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Muscle attachment site (MAS) patterns for species determination in five species of *Sarcophaga* (Diptera: Sarcophagidae)

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Abstract To further establish species determination using the muscle attachment site (MAS) pattern method, third instar larvae of five forensically important species of *Sarcophaga* Meigen were investigated: *Sarcophaga argyrostoma* (Robineau-Desvoidy), *Sarcophaga caerulescens* Zetterstedt, *Sarcophaga melanura* Meigen, *Sarcophaga albiceps* Meigen and *Sarcophaga similis* Meade. As in the previously investigated Calliphoridae, patterns were found to be species specific. The main feature of the *Sarcophaga* patterns is the divided central horizontal row of segment four. A genus pattern was established to be used as base for comparison in further species determination.

Keywords Muscle attachment sites · Flesh flies · *Sarcophaga* · Species determination · Genus pattern

Introduction

The fly family Sarcophagidae, also known as flesh flies, is globally distributed and comprises almost 3000 species, subdivided into only three subfamilies (Pape 1996). The taxon is monophyletic, although internal relationships among subfamilies are still unresolved (Piwczyński et al. 2014). Most species of medical and veterinary importance belong to the traditionally recognized subfamilies Sarcophaginae and Paramacronychiinae and are scavengers, coprophages, or myiasis agents. The third subfamily, Miltogramminae, contains predominantly kleptoparasites of solitary wasps and bees, with a few scavengers, reptilian egg predators, and true parasites of adult insects (Pape 1996; Szpila et al. 2010).

Most flesh flies considered of high forensic importance are species belonging to the genus *Sarcophaga* Meigen, 1826 (Povolný and Verves 1997; Szpila et al. 2015a). In Central Europe, a large carrion attracts a relatively high number of species of adult flesh flies but only few are successful in colonizing this breeding substrate (Szpila et al. 2015a). Successional experiments and forensic case reports indicate *Sarcophaga argyrostoma*, *S. caerulescens*, *S. melanura*, and *S. similis* to be the most successful species in colonizing a large animal carrion and human corpses (Anton et al. 2011; Cherix et al. 2012; Grassberger and Frank 2004; Mądra et al. 2015; Matuszewski et al. 2013; Pohjoismäki et al. 2010; Szpila et al. 2015a). In Mediterranean parts of Europe, this list would include several additional species (Szpila et al. 2015a).

Unfortunately, species determination for *Sarcophaga* is especially difficult in larvae (Szpila et al. 2015b) as they are typical maggots with a narrow morphological diversity (Pape 1987). Forensic entomology manuals recommend mass rearing of collected larvae to adults or the use of molecular methods (Byrd and Castner 2009; Smith 1986). Two recent publications partly solve this problem at least for the European ecozone by providing a large amount of new information about larval stages of flesh flies (Richet et al. 2011) and a key to third instar larvae of forensically important species (Szpila et al. 2015b). Characters proposed for determination of *Sarcophaga* larvae, however, are often subtle and may pose problems for unexperienced users. Therefore, new and easy methods for species identification in larval Sarcophagidae are still required.

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The method of muscle attachment site (MAS) patterns proved to be powerful in species determination of forensically important Calliphoridae larvae (Niederegger and Spieß 2012; Niederegger and Spieß 2014; Niederegger et al. 2015). The aim of this study was to further establish the method for five of the most frequent visitors and successful colonizers of large carrions: *S. argyrostoma*, *S. caerulescens*, *S. melanura*, *S. albiceps*, and *S. similis*.

Materials and methods

Animals

Adult females were attracted to decomposing pig or chicken liver and collected by sweep net. Detailed data regarding collecting locations in Poland are presented in Szpila et al. (2015b). The first instar larvae were obtained by gently squeezing the abdomen of living females. Larvae from different females were reared separately at room temperature in plastic containers (150 ml) with small portions of pig or chicken liver (20–30 g) as feeding medium. When the larvae reached the post-feeding stage of the third instar, they were killed by dousing with boiling water and stored in 70% ethanol.

