Morphology successfully separates third instar larvae of *Muscina*

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Abstract

Three representatives of the muscid genus *Muscina* Robineau-Desvoidy, *M. levida* (Harris), *M. prolapsa* (Harris) and *M. stabulans* (Fallén), are well known from their medical, veterinary and forensic importance. Nevertheless data in the literature provide contradictory information concerning identification of the third instar larvae of these species. This hinders easy species differentiation because it requires rearing of material to the adult stages to obtain a reliable species identification. Third instar larvae of three *Muscina* species were studied here in detail and thorough re-descriptions of their morphology are provided using light and scanning electron microscopy. Existing information concerning third instar morphology, particularly 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., since 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 pattern of their abdominal segments.

Key words

Muscidae, *Muscina levida*, *Muscina prolapsa*, *Muscina stabulans*, third instar larva, spiracular distance factor, SEM, forensic entomology

Introduction

Muscina is a small genus of the family Muscidae and comprises currently 14 valid species (Pape & Evenhuis 2014). Three representatives of the genus, *M. levida*, *M. prolapsa* and *M.*

stabulans, are well known from their medical and veterinary importance. Immature stages of *Muscina* spp. have been reported from nests of insects and birds 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 myiasis cases in humans and animals (Zumpt 1965). In addition to their medical and veterinary importance, larvae of *Muscina* spp. are also known for their forensic importance, since they can colonise both exposed and buried decomposing human bodies (Greenberg & Kunich 2002; Schroeder *et al*., 2003; Gaudry 2010). Under certain circumstances *Muscina stabulans* may exclusively colonize a dead body, or may even colonize the body before death due to myiasis cases (Smith 1986). *Muscina levida* and *M. prolapsa* have both been reported from the Holarctic region, however the latter is known also from St. Helena Island (Pont 1986). In contrast, *M. stabulans* is a cosmopolitan insect spread by commerce and currently is known from 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 *M. assimilis* (Fallén) and *M. pabulorum* (Fallén) refer to *M. levida* and *M. prolapsa* respectively.

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 from medical, veterinary and forensic cases. The literature concerning the larval morphology of *Muscina* spp. is large. Not surprisingly, the quality of data published varies from superficial to exhaustive, but unfortunately with a predominance of the former. Earlier students of larval morphology often described only a small number of characters, like general body shape, arrangement of anterior and posterior spiracles or outline of the cephaloskeleton (Bouché 1834; Portchinsky 1910; Engel 1915; Séguy 1923), but these can be found in more recent studies as well (Queiroz & Carvalho 1987; Liu & Greenberg 1989). The third instar larva of *M. levida* has been described several times (Keilin 1917, as *M. assimilis*; Séguy 1923, as *M. assimilis*; Zimin 1948, as *M. assimilis*; Liu & Greenberg 1989, as *M. assimilis*; Skidmore 1985). That of *M. prolapsa* has been studied by Thomson (1937, as *M. pabulorum*), Ishijima (1967, as *M. pabulorum*) 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; Schumann 1954; Zumpt 1965; Ishijima 1967; Skidmore 1985; Queiroz & Carvalho 1987; Liu & Greenberg 1989). Zumpt (1965) suggested that morphological differences between *Muscina* spp. are only superficially or inadequately studied and recommended 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, Liu & Greenberg 1989), but these new data still do not enable species discrimination and they 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 and scanning electron microscopy. Detailed redescriptions of third instar larval morphology are provided as well as comprehensive documentation of the morphology. Published data concerning third instar morphology are revised and discussed with a focus on their value for taxonomy and identification.

Material 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. They were transported to the laboratory in separate 2 ml eppendorf tubes, each with a perforated cap to allow for aeration. They were supplied with ???? liver and kept alive until they oviposited. Laboratory rearing and killing of larvae was 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 CO_2 using a CPD 030 (Bal-Tec GmbH, Balzers, Lichtenstein) or Autosamdri®-815, Series A (tousimis®, Rockville, MD, USA) 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). SEM images of *Muscina prolapsa* and *M. stabulans* were taken with a JEOL scanning microscope (JSM-6335F; JEOL Ltd, Tokyo, Japan) and images of *M. levida* with a variable pressure SEM LEO 1455 (Carl Zeiss Microscopy, Germany).

