, cleft; cut, cutaneous teeth; ko, Keilin’s organ; mp, maxillary palpus; ob, oral bar; or, oral ridges; lo, labial organ; ll, labial lobe; mh, mouthhook; mp, maxillary palpus; ns1–2, first and second additional sensillum coeloconicum; sb1–3, sensilla basiconica 1–3; sc1–3, sensilla coeloconica 1–3; sub, suprabuccal teeth; vo, ventral organ.

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and arranged in short rows (Figs 4A, 7A, 9A). In *M. levida* and *M. prolapsa* the anterior spinose band on *aIII* is interrupted dorsally, although it reaches above the upper margin of *lcw* (Figs 1E, F; 4A; 7A), and the anterior spinose band on the subsequent segment (*aIV*) reaches at least the middle of *lcw* (Fig. 1E, F). In *M. stabulans* the anterior spinose band on *aIII* reaches to or at most slightly above the upper margin of the *lcw* (Figs 1G, 9A); if it extends slightly above this margin, the anterior spinose band on *aIV* is hardly developed (Fig. 1G). Abdominal segments carry anteroventral creeping welts (*vcw*) (Figs 1E–G; 4A, B; 7A, B; 9A, B) with the welt on the anal division termed the pre-anal welt (*pre*) (Figs 1E, 5C). Each welt consists of a few (c. six) rows of spines of variable shape, but the welt is well differentiated from the remaining part of any particular spinose band. Welts are placed solely at the anterior margin of a segment and do not involve the posterior margin of the preceding segment (Figs 4A; 7A, B; 9A, B). The first welt, placed on *aI*, is weakly developed (Fig. 1E–G). Spines of *vcw* are relatively massive, preceded and/or followed with smaller ones, yet still distinct. Massive spines are robust and flattened basally with hook-like apical parts and are somewhat triangular in shape in the lateral view (Figs 4D, 7B). The shape and arrangement of smaller spines generally resemble those present in the anterior spinose bands of particular species, and are colourless, arranged individually or clustered in groups or short rows.

The anal division has a ventrally located anal opening (*ao*) surrounded by a porous anal plate (*ap*) (Figs 5D, 7I, 9H). The *ap* is W-shaped, distinctly expanded laterally and shows two bulges posterolaterally to *ao* that are well visible in lateral view (Figs 5C, D, F, G; 7G–I; 9F–H). An unpaired post-anal papilla (*pa*) lies directly behind the anal opening, and pairs of sub-anal (*sa*) and extra-anal (*ex*) papillae are present laterally to the *pa* (Figs 5C, D, F, G; 7G–I; 9F–H). The surface of the *ad* lateral and posterior to *ap*, and *pre*, *pa* and *sa* are covered by spines (Figs 5B–D, F, G; 7E, G–I; 9E–H), with those

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**Fig. 4.** Third instar larva of *Muscina levida*. (A) Abdominal segments, bubble membrane on the first abdominal segment (arrow), lateral view. (B) Abdominal segments, ventral view. (C) Bubble membrane. (D) Abdominal segments with spines at the posteroventral margin (arrow), lateral view. *aI–aIII, aVI, aVII*, abdominal segments I–III, VI, VII; *cr*, transverse crevice; *lcw*, lateral creeping welt; *vcw*, ventral creeping welt.
Fig. 5. Third instar larva of *Muscina levida*. (A) Posterior spiracles, posterior view. (B) Sub-anal papilla, ventral view. (C) Posterior end of body, lateral view. (D) Anal division with anal opening and anal plate with bulges (asterisks), ventral view. (E) Anal division, papilla p3. (F) Anal division of young larva, posterior view. (G) Anal division of mature larva, posterior view. ao, anal opening; ap, anal plate; ex, extra-anal papilla; p1–p7, papillae 1–7 surrounding spiracular field; pa, post-anal papilla; pre, pre-anal welt; rs, respiratory slit; sa, sub-anal papilla; ss, spiracular scar; st, spiracular tuft.
Fig. 6. Third instar larva of *Muscina prolapsa*. (A) Anterior end of body with anterior spinose band further ramified ventrally (arrow), lateral view. (B) Anterior end of body, ventral view. (C) Antennal complex with basal pores (asterisk). (D) Maxillary palpus. (E) Facial mask, ventral view. (F) Ventral organ. (G) Second thoracic segment, anterior spinose band, lateral view. abr, antennal basal ring; and, antennal dome; as, anterior spiracle; cl, cleft.
Fig. 7. Third instar larva of *Muscina prolapsa*. (A) Abdominal segments, bubble membrane on the first abdominal segment (arrow), lateral view. (B) Abdominal segments without short rows of spines at the posterior margin (arrow), ventral view. (C) Bubble membrane. (D) Posterior spiracles, posterior view. (E) Sub-anal papilla, ventral view. (F) Anal division, papilla p2. (G) Posterior end of body, lateral view. (H) Anal division, posterior view. (I) Anal division, ventral view. al–alII, VI, VII, abdominal segments I–III, VI, VII.
Fig. 8. Third instar larva of *Muscina stabulans*. (A) Anterior end of body with anterior spinose band further ramified ventrally (arrow), lateral view. (B) Antennal complex. (C) Maxillary palpus. (D) Facial mask, ventral view. (E) Ventral organ. (F) Trichoid sensilla of Keilin’s organ. (G) Second thoracic segment, anterior spinose band, lateral view.
Fig. 9. Third instar larva of *Muscina stabulans*. (A) Abdominal segments with open apertures of probably non-functional spiracle (arrow), lateral view. (B) Abdominal segments with short rows of spines at the posterior margin (arrow), ventral view. (C) Bubble membrane. (D) Posterior spiracles, posterior view. (E) Sub-anal papilla with sensillum basiconicum (asterisk) and sensilla ampullacea (arrows), ventral view. (F) Anal division, lateral view. (G) Anal division, posterior view. (H) Anal division, ventral view. al–aIII, aVI, aVII, abdominal segments I–III, VI, VII; lcw, lateral creeping welt.

