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Key for the identification of third instar larvae of African blowflies (Diptera: Calliphoridae) of forensic importance in death investigations

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Abstract. Blowfly larvae are the insects primarily responsible for the active stage of decomposition of exposed vertebrate remains and are the most frequently collected entomological evidence during forensic investigations of death. The necrophagous calliphorids in continental Africa that consistently develop on large vertebrate carrion include 11 species belonging to four genera: *Calliphora*, *Chrysomya*, *Hemipyrellia* and *Lucilia*. Most of these species are widespread in Africa and frequently reported on large animal carcasses and carrion and human corpses. A few keys have been compiled for identification of their third instar larvae, but none of them covers the complete set of taxa. Therefore, we provide a new comprehensive key original

illustrations of all taxonomically significant characters. The key is based on characters that should be easily observable even in poorly equipped local laboratories and is a reliable taxonomic tool for material collected in either urban or rural areas where synanthropic species predominate. However, it should be used with some caution in areas with well-preserved natural habitats, where additional carrion-breeding species may occur. The publication of the key will significantly facilitate both medical and forensic entomological research and practice in Africa.

Introduction

Blowfly larvae are the primary group of insects responsible for the active stage of decomposition of exposed vertebrate remains, including human corpses and animal carcasses and carrion [1-3]. They are used as evidence in forensic entomology because blowfly larvae are the most frequently collected entomological material in actual cases [4]. Their species identification, crucial for further forensic inferences, is often characterized as challenging [5, 6]. Fortunately, there is sufficient knowledge of the morphology of third instars of Calliphoridae for the preparation of a comprehensive identification key, for most African countries [7-15].

The necrophagous blowfly fauna of Africa is relatively rich [9, 16-22]. As summarized by Szpila & Villet [20] and Lutz et al. [22], the set of continental African species that regularly develop on large vertebrate carrion is consistent and largely restricted to the following nine species: *Calliphora croceipalpis* Jaennicke, *Chrysomya albiceps* (Wiedemann), *Ch. chloropyga* (Wiedemann), *Ch. marginalis* (Wiedemann), *Ch. megacephala* (Fabricius), *Ch. putoria* (Wiedemann), *Hemipyrellia fernandica* (Macquart), *Lucilia cuprina* (Wiedemann) and *L. sericata* (Meigen) [9, 19, 23-27]. However, a few other species of proven forensic importance in death investigations also occur in the continent [22]. *Calliphora vomitoria* (Linnaeus, 1758)

occurs locally in mountainous areas of North Africa [22, 28], and *Calliphora vicina* Robineau-Desvoidy is common and widespread in countries along the coast of the Mediterranean Sea [17, 25], but recently also reported in South Africa [29-31]. The forensic importance in death investigations, understood as feeding of larvae on human corpses [32], is not obvious for a few other species like *Chrysomya inclinata* (Walker), *Ch. laxifrons* (Villeneuve), *Lucilia infernalis* Villeneuve and *Hemipyrellia pulchra* (Wiedemann). Adult forms of these species are attracted to vertebrate remains [21, 27], although there are no reliable breeding records from human corpses.

The third instar larvae for the basic set of African blowflies of forensic importance are already described and relevant information is scattered in many papers [5, 7, 9, 10, 14, 28, 33-45]. However, only some of them are based on material collected directly in African countries [9, 28, 36, 46-48]. A few keys have been compiled so far, but none of them covers the complete set of species presented above [9, 28, 36, 47, 48]. The most comprehensive work on this list is the key of Prins [9], which includes only six species.

The present key for the identification of third instar larvae is the first to cover all of the forensically most important species of blowflies in Africa. The list of taxa included 11 species of confirmed forensic relevance. Most of them are widespread in Africa and were frequently reported on large animal carrion and human corpses [22]. All taxonomically significant characters are illustrated in the form of colour pictures produced using digital cameras mounted on a compound microscope, stereomicroscope or scanning electron microscope (SEM). The identification key, based on characters that should be easily observable even in poorly equipped laboratories, was tested in practice before publication by students from the KS research group.

Material and Methods

Third instar larvae of *Calliphora croceipalpis*, *C. vicina*, *C. vomitoria*, *Chrysomya albiceps*, *Ch. chloropyga*, *Ch. marginalis*, *Ch. megacephala*, *Ch. putoria*, *Lucilia cuprina*, *L. sericata* and *Hemipyrellia fernandica* were reared from eggs deposited by females collected in various localities around Africa and Poland (Table 1). Some of the larvae of *Ch. chloropyga*, *Ch. marginalis* and *Ch. putoria*, were also collected during autopsy in Salt River Forensic Pathology Laboratory, Cape Town. All larvae were killed by soaking in hot water (about 95° Celsius) for ~ 1 minute and next stored in 80% ethanol. This method of killing and preserving insects is often recommended to forensic entomologists because of its convenience and ease of use, even in poorly equipped laboratories [49-52].

