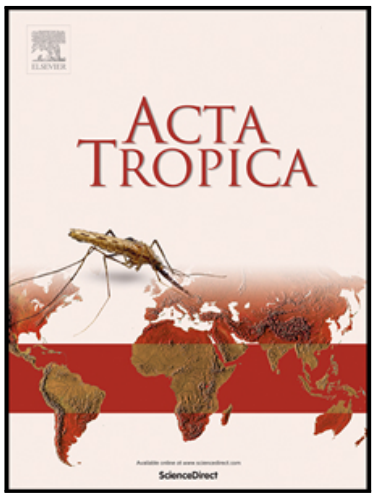

Comparative larval anatomy of the digestive system of three Calliphoridae (Diptera) species that cause different types of myiasis

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Highlights

- The anatomy of the digestive organs of larvae of three Calliphoridae species differ.
- Large crop and salivary glands appear as adaptations for the necrophagous species.
- Small salivary glands and medium sized crop characterized hematophagous larvae.
- The species causing subcutaneous myasis has small crop and large salivary glands.
- Feeding habits of the three species explain these anatomical differences.

Comparative larval anatomy of the digestive system of three Calliphoridae (Diptera) species that cause different types of myiasis

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Abstract

The Calliphoridae are one of the main Diptera families that include agents of the parasitic disease condition known as myiasis. Parasitism seems to have evolved multiple independent times within the Calliphoridae; consequently, this family includes a diversity of myiasis-causing species, varying in their obligate or facultative habits and in their specific location in the host. Larval morphological studies can provide novel and relevant insights into the biology of those species, as well as on the pathogenesis and evolution of myiasis; however, the anatomy of internal organs and structures —particularly those within the digestive system— has generally been overlooked, despite potentially reflecting parasitic adaptations. We use here non-invasive X-ray micro-computed tomographic techniques to study the anatomy of the digestive system of the third instar larvae of three Calliphoridae species: *Protocalliphora azurea*, an obligate agent of sanguinivorous myiasis in passerine bird nestlings; *Cordylobia anthropophaga*, an obligate agent of subcutaneous myiasis in mammals; and *Lucilia sericata*, a facultative agent of traumatic myiasis in mammals. The three species are relatively uniform in the internal anatomy of their digestive organs, although they differ in the shape and size of the salivary glands —a main source of larval antigens—, which are considerably smaller in *P. azurea*. Moreover, the three species differ from the larvae of Oestridae, a close family that exclusively includes obligate myiasis-causing species, in the presence of gastric caeca and a crop, which shows a remarkable storage capacity in *L. sericata*. The observed differences are discussed from a functional perspective and in relation to the type of myiasis caused.

Keywords: *Cordylobia anthropophaga*, larval morphology, *Lucilia sericata*, micro-computed tomography, parasite anatomy, *Protocalliphora azurea*

1. Introduction

Myiasis is a disease condition caused by Diptera larvae that infest the living and/or necrotic tissues of live vertebrates, including humans (Zumpt, 1965; Hall and Wall, 1995). The pathology and the severity of damage to the host vary depending on several factors, such as the location of the infestation, the density of larvae and the species involved (Hall and Wall, 1995). Several Diptera families can act as agents of myiasis, sometimes just as a result of an accidental infestation; however, only three families have a major impact on human health and animal welfare and productivity: Oestridae, Calliphoridae and Sarcophagidae, all of them belonging to the superfamily Oestroidea (Zumpt, 1965; Hall and Wall, 1995; Hall et al., 2016). Both Oestridae and Calliphoridae include the greater diversity of species that act as agents of myiasis, as well as the greater diversity of life strategies and host locations. Within the Oestridae, all species are obligatory parasites, i.e., the larval stage must develop in a live vertebrate host, causing myiasis, to successfully complete the life cycle (Hall and Wall, 1995). Depending on the oestrid subfamily and species, the location in the host varies and the myiasis can be nasopharyngeal, gastrointestinal or subcutaneous (Colwell et al., 2006).

On the other hand, the Calliphoridae include a number of species that are obligatory parasites, but also several species that are facultative parasites: their larvae primarily feed on carrion but can also develop on the necrotic or living tissues of a live host (Zumpt, 1965; Hall and Wall, 1995). Among the Calliphoridae species that are obligatory parasites, there are some agents of: (i) sanguinivorous myiasis, if the larvae are haematophagous ectoparasites of birds or mammals; (ii) subcutaneous myiasis, if the larvae penetrate the skin of the host producing furuncle-like swellings; and (iii) wound or traumatic myiasis, if the larvae invade either a pre-existing wound on the body of the host, or infest the natural body orifices, thus creating a wound (Zumpt, 1965; Hall and Wall, 1995; Hall et al., 2016). All the facultative parasite species within the Calliphoridae are agents of traumatic myiasis (Hall et al., 2016).