Unambiguous species identification was possible by breeding some larvae of each species to the adult form. Obtained male specimens were identified using keys of Pape (1987), Povolný and Verves (1997), and Richet et al. (2011). All maternal females and laboratory-bred adult specimens were labeled and are available as voucher specimens in the insect collection of the Chair of Ecology and Biogeography, Faculty of Biology and Environmental Protection, Nicolaus Copernicus University in Toruń, Poland.

Preparation

The larvae were measured to the nearest 0.1 mm using a dissecting microscope (Zeiss Stemi 2000C) with digital camera (Zeiss AxioCam ICc1) and measuring software (AxioVision). All preparation and evaluation steps leading to the condensed patterns were performed as given in our previous publications (Niederegger et al. 2013; Niederegger and Spieß 2012).

Data evaluation

All rows in segments 2–4 were labeled according to our previous publication on *Lucilia* Robineau-Desvoidy species (Niederegger et al. 2015), except for 4.1. This part of the MAS pattern is doubled in comparison to *Lucilia* and was thus labeled 4.1a and 4.1b (Fig 1). The patterns were evaluated using Inkscape (freeware) and Adobe Photoshop; means and standard deviations were calculated using Microsoft Excel 2010.

Results

Transversal muscles of brachyceran larvae are attached to the inside of the cuticula in bilaterally symmetrical rows. These muscle attachment site (MAS) rows can be stained and subsequently easily distinguished. The range of MAS for all three hemi segments was quite vast (Table 1). The lowest total

Fig. 1 Genus pattern for *Sarcophaga* composed of 50 individual MAS patterns of *S. argyrostoma*, *S. caerulescens*, *S. melanura*, *S. albiceps*, and *S. similis*. Rows are labeled according to their location on and affiliation with a segment (e.g., 2.1=central row in segment 2, 4.5=most distal row in segment 4)
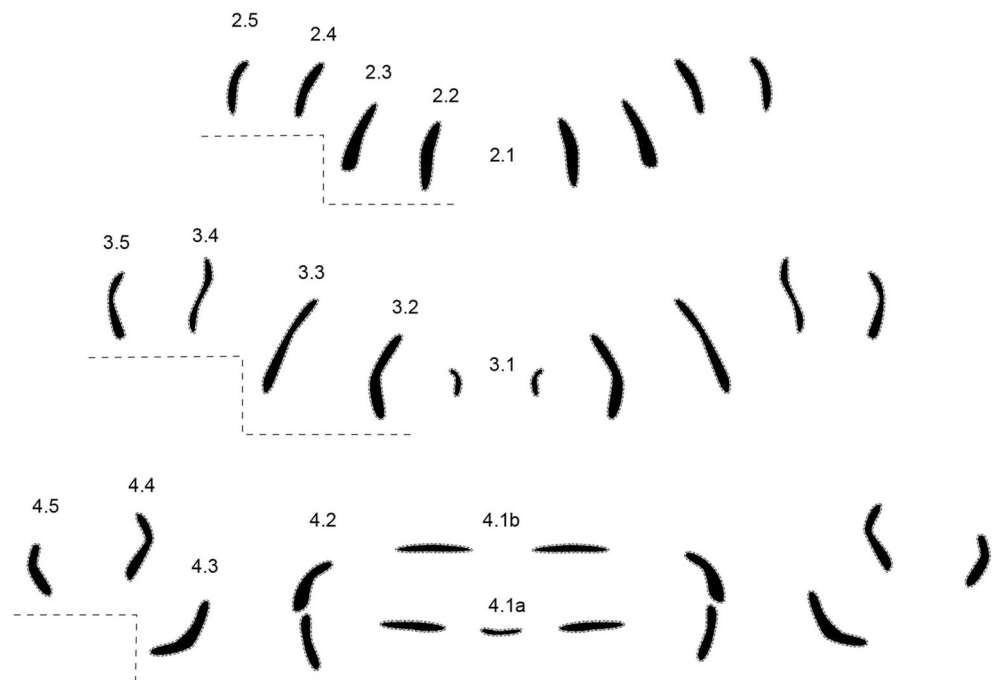
Table 1 Average size of larvae (±STD) compared to the ranges of total MAS numbers in all hemi segments