For preparation of slides, larvae were macerated for 24 hours in a cold solution of 5% KOH. Next the particular parts of the body were mounted in Hoyer's medium or dehydrated through 80, 90 and 99.5% ethanol and mounted in Euparal [14]. Concave slides were used for cephaloskeletons and flat slides for other morphological details. Larvae and slides are deposited in the Department of Ecology and Biogeography, Nicolaus Copernicus University.

A Nikon 8400 digital camera mounted on a Nikon Eclipse E200 microscope was used for photomicrography of slides. Image-stacking was done using an M205C Leica Stereomicroscope with an integrated high-resolution Leica DFC495 digital camera and associated software (Leica Application Suite 4.4.0). For the compilation of final pictures, 20-30 images were stacked. To improve contrast of spines against cuticule, larvae were coloured with a Stabilo marker (e.g. Fig. 1F, G; [57]). Preparation for scanning electron microscopy (SEM) involved critical-point drying in CO_2, after which larvae were coated with ~100 nm of platinum/palladium. SEM images were taken with a JEOL JSM 6335F field emission microscope.

Larval terminology follows Courtney et al. [53] and Szpila [14].

Results

Key to the third instar larva of African blowflies of forensic importance in death investigations.

1. – abdominal segments of larva with numerous large fleshy protuberances with groups of spines on their apex (Fig. 1H; 2K) ..... *Chrysomya albiceps* (Wiedemann, 1819)

– abdominal segments of larva without fleshy protuberances (Fig. 4A–J) 2

2. – oral sclerite at least partly sclerotized (Fig. 1A, B; 2A; 3A, K, M) 3

– oral sclerite totally unsclerotized (Fig. 1C; 2G, M; 3E) 8

3. – spines large, robust, strongly sclerotized, with single or multiple (serrated) tips, arranged separately (most distinctly observable on anterior spinose bands of dorsal surface of thoracic segments) (Fig. 2L, O; 3C, I; 5A, B) 4

– spines small, with single tips, arranged in short rows (most distinctly observable on anterior spinose bands of dorsal surface of thoracic segments) (Fig. 2F; 3N; 5C) 6

4. – at least some spines with multiple (serrated) tips (most distinctly observable on anterior spinose bands of lateral surface of thoracic segments) (Fig. 2O, 5B) .... *Chrysomya megacephala* (Fabricius, 1794)

– all spines with single tips (most distinctly observable on anterior spinose bands of dorsal and lateral surfaces of thoracic segments) (Fig. 3C; 5A) 5

5. – posterior spinose bands present on segments a4–a7 (Fig. 4C); oral sclerite oblong in ventral view (observed precisely perpendicular to long axis of body) (Fig. 1A); spiracular

peritreme of fully grown larvae complete (Fig. 2C) .... *Calliphora vomitoria* (Linnaeus, 1758)

– posterior spinose band present on dorsal surface of a7 only, absent on other abdominal segments (Fig. 4); oral sclerite short, triangular or square in ventral view (observed precisely perpendicular to long axis of body) (Fig. 1B); spiracular peritreme of fully-grown larva incomplete (Fig. 3D) ..... *Chrysomya marginalis* (Wiedemann, 1830)

6. – posterior spinose bands on segmenta6 complete, encircling entire segment (Fig. 4B) *Calliphora vicina* Robineau-Desvoidy, 1830

– posterior spinose bands on segment a6 incomplete, interrupted on entire dorsal surface (Fig. 4A, J) 7

7. – anterior spinose bands on segment a5 incomplete, interrupted on entire dorsal surface (Fig. 4A); oral sclerite oblong in ventral view (observed precisely perpendicular to long axis of body) (Fig. 1A) ..... *Calliphora croceipalpis* Jaennicke, 1867

– anterior spinose bands on segments a5 complete, encircling entire segment (Fig. 4J); oral scleritetriangular or square in ventral view (observed precisely perpendicular to long axis of body) (Fig. 1B; 3K, M) ..... *Hemipyrellia fernandica* (Macquart, 1855)

8. – spines on thoracic segments predominantly with multiple (serrated) tips (Fig. 2O; 3I; 5B) . 9

– spines on thoracic segments with single tips (Fig. 3N; 5C) 10

9. – anal opening with spined areas laterally (Fig. 1D); dorsal surface of abdominal segments without hair-like spines (best observable after colouring with marker) (Fig. 1F)*Chrysomya chloropyga* (Wiedemann, 1818)

– anal opening lacking spined areas laterally(Fig. 1E); dorsal surface of abdominal segments with large areas with hair-like spines (best observable after colouring with marker)(Fig. 1G; 3J;

5D, E, F) .... *Chrysomya putoria* (Wiedemann, 1830)

10. – in posterior view distance between each p1 larger than distance between p1 and p2 (Fig. 5G)and distance between p1 and p2 shorter than distance between p2 and p3 (Fig. 5G); posterior spinose band on a7 present on dorsal surface (best observable after colouring with marker) (Fig. 4H; 5G) .... *Lucilia cuprina* (Wiedemann, 1830)

– in posterior view distance between each p1 similar to distance between p1 and p2 (Fig. 5Hand distance between p1 and p2 similar to distance between p2 and p3 (Fig. 5H); posterior spinose band on a7 entirely absent on dorsal surface (best observable after colouring with marker) (Fig. 4I; 5H) ..... *Lucilia sericata* (Meigen, 1826)

Discussion

Critical reviews of morphological characters of third instar larvae of Calliphoridae were provided by Erzinçlioğlu [10], Wallman [42] and subsequently Szpila [14]. Experience from on-going work by KS on larval morphology of African blowflies revealed a few morphological details that should be used for taxonomic purposes with some caution. These are: 1) the sclerotisation of the oral sclerite, 2) presence of spines with multiple (serrated) tips, 3) presence of hair-like spines on the abdominal segments, 4) position of papillae around the spiracular field, and 5) the level of sclerotisation of the peritreme and the spiracular distance factor (SDF).

