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Third instar larva morphology of *Hydrotaea cyrtoneurina* (ZETTERSTEDT, 1845) (Diptera: Muscidae) – a species of forensic interest

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ABSTRACT. The muscid *Hydrotaea cyrtoneurina* (ZETTERSTEDT, 1845) is a Palaearctic species whose adults are known for their forensic importance. The third instar larva morphology of *H. cyrtoneurina*, hitherto unknown, is described herein with the application of light and scanning electron microscopy. The characters of larval morphology enabling this species to be distinguished from other forensically important third instar larvae of Muscidae are discussed as well. Some details of the cephaloskeleton are discussed, and modifications in terminology are proposed based on the literature data and the results of this study.

KEY WORDS: Muscidae, *Hydrotaea cyrtoneurina*, third instar larva, SEM, forensic entomology.

INTRODUCTION

Among the houseflies (Muscidae) found on human cadavers and animal carrion, some of the species most often recorded are representatives of the genus *Hydrotaea* ROBINEAU-DESVOIDY, 1830 (e.g. SMITH 1986, BYRD & CASTNER 2010). One of these species is *Hydrotaea cyrtoneurina* (ZETTERSTEDT, 1845), a species whose distribution is restricted to the Palaearctic region (GREGOR et al. 2002), where is widespread throughout Europe and eastwards to Siberia, India and the Korean Peninsula (PONT 1986, SOROKINA & PONT 2010). The literature information on the biology of *H. cyrtoneurina* is scarce. According to GREGOR et al. (2002) *H. cyrtoneurina* is closely associated with badger setts. PAPP (2002) reported a single representative of the species attracted to fox faeces. On the other hand, adults of *H. cyrtoneurina* have recently been reported from carrion succession experiments

(FIEDLER et al. 2008, MATUSZEWSKI et al. 2008, ANTON et al. 2011). The larvae of *H. cyrtoneurina* have not yet been described. Therefore, even if they had previously been collected in legal cases or in carrion succession experiments, the larvae could not have been correctly identified, except those specimens bred to the adult stage. Despite the lack of *H. cyrtoneurina* breeding records from dead human bodies or vertebrate carrion, the species was stated as being forensically important because of its usefulness for succession-based post-mortem interval (PMI) calculations in the forests of central Europe (MATUSZEWSKI et al. 2010).

The variable state of knowledge of third instar larval morphology of Muscidae of forensic importance hinders easy species identification. In some cases even detailed descriptions do not provide the necessary information allowing students to distinguish species according to larval morphology characters. Furthermore, in some species the immature stages are not described at all. The aim of this study is to provide the first description and thorough documentation of the third instar larva morphology of *H. cyrtoneurina* with the application of combined light and scanning electron microscopy.

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MATERIAL AND METHODS

Material for the present study was obtained by keeping field-collected females in the laboratory until oviposition. Gravid females were attracted to decomposing chicken liver and were collected with an entomological net at sites in the forest area adjoining the edge of the airfield of the Pomeranian Aeroclub in Toruń (Poland) in July 2010 and June 2011. Laboratory breeding and killing of larvae was performed according to GRZYWACZ et al. (2012b). Adults were subsequently killed with ethyl acetate vapour, pinned, labelled and identified according to GREGOR et al. (2002). Voucher specimens were deposited in the collection of the Chair of Ecology and Biogeography, Nicolaus Copernicus University, Toruń, Poland. Preparation for scanning electron microscopy examination involved dehydration through 80.0%, 90.0% and 99.5% ethanol, critical point drying in CO_2, mounting on aluminium stubs and sputter-coating with platinum for 140 s (20 nm of coating). SEM images were taken with a variable pressure SEM LEO 1455.

For light microscopy examination larvae were mounted in Hoyer's medium prepared according to CIELECKA et al. (2009). Slides were examined with a Nikon Eclipse E200 microscope and images were taken with a Nikon 8400 digital camera mounted on the microscope.