	S. melanura	S. similis	S. albiceps	S.caerulescens	S.argyrostoma
Mean body length [mm]	13.04 (±0.8)	13.31 (±1.2)	15.56 (±0.4)	17.08 (±1.3)	18.43 (±0.9)
No. of MAS in three hemi segments	153–168	129–182	127–163	138–202	138–194

number of MAS was found in an individual of *S. albiceps* with 127 MAS, and the highest number was found in *S. caerulescens* with 202 MAS. The smallest individuals investigated belonged to *S. melanura* with an average size of 13.04 mm, closely followed by *S. similis* with 13.31 mm; *S. albiceps* had a mean size of 15.56 mm and *S. caerulescens* with 17.08 mm and *S. argyrostoma* with an average length of 18.43 mm were the largest (Table 1).

The average number of MAS per hemi segment over all individual larvae investigated was 44 (±8) for segment 1, 54 (±8) for segment 2, and 62 (±10) for segment 4 (Table 2). Because the numbers of MAS in pattern rows are very similar in most cases, deviations (framed in Table 2) can be of important assistance for species determination.

Most deviations showed three or four MAS above the average genus count. Predominantly, they were found in *S. argyrostoma*, which comprised the largest larvae investigated. Interestingly, one such deviation was found in *S. melanura*, comprising the smallest larvae. Only two deviations with three MAS less than the average genus count were found (Table 2).

The genus pattern, generated for the five species of the genus *Sarcophaga* investigated in this study, is the base of

comparison for the researcher in order to further determine the species once the genus is defined.

Genus pattern (*n*=50)

The genus pattern (Fig. 1) contains no MAS at position 2.1 at the center of segment 2. Rows 2.2, 2.3, 2.4, and 2.5 are short vertical rows with slight curves in the middle like closing brackets. Rows 2.4 and 2.5 are positioned more anteriorly, giving the pattern a step-like appearance.

In segment 3, the central rows 3.1 are pointing their convex parts at each other like inverted brackets while rows 3.2 enclose the two small ones like big closing brackets. Rows 3.3 are almost straight with an angle of about 30° to the midline. 3.4 is z-shaped on the left hemi segment and s-shaped on the right hemi segment, whereas 3.5 again follows the bracket-like shaping of the rows in segment 2. The step-like appearance is maintained as in segment 2.

Compared to the genus pattern found for *Lucilia* in a previous study (Niederegger et al. 2015), the pattern for central horizontal row of segment 4 is divided; the parts

Table 2 Average numbers of MAS per row structure for five *Sarcophaga* species and the genus (±STD). Frames indicate most obvious deviations from genus pattern numbers

Row	2.1	2.2	2.3	2.4	2.5	3.1	3.2	3.3.	3.4	3.5
Genus	0 (±0.5)	12 (±2.6)	13 (±2.9)	11 (±2.7)	8 (±1.8)	4 (±1.1)	14 (±2.4)	14 (±2.9)	12 (±2.8)	10 (±2.0)
S. melanura	0 (±0.0)	11 (±1.6)	12 (±1.8)	10 (±1.3)	8 (±1.0)	4 (±0.6)	15 (±1.9)	14 (±1.8)	10 (±1.7)	9 (±1.9)
S. similis	0 (±0.0)	13 (±2.4)	13 (±2.5)	10 (±2.2)	8 (±2.3)	5 (±1.0)	13 (±3.4)	14 (±2.7)	12 (±2.5)	10 (±1.5)
S. albiceps	0 (±0.0)	10 (±0.7)	10 (±1.6)	10 (±1.7)	8 (±1.7)	3 (±0.6)	12 (±0.7)	12 (±1.6)	12 (±1.6)	9 (±1.3)
S. caerulescens	1 (±0.5)	14 (±2.0)	15 (±2.5)	10 (±1.7)	9 (±2.3)	5 (±0.7)	16 (±1.8)	16 (±3.2)	11 (±2.0)	11 (±2.4)
S. argyrostoma	1 (±0.3)	15 (±2.7)	15 (±2.0)	15 (±2.5)	8 (±1.4)	5 (±0.7)	15 (±1.3)	17 (±1.7)	15 (±3.5)	10 (±1.9)