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on pa being somewhat dark in colour. All spines are directed posteriorly except for those in pre. Each sa is equipped with a sensillum basiconicum and two sensilla resembling sensilla ampullacea (Fig. 9E). The spiracular field carries posterior spiracles and is surrounded by seven pairs of sensilla. Each pair of sensilla together with the immediately adjoining cuticle is termed a ‘papilla’ (p1–p7) (Figs 5C, F; G; 7G, H; 9F, G). Papillae p1, p3, p5 and p7 are placed at the margin of the spiracular field, and papillae p2, p4 and p6 are shifted more or less anteriorly. Papillae p1, p3, p5 and p7 are in the form of cuticular bulges and each is equipped with a sensillum resembling a sensillum basiconicum (Fig. 5E), whereas papillae p2, p4 and p6 are indistinct and are equipped with a sensillum resembling a sensillum ampullaceum placed level with the adjacent integument (Fig. 7F). Posterior spiracles are slightly raised above the surface of the ad (Figs 5C, 7G, 9). A spiracular scar (ss) is located in the middle position; the respiratory slits (rs) are crescent-shaped and arranged in a radiating configuration (Figs 5A, 7D, 9D). The peritreme is complete and sclerotized like the adjacent surface (Fig. 2B, D, F). The sclerotization of the posterior spiracles and the adjoining area intensifies during larval growth (i.e. the peritreme is blackish brown in the mature larva) (cf. Fig. 2B, D with F).

Key for identification of third instar Muscina spp

1 Posterior spiracles rounded (Fig. 2B, D), heavily sclerotized in mature larvae (Fig. 2F), never raised on distinct stalks, respiratory slits crescent-shaped (Figs 2B, D; F; 5A; 7D; 9D), extra-anal, post-anal and sub-anal papillae well developed and covered by spines; para-anal papillae absent (Figs 5C, F; G; 7G–I; 9F–H). Anal plate broad, W-shaped and with two bulges posterolaterally to the anal opening (Figs 5D, 7I, 9H). Mouth-hooks symmetrical, anterior rods, oral bars and suprabuc-...
Identification of third instars of Muscina

Precise species identification is a vital first step in the analysis of entomological material of medical and veterinary importance. However, the identification of immature stages of some insect groups causes severe problems. An analysis of the illustrations of entomological material from two recent reports of cases of human intestinal myiasis in India caused by *M. stabulans* (Shivekar *et al.*, 2008; Udgaonkar *et al.*, 2012) raises serious doubts about the species identification. We conclude that the reports of Shivekar *et al.* (2008) and Udgaonkar *et al.* (2012) present misidentifications. The larva figured by Udgaonkar *et al.* (2012) represents a first instar, not a second as the authors state, and depicts a flesh fly larva (Sarcophagidae), not that of a muscid. The larvae obtained by Shivekar *et al.* (2008) also correspond to first instar flesh flies. Thus it is necessary to provide keys for easy species identification which can be used with standard light microscopy, particularly by students without specialist entomological training. Keys that are properly illustrated and provide a set of reference images for all species of interest will facilitate precise species identification.