Light microscopy observations were performed with a Stemi 2000 stereomicroscope (Carl Zeiss Light Microscopy, Germany). Because the larval spines are colourless, the 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 according to Niederegger *et al*. (2011). 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 either on a Nikon Eclipse E200 microscope or Nikon SMZ 1500 stereomicroscope (Nikon Corp., Tokyo, Japan).

Terminology follows 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 both in young and mature larvae: maximum body length and width, distance between posterior spiracles (*dps*), width of posterior spiracle in horizontal plain (*wps*) and spiracular distance factor (SDF) defined after 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 was 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

Larval morphology of third instars of *M. levida*, *M. prolapsa* and *M. stabulans* is jointly described below to avoid repetition, since in most aspects the examined species were identical, unless emphasized otherwise.

Muscina levida (Harris), syn. *M. assimilis* (Fallén), (Figs 1A, B, E; 2A, B; 3; 4; 5)
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 (n = 40).

Muscina prolapsa (Harris), syn. *M. pabulorum* (Fallén), (Figs 1C, F; 2C, D; 6; 7)
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 (n = 10).

Muscina stabulans (Fallén), (Figs 1D, G; 2E, F; 8; 9)
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 (n = 30).

The third instar larva of the three species has a long, slender body that tapers gradually anteriorly and when viewed laterally has an obliquely truncated anal division (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 spiracles (*ps*), respectively. Minute open apertures of probably non-functional spiracles are present in each of the antero-lateral margins of *tIII–aVII* (Fig. 9A). The body surface is smooth, neither equipped with distinct sculpture nor covered with longitudinal ridges.
Pseudocephalon. Lobes of the bilobate pseudocephalon carry an antennal complex (*an*), maxillary palpus (*mp*) and ventral organ (*vo*) (Figs 3A, B, D). The antennal complex consists of an antennal dome (*and*), encircled with seven basal pores located equidistantly from each other, 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 palpus 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 latero-dorsally (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 antero-

lateral 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 (*sub*) are present laterally to the anterior end of the functional mouth opening, as well as two rows of minute cutaneous teeth (*cut*) on its sides (Figs 3D; 6E; 8D).

Cephaloskeleton. Mouthhooks (*mh*) are well separated, robust basally with slender, symmetric distal parts (Fig. 1B). The apical part of the *mh* is more or less curved ventrally and sharply pointed. An unpaired longitudinal sclerite (*us*) in the form of a spicule 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 antero-dorsal 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 in mature larvae, thus forming an inverted arch (Fig. 1B), however in newly moulted specimens these sclerites are not fused. The intermediate sclerite (*is*) is H-shaped with a broad crossbeam (Fig. 1B). An epistomal sclerite (*es*) and a pair of labial sclerites (*ls*) lie freely between the anterior part of the arms of the *is*. The epistomal sclerite is equipped with two pairs of rounded perforations. Longitudinal labial sclerites are equipped with about four circular openings of uneven size (Fig. 1B).

The long basal sclerite consists of paired vertical plates (*vp*) with dorsal (*dc*) and broad ventral cornua (*vc*), connected antero-dorsally by a perforated dorsal bridge (*db*) and antero-ventrally by a narrow ventral bridge (*vb*) (Fig. 1A, C, D). The dorsal cornu is shorter than the ventral cornu and the latter bears a well developed dorsal expansion in the postero-dorsal 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 *Muscina 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 as in *M. levida*. The degree of sclerotization differs intraspecifically and depends on the stage of larval maturity.

Thorax. Anterior spiracles are equipped with four to six (Fig. 3A, B), four to five (Fig. 6A) and six to 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 second and third thoracic 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 thoracic segments.