Row	4.1a	4.1b_side	4.1b_center	4.2	4.3	4.4	4.5
Genus	6 (±2.2)	7(±2.1)	5(±2.3)	17 (±3.4)	11 (±2.2)	9 (±2.5)	9 (±1.9)
S. melanura	6 (±1.1)	8(±0.9)	6(±1.3)	20 (±2.4)	10 (±2.2)	9 (±1.6)	9 (±1.3)
S. similis	6 (±1.5)	7(±1.4)	6(±1.0)	14 (±4.0)	11 (±1.7)	8 (±2.3)	8 (±2.5)
S. albiceps	5 (±0.7)	6(±0.7)	4(±0.9)	18 (±1.8)	9 (±0.8)	11 (±0.8)	8 (±1.2)
S. caerulescens	5 (±3.8)	10(±1.8)	5(±3.2)	19 (±3.4)	13 (±2.3)	9 (±2.5)	11 (±1.7)
S. argyrostoma	8 (±1.3)	7(±2.5)	3(±2.7)	15 (±1.2)	12 (±1.7)	8 (±3.2)	8 (±1.7)

where therefore named 4.1a and 4.1b. 4.1a comprises two short mirrored horizontal rows at the center. 4.1b is composed of two short mirrored horizontal rows divided by a small horizontal row in the middle. 4.2 is the only divided vertical row in the genus pattern, where the top part is more curved than the bottom part. 4.3 is an L-shape positioned in the lower part of the segment. 4.4 and 4.5 are almost mirrored bended vertical rows but 4.4 is positioned higher in the segment than 4.5. The step-like appearance is maintained in this segment as well.

Sarcophaga (Parasarcophaga) albiceps Meigen, 1826 (n=10)

All rows of segment 2 were shorter in *S. albiceps* (Fig. 2) compared to the genus pattern. Rows 2.2 were bent rather than just curved, as were rows 3.2. The other rows of segment 3 were more or less concordant to the genus pattern when both hemispheres were taken into account. In segment 4, rows 4.3 were less curved than the L-shapes of the genus pattern and rows 4.4 were longer throughout all larvae investigated.

Sarcophaga (Pandelleisca) similis Meade, 1876 (n=10)

At the first glance, several rows seemed to differ in *S. similis* (Fig. 3) compared to the genus pattern. Over all larvae investigated, however, most differences were not consistent on both hemispheres and were therefore omitted. Only one difference was present in both hemispheres and in all larvae investigated:

rows 4.2 were not divided in *S. similis* which is a distinctive discrepancy compared to the genus pattern.

Sarcophaga (Liopygia) argyrostoma (Robineau-Desvoidy, 1830) (n=10)

S. argyrostoma (Fig. 4) had 2.1 rows, or dots rather. In 9 of 10 larvae 2.1, on each side consisted of one MAS point and one larva lacked them entirely. Furthermore, there were divisions in rows 2.4 in both hemispheres. Similar disruptions could be found in rows 3.4, where the upper part of the row corresponded to the 3.4 row of the genus pattern, but an additional small row part was found below. Overall, most rows were longer than in the genus pattern.

Sarcophaga (Helicophagella) melanura Meigen, 1826 (n=10)

In *S. melanura*, (Fig. 5) most of the rows seem a tad shorter than in the genus pattern. In the second segment, rows 2.3 had an additional small piece above the main row in half of the larvae. Rows 3.4 lacked the bending into an s- or z-shape and presented themselves as short straight rows instead.