The oral sclerite maybe described by three character states: sclerotised oblong; sclerotised rounded or triangular; and unsclerotized (invisible). The shape of the oral sclerite may be assessed on both intact larvae (Fig. A–C) and those dissected for preparation of microscopic

slides (Fig. 2A, G, M). Misinterpretation of the shape of the oral sclerite, which is easily done when considering "long" versus "short" shape, may result in a serious species misidentification. For instance, interpretation of the oral sclerite of *Ch. marginalis* as "long" may lead to its identification as *C. vomitoria*, which creates difficulty for verification as both species possess similar shape of spines on their anterior spinose bands (Fig. 3C; [14]: fig. 3.5c). Users of the key should ensure that they are viewing the pseudocephalon of each larva exactly perpendicularly to its axis to avoid parallax that shortens the apparent length of this structure. The taxonomic importance of the oral sclerite in species of *Lucilia* is also complicated by progressive darkening during prolonged storage [54].

The presence of spines with multiple (serrated) tips on the spinose bands is used for differentiation of larvae of Chrysomyinae from other blowflies [11, 14, 28, 55]. This character is obvious and easily observable, even with a stereomicroscope, in species where the abundance of these spines is high, e.g. *Ch. chloropyga* and *Ch. putoria* (Fig. 2O, 3I). However, the abundance of spines with multiple tips is much lower in *Ch. megacephala* and they may be easily overlooked [14]. In this particular case, observers should also examine the anterior spinose bands on the dorso-lateral surfaces of the segments, where serrated spines are more abundant.

The presence of hair-like spines in *Ch. putoria* (as "*Ch. chloropyga*") was first reported by Wells et al. [41] in a key dedicated to larvae of Chrysomyinae of the United States of America. Interestingly, this unique form of spinulation was also reported in some other Chrysomyinae by Sukontason et al. [56] for the Oriental/Australasian species *Ch. nigripes* Aubertin and by Szpila & Grzywacz [15] for *Phormia regina* Meigen. This character is difficult to observe using a stereomicroscope, and the integument of larvae may require colouring using ink markers for better contrast [57]. Alternative methods such as preparing compound

microscope slides or specimens for SEM (Fig. 3J, 5D–F) are far more time-consuming and expensive.

Assessment of the position of papillae p1–p3 along the dorsal margin of the spiracular field may by hindered by inadequate viewing angles or inadequate methods of killing, preservation and/or storage of larvae [50-52]. The observation with standardized, exactly posterior or dorsal views is recommended [14, 45]. Inappropriate killing and preservation techniques may result in deformation of part or all of the anal division by invagination and shrinking. The natural shape and position of papillae are affected, which prohibits reliable measurement and comparison of distances between them.

The presence of interruptions of the peritreme of the posterior spiracles was often used in the past to separate larvae of Chrysomyinae from those of other blowflies [8, 9, 11, 34, 38, 41]. However, more recent contributors questioned the taxonomic significance of this character for identification of larvae of necrophagous blowflies [10, 14, 42] and some other families [32, 57]. An interrupted peritreme is also reported for the early third instar larvae of *Calliphora*, but it is complete in the late third instar larvae (Fig. 2B; [10, 14]).

The value of the spiracular distance factor (SDF) may vary according to the sizes of larvae and techniques of preparation [14, 42, 57]. Like the interrupted peritreme, this measure should be used only for full-grown third instar larvae. Additionally, Wallman [42] recommend using this measure only for freshly killed larvae and, therefore, it is not used in this key.

The key should be used with caution in geographical regions where additional species of necrophagous blowflies occur that are still of unknown forensic importance. This primarily concerns areas with undisturbed natural habitats with recorded high overall species diversity. However, our key is certainly reliable for both urban and rural environments strongly affected by

human activities. The colonization of human corpses by insects most often takes place in these habitats, where human populations are dense and the dominance of synanthropic flies is overwhelming. We are confident that our key will accelerate forensically-oriented studies of the African entomofauna, and facilitate the work of local investigators involved in solving real cases using entomological evidence.