Terminology

The terminology follows COURTNEY et al. (2000) and SZPILA & PAPE (2005) for general larval morphology, and SKIDMORE (1985) for family-specific structures, with a few modifications as described below. For a pair of longitudinal sclerites articulating antero-dorsally with oral bars, the term "anterior rods" is applied after THOMSON (1937) rather than KEILIN'S (1917) "anterior ribbon". For a sclerite placed between the mouthhooks and termed "unpaired mouth-sclerite" by THOMSEN (1935), the term "unpaired sclerite" is proposed here. For a pair of small structures occurring posterior to accessory stomal sclerites and observed only in mature larvae, named "chitinous spicules" by SIDDONS & ROY (1942), the term "supplementary accessory stomal sclerites" is applied here after VELASQUEZ et al. (2013). For a paired sensory organ present in the lower posterior part of each of the ventral cornua, the term "sensory organ X" is applied after KEILIN (1915). The spiracular distance factor (SDF) defined after EMDEN (1965) as the ratio of the distance between posterior spiracles to the width of a posterior spiracle was calculated in order to demonstrate the relative differences in distance between posterior spiracles. However, unlike EMDEN (1965), the latter variable was expressed as the maximum horizontal diameter of the spiracle rather than its greatest width.

RESULTS

Six of the collected females oviposited in the laboratory and a total of 236 third instar larvae were studied. The larval body length ranged from 5.28 mm to 12.72 mm, the distance between posterior spiracles from 0.02 mm to 0.07 mm; both measurements were made in young and postfeeding larvae. The maximum horizontal width of the posterior spiracles varied from 0.13 mm to 0.14 mm (n=15) and SDF from 0.15 to 0.54. The larva has a maggot-shaped body comprised of 12 visible segments: a bilobate pseudocephalon, three thoracic segments, seven abdominal segments and an anal division (Figs 1A, 2A).

Pseudocephalon. Each lobe of the pseudocephalon carries an antennal complex, maxillary palpus, ventral organ and three pairs of sensilla resembling sensilla ampullacea. The antennal complex consists of a conical antennal dome situated on a basal ring and the length of the former is similar to the height of the latter (Fig. 3E). The maxillary palpus consists of three sensilla coeloconica, three sensilla basiconica, a few small additional sensilla arranged in a tight cluster and two typical sensilla coeloconica of nonmaxillary origin located latero-dorsally (Fig. 3C). The facial mask, surrounding the functional mouth opening, is composed of numerous oral ridges. A pair of labial lobes is equipped apically with sensilla of the labial organ (Fig. 3B) and internally their basal parts are covered by dark spines directed anteriorly. The ventral organ is bulge-shaped and equipped with four sensilla (Fig. 3D). A paired complex of scarcely sclerotized suprabuccal teeth is placed laterally to the anterior end of the mouth opening, and two rows of minute cutaneous teeth are present on its sides as well.