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 postero-lateral 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 be also present in the posterior margin of the ventral surface of some segments (Figs 1E, G; 4D). In *M. levida* and *M. stabulans* the postero-ventral margin of *aII*–a*VI* (cf. Figs 4D; 9B) are covered by spines randomly arranged in several short rows, while in *M. prolapsa* the postero-ventral 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 and arranged in short rows (Figs 4A; 7A; 9A). In *M. levida* and *M. prolapsa* the anterior spinose band on *aIII* is interrupted dorsally, albeit it reaches above the upper margin of *lcw* (Figs 1E, F; 4A; 7A), and 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 reaching slightly above this, then the anterior spinose band on *aIV* is hardly developed (Fig. 1G). Abdominal segments carry antero-ventral 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 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 in lateral view are somewhat triangular in shape (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 with two bulges postero-laterally to *ao* well visible in lateral view (Figs 5C, D, F, G; 7G–I; 9F–H). An unpaired postanal papilla (*pa*) lies directly behind the anal opening, and pairs of subanal (*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 anal division lateral and posterior to *ap* as well as *pre*, *pa*, and *sa* are covered by spines (Figs 5B–D, F, G; 7E, G–I; 9E–H), with those on *pa* being somewhat dark coloured. 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 more or less shifted 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 anal division (Figs 5C; 7G; 9F). A spiracular scar (*ss*) is in middle position, the respiratory slits (*rs*) are crescent-shaped and arranged in a radiating configuration (Figs 5A; 7D; 9D). The peritreme is complete and scletorized 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 (compare Fig. 2B, D with 2F).

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, postanal and subanal papillae well developed and covered by spines, para-anal papillae absent (Fig. 5C, F, G; 7G–I; 9F–H). Anal plate broad, W-shaped and with two bulges postero-laterally to the anal opening (Figs 5D; 7I; 9H). Mouthhooks symmetric, anterior rods, oral bars and suprabuccal teeth distinct and dark, optic depression not sclerotized (Figs 1A–D; 2A, C, E) → *Muscina* spp. 2

Other combination of characters → other Muscidae

2. Ventral surface of abdominal segments *aV–aVI* without numerous spines at the posterior margin (Figs 1F; 7B), at most with two rows of about five spines on 1-2 segments→ *Muscina prolapsa*

   Ventral surface of abdominal segments *aV–aVI* with small lightly sclerotized spines at the posterior margin, randomly arranged in about 10 short rows of about five spines (Figs 1E, G; 4D; 9B) → 7

3. Spinose band on *aIII* reaches well above the upper margin of the lateral creeping welt (Figs 1E; 4A) and anterior spinose band on the subsequent segment (*aIV*) reaches at least the middle of the lateral creeping welt (Fig. 1E); cephaloskeleton sometimes with additional longitudinal dorsal sclerite articulating with the dorsal cornu of basal sclerite and dorsally forming a bar-like structure (Figs 1A; 2A) → *Muscina levida*

   Spinose band on *aIII* reaches to or at most slightly above the upper margin of the lateral creeping welt (Figs 1G; 9A), if reaching slightly above this, then anterior spinose band on *aIV* hardly developed (Fig. 1G); cephaloskeleton never with an additional sclerite articulating with basal sclerite (Figs 1D; 2E) → *Muscina stabulans*

Discussion

Comparative morphology

Except for the most recent studies (e.g., Velasquez *et al*., 2013; Grzywacz 2013; Grzywacz & Pape 2014; Grzywacz *et al*., 2013, 2014) none of the previous authors observed sensilla surrounding the spiracular field in the third instars of Muscidae. However the study of Velasquez et al. (2013) and Grzywacz et al. (2013) are incomplete since these authors did not recognize the sensilla resembling sensilla ampullacea (i.e., *p2, p4*, and *p6*) in *Synthesiomyia nudiseta* (van der Wulp) and *Atherigona reversura* Villeneuve, respectively. However, re-examination of this material (Grzywacz unpubl.) confirms the occurrence of *p1–p7* with concomitant sensilla in these species. Because sensilla on *p1–p7* are indistinguishable under a stereomicroscope, they could not be recognized without the application of SEM. Thus, papillae surrounding the spiracular field, if distinguishable, were considered only as papillae