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Captions to figures:

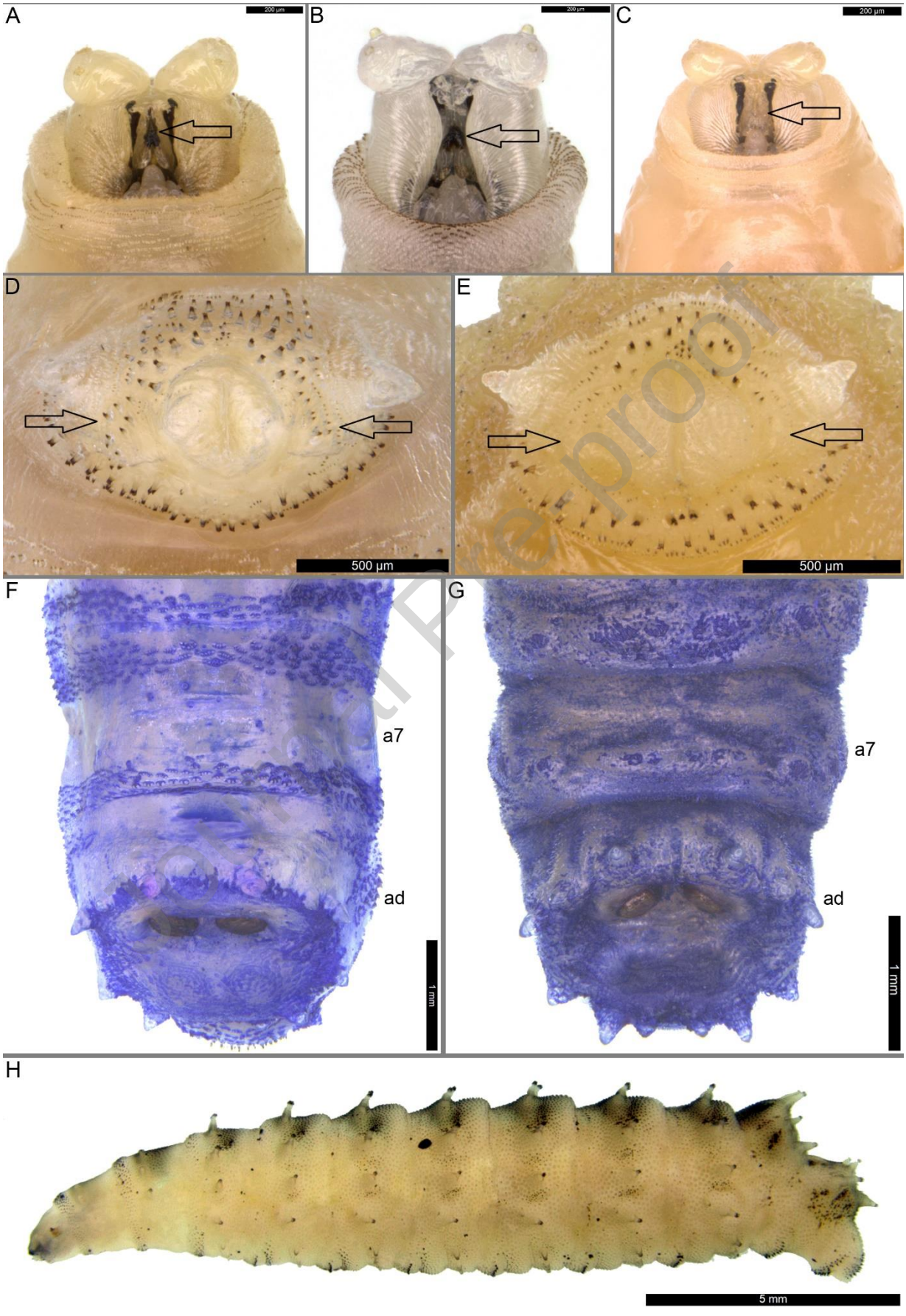

Figure 1. Stereoscope images of third instar larvae of necrophagous blowflies of Africa. (a) *Calliphora vomitoria*, pseudocephalon, ventral view, oral sclerite indicated by arrow; (b) *Chrysomya megacephala*, pseudocephalon, ventral view, oral sclerite indicated by arrow; (c) *Lucilia sericata*, pseudocephalon, ventral view; (d) *Chrysomya chloropyga*, anal region, arrows point to uninterrupted circle of spines around anal opening; (e) *Chrysomya. putoria*, anal region, arrows point on breaks in circle of spines around anal opening; (f) *Ch. chloropyga*, posterior end of body, dorsal view; (g) *Ch. putoria*, posterior end of body, dorsal view; (h) *Ch. albiceps*, habitus, lateral view. ad, anal division; a7, seventh abdominal segment.