As holometabolous insects, the larval stage of Diptera differs radically from the adult both in morphology and biology (Rolff et al., 2019). The larva is primarily a feeding stage, which, in the case of the Oestroidea and other cyclorrhaphous Diptera — i.e., those flies that undergo metamorphosis inside a puparium formed by the hardened cuticle of the third-instar larva—, shows a rather simple morphology with extensive reductional specialisations (Li et al., 2021). In the larvae of the species that cause myiasis, those specialisations may reflect adaptations to parasitism (Jánošková et al., 2010; Szpila et al., 2014; Li et al., 2021); hence, larval morphological studies can provide novel and relevant information on the biology of those species, as well as on the pathogenesis and evolution of the different types of myiasis. Structures like the cuticular spines, the posterior spiracles and the cephalopharyngeal skeleton have classically been the focus of larval morphological studies of both obligatory and facultative myiasis agents, mostly due to their usefulness for species identification (e.g., Gómez-Fernández, 1958; Zumpt, 1965; Jánošková et al., 2010; Szpila et al., 2013, 2014, 2024). However, apart from the cephalopharyngeal skeleton, the anatomical study of internal organs and structures of myiasis-causing species has largely been overlooked, in spite of its potential for providing new insights into different biological and physiological aspects (Martín-Vega et al., 2021; Caleffe et al., 2024).

Among the larval internal structures that are potentially informative, the digestive organs are clearly of upmost importance, given the feeding role of this life stage. In fact, the larval alimentary canal and salivary glands have been the focus of a limited number of anatomical studies on myiasis-causing species, either through dissection, extraction and morphological analysis of the organs, or through the study of serial histological sections (e.g., Boonsriwong et al., 2007; Evangelista and Leite, 2007; Roelfstra et al., 2010; Caleffe et al., 2024). In recent years, the use of X-ray micro-computed tomography (micro-CT) has provided further insights into the functional anatomy of the digestive system in Oestridae larvae in relation to their role as obligate agents of myiasis (Martín-Vega et al., 2021, 2023). As a non-invasive technique, micro-CT enables the visualisation and analysis of internal soft tissues and organs *in situ*, gathering both quantitative and qualitative 3D data of complex anatomical structures (Lauridsen et al., 2011). Comparative micro-CT studies on the larvae of different

Oestridae species have revealed interesting differences in the anatomy of the digestive organs, depending on the subfamily and type of myiasis they cause (Martín-Vega et al., 2021, 2023). Considering that obligate and facultative parasitism seem to have evolved multiple independent times within the Calliphoridae (Stevens and Wallman, 2006; Stevens et al., 2006; Cardoso et al., 2025), it is desirable to explore if similar anatomical differences occur between the larvae of calliphorid species from different subfamilies and that cause different types of myiasis.

The aim of the present study is to describe and compare the anatomy of the larval digestive system of three Calliphoridae species that are agents of myiasis: the 'bird blow fly', *Protocalliphora azurea* (Fallén, 1816) (subfamily Chrysomyinae); the 'tumbu fly' or 'mango fly', *Cordylobia anthropophaga* (Blanchard, 1893) (subfamily Bengaliinae); and the 'green bottle blow fly', *Lucilia sericata* (Meigen, 1826) (subfamily Luciliinae). The larvae of *P. azurea* are obligate agents of sanguinivorous myiasis; they feed on the blood of nestlings of a variety of passerine bird species throughout the Palaearctic region, affecting the host survival in cases of severe infestations (Zumpt, 1965; Merino and Potti, 1995). Like *P. azurea*, the larvae of *C. anthropophaga* are also obligate agents of myiasis; however, they cause furuncular subcutaneous myiasis in many mammals, including humans, in sub-Saharan Africa (Zumpt, 1965) and southwestern Saudi Arabia (Sundharam and Al-Gamal, 1994, Magram et al, 2023). Human infestations by *C. anthropophaga* in travellers returning from endemic areas are not rare (see recent recount of cases identified by both morphological and molecular techniques in Alvaro et al., 2024 and Biernat et al, 2025). Even infestations outside Africa/Arabia may occur, as female flies can lay their eggs on drying clothes which can then be transported beyond the normal habitat (Hall and Wall, 1995; Whitehorn et al., 2010). On the other hand, *L. sericata* larvae are primarily sarcosaprophagous and feed on the soft tissues of cadavers during the early stages of decomposition, but they are also frequently reported as facultative agents of traumatic myiasis in humans and domestic animals (Hall and Wall, 1995). Due to its synanthropy, feeding habits and nearly cosmopolitan distribution, *L. sericata* is considered an ideal forensic indicator, both for minimum postmortem interval estimations in suspicious death cases and for estimating a minimum period of neglect in cases of traumatic myiasis in domestic animals or in elderly, disabled and other types of dependent people (Amendt et al., 2011; Hall et al., 2016). In addition, the larvae of *L. sericata* can be used in clinical practice for removing necrotic tissue and cleaning wounds, which is known as 'maggot debridement' or 'maggot therapy' (Sherman et al., 2000; Gazi et al., 2021). *Lucilia sericata* and *P. azurea* are among the few species of myiasis causing flies to have had their genomes published (Davis et al., 2021; Falk et al., 2023). The three studied species are thus an example of the different types of myiasis caused by the Calliphoridae (sanguinivorous, subcutaneous and traumatic). Potential differences between the three species will be discussed from the perspective of the functional anatomy of the digestive organs.