Sarcophaga (Robineauella) caerulescens Zetterstedt, 1838 (n=10)

S. caerulescens (Fig. 6) showed the most conspicuous deviations from the genus pattern as there were divisions as

Fig. 2 Condensed MAS pattern for *Sarcophaga albiceps* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). Numbers indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern
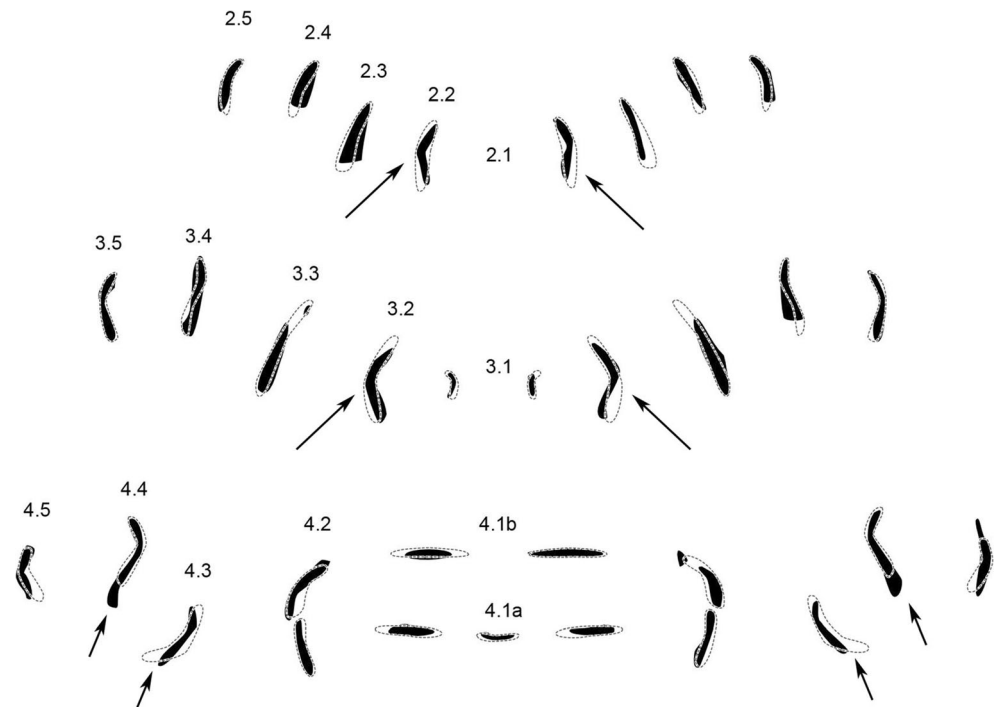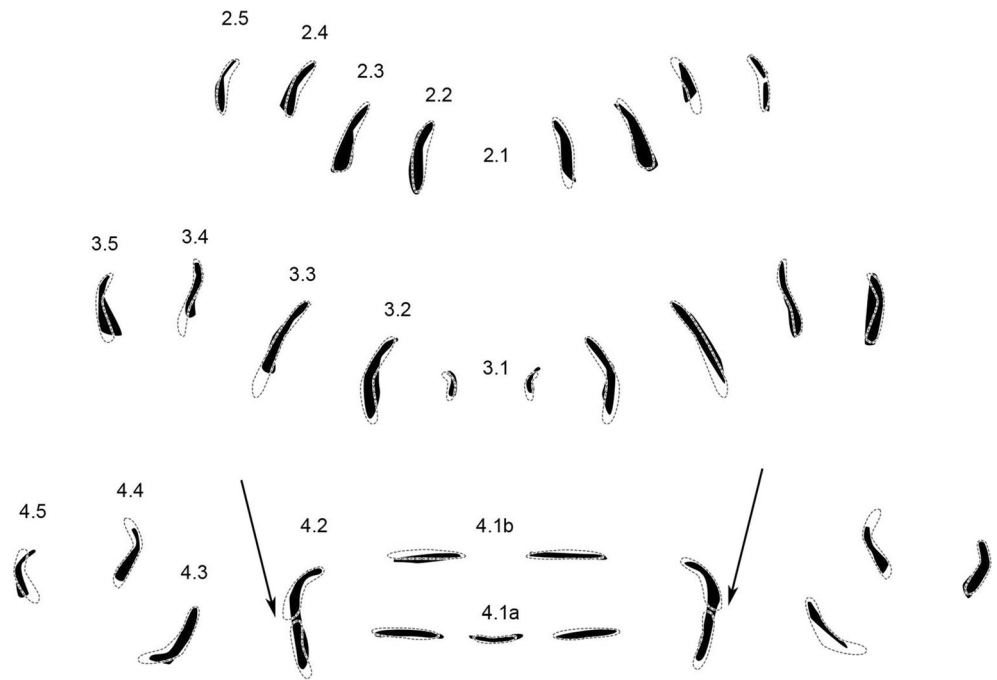
well as additions in the rows. Rows 2.3 were divided in
the upper middle part and had an additional piece on top
of the divided row. Rows 3.3 had divisions but lacked an
additional piece. Furthermore, the upper parts of rows 4.2
and 4.4 were bent more than corresponding rows in the
genus pattern. This, however, should not be taken into
account as indicative discrepancy.