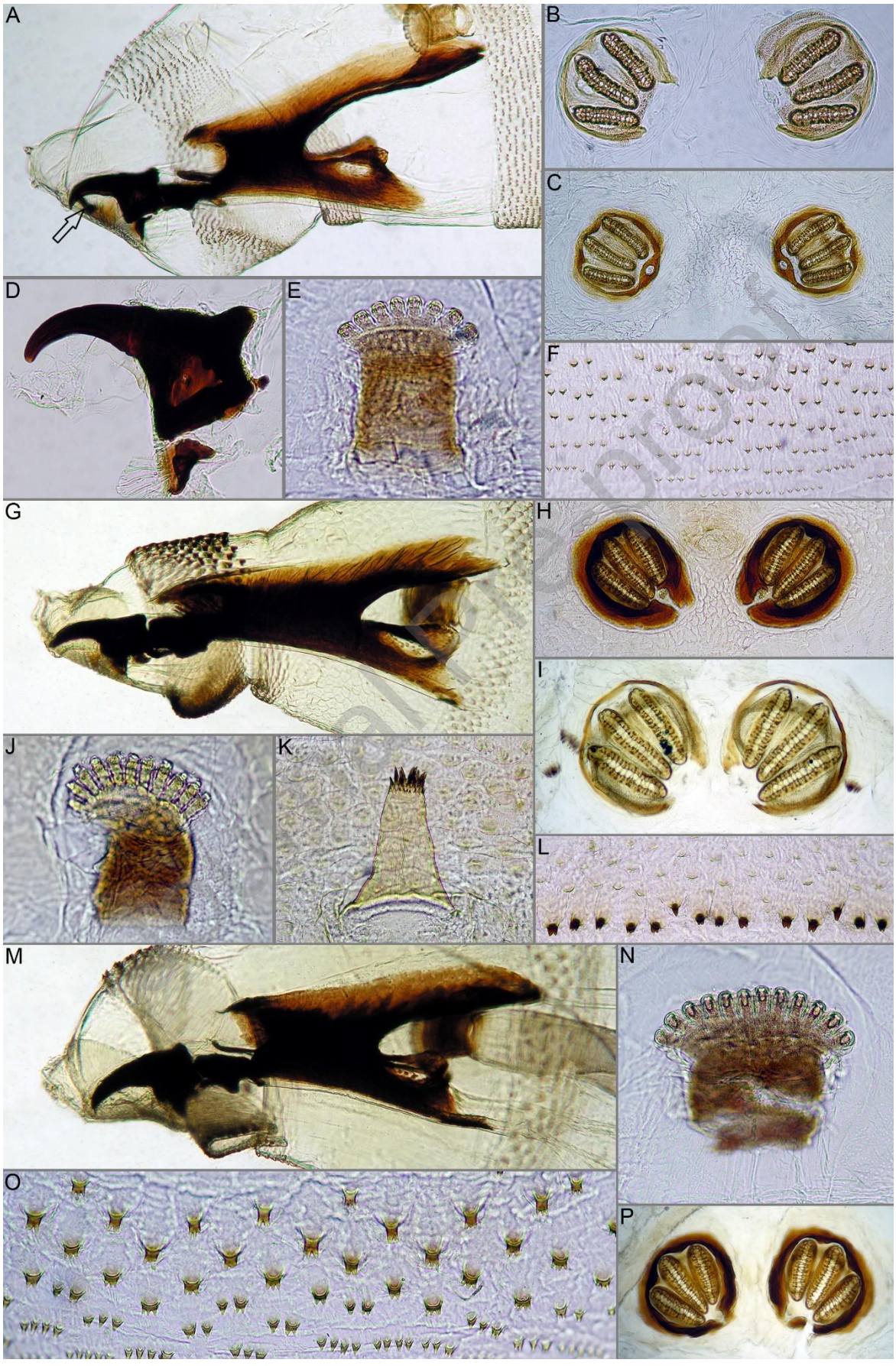
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Figure 2. Compound microscope images of third instar larvae of necrophagous blowflies of Africa. (a) *Calliphora croceipalpis*, cephaloskeleton, lateral view, oral sclerite indicated by arrow; (b) *C. croceipalpis*, posterior spiracles, early third instar larva; (c) *C. croceipalpis*, posterior spiracles, late third instar larva; (d) *C. croceipalpis*, mouthhook, lateral view; (e) *C. croceipalpis*, anterior spiracle; (f) *C. croceipalpis*, spines of anterior spinose band, third thoracic segment; (g) *Chrysomya albiceps*, cephaloskeleton, lateral view; (h) *Ch. albiceps*, posterior spiracles, late third instar larva; (i) *Ch. albiceps*, posterior spiracles, early third instar larva; (j) *Ch. albiceps*, anterior spiracle; (k) *Ch. albiceps*, fleshy process of the integument, first abdominal segment; (l) *Ch. albiceps*, spines of anterior spinose band, third thoracic segment; (m) *Chrysomya chloropyga*, cephaloskeleton, lateral view; (n) *Ch. chloropyga*, anterior spiracle; (o) *Ch. chloropyga*, spines of anterior spinose band, third thoracic segment; (p) *Ch. chloropyga*, posterior spiracles.