2. Material and methods

Third-instar larvae of *P. azurea* were collected alive from wooden nestboxes of a population of the Eurasian blue tit, *Cyanistes caeruleus* (Linnaeus, 1758) (Passeriformes: Paridae), which has been monitored since 1991 in Valsaín (Segovia, central Spain). Further details on that Eurasian blue tit population and its long-term study can be found in García-del Río et al. (2025). The *P. azurea* larvae were fixed in near-boiling water for approximately 30 s (Adams and Hall, 2003) and then preserved and stored in 70% ethanol at 4 °C. For *C. anthropophaga*, one third-instar larva was collected from a black rat, *Rattus rattus* (Linnaeus, 1758) (Rodentia: Muridae), in Santiago Island (Cape Verde), preserved directly in 70% ethanol and stored at 4 °C. Further details on the collection of the specimen can be found in Fernández-Álvarez et al. (2022). Finally, a laboratory colony of *L. sericata* established from wild adults collected at the scientific campus of the University of Alcalá (Alcalá de Henares, Madrid, Spain) was used for obtaining third-instar larval specimens. The larvae were reared in an incubator under a constant temperature of 25 °C using fresh pig liver as a feeding substrate, following a standard rearing protocol (Martín-Vega et al., 2017). Ten third-instar larvae were collected from the feeding substrate 144 h after hatching, fixed in near-boiling water for approximately 30 s and then preserved and stored in 70% ethanol at 4 °C. Prior to micro-CT scanning, the larva of *C. anthropophaga* and 2 larvae collected at random from each *P. azurea* and *L. sericata* species were immersed in 0.5 M iodine in aqueous solution and stored at 4 °C for two weeks, following the procedure described in previous publications (Martín-Vega et al., 2021, 2023). Twenty-four hours before scanning, the specimens were transferred to 70% ethanol to wash out any excess of the iodine staining solution.

For micro-CT scanning, each larval specimen was transferred into a microcentrifuge tube containing 70% ethanol and placed into a Zeiss Versa 520 system at the Imaging and Analysis Centre of the Natural History Museum, London. All the specimens were scanned using 0.4 x magnification. Due to differential contrast of the internal soft tissues among specimens, exposure time was set to 1.5 s, voltage to 110 kV, and current to 91 μA for *P. azurea* and *C. anthropophaga* specimens, whereas, for *L. sericata* specimens, exposure time was set to 6 s, voltage to 130 kV and current to 77 μA. The resulting projections from each scanned specimen were reconstructed as TIFF stacks using the software Scout-and-Scan Control System and Reconstruction (Carl Zeiss AG, Jena, Germany), with a voxel size of 8.86–11.49 μm^3. The TIFF stacks were imported into the software VG Studio Max 2.2 (Volume Graphics Gmbh, Heidelberg, Germany), where they were rendered, reoriented and visualised in cross and sagittal anatomical reference planes. The TIFF stacks were also imported into the software Avizo 3D 2021.2 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 3D reconstruction and visualisation. The "Material statistics" module from Avizo software was used for volumetric measurements after manual segmentation of the digestive organs (Martín-Vega et al., 2021, 2023).

3. Results

The digestive system —and, more specifically, the alimentary canal— is clearly the most conspicuous structure within the larval body (Figs. 1–4). The alimentary canal opens at the oral orifice and is divided into three sections. The first section of the canal is the foregut or stomodaeum, which comprises the pharynx and the oesophagus (Figs. 2–4). The oesophagus shows a dorsal diverticulum for food storage, the crop, which was strikingly enlarged in the scanned *L. sericata* larvae (Figs. 1I–K, 4A, C, D). In those larvae, the crop was filled with food content and greatly distended (Fig. 1I–K), pushing other digestive structures against the ventral side of the body (Fig. 4). In the scanned *P. azurea* larvae (Figs. 1A, B, 2A–C), the crop was enlarged —but not comparable to the enlarged crop of *L. sericata* larvae—, whereas, in the larva of *C. anthropophaga* (Figs. 1E, 3A–C), the crop was deflated and apparently empty of food.

After the divergence that originates at the crop, the oesophagus runs between the larval brain hemispheres and dorsal to the thoracic ganglion (Fig. 1B, F, J), ending by entering the cardia or proventriculus (Figs 2C, 3C, 4B, D), a bulb-shaped organ involved in the synthesis of the peritrophic matrix. The cardia marks the start of the longest section of the alimentary canal, the midgut or mesenteron, a long and convoluted tubular structure that occupied a significant part of the abdominal segments of the larval body cavity in the scanned *P. azurea* (Fig. 2A–C) and *C. anthropophaga* (Fig. 3A–C) specimens. In the scanned *L. sericata* larvae, however, the midgut was largely restricted to the posterior region of the body cavity, leaving room for the distended crop in the anterior and middle segments (Fig. 4A–D). It must be noted that, whereas the midgut of *L. sericata* was thin and mostly empty of food throughout its entire length (Fig. 1I), the scanned *P. azurea* and *C. anthropophaga* larvae showed food content in several distended portions of the midgut (Fig. 1A, C, E, G). The contrast between distended and non-distended midgut portions was particularly obvious in the virtual sections of the midgut wall of *C. anthropophaga*, being folded in the non-distended portions but thinner and extended in the distended ones (Fig. 1E, G). The larval midgut of the three species showed four finger-like gastric caeca originating in its anterior portion, just after the constriction that follows the cardia (Figs. 2C, 3C, 4B, D).