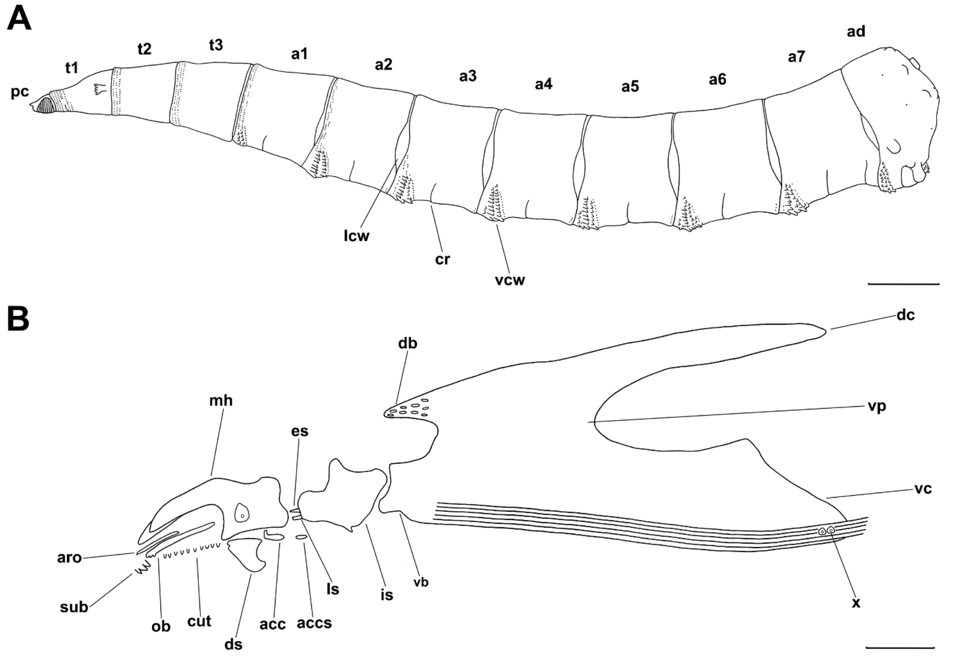

Fig. 1A, B. Third instar larvae of *Hydrotaea cyrtoneurina*: A – habitus, B – cephaloskeleton, lateral view. Scale bars = 1 mm for A and 0.1 mm for B.

Abbreviations: aI-aVII – abdominal segments I-VII, acc – accessory stomal sclerite, accs – supplementary accessory stomal sclerite, ad – anal division, aro – anterior rod, cr – transverse crevice, cut – cutaneous teeth, db – dorsal bridge, dc – dorsal cornu, ds – dental sclerite, es – epistomal plate, is – intermediate sclerite, lcw – lateral creeping welt, ls – labial sclerite, mh – mouthhook, ob – oral bar, pc – pseudocephalon, sub – suprabuccal teeth, tI-tIII – thoracic segments I-III, vc – ventral cornu, vcw – ventral creeping welt, vb – ventral bridge, vp – vertical plate, x – sensory organ X.

Cephaloskeleton. Mouthhooks are robust basally with slender, sharply pointed and asymmetric distal parts, i.e. the left mouthhook is shorter (Fig. 1B). Apical parts of the mouthhooks closely appose each other and basal parts are joined via an unpaired sclerite that is basally broadened but narrow apically. Accessory oral sclerites include the paired complex of an anterior rod articulating with the antero-dorsal margin of an oral bar. Both anterior rods are unconnected anteriorly and each of the oral bars is toothed at the anterior end. Ventrally to the basal parts of the mouthhooks are situated a pair of dental sclerites, not fused ventrally, and a pair of accessory stomal sclerites. In the mature larva a pair of small supplementary accessory stomal sclerites occurs. An epistomal sclerite and a pair of labial sclerites lie freely between the anterior arms of an intermediate sclerite. The intermediate sclerite is H-shaped in ventral view and in mature larvae an additional patch or irregular sclerotization can be found in its antero-dorsal part (Fig. 2B). The epistomal sclerite is equipped with two pairs of rounded perforations, whereas the longitudinal labial sclerites have about four circular openings each. The long basal sclerite consists of paired vertical plates with dorsal and ventral cornua, both of similar length. These are connected antero-dorsally by a perforated dorsal bridge and antero-ventrally by a narrow ventral bridge. The hypopharynx bears distinctly developed longitudinal ridges. The optic depression situated below the dorsal bridge is devoid of any sclerotization. Each of the ventral cornua has a dorsal expansion and in its lower posterior part carries a sensory organ X.