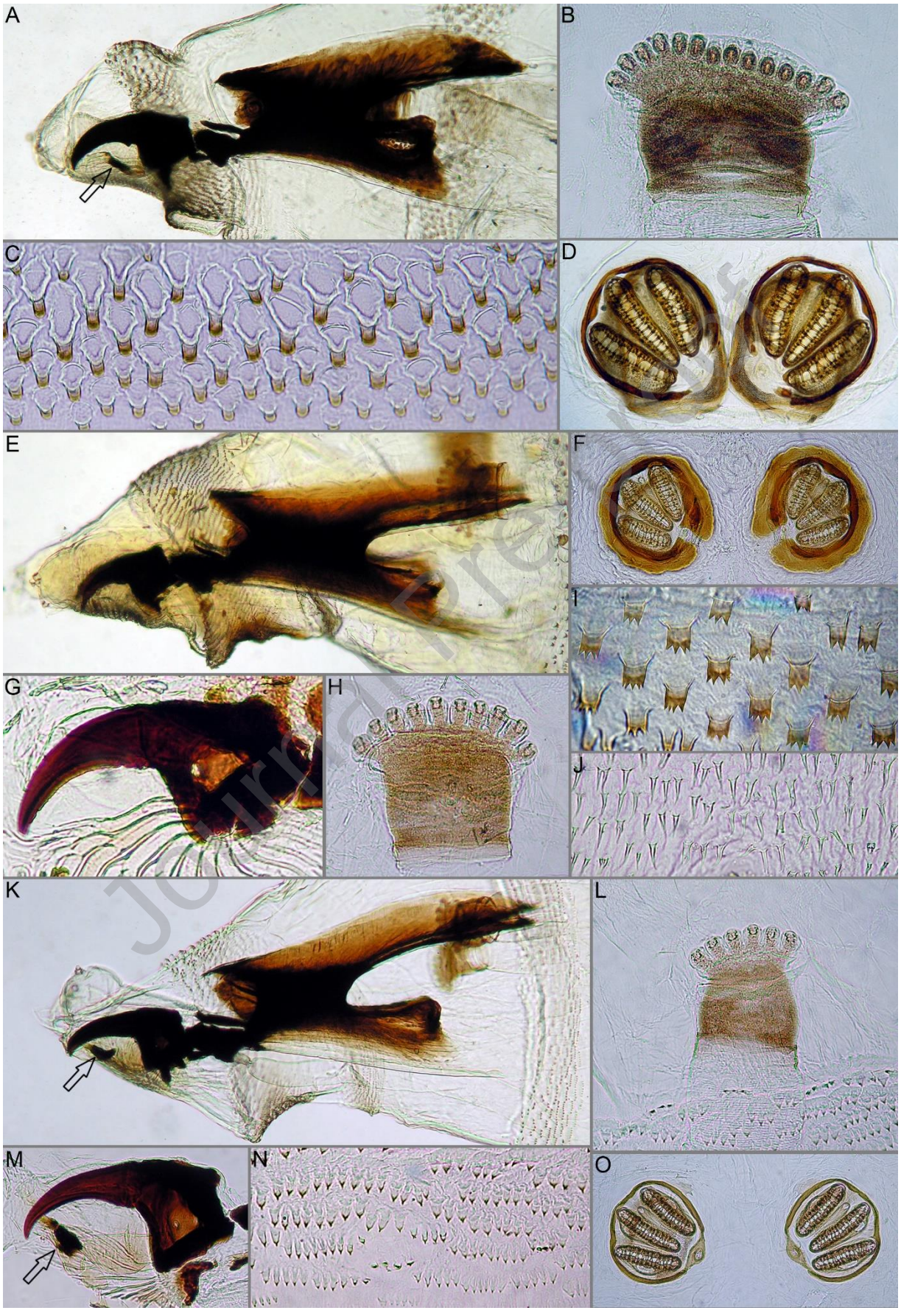
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Figure 3. Compound microscope images of third instar larvae of necrophagous blowflies of Africa. (a) *Chrysomya marginalis*, cephaloskeleton, lateral view, oral sclerite pointed by arrow; (b) *Ch. marginalis*, anterior spiracle; (c) *Ch. marginalis*, spines of anterior spinose band, third thoracic segment; (d) *Ch. marginalis*, posterior spiracles; (e) *Chrysomya putoria*, cephaloskeleton, lateral view; (f) *Ch. putoria*, posterior spiracles; (g) *Ch. putoria*, mouthhook, lateral view; (h) *Ch. putoria*, anterior spiracle; (i) *Ch. putoria*, spines of anterior spinose band, third thoracic segment; (j) *Ch. putoria*, hair-like spines, seventh abdominal segment; (k) *Hemipyrellia fernandica*, cephaloskeleton, lateral view, oral sclerite indicated by arrow; (l) *H. fernandica*, anterior spiracle; (m) *H. fernandica*, mouthhook, lateral view, oral sclerite indicated by arrow; (n) *H. fernandica*, spines of anterior spinose band, third thoracic segment; (o) *H. fernandica*, posterior spiracles.