or protuberances, not as carriers of concomitant sensory sensilla. For example, third instars of *Muscina* spp. were hitherto reported to have four pairs of papillae (Keilin 1917; Ishijima 1968; Skidmore 1985), six pairs were reported in *M. autumnalis* de Geer (Zimin 1948), whereas the spiracular field of *Musca domestica* Linnaeus was considered as completely smooth (Zimin 1948). Furthermore, according to Schumann (1963), an apomorphic character state for the Muscidae is the lack of papillae along the margin of the spiracular field. Third instars of *Muscina* and other representatives of Muscidae (Grzywacz 2013; Grzywacz & Pape 2014) possess seven pairs of sensory sensilla, although often indistinctly raised on protuberances, and therefore Schumann's (1963) statement is misleading. The appearance of the papillae surrounding the spiracular field in the third instars of representatives of other Calyptratae differs from those that we observed, yet still seven pairs are present. According to the present state of knowledge, the seven pairs of papillae surrounding the spiracular field may be classified into two types by means of their position and most of all by the type of concomitant sensory sensilla, *p1*, *p3*, *p5*, *p7* are each equipped with a sensillum resembling a sensillum coeloconicum or basiconicum, whereas *p2*, *p4*, *p6* are each equipped with a sensillum resembling a sensillum ampullaceum.

Emden (1965) defined the 'spiracular distance factor' (SDF) to allow species identification based on the distance separating the posterior spiracles in a given species. The use of this ratio gained acceptance and has subsequently been incorporated into identification keys (Erzinçlioğlu 1985; Szpila 2010a). However, Wallman (2001) revealed SDF in *Calliphora* Robineau-Desvoidy reliable for taxonomic purposes only in freshly killed specimens. In preserved specimens the distance between the posterior spiracles has been affected by the degree of cuticular shrinkage and hardening (Wallman 2001). We recognized that the SDF shows variable, age-related, values in the species studied here. In *Muscina* spp. and also some *Hydrotaea* Robineau-Desvoidy (Grzywacz 2013; Grzywacz *et al*., 2014) SDF ratios overlap, strongly reducing the application of this metric for species identification purposes. The ratio has been observed to increase during the maturation of larvae since the distance between both spiracles increases during larval growth while the spiracle size remains constant. In conclusion, the spiracular distance factor is inappropriate for taxonomic purposes in third instar larvae of *Muscina* spp. SDF and its usefulness in species from other genera should be re-examined by means of determining SDF values in both young and mature larvae.

Identification of *Muscina* species

Precise species identification is a vital first step in the analysis of entomological material of medical and veterinary importance as well as insects inhabiting both live and dead bodies in any forensic case. However, identification of immature stages of some insect groups cause severe problems. An analysis of the illustrations of entomological material from the two recent reports of cases of human intestinal myiasis in India caused by *M. stabulans* (Shivekar *et al*., 2008; Udgaonkar *et al*., 2012) raise serious doubts about the species identification. We conclude that the reports of Shivekar et al. (2008) and Udgaonkar et al. (2012) present misidentifications. Larva figured by Udgaonkar et al. (2012) represent a first instar, not a second as stated by the authors, and a flesh fly larva is depicted (Sarcophagidae), not that of a muscid.. Also the larvae obtained by Shivekar et al. (2008) correspond to a first instar flesh fly. Thus it is necessary to provide keys for easy species identification which can be used with standard light microscopy particularly by students without a specialist entomological training. Particularly well illustrated keys providing a set of reference images for all species of interest will facilitate precise species identification.

In spite of 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 of the previous studies focused only of brief descriptions, without any species specific characters, possibly because of a conviction that immature stages carry only few morphological characters, most often reflecting only adaptations to a certain habitat. That is not the case, however, since recently immature dipterans have been revealed as a valuable source of data for both taxonomic (Szpila 2010a; Uber-Pascal *et al*. 2014) and systematic purposes (Skidmore 1985, Szpila 2010b).