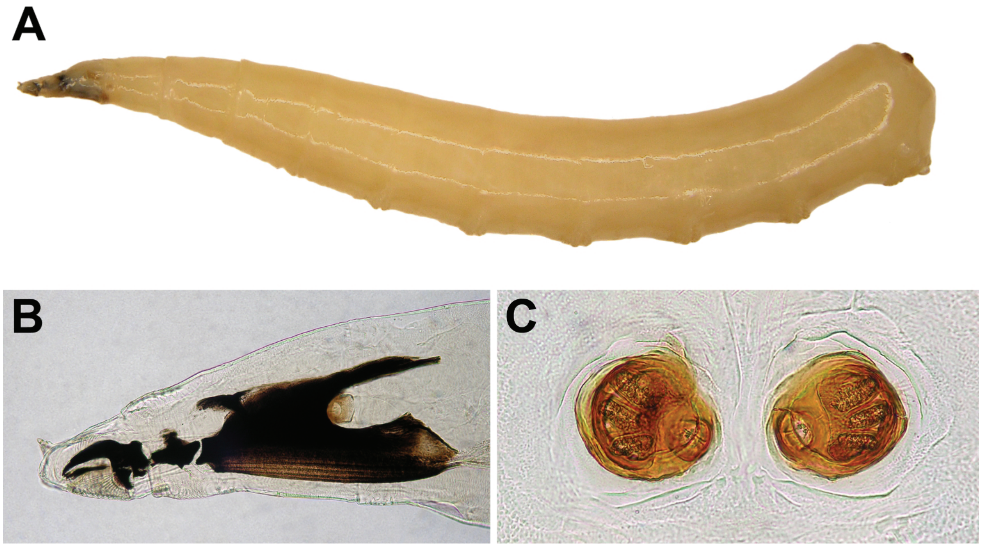

Fig. 2A-C. Third instar larvae of *Hydrotaea cyrtoneurina*: A – habitus, B – cephaloskeleton, lateral view, C – posterior spiracles.