Discussion

Sarcophaginae larvae, including the genus *Sarcophaga*, can
easily be recognized from the large and deep spiracular cavity
on the last segment (Szpila et al. 2015b). Species identification
however is much more difficult. Previous studies on MAS
patterns for Calliphoridae species (Niederegger et al. 2013;

Fig. 5 Condensed MAS pattern for *Sarcophaga melanura* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern
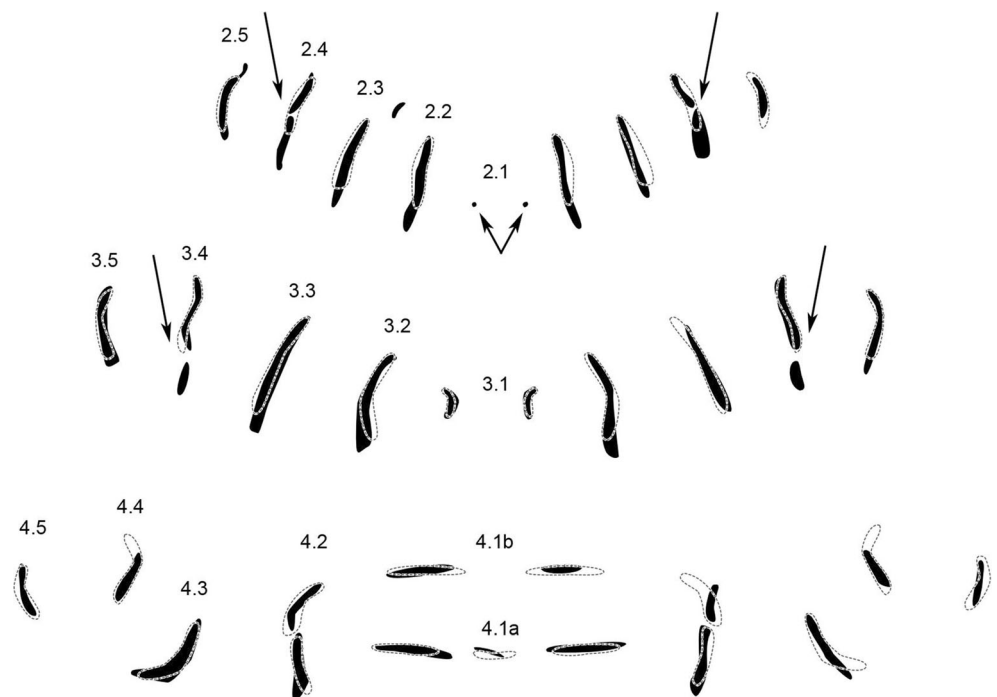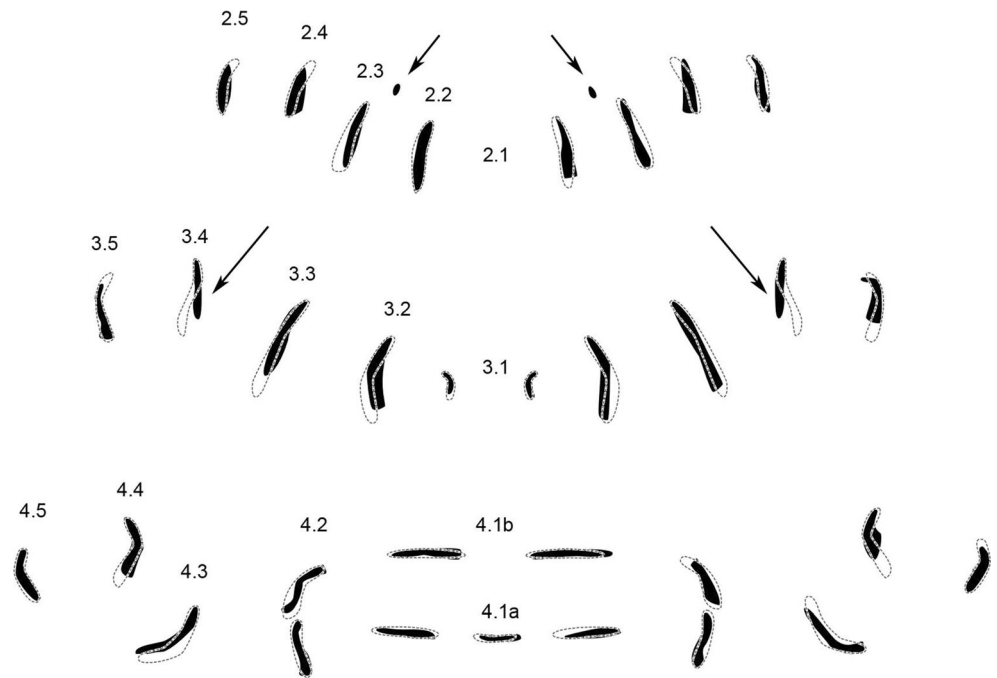

Niederegger and Spieß 2012; Niederegger and Spieß 2014; Niederegger et al. 2015) motivated us to exploring the method for this forensically important genus.

As was previously shown for Calliphoridae larvae, MAS patterns only expand in size during growth and development of the larvae, while maintaining their shape and numerical characteristics (Niederegger et al. 2013). Even though behavior of the larvae changes within the third instar from feeding to

migration, their muscular and cuticular equipment remains the same as a further ecdysis would be necessary to alter it. These assumptions were adapted for Sarcophagidae larvae. For this study, therefore, only third instar larvae were investigated.

Five species of the genus *Sarcophaga* from five different subgenera were examined, and a characteristic genus pattern as well as species-specific pattern variations was found. The previously defined field of analysis comprising segments 2–4