Although previous descriptions of *Muscina* spp. third instars provide valuable information, they are incorrect or contradictory in many details, a few of which are highlighted below (see Supporting material 2 for full details). According to Skidmore (1985) the third instars of *Muscina* spp. often have suprabuccal teeth but are devoid of cutaneous teeth. This is not the case, since *sub* as well as *cut* have been observed in all specimens currently studied. In Thomson's (1937) and Zimin's (1948) figures showing the posterior spiracles of young versus mature larvae of *M. prolapsa* and *M. levida*, respectively, each spiracle is distinctly broader in the mature larvae, even taking into account differences in scale. During growth of *Muscina* larvae, posterior spiracles do not grow but become increasingly sclerotized, with the spiracles and adjoining cuticle turning distinctly black (compare Fig. 2B, D, F). This phenomenon may cause confusion and make the boundaries of posterior spiracles difficult to distinguish.

Zimin (1948) stated that the apical part of the mouthhook is narrower in *M. levida* than in *M. stabulans*, yet such differences were not observed in the present work. According to Zimin (1948), *M. levida* possesses a unique character in comparison with *M. stabulans* – the shape of the anal plate protruding laterally, and forming 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). Moreover, Zimin (1948) most probably studied only young larvae of *M. levida* since his drawing of the posterior spiracle shows no indication of sclerotization of the area adjoining the spiracles.

According to Ishijima (1967), third instars of *Muscina 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 coincide. Possibly Ishijima (1968) made his measurements on 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. from three to five in *M. levida* and from four to six in *M. stabulans*. Liu & Greenberg (1989) also showed that this character does not provide species-specific states for *M. levida* and *M. stabulans* because the values overlap. 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, since 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. On the other hand none of the previous authors provided comprehensive descriptions of spinulation patterns of these species, though 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, with few exceptions (e.g., Zimin 1948; Ishijima 1967). Despite Skidmore's (1985) suggestion that third instar larvae of the examined *Muscina* species are not distinguishable, they were shown here to differ reliably in their spinulation. Although it may be difficult to observe initially, staining reveals spinulation to be a stable character for taxonomic purposes.

In spite of 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 revealed significant diversity of morphological characters overlooked by previous authors. We urge dipterists to collect and study the morphology of immature stages, since even species commonly regarded as indistinguishable as larvae may be revealed as differing in some morphological aspects. It is important to revise previous studies and provide exhaustive descriptions based on offspring of at least several females to study intra and interspecific variation, for example, 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 selection of characters useful for taxonomic purposes rather than those linked to larval maturation.

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Fig. 1. Third instar larvae of *Muscina*. (A) *M. levida*, cephaloskeleton, lateral view; (B) *M. levida*, cephaloskeleton, dorsal view; (C) *M. prolapsa*, cephaloskeleton, lateral view; (D) *M. stabulans*, cephaloskeleton, lateral view; (E) *M. levida*, habitus; (F) *M. prolapsa*, habitus; (G) *M. stabulans*, habitus. Scale bars 0.1mm for A–D, 1mm for E–G. Abbreviations: aI–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; is, intermediate sclerite; lcw, 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.

Fig. 2. Third instar larvae of *Muscina*. (A) *M. levida*, cephaloskeleton, lateral view; (B) *M. levida*, posterior spiracles of young larva;(C) *M. prolapsa*, cephaloskeleton, lateral view; (D) *M. prolapsa*, posterior spiracles of young larva; (E) *M. stabulans*, cephaloskeleton, lateral view; (F) *M. stabulans*, posterior spiracles of mature larva.

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. Abbreviations: 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.

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, spines on the postero-ventral margin (arrow), lateral view. Abbreviations: 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) subanal papilla, ventral view; (C) posterior end of body, later view; (D) anal division with anal opening (arrow) and anal plate with bulges (asterisks), ventral view; (E) anal division with bulges of the anal plate (arrow), papilla p3; (F) anal division of young larva, posterior view; (G) anal division of mature larva, posterior view. Abbreviations: ao, anal opening; ap, anal plate; ex, extra-anal papilla; p1–p7, papillae 1–7 surrounding spiracular field; pa, postanal papilla; pre, pre-anal welt; rs, respiratory slit; sa, subanal 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. Abbreviations: 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, ventral view; (C) bubble membrane; (D) posterior spiracles, posterior view; (E) subanal papilla, ventral view; (F) papilla p2; (G) posterior end of body, lateral view; (H) anal division, posterior view; (I) anal division, ventral view. Abbreviations: aI–aIII, VI, VII, abdominal segments I–III, VI, VII.