The last section of the alimentary canal, the hindgut or proctodaeum, starts at the point where the Malpighian tubules —typically two pairs in adult and larval Diptera— originate. In all the scanned specimens, those tubules were very thin throughout their entire length and hardly traceable throughout the micro-CT-based virtual sections (Fig. 1C, G); hence, it was not possible to segment and reconstruct them for 3D visualisation. Anyhow, the origin of the Malpighian tubules and the transition from the midgut to hindgut take place amidst the tangled mass formed by the several midgut loops. From its origin, the hindgut runs towards the dorsal side of the larval body, emerging from the tangled midgut mass and then running dorsally and posteriorly to the anal segment, where it opens into the anus (Figs. 3A, B, 4A–C). Interestingly, in the scanned *P. azurea* larvae, the hindgut ran ventrally around the midgut mass after emerging from it, and before running dorsally and posteriorly to the anal segment (Fig. 2A–C). In the three

studied species, the final run of the hindgut is surrounded by a profusion of fat body cells within the posterior abdominal segments of the larva (Fig. 1D, H, L). A distinctly swollen portion of the larval hindgut within those posterior body segments was observed in *P. azurea* (Fig. 2A) and, even more markedly, in *C. anthropophaga* (Fig. 3A–C), prior to abruptly narrowing for opening into the anus (Fig. 1E). A somewhat distended final portion of the hindgut, which narrowed near the anus, was also observed in the scanned *L. sericata* larvae; however, that distended portion showed a flattened appearance, rather than being clearly swollen (Fig. 4A–C).

While the alimentary canal showed many similarities and some more or less subtle differences between the three species, the most distinctive anatomical differences between the digestive organs of the three species were observed in the morphology of the pair of salivary glands (Figs. 2D, 3D, 4E). The larval salivary glands of *P. azurea* are thin and relatively short, running ventrally up to the first abdominal segment (Fig. 2D). The larval salivary glands of *C. anthropophaga* are also tubular in shape, but thicker, longer and more convoluted, running ventrally up to the fourth abdominal segment (Fig. 3D). In contrast, the larval salivary glands of *L. sericata* are non-convoluted, relatively large sack-like organs, which progressively narrow towards their posterior ends and run ventrally up to the third abdominal segment (Fig. 4E). Moreover, the salivary ducts of *L. sericata* larval salivary glands are distinctly shorter than those of *P. azurea* and *C. anthropophaga* (Figs. 2D, 3D, 4E).

Finally, the volume measurements performed on the segmented and 3D-reconstructed specimens confirmed the observations on the relative size of the different digestive organs and structures. The midgut was the largest organ within the larval body of *P. azurea* (31.94 mm^3; 36.4% of the total body volume) and *C. anthropophaga* (20.37 mm^3; 24.85% of the total body volume), whereas in *L. sericata* the midgut volume was only 3.94 mm^3 (5.77% of the total body volume), as the distended crop was the largest organ in this species (22.09 mm^3, 32.37% of the total body volume). The crop volume was 1.4 mm^3 (1.6% of the total body volume) in *P. azurea* and 0.07 mm^3 (0.08% of the total body volume) in *C. anthropophaga*. The volume of the hindgut was distinctly greater in *C. anthropophaga* (2.58 mm^3; 3.15% of the total body volume) than in *P. azurea* (0.94 mm^3; 1.07% of the total body volume) and *L. sericata* (0.43 mm^3; 0.63% of the total body volume), due to the aforementioned enlarged portion of that organ in the first species (Fig. 3A–C). Also, in accordance with the descriptions above (Figs. 2D, 3D, 4E), the size of the larval salivary glands was larger in *L. sericata* (1.16 mm^3; 1.7% of the total body volume) and *C. anthropophaga* (1.13 mm^3; 1.38% of the total body volume) than in *P. azurea* (0.6 mm^3; 0.68% of the total body volume).

4. Discussion

Based on the hypothesis from Zumpt (1965) and on morphological, molecular and biogeographical evidences, Stevens et al. (2006) provided support for a polyphyletic origin of myiasis among the Calliphoridae following at least two evolutionary

pathways: one sanguinivorous and one saprophagous. Whether developing on living or necrotic host tissues, Cardoso et al. (2025) highlighted the relevance of facultative parasitism as a transitional stage in the evolution of myiasis among the Calliphoridae. In this sense, Stevens et al. (2006) pointed out that the facultative parasitic habits observed in *L. sericata*, and other primarily necrophagous blow flies, *«could be more a result of changes in the conformation of the domesticated host* [given that a greater susceptibility to parasitic infestations might be a consequence of artificial selection] *than a marked evolutionary change in the adult or larval forms of the potentially parasitic fly»*. In fact, the general morphology of the different digestive organs and structures observed in *L. sericata* larvae (Figs. 1I–L, 4A–E) is very similar to that described from other primarily necrophagous blow fly species that are also facultative agents of myiasis, like *Calliphora vicina* Robineau-Desvoidy, 1830 (subfamily Calliphorinae) (Lowne, 1892; Schoofs and Spieβ, 2007) and *Chrysomya megacephala* (Fabricius, 1794) (subfamily Chrysomyinae) (Boonsriwong, 2007; Caleffe et al., 2024). Because carrion is a nutrient-rich yet ephemeral resource, the crop is a particularly important organ for the larvae of these and other necrophagous blow fly species, as it enables ingestion of a great amount of food in a short time, and its storage before pumping it into the midgut for digestion (Knight, 1962; Charabidze et al., 2013). Several studies have shown that *L. sericata* might be an inferior competitor when coexisting on carrion with the larvae of other blow fly species (Smith and Wall, 1997; MacInnis and Highley, 2020; Ivorra et al., 2022); hence, the high storage capacity of its larval crop (Figs. 1I, 4A–D) enables ingestion of great amounts of food, thereby playing a significant role in the discontinuous foraging behaviour observed in this species (Charabidze et al., 2013). Charabidze et al. (2011, 2013) hypothesised that such behaviour may favour the turnover of *L. sericata* larvae within a mass aggregation and their metabolic heat emission, favouring in turn the larval fitness and development. In future studies, non-invasive micro-CT imaging techniques might be a powerful tool for providing novel insights into the functioning of the crop during the discontinuous feeding of *L. sericata* larvae, as well as to unveil potential differences in crop usage between larvae feeding on carrion or on live hosts, between different larval mass densities, or between *L. sericata* larvae and the larvae of other necrophagous Calliphoridae species that show a more continuous foraging behaviour (Hückesfeld et al., 2010).