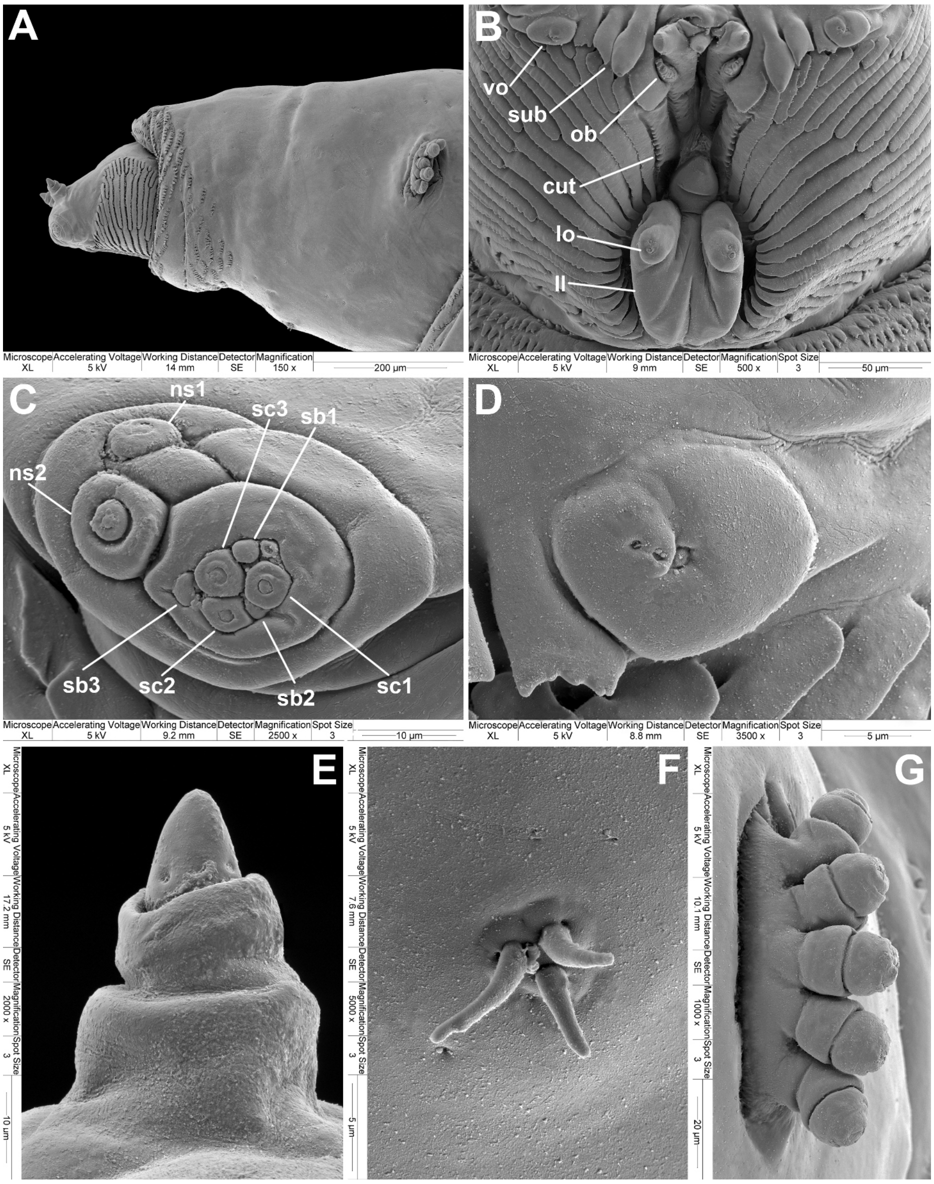

Fig. 3A-G. Third instar larva of *Hydrotaea cyrtoneurina*: A – anterior end of body, lateral view, B – facial mask, C – maxillary palpus, D – ventral organ, E – antennal complex, F – trichoid sensilla of Keilin's organ, G – anterior spiracle.

Abbreviations: cut – cutaneous teeth, ob – oral bar, lo – labial organ, ll – labial lobe, 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.