Fig. 8. Third instar larva of *Muscina stabulans*. (A) anterior end of body, 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 a group of spines at the posterior margin (arrow), ventral view; (C) bubble membrane; (D) posterior spiracles, posterior view; (E) subanal 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. Abbreviations: aI–aIII, aVI, aVII, abdominal segments I–III, VI, VII; lcw, lateral creeping welt.

Table 1. Origin in Poland of *Muscina* females from which larvae were obtained.

Species	Number of females	Number of 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 Pławin (2011)
Muscina prolapsa	3	7-19	Lotnisko airport near Toruń (2010) and Pławin (2009)
Muscina stabulans	4	19-60	Pławin (2009-2011)

Supplementary information:

Discrepancies between the previous descriptions of third instar larva morphology of *Muscina* spp. and the present study.

Author	*Muscina levida*	*Muscina prolapsa*	*Muscina stabulans*
Bouché (1834), Engel (1915)	-	-	Superficial descriptions that do not present any species specific characters.
Portchinsky (1910)	-	-	Did not describe spinulation pattern, yet perceived transverse folds in each anterior margin of the abdominal segments (*vcw* of *aI–VII* and the *pre* herein). Dufour (in Portchinsky 1910) described *M. stabulans* third instar larva with ten lobes in *as*, albeit in the enclosed drawing he presented only six lobes.
	-		
Keilin (1917)	Observed *ad* with seven "sub-anal papillae" and projections surrounding the posterior edge of the segment. The former correspond to anal papillae and the latter to *p1*, *p3*, *p5* and *p7*. Anal papillae comprise five papillae (paired *ex* and *sa* and single *pa*). Hence Keilin probably misidentified bulges on	-	-

	the *ap* as two additional papillae. Correctly reported presence as in the present study. In the cephaloskeleton details he did not observe *cut*, *us*, *acc*, *accs*, *ls* and *es*.		
Séguy (1923)	In his original illustration of the anterior body end with the cephaloskeleton omitted the presence of *cut*, *rp*, *us*, *acc*, *accs*, *ls*, *es* and additional patches of sclerotization.	-	In original drawing of the entire larva did not present spinulation pattern. Omitted the presence of *cut*, *rp*, *us*, *acc*, *accs*, *ls*, and *es*. The illustration of the left posterior spiracle is presented upside down.
Thomson (1937)	-	From five to seven lobes in the anterior spiracles (in the present study the number ranges from four to five). Did not detect *cut*, *us*, *acc*, *accs*, *ls* and *es*, yet he pictured traces of the accessory rectangular process.	-
Zimin (1948)	Anterior spiracle equipped with five lobes. The author neither recognized nor pictured *cut*, *us*, *accs*, *ls* or *es*. Additional sclerites in the posterior part of the *dc* and *vc*. An additional longitudinal dorsal sclerite, arising from the anterior part of the *dc* is not apparent in Zimin's illustration.	-	Anterior spiracles equipped with six lobes. Omitted the presence of *cut*, *us* and *accs*. Did not describe *ls* and *es* (their traces may be found in drawing "Рис. 36 А"). Erroneously discerned *acc* as *ls*. He observed the presence of *sub*, yet did not illustrate them. Spinulation pattern similar to that described