The crop must also play an important role in *P. azurea* larval feeding, considering the temporal relationship between this sanguinivorous parasite and its host: the larva only attaches to the nestling to feed, but then it drops and hides in the nest to digest the ingested blood (Scholl et al., 2019). Once detached from the host, the blood stored in the crop (Figs. 1A–B, 2A–C) should be pumped into the midgut for digestion, as in necrophagous Calliphoridae larvae (Knight, 1962), and in a similar way to the functioning of the analogous crop of adult mosquitoes (Calkins et al., 2017).

The presence of a crop in the larval digestive system of *C. anthropophaga* (Figs. 3A–C) might also suggest that the feeding of this species may be discontinuous or intermittent and that the ingested food is also stored in this species, even if just for a

short period. However, larvae of *C. anthropophaga* remain in close association with the host throughout the different larval instars and within individual furuncular lesions, i.e., not directly competing with other larvae. Therefore, storing a great amount of food in the crop does not seem essential in this case, and perhaps the direct passage of ingested food into the midgut, which seems to be exceptional in necrophagous blow flies (Knight, 1962), is more frequent in *C. anthropophaga*, rendering the crop almost redundant. Indeed, the single larval specimen analysed here showed an empty crop (Figs. 1E, 3A–C) and one that was very small compared to those of *P. azurea* (Fig 2 A) and *L. sericata* (Fig 3A); nonetheless, the study of more specimens would be desirable to determine the crop storage capacity and usage in this species, further understanding its feeding behaviour. Time and financial constraints limited the number of scanned specimens in the present study, but further analyses with larger sample sizes will enable exploration of potential volumetric differences between the crop and other digestive organs at different stages of food intake. Interestingly, the larvae of the Oestridae subfamilies Hypodermatinae and Cuterebrinae, which also cause subcutaneous myiasis, lack a crop (Evangelista and Leite, 2007; Martín-Vega et al., 2021). The crop is also absent in the larvae of the other two Oestridae subfamilies, Gasterophilinae (Roelfstra et al., 2010) and Oestrinae (Martín-Vega et al., 2021, 2023), which cause gastrointestinal and nasopharyngeal myiasis, respectively (Colwell, 2006). Like *C. anthropophaga*, all Oestridae are attached to the host tissues throughout the larval stage (Colwell, 2006) and, whether they feed continuously or intermittently, the storage of ingested food is definitely not essential to successfully complete their life cycle.

The larval digestive system of the three studied Calliphoridae species does not only differ from that of Oestridae in the presence of a crop, but also in the presence of gastric caeca in the anterior portion of the midgut (Figs. 2C, 3C, 4D). Gastric caeca are involved in the absorption of nutrients and occur in the larval midgut of many Diptera in a variable number, from four in Calliphoridae and other flies (Boonsriwong, 2007; Chen and Johnston, 2022; Caleffe et al., 2024) to eight in mosquitoes (Jones and Zeve, 1968; Volkmann and Peters, 1989). Gastric caeca are, however, absent in the larval alimentary canal of Oestridae (Evangelista and Leite, 2007; Martín-Vega et al., 2021, 2023) and other Diptera, like the 'black soldier fly', *Hermetia illucens* (Linnaeus, 1758) (Bonelli et al., 2019). No differences were observed in the number and morphology of the gastric caeca, nor in the general morphology of the rest of the midgut, between the three species studied here (Figs. 2A–C, 3A–C, 4A–D) and compared to other Calliphoridae species (Lowne, 1892; Boonsriwong, 2007; Caleffe et al., 2024). In Calliphoridae and other cyclorrhaphous flies, the larval midgut is generally a uniformly long and convoluted tube (Caleffe et al., 2024), with some exceptions among the Oestridae (Martín-Vega et al., 2021). Although the larval midgut shows functional and ultrastructural regionalisation (Buchon and Osman, 2015), there are generally no distinct morphological regions, other than distended portions in fed larvae (Hobson, 1931). Distended midgut portions containing food were observed here in both *P. azurea* and *C. anthropophaga* (Fig. 1A–G), but not in *L. sericata*, where most ingested food was stored in the crop (Fig. 1I–K).