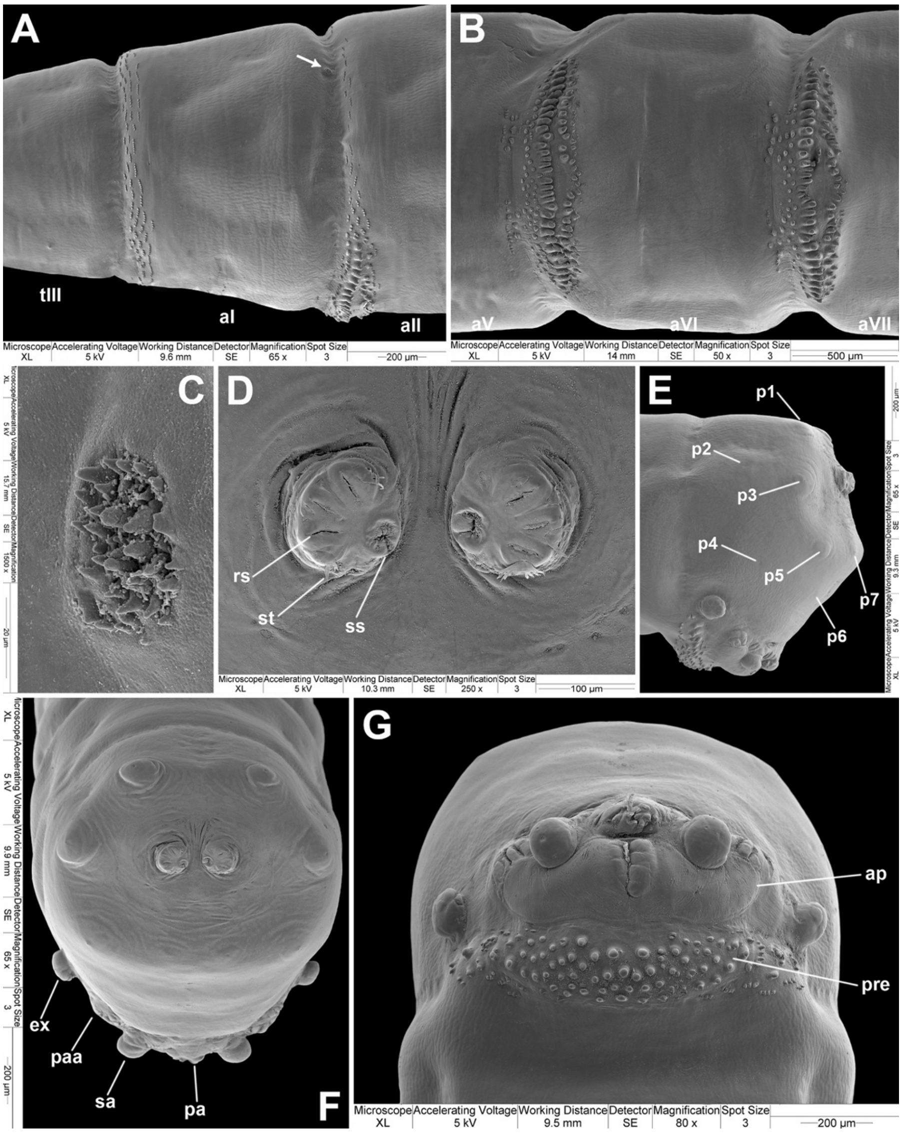

Fig. 4A-G. Third instar larva of *Hydrotaea cyrtoneurina*: A – thoracic and abdominal segments with bubble membrane (arrow), lateral view, B – abdominal segments, ventral view, C – bubble membrane, D – posterior spiracles, posterior view, E – anal division, F – anal division, posterior view, G – anal division, ventral view.

Abbreviations: aI, II, V-VII – abdominal segments I, II, V-VII, ap – anal plate, bm – bubble membrane, ex – extra-anal papilla, p1-p7 – papillae 1-7 surrounding spiracular field, pa – postanal papilla, paa – para-anal papilla, pre – pre-anal welt, rs – respiratory slit, sa – subanal papilla, ss – spiracular scar, st – spiracular tuft, tIII – thoracic segment III.

Thoracic and abdominal segments. Anterior spiracles are equipped with five lobes (Fig. 3G). Minute apertures of probably non-functional spiracles are present in the antero-lateral margins of tIII-aVII. A pair of Keilin's organs, each consisting of three clustered trichoid sensilla, is present in the middle ventral part of each of the thoracic segments (Fig. 3F). A transverse crevice is present ventrally in the middle part of abdominal segments 1-7. Lateral creeping welts are present between abdominal segments and are never covered with spines. A bubble membrane is situated in each of the postero-lateral margins of the first abdominal segments and consists of clustered globules lying level with the adjacent integument (Figs 4A, C). A spinose band on the first thoracic segment is covered with colourless, single or double pointed spines and is further ramified ventrally by an additional patch of spines (Fig. 3A). Segments tII-aII are covered by narrow, complete bands consisting of colourless spines, arranged in short rows fused basally (Figs 1A, 4A). Those spines are blunt and both may be single or double ended. The spines on the ventral creeping welts are massive, flattened basally, with slightly curved apical parts and are preceded and followed by smaller ones arranged in short rows (Figs 3, 4A, B). The postero-ventral margin of each of the abdominal segments 2-6 is covered by a few minute spines arranged in short rows (Fig. 4B).

Anal division. The anal plate is W-shaped, expanded laterally and somewhat visible in the lateral view (Figs 4E, G). An unpaired postanal papilla covered by darkly pigmented spines lies directly behind the anal opening (Fig. 4F). Among other anal papillae, pairs of subanal, para-anal and extra-anal papillae occur subsequently. Para-anal papillae are distinctly less developed than others (Figs 4E-G). Each subanal papilla is equipped with a sensillum basiconicum and two sensilla resembling sensilla ampullacea. The surface posterior to the anal papillae is smooth (Figs 4E-G). The spiracular field is equipped with posterior spiracles and is surrounded by seven pairs of sensilla (Fig. 4E). Following the existing terminology, all these sensilla together with the adjoining area are termed papillae (*p1*-*p7*). Papillae *p1*, *p3*, *p5* and *p7* form indistinct bulges, whereas papillae *p2*, *p4* and *p6* are indistinct, positioned at the same level as the adjacent integument and are shifted more or less below the disc of the spiracular field. Papillae with odd numbers (*p1*, *p3*, *p5* and *p7*) take the form of small bulges, and each has a sensillum resembling a sensillum basiconicum. Those with even numbers (*p2*, *p4* and *p6*) are indistinguishable using standard light microscopy and have sensilla ampullacea positioned at the same level as the adjacent integument. The posterior spiracles are slightly raised (Fig. 4D, E) and equipped with three straight respiratory slits. The latter are arranged obliquely and are convergent (Figs 2C, 4D). The spiracular scar is placed in inferior position and the peritreme is yellowish to brownish in colour (Fig. 2C).