Despite the extensive literature concerning third instar larval morphology of *Muscina* species, very few authors have provided comprehensive descriptions (Keilin, 1917; Thomson, 1937; Zimin, 1948). Most previous studies focused only on brief descriptions and did not give species-specific characters, possibly out of conviction that immature stages carry few morphological characters and that these usually reflect only adaptations to a certain habitat. However, this is not the case; immature dipterans have recently been revealed as a valuable source of data for both taxonomic (Szpila, 2010a; Ubero-Pascal *et al.*, 2015) and systematic (Skidmore, 1985; Szpila, 2010b) purposes.

Although previous descriptions of *Muscina* spp. third instars provide valuable information, they are incorrect or contradictory in many details. Some of these are highlighted below (see Table S1 for full details). According to Skidmore (1985), third instars of *Muscina* spp. often have supra buccal teeth but are devoid of cutaneous teeth. This is not the case: sub as well as cut were observed in all specimens currently studied. In Thomson’s (1937) and Zimin’s (1948) figures showing the posterior spiracles of young vs. mature larvae of *M. prolapsa* and *M. levida*, respectively, each spiracle is distinctly broader in the mature larvae, even when differences in scale are taken into account. During growth of *Muscina* larvae, posterior spiracles do not grow, but become increasing sclerotized, and the spiracles and adjoining cuticle turn distinctly black (cf. Fig. 2B, D, F). This phenomenon may cause confusion and make the boundaries of posterior spiracles difficult to distinguish.

According to Zimin (1948), *M. levida* possesses a unique character in comparison with *M. stabulans*, which refers to the shape of the anal plate, which protrudes laterally and forms a structure resembling one of the anal papillae (Fig. 5F). Although this feature was observed in the studied material, it was apparent only in some of the young larvae (Fig. 5F), whereas in mature larvae its presence is dubious (Fig. 5G). It is likely that Zimin (1948) studied only young larvae of *M. levida* as his drawing of the posterior spiracle shows no indication of sclerotization of the area adjoining the spiracles.

According to Ishijima (1967), third instars of *M. stabulans* and *M. prolapsa* differ in the distance between posterior spiracles. Posterior spiracles of the former should be separated by a distance of about half of the diameter of one spiracle, whereas in the latter this value is similar to its diameter. In the present study, the distance between posterior spiracles in *Muscina* spp. was found to increase during larval growth, and the SDF ranges in both species to coincide. It is possible that the measurements obtained by Ishijima (1967) refer to larvae of different ages.

Liu & Greenberg (1989) stated that *M. levida* and *M. stabulans* are very similar except for the number of lobes in the anterior spiracles and details of the posterior spiracles (i.e. three to five in *M. levida* and four to six in *M. stabulans*). Liu & Greenberg (1989) also showed that this character does not provide species-specific states because the values overlap across both species. Nonetheless, this finding did not prevent them from including the feature in their identification key. According to Liu & Greenberg (1989), *M. levida* and *M. stabulans* can also be identified on the basis of a small non-sclerotized triangular area present between the upper and middle respiratory slits (cf. Fig. 2B, D) in the latter species. Thomson (1937) observed a similar lightly sclerotized triangular area in *M. prolapsa*. Examination of a long series of specimens revealed the occurrence of a bright yellowish-orange area in all species (Fig. 2B, F); however, as the sclerotization process of the posterior spiracles takes place throughout larval development, the size of the triangular area decreases with age and may be hardly distinguishable or even absent in the mature larva (cf. Fig. 2F). Liu & Greenberg (1989) apparently compared young specimens of *M. stabulans* with mature larvae of *M. levida*. Characters proposed for the discrimination of *Muscina* species by other authors (Zimin, 1948; Ishijima, 1967; Liu & Greenberg, 1989) have been revealed as insufficient. None of the previous authors provided comprehensive descriptions of spination patterns of these species, although Zimin (1948) and Liu & Greenberg (1989) attempted to describe some details. The presence or absence of spines covering thoracic and abdominal segments in Muscidae may be difficult to observe because of their lack of contrast (i.e. they are non-sclerotized); thus such characters have not been included in identification keys. Despite previous suggestions that third instar larvae of the examined *Muscina* species are not distinguishable, they were shown here to differ reliably in their spination. Although it may be difficult to observe initially, staining reveals spination to be a stable character for taxonomic purposes.