Like the midgut, the larval hindgut of Diptera is a relatively uniform tube that shows ultrastructural and functional regionalisation (Murakami and Shiotsuki, 2001); however, it is much shorter and less convoluted than the midgut, and it usually narrows within its anal or final portion (Martín-Vega et al., 2021, 2023; Caleffe et al., 2024). The apparently longer hindgut of *P. azurea*, which runs ventrally around the tangled mass formed by the larval midgut (Fig. 2A–C), might indicate the need for a greater surface for reabsorbing salts and/or maintaining the osmotic pressure in the haemolymph in this species, but this aspect needs to be further studied. On the other hand, some studies had recorded that the larvae of sarcosaprophagous Calliphoridae reared on calcium-rich feeding substrates show enlarged Malpighian tubules containing calcium granules (Waterhouse, 1950), seen as highly radio-dense concretions in micro-CT scans (Bell et al., 2021). Greatly enlarged anterior Malpighian tubules containing radio-dense concretions occur in the larvae of Oestridae species within the subfamily Oestrinae, likely related to high calcium concentrations in the host tissues they typically infest (Martín-Vega et al., 2021, 2023). The absence of enlarged portions of the Malpighian tubules in the three species studied here might just be an indicator of non-calcium-rich diets. In fact, non-enlarged Malpighian tubules also occur in the larvae of oestrid species within subfamilies Cuterebrinae and Hypodermatinae, which, like *C. anthropophaga*, are obligate agents of subcutaneous myiasis (Evangelista and Leite, 2007; Martín-Vega et al., 2021). In the case of *L. sericata* and other facultative agents of myiasis, it would be interesting to analyse larval specimens collected from live vertebrate hosts and compare their Malpighian tubules to those from individuals reared in the laboratory on different feeding substrates (Waterhouse, 1950; Bell et al., 2021).

Perhaps the greatest morphological differences between the digestive organs of the three studied species were observed in the salivary glands, with *P. azurea* showing significantly smaller glands than *C. anthropophaga* and *L. sericata* (Figs. 2D, 3D, 4E). To feed, the larvae of *Lucilia* Robineau-Desvoidy, 1830 species secrete proteolytic saliva that digest collagen and other proteins, liquefying the live or necrotic tissues of the host (Kerlin and Hughes, 1992). To the best of our knowledge, the excretory/secretory (ES) products of *C. anthropophaga* have not been characterised yet, but serine protease activity has also been identified in the larval saliva of Oestridae species that, like *C. anthropophaga*, cause subcutaneous myiasis (Brant et al., 2010; Ahmed et al., 2010). Therefore, it is reasonable to think that the saliva of *C. anthropophaga* larvae shows a similar proteolytic activity to other Calliphoridae and Oestridae species. On the other hand, the serine proteases secreted by *L. sericata* larvae have been shown to induce the coagulation of human blood and plasma (Gazi et al., 2021). The clotting of host blood would be counter-productive for *P. azurea* and, anyhow, feeding on a fluid means that proteolytic digestion prior to ingestion might not be needed, so the small salivary glands observed in this species may indicate an adaptation to a reduced need for salivary production and secretion. Extremely low levels of protease activity are typical of the saliva of other haematophagous arthropods (Kerlin and Hughes, 1992), so it would be interesting to characterise the larval ES products of *P. azurea* —and other species within the genus *Protocalliphora* Hough, 1899— and

compare them to those from other Calliphoridae. Particularly interesting would be to characterise the larval ES products and to image the salivary glands of the 'Congo floor maggot', *Auchmeromyia senegalensis* (Macquart, 1851) (subfamily Bengaliinae), and other species within the genus *Auchmeromyia* Brauer and Bergenstamm, 1891, for comparison with *P. azurea*. The larvae of *A. senegalensis* and other *Auchmeromyia* species feed intermittently on the blood of mammal hosts, including humans (Scholl et al., 2019); hence, they might share some anatomical features and convergent adaptations with the larvae of *Protocalliphora* species.

It had been highlighted that most Calliphoridae and Sarcophagidae species are rather uniform in larval external morphology, in contrast to Oestridae, which show major morphological differences between subfamilies (Stevens et al., 2006). Recent studies have revealed that there are also significant differences in the internal larval anatomy between Oestridae subfamilies, which might be explained from a functional perspective as adaptations to their specific parasitic habits (Martín-Vega et al., 2021, 2023). The present study suggests that, like in their external morphology, the larvae of the Calliphoridae species that cause myiasis are relatively uniform in internal anatomy, although there are some interesting differences —particularly those in the morphology of the salivary glands, as they are a main source of antigens— that need to be further explored. In this sense, micro-CT is a powerful tool that, together with other novel imaging techniques, provides unparalleled opportunities for functional and comparative studies of the anatomy of Diptera larvae, contributing to a better knowledge and understanding of the evolution, diversity and biology of myiasis-causing species, as well as their impact on human and animal health.