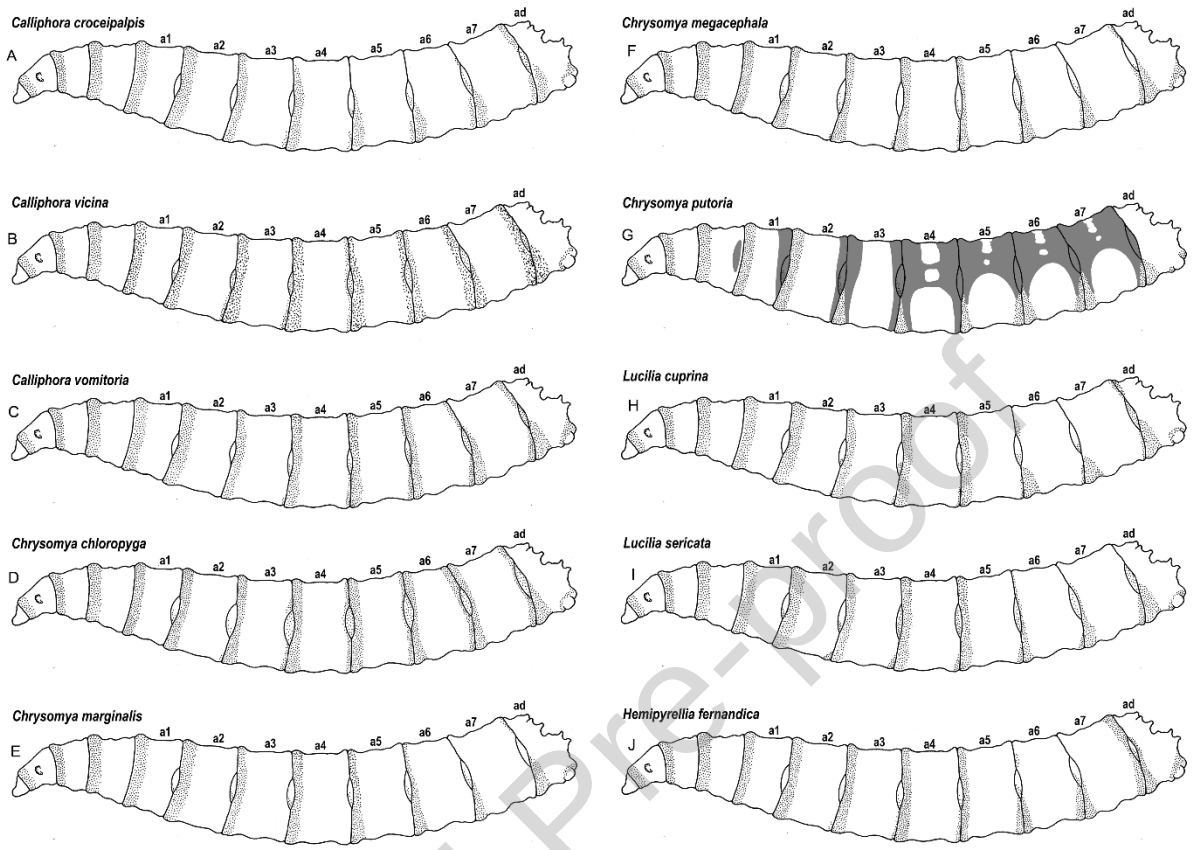

Figure 4. Third instar larvae of necrophagous blowflies of Africa, schematic distribution of spinose bands. (a) *Calliphora croceipalpis*; (b) *C. vicina*; (c) *C. vomitoria*; (d) *Chrysomya chloropyga*; (e) *Ch. marginalis*; (f) *Ch. megacephala*; (g) *Ch. putoria*; (h) *Lucilia cuprina*; (i) *L. sericata*; (j) *Hemipyrellia fernandica.*