Fig. 6 Condensed MAS pattern for *Sarcophaga caerulescens* (*solid structures*) superimposed with outlines of the genus pattern (*dotted lines*). *Numbers* indicate rows of transversal muscle patterns according to the location on and affiliation with a segment. *Arrows* indicate differences compared to genus pattern
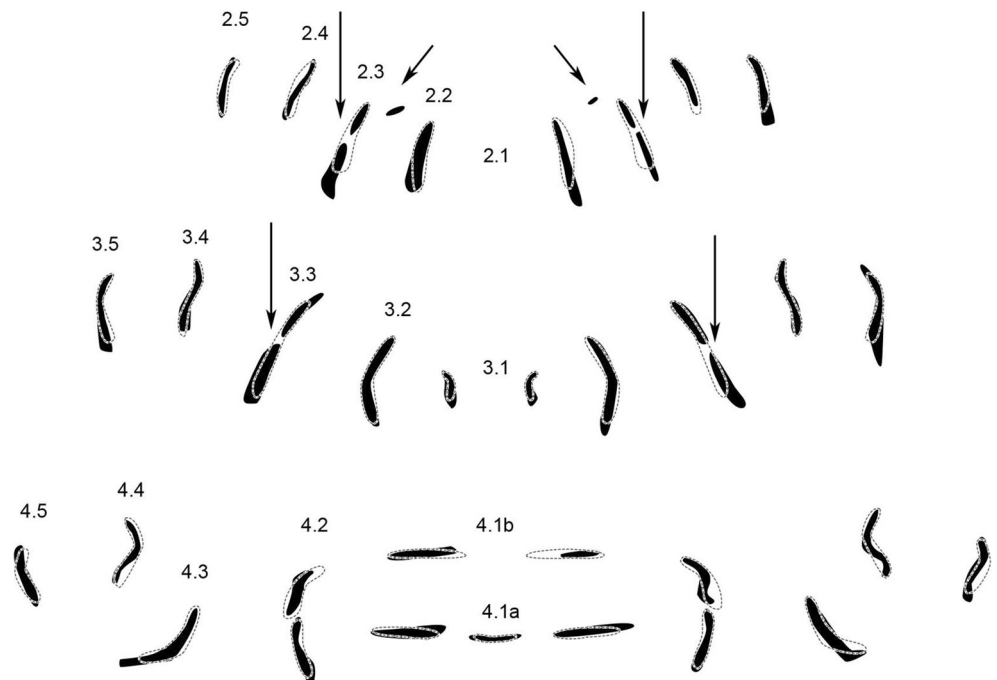
of the third-stage larvae did not have to be extended. Size differences between species were only rarely reflected in the patterns: if deviations were found, the number of MAS per row was never more than four above or below the number for the genus.

Sarcophaga argyrostoma, S. caerulescens, and S. similis are known mostly as carrion breeders with potential facultative parasitism or predation (Pape 1987; Povolný and Verves 1997; Richet et al. 2011). Sarcophaga albiceps and S. melanura are ubiquitous and can develop successfully in carrion but their preferred breeding substrates are feces (S. albiceps) or undefined remains with probable facultative predation on other fly larvae (S. melanura) (Bänziger and Pape 2004; Povolný and Verves 1997; Richet et al. 2011). Larval behavior of these species was not studied in detail, but similarity can be assumed. Only obvious differences in behavior or habitat would be expected to lead to dramatic alterations in the larval muscular equipment and thus in MAS numbers or patterns. Obligatory parasitic larvae for instance might have a reduced number of muscles due to diminished motility. Confirmation of this statement however needs further studies of larvae representing different breeding behaviors.

As the genus Sarcophaga comprises a large number of species (Pape 1996), this study of five species can only be preliminary. These first results however are encouraging for the continuation of this method within the genus Sarcophaga and other genera of the vast family of Sarcophagidae. In further studies, it would be interesting to investigate whether the genus pattern found here is characteristic for all or most species of Sarcophaga or if MAS patterns from species with different food preferences or habitats deviate from necrophagous species.

With expansion of the MAS method for more species and genera, it becomes apparent that it is a useful and applicable tool for species determination in brachyceran larvae. The first concept of "key features" (Niederegger and Spieß 2012), however, had to be abandoned with the examination of more species. The risk of false determinations when relying solely on "key features" rose with the broadening of the field of analysis to include all rows of three segments. The consideration of the entirety of features is necessary for correct species determination.

A previous experiment in species determination using MAS patterns performed by impartial and inexperienced test subjects showed good results (Niederegger and Spieß 2012). As with all determination keys and methods however, some level of experience and practice is necessary for successful identifications. A long-term aim for further facilitation of the method and elimination of researcher bias is the development of recognition and comparison software for MAS patterns.

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