Despite the extensive literature concerning third instar larval morphology of *Muscina* species, previously published data did not allow for unambiguous identification of those species. However, the present study reveals significant diversity of morphological characters overlooked by previous authors. We urge dipterists to collect and study the morphology of immature stages as even species commonly regarded as indistinguishable as larvae may be revealed to differ in some morphological aspects. It is important to revise previous studies and provide exhaustive descriptions based on offspring of at least several females in order to study intra- and interspecific variation; for example, the comparison of larvae of different ages led previous authors to incorrect conclusions concerning species identification (Zimin, 1948; Ishijima, 1967; Liu & Greenberg, 1989). Hence examination of both young and mature larvae of
a given species will enable the selection of characters that are more useful for taxonomic purposes than those linked to larval maturation.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12117

Table S1. Discrepancies between previous descriptions of third instar larva morphology of Muscina spp. and those in the present study.

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References

Bouché, P.F. (1834) Naturgeschichte der Insekten besonders in Hinsicht ihrer ersten Zustände als Larven und Puppen. Erste Lieferung, Berlin.

Courtney, G.W., Sinclair, B.J. & Meier, R. (2000) Morphology and terminology of Diptera larvae. Contributions to a Manual of Palaearctic Diptera (with Special Reference to Flies of Economic Importance), Vol. 1. General and Applied Dipterology (ed. by L. Papp & B. Darvas), pp. 75–163. Science Herald, Budapest.

Duarte, J.L.P., Krüger, R.F. & Ribeiro, P.B. (2013) Interaction between Musca domestica L. and its predator Muscina stabulans (Fallén) (Diptera, Muscidae): effects of prey density and food source abundance. Revista Brasileira de Entomologia, 57, 55–58.

van Emden, F.I. (1965) The Fauna of India and Adjacent Countries. Diptera 7, Muscidae, Part 1. Government of India, Delhi.

Engel, E.O. (1915) Beiträge zur Kenntnis einiger Diptereneren. Mitteilungen der Münchner Entomologischen Gesellschaft, 7, 68–76.

Erzinçlioğlu, Y.Z. (1985) Immature stages of British Calliphora and Cynomya, with re-evaluation of the taxonomic characters of larval Calliphoridae (Diptera). Journal of Natural History, 19, 69–96.

Gaudry, E. (2010) The insects colonization of buried remains. Current Concepts in Forensic Entomology (ed. by J. Amendt, M.L. Goff, C.P. Campobasso & M. Grassegger), pp. 273–311, Springer, Dordrecht.

Gestmann, F., Förster, M., Mehlhorn, H. et al. (2012) Flies as vectors of microorganisms potentially inducing severe diseases in humans and animals. Arthropods as Vectors of Emerging Diseases (ed. by H. Mehlhorn), pp. 195–226. Springer, Heidelberg.

Gregor, F., Rozkošný, R., Barták, M., Vathara, J. (2002) The Muscidae (Diptera) of Central Europe. Folia Facultatis Scientiarum Naturatium Universitatis Masarykianae Brunensis, Biologia, 107, 1–280.

Greenberg, B. & Kunich, J.C. (2002) Entomology and the law: Flies as forensic indicators. Cambridge University Press, Cambridge.

Grzywacz, A. (2013) Third instar larva morphology of Hydrotaea cypriocentaurina (Zetterstedt, 1845) (Diptera: Muscidae) – a species of forensic interest. Polish Journal of Entomology, 82, 303–315.

Grzywacz, A., Pape, T. (2014) Larval morphology of Atherigona orientalis (Schiner) (Diptera: Muscidae) – a species of sanitary and forensic importance. Acta Tropica, 137, 174–184.

Grzywacz, A., Pape, T., Hudson, W.G. & Gomez, S. (2013) Morphology of immature stages of Atherigona reversura (Diptera: Muscidae), with notes on the recent invasion of North America. Journal of Natural History, 47, 1055–1067.

Grzywacz, A., Lindström, A. & Hall, M.J.R. (2014) Hydrotaea similis Meade (Diptera: Muscidae) newly reported from a human cadaver. Forensic Science International, 242, e34–e43.

Iancu, L. & Păru, C. (2013) Necrophagous entomofauna (Diptera, Coleoptera) on Meles meles (L.) (Mammalia: Mustelidae) carcasses within different conditions of exposure in Bucharest (Romania). Travaux du Muséum National d’Histoire Naturelle ‘Grigore Antipa’, 56, 45–63.

Ishijima, H. (1967) Revision of the third stage larvae of synanthropic flies of Japan (Diptera: Anthomyiidae, Muscidae, Calliphoridae and Sarcophagidae). Japanese Journal of Sanitary Zoology, 18, 47–100.

Keilin, D. (1917) Recherches sur les Anthomyiidae a larves carnivores. Parasitology, 9, 325–450.

Liu, D. & Greenberg, B. (1989) Immature stages of some flies of forensic importance. Annales of the Entomological Society of America, 82, 80–93.