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Figure legends

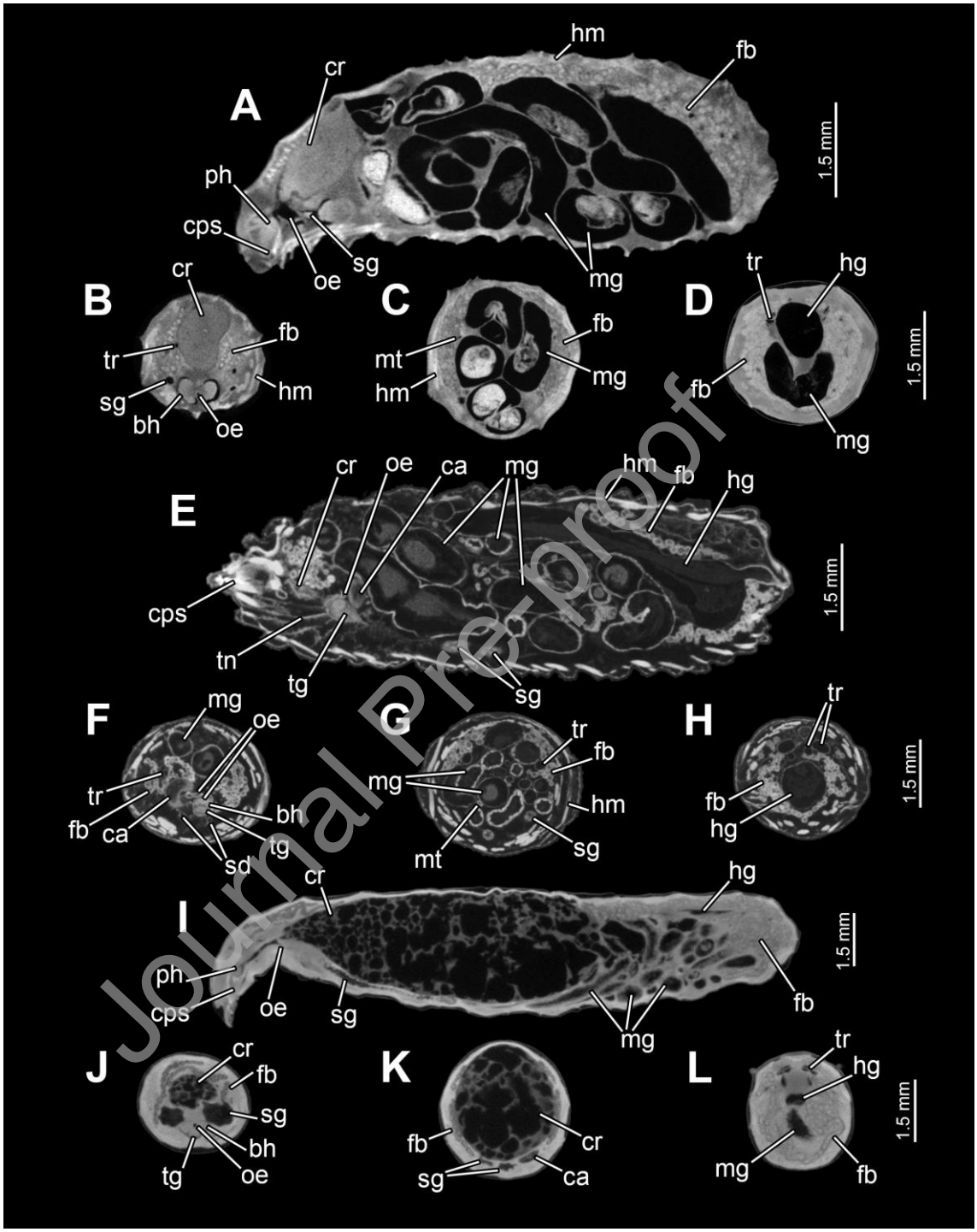

Fig. 1. Micro-CT-based virtual sections of third-instar larvae of three Calliphoridae species that cause myiasis. *Protocalliphora azurea*: (A) medial sagittal section, (B) anterior cross section, (C) medial cross section, (D) posterior cross section. *Cordylobia anthropophaga*: (E) medial sagittal section, (F) anterior cross section, (G) medial cross section, (H) posterior cross section. *Lucilia sericata*: (I) medial sagittal section, (J) anterior cross section, (K) medial cross section, (L) posterior cross section.

Abbreviations: bh, brain hemisphere; ca, cardia; cps, cephalopharyngeal skeleton; cr, crop; fb, fat bodies; hg, hindgut; hm, hypodermal muscles; mg, midgut; mt, Malpighian tubule; oe, oesophagus; ph, pharynx; sd, salivary duct; sg, salivary gland; tg, thoracic ganglion; tn, thoracic nerve; tr, trachea.