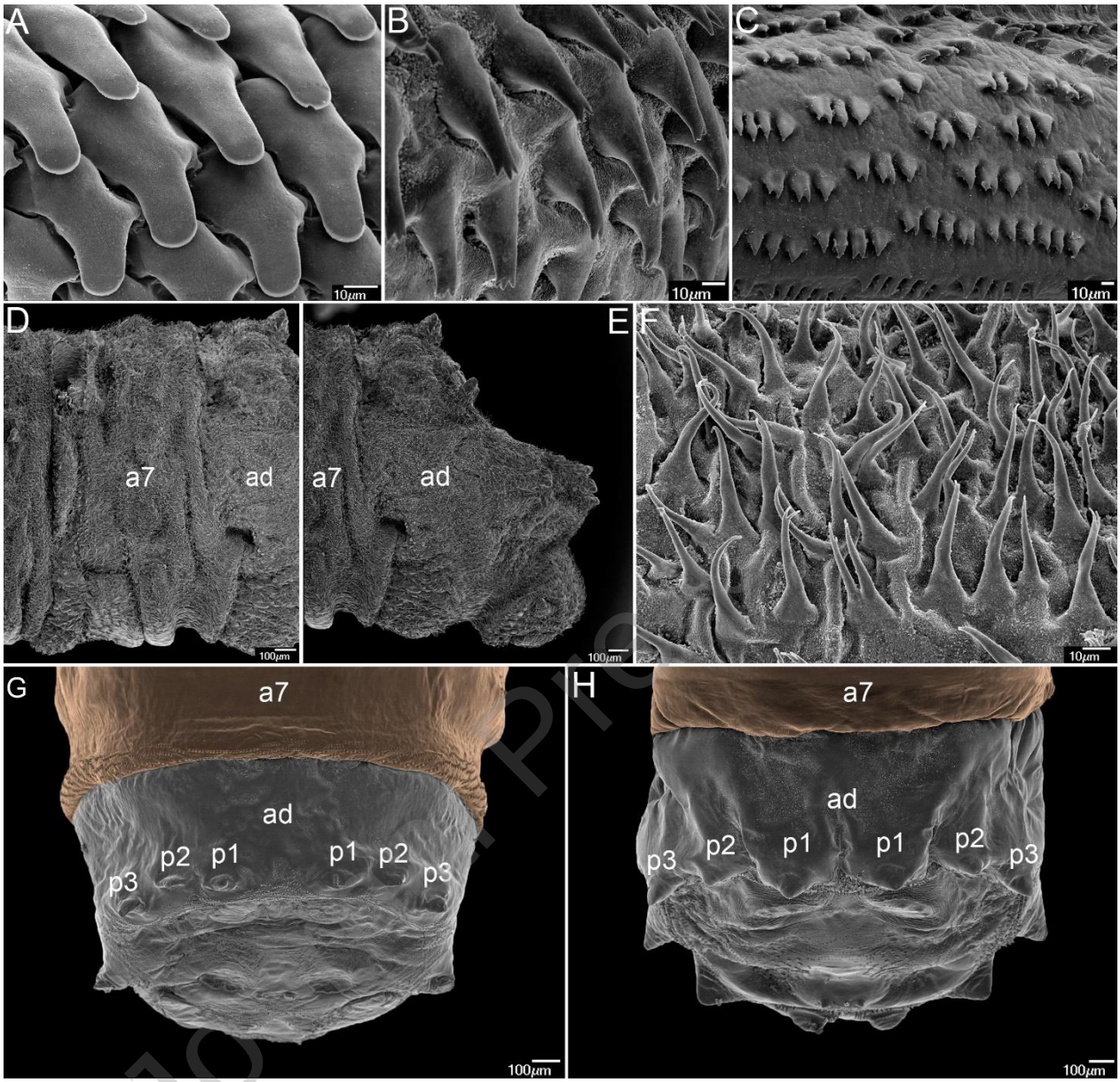

Figure 5. SEM micrographs of third instar larvae of necrophagous blowflies of Africa. (a) *Chrysomya marginalis*, spines of anterior spinose band, third thoracic segment; (b) *Ch. putoria*, spines of anterior spinose band, third thoracic segment; (c) *Calliphora vicina*, spines of anterior spinose band, third thoracic segment; (d) *Chrysomya putoria*, seventh abdominal segment, lateral view; (e) *Ch. putoria*, anal division, lateral view; (f) *Ch. putoria*, hair-like spines, seventh abdominal segment; (g) *Lucilia cuprina*, posterior end of body, dorsal view; (h) *L. sericata*,

posterior end of body, dorsal view. ad, anal division; a7, seventh abdominal segment; p1–p3, papillae 1–3.

CRediT authorship contribution statement

K. Szpila: Conceptualization, Resources, Writing - Original Draft, Supervision, Funding acquisition, Data Curation, Investigation
K. Williams: Resources, Writing - Review & Editing
A. Soszyńska: Writing - Review & Editing
M. Ekanem: Resources
M. Heyns: Resources, Writing - Review & Editing
M. Daba Dinka: Resources, Writing - Review & Editing
M. Villet: Conceptualization, Resources, Writing - Review & Editing, Funding acquisition

Declaration of Competing Interest

All authors declare no conflict of interest

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Highlights

- Blowfly larvae are the main group of insects collected in forensic investigations of death.
- This is the first complete key for the identification of third instar larvae of forensically important African blowflies.
- The key will greatly facilitate both research and practice of forensic entomology in African countries. .

Table 1. Localities of females of Calliphoridae from which larvae were obtained.

Species	Location	Coordinate
Calliphora croceipalpis	Addis Ababa, Ethiopia	9°01′N 38°44′E
Calliphora croceipalpis	Makhanda, RSA	33°17′S 26°32′E
Calliphora vicina	Mała Nieszawka, Poland	52º59’N, 18º32’E
Calliphora vomitoria	Mała NieszawkaPoland	52º59’N, 18º32’E
Chrysomya albiceps	Addis Ababa, Ethiopia	30º48’N, 34º48’E
Chrysomya chloropyga	Adaba, Ethiopia	7°02′N 39°31′E

Chrysomya chloropyga	Cape Town, RSA	33°55′S 18°25′E
Chrysomya marginalis	Cape Town, RSA	33°55′S 18°25′E
Chrysomya marginalis	Makhanda, RSA	33°17′S 26°32′E
Chrysomya megacephala	Sde Boqer, Israel	49º57’N, 20º52’E
Chrysomya putoria	Uyo, Nigeria	5°01′N 7°55′E
Chrysomya putoria	Cape Town, RSA	33°55′S 18°25′E
Chrysomya putoria	Adaba, Ethiopia	7°02′N 39°31′E
Chrysomya putoria	Makhanda, RSA	33°17′S 26°32′E
Hemipyrellia fernandica	Uyo, Nigeria	5°01′N 7°55′E
Lucilia cuprina	Bulbula, Ethiopia	7°40′N 38°39′E
Lucilia sericata	Toruń, Poland	53º01’N, 18º33’E