Matheson, R. (1950) Medical Entomology. Comstock Publishing, New York, NY.

Pape, T. & Evenhuis, N.L. (2014) Systema Dipterorum, Version 1.5. http://www.diptera.org/ [accessed on 30 September 2014].

Pont, A.C. (1986) Family Muscidae. Catalogue of Palaearctic Diptera, Scaphotagidae – Hypodermatidae, Vol. 11 (ed. by A. Soós & L. Papp), pp. 1–345. Amsterdam, Elsevier.

Portchinsky, J.A. (1910) Osennaya zhigalka (Stomoxys calcitrans L.), yeya biologiya v’ svyazi s’ drugimi mukhami i bor’ba s’ neyu. Trudy Byuro po Entomologii, 8, 1–90 (in Russian).

Queiroz, S.M.P. & de Carvalho, C.J.B. (1987) Chave pictórica e descrições de larvas de 3º instar de Diptera (Calliphoridae, Muscidae e Fanniidae) em vazadouros de resíduos sólidos domésticos em Curitiba, Paraná. Anais da Sociedade Entomológica do Brasil, 16, 265–288 (in Portuguese with English abstract).

Robak, S.S. (1951) A classification of the muscid Calyptrate, Diptera. Annales of the Entomological Society of America, 44, 327–361.

Schumann, H. (1963) Zur larvalsystematik der Muscinae nebst beschreibung einiger Muscidaen- und Anthomyiadenlarven. Deutsche Entomologische Zeitschrift, 10, 134–164 (in German).

Séguy, E. (1923) Diptéres anthomyides. Faune de France, 6, 1–393.

Shanmugam, J. & Gopal, R. (2008) Intestinal myiasis caused by Muscina stabulans (Muscidae, Calliphoridae) newly reported from a human cadaver. Entomologica, 29, 83–85.

Skidmore, P. (1985) The biology of the Muscidae of the world. Series Entomologica, 29, 1–550.

Smith, K.G.V. (1986) A Manual of Forensic Entomology. British Museum (Natural History), London; Cornell University Press, Ithaca, NY.

Sukontason, K., Sukontason, K.L., Piangjai, S., Boonchu, N., Kuraishi, H., Hope, M. & Olson, J.K. (2004) Identification of forensically important fly eggs using a potassium permanganate staining technique. Micron, 35, 391–395.

Szpila, K. (2010a) Key for the identification of third instars of European blowflies (Diptera: Calliphoridae) of forensic importance. Current Concepts in Forensic Entomology (ed. by J. Amendt, M.L. Goff, C.P. Campobasso & M. Grassegger), pp. 43–56. Springer, Dordrecht.

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Szpila, K. (2010b) The First Instar of European Miltogramminae (Diptera: Sarcophagidae). Wydawnictwo Naukowe UMK, Toruń.
Szpila, K. & Pape, T. (2005) The first instar larva of Apodacra pulchra (Diptera: Sarcophagidae, Miltogrammidae). *Insect Systematics & Evolution*, 36, 293–300.
Thomson, R.C.M. (1937) Observations on the biology and larvae of the Anthomyiidae. *Parasitology*, 29, 273–358.
Ubero-Pascal, N., Paños, A., García, M.D., Presa, J.J., Torres, B. & Arnaldos, M.I. (2015) Micromorphology of immature stages of Sarcophaga (Liopygia) cultellata Pandellé, 1896 (Diptera: Sarcophagidae), a forensically important fly. *Microscopy Research and Technique*, 78, 148–172.
Udgaonkar, U.S., Dharamsi, R., Kulkarni, S.A. *et al.* (2012) Intestinal myiasis. *Indian Journal of Medical Microbiology*, 30, 332–337.
Velasquez, Y., Ivorra, T., Grzywacz, A., Martinez-Sanchez, A., Magna, C., Garcia-Rojo, A. & Rojo, S. (2013) Larval morphology, development and forensic importance of Synthesiomyia nudiseta (Diptera: Muscidae) in Europe: a rare species or just overlooked? *Bulletin of Entomological Research*, 103, 98–110.
Wallman, J. (2001) Third instar larvae of common carrion-breeding blowflies of the genus Calliphora (Diptera: Calliphoridae) in South Australia. *Invertebrate Taxonomy*, 15, 37–51.
Zimin, L.S. (1948) Opredeliteli lichinok sinantropnyh mukh Tadzhikistana. *Opredeliteli po Faune CCCP*, 28, 1–116 (in Russian).
Zumpt, F. (1965) *Myiasis in Man and Animals in the Old World: A Textbook for Physicians, Veterinarians and Zoologists*. Butterworths, London.

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