DISCUSSION

Despite the fact that many Muscidae species have been reported as visiting decomposing human bodies and animal carrion (e.g. KENTNER & STREIT 1990, HWANG & TURNER 2005, MATUSZEWSKI et al. 2008, 2010), only a minority of them breed in this medium, either feeding on putrefying tissues or preying on other necrophagous larvae. Adults of *Hydrotaea cyrtoneurina* are known from their association with decomposing carrion (FIEDLER et al. 2008, MATUSZEWSKI et al. 2008, ANTON et al. 2011), but the biology of the immature stages has not yet been described. Because of the morphological similarity between *H. cyrtoneurina* third instars and the closely related *H. dentipes* (FABRICIUS, 1805) and *H. similis* MEADE, 1887 larvae, the former could earlier have been identified erroneously if they were present in carrion. *Hydrotaea dentipes* and *H. similis* are both well known for their forensic importance (WYSS & CHERIX 2006, FIEDLER et al. 2008, MATUSZEWSKI et al. 2008, 2010). The larvae of the former have already been described (e.g. KEILIN 1917, ZIMIN 1948, ISHIJIMA 1967, SKIDMORE 1985) whereas the latter has only recently been studied (GRZYWACZ et al. 2012a). Based on literature data (SKIDMORE 1985, GRZYWACZ et al. 2012a) and the results of the present study, differentiation of *H. cyrtoneurina* from the third instar larvae of other forensically important Muscidae, including the closely related *H. dentipes* and *H. similis*, is possible because of the following combination of characters: respiratory slits in posterior spiracles straight and spiracular scar placed inferior to the respiratory slits, peritreme never pigmented dark, surface of the anal division posterior to the anal papillae devoid of spines, and para-anal papillae distinctly less developed than extra-anal and subanal papillae. It is not always possible to determine larval natural history based exclusively on the presence in a certain type of breeding medium. Occurrence in decomposing organic matter, for example, does not resolve doubts as to a saprophagous or carnivorous mode of life. However, it is possible to deduce whether a larva is saprophagous, or facultatively or obligatorily carnivorous from details of the cephaloskeleton (KEILIN 1917, THOMSON 1937, FERRAR 1987). Based on the results of the current study and literature data (KEILIN 1917, SKIDMORE 1985), *H. cyrtoneurina* larvae can be described as facultative predators because of the presence of anterior rods, oral bars and sharply pointed mouthhooks. Thus the possible occurrence of immature stages of *H. cyrtoneurina* in carrion can be of great importance since it can limit the abundance of other necrophagous larvae.