			herein. He observed short rows of spines in posterior ventral margins of abdominal segments. He presented figures of *ps* of both young and mature larvae. In the latter erroneously included also the darkly pigmented area below the cuticle surface.
Matheson (1950)	-	-	Barely recognized the occurrence of *mh*, *ds*, *is* and *bs*.
Roback (1951)	-	Reprinted Thomson's (1937) figure of *M. prolapsa ps* and erroneously captioned it as "*M. pascuorum* Linn.".	-
Ishijima (1967)	-	Did not observe a spinose band in the anterior margin of a*II – III* at all, as well as protuberances surrounding the spiracular field (as "circumspiracular papillae"). Figure of the cephaloskeleton ("Plate 2. 2") is blurred, he did not discern the occurrence of *aro*, *ob*, *sub*, *cut*, *us*, *acc*, *accs*, *ls* or *es*.	Did not observe the spinose band in the anterior margin of *aII*, as well as protuberances surrounding the spiracular field (as "circumspiracular papillae"). The author considered the *ob* and *aro* as a single structure. Figure of the cephaloskeleton ("Plate 2. 3") is blurred, he omitted *cut*, *us*, *acc*, *accs*, *ls* and *es*.
Aspöck (1972)	-	-	Vague and imprecise general outlines of the larval body, cephaloskeleton and *ps*.

			The *is* is presented as broadly fused posteriorly with the basal sclerite. The *ob* and *aro* illustrated joined respectively with the *rp* and *sub*. Did not describe spinulation pattern. Drawing of the *ps* is turned 180-degree.
Skidmore (1981)	The author provided a drawing of the cephaloskeleton, anal plate and *ps* from the puparium, but without a description. He did not recognize *us*, *accs*, *ls*, *es* or additional sclerites.	The author provided a drawing of the cephaloskeleton, *ps* and the posterior body end, but without a description. In the former case the author showed sclerites recognized in the present study, barring *cut*, *us*, *acc*, *accs*, *ls* and *es*.	The author provided a drawing of the cephaloskeleton from the puparium, but without a description. The figure is incomplete since the author did not recognize *cut*, *acc*, *us*, *accs*, *ls* and *es*.
Queiroz & Carvalho (1987)	-	-	The anterior spinose bands are present until the *aII*. The authors pictured three pairs of papillae surrounding the spiracular field (corresponding with *p1*, *p3* and *p7*). Distance between both *ps* is equal to half of the width of one spiracle. The arrangement of *rs* presented by the authors only slightly resembles that observed in this study. In details of the cephaloskeleton only discerned the *mh*, erroneously fused ventrally with *ds*, the *is*

			fused posteriorly with the basal sclerite.
Liu & Greenberg (1989)	A range of lobes in the *as* from three to five with four as the most common. Authors did not describe details of the cephaloskeleton, providing only rough drawings, presenting all sclerites as not separated from each other. Complete bands of spines present from the *tI* to the *aII*, from the *aIII* dorsal spines are missing. Authors did not explain what level the spinose band reaches on *aIII*.	-	A range of lobes in the *as* from four to six lobes with five as the most common. Authors did not describe details of the cephaloskeleton, providing only rough drawings, presenting all sclerites as not separated from each other. Complete bands of spines present from the *tI* to the *aII*, from the *aIII* dorsal spines are missing. Authors did not explain what level the spinose band reaches on *aIII*.
Iancu & Pârvu (2013)	-	-	The authors provided a general outline of larval body, the cephaloskeleton and *ps* without a description. *Rs* in *ps* are almost parallel. This report is a misidentification, and a representative of *Hydrotaea* is depicted, not *M. stabulans*.

Abbreviations: *aI–aVII*, abdominal segments I–VII; *acc*, accessory stomal sclerite; *accs*, supplementary accessory stomal sclerite; *ad*, anal division; *aro*, anterior rod; *as*, anterior spiracle; *cut*, cutaneous teeth; *dc*, dorsal cornu; *ds*, dental sclerite; *es*, epistomal sclerite; *is*, intermediate sclerite; *ls*, labial sclerite; *mh*, mouthhook; *ob*, oral bar; *p1–p7*, papillae 1–7 surrounding spiracular field; *pre*, pre-anal welt; *rp*, accessory rectangular process; *rs*, respiratory slits; *sub*, suprabuccal teeth; *tI*, first thoracic segment; *us*, unpaired sclerite; *vc*, ventral cornu; *vcw*, ventral creeping welt.