Several modifications in the terminology of Muscidae third instar larval morphology are proposed herein. These terminological modifications concern details of the cephaloskeleton and are based on the results of the current study and literature data. PORTCHINSKY (1910, 1911) observed a distinct separation between the sclerites herein recognized as anterior rods, but he did not name them. KEILIN (1917) described the structure articulating antero-dorsally with the oral bar as an “anterior ribbon” (“ruban antérieur”). However, he pointed out that two separate sclerites are present in some species, whereas in others they may be connected into a single sclerite. In both cases that author applied the aforementioned term, but used the plural or singular form respectively. KEILIN'S (1917) terminology was accepted by later authors (e.g. SKIDMORE 1985), but the usage of this term does not clearly differentiate between the presence or absence of a connection between the sclerites. The mere statement of the presence of an "anterior ribbon" in a species does not indicate whether a connection between sclerites is present or absent, i.e. whether a single sclerite or a pair of sclerites occurs. SKIDMORE (1985) did not differentiate these states and applied the term "ribbon" in both cases ("Figure 3. b; Figure 5. c, d"). THOMSON (1937) distinguished these two features: he restricted the term "ribbon" to sclerites connected with one another, and named those devoid of a connection "rods". Although a connection between these sclerites has been reported in some species of Muscidae (SKIDMORE 1985), this is not the case in the present study. Despite the fact that THOMSON'S (1937) suggestion was not accepted by subsequent authors (SKIDMORE 1985), I suggest that his proposal be restored in order to differentiate between the presence of either anterior rods or an anterior ribbon in certain species. FERRAR (1987) showed that the presence of accessory stomal sclerites (probably "ligulate sclerite"), labial sclerites ("subhypostomal sclerite") and the epistomal sclerite was reported in some species of Muscidae but did not give any details. Most students of the larval morphology of Muscidae have made no mention of the occurrence of these sclerites; the exceptions are THOMSEN (1935), BOLWIG (1945) and SCHUMANN (1954) in *Musca domestica* LINNAEUS, 1758, SCHUMANN (1963) in *M. autumnalis* DE GEER, 1776 and SCHUMANN (1982) in *Hydrotaea aenescens* (WIEDEMANN, 1830). Some authors (ZIMIN 1948) provided vague drawings in which traces of these sclerites can be discerned. The existence of accessory stomal sclerites, labial sclerites and the epistomal sclerite is described here in the same way as GRZYWACZ et al. (2013) did in *Atherigona reversura* VILLENEUVE, 1936 and as VELASQUEZ et al. (2013) did in *Synthesiomyia nudiseta* (VAN DER WULP, 1883). Therefore, the previously noted lack of these elements is probably due to imprecise observations rather than to their actual absence in particular species. Moreover, a description of the cephaloskeleton details extracted from the puparium may facilitate the omission of these small sclerites, as in the case of SKIDMORE (1985). On the other hand, examination of the cephaloskeleton solely in the lateral position makes them hard to detect. These methods of study also preclude the detection of the unpaired sclerite, which was the case in the majority of previous studies, except for THOMSEN (1935), BOLWIG (1945) and SCHUMANN (1954, 1963). Supplementary accessory stomal sclerites have been shown in maturing larvae of *H. cyrtoneurina*. Prior to this study, these sclerites were reported by SIDDONS & ROY (1942) and subsequently by VELASQUEZ et al. (2013) in the third instar of *S. nudiseta*. Because they appear in the mature larvae and lie freely, like other accessory stomal sclerites, their detection is possible only by examining the anterior end of the larva with the application of, for instance, Hoyer's medium or methyl salicylate (SKIDMORE 1973, NIEDEREGGER 2011). KEILIN (1915) described and illustrated the sensory organ X (as "l'organe X") for the first time in the third instar of *M. domestica*, but did not reveal its presence in other studied genera, including *Muscina* ROBINEAU-DESVOIDY, 1830. In his next paper, KEILIN (1917) illustrated the sensory organ X in *M. domestica* once again, although he did not discuss its role and yet again omitted its presence in other studied species. THOMSEN (1935) confirmed KEILIN'S (1915) observations in *M. domestica* (as the "pharyngeal sense organ"), a few species of Stomoxyini, *Calliphora vicina* ROBINEAU-DESVOIDY, 1830 (as *C. erythrocephala* (MEIGEN, 1826)) and *Lucilia* sp. ROBINEAU-DESVOIDY, 1830. Except for the aforementioned authors, no other workers have described or illustrated the sensory organ X in any of the muscid species. According to ZACHARUK & SHIELDS (1991), the sensory organ X is equipped with uniporous gustatory chemosensilla or chemo-mechanosensilla. Recent studies (PYTHON 2002) of the neuronal organization of sensilla in the third instar larva of *Drosophila melanogaster* MEIGEN, 1830 (Drosophilidae) confirmed the presence of sensilla in a position equivalent to KEILIN'S (1915) sensory organ X. The terms "posterior pharyngeal sensilla" or "hypopharyngeal organ" (in PYTHON 2002) proposed for this structure by some recent authors are in contradiction with current opinions on the homology between elements of the cephaloskeleton and pharynx (COURTNEY et al. 2000). The sensory organ X is of intercalary origin (COURTNEY et al. 2000), and thus KEILIN'S (1915) neutral terminology, not implying any homology, has been applied in the present work.

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