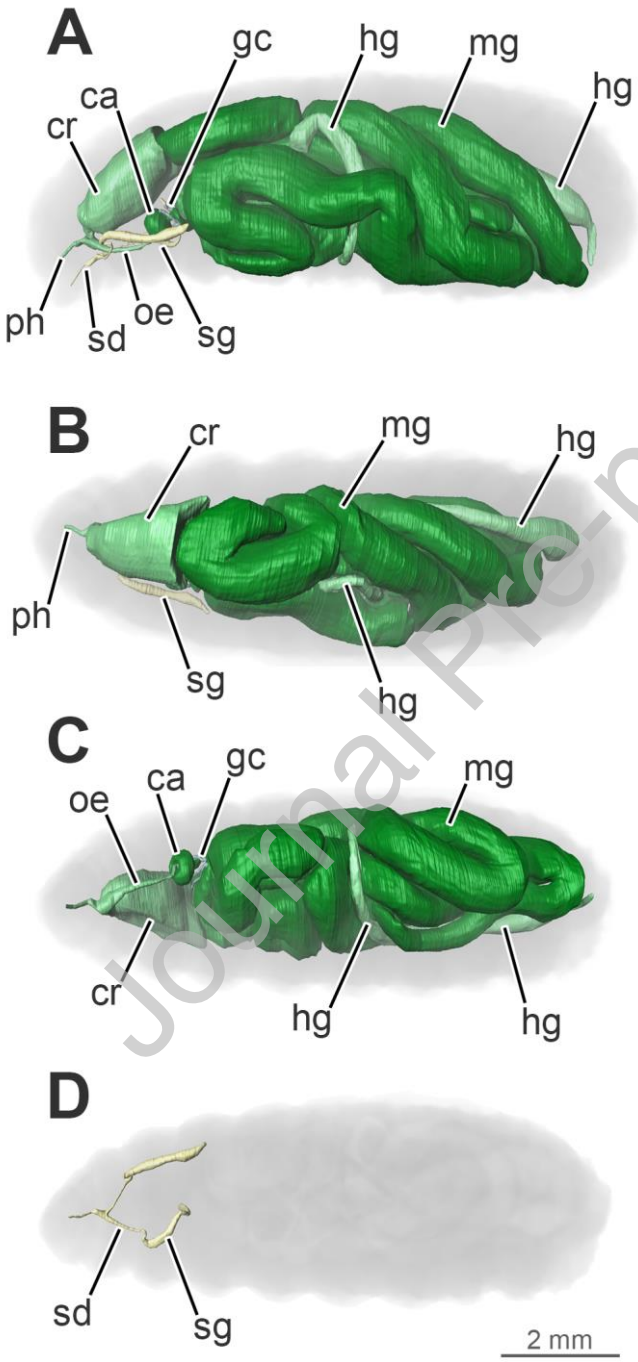

Fig. 2. *Protocalliphora azurea*, false coloured 3D surface models of a third-instar larva showing different organs of the digestive system. (A) lateral view. (B) dorsal view. (C)

ventral view showing the alimentary canal. (D) ventral view showing the salivary glands. Abbreviations: ca, cardia (shown in dark green); cr, crop (light green); gc, gastric caeca (blue); hg, hindgut (light green); mg, midgut (dark green); oe oesophagus (light green); ph, pharynx (light green); sd, salivary duct (yellow); sg, salivary gland (yellow).

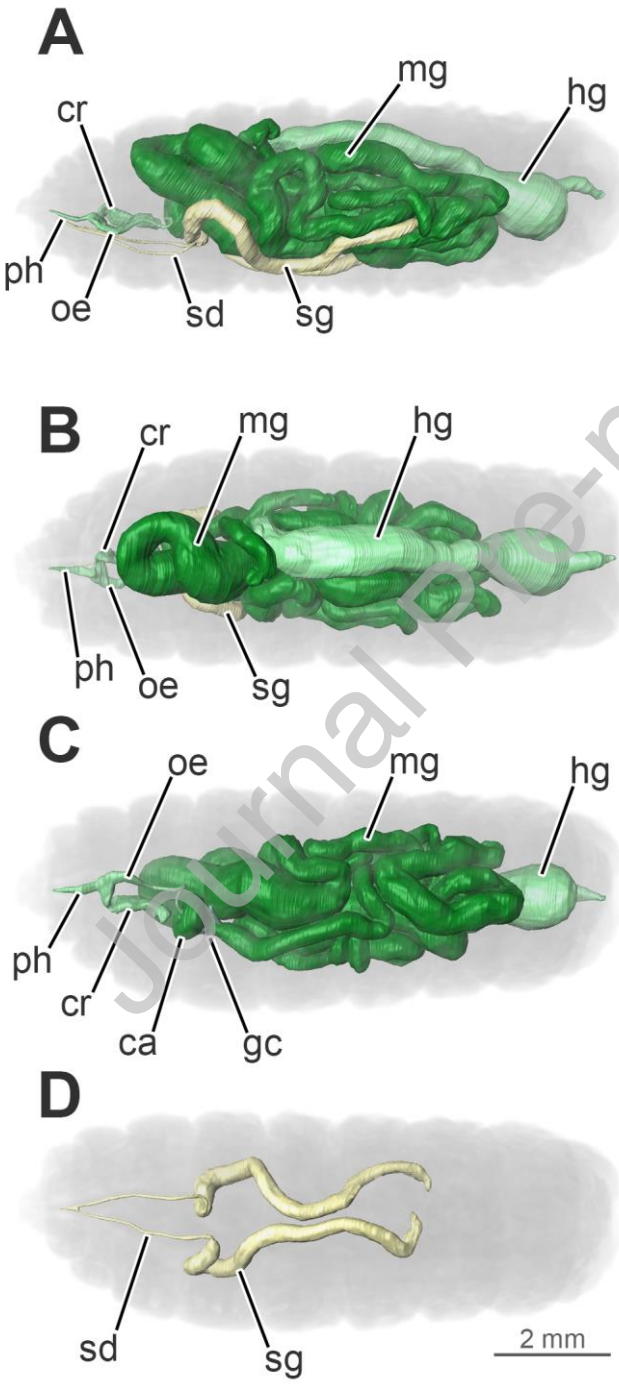

Fig. 3. *Cordylobia anthropophaga*, false coloured 3D surface models of a third-instar larva showing different organs of the digestive system. (A) lateral view. (B) dorsal view. (C) ventral view showing the alimentary canal. (D) ventral view showing the salivary glands. Abbreviations: ca, cardia (shown in dark green); cr, crop (light green); gc, gastric caeca (blue); hg, hindgut (light green); mg, midgut (dark green); oe oesophagus (light green); ph, pharynx (light green); sd, salivary duct (yellow); sg, salivary gland (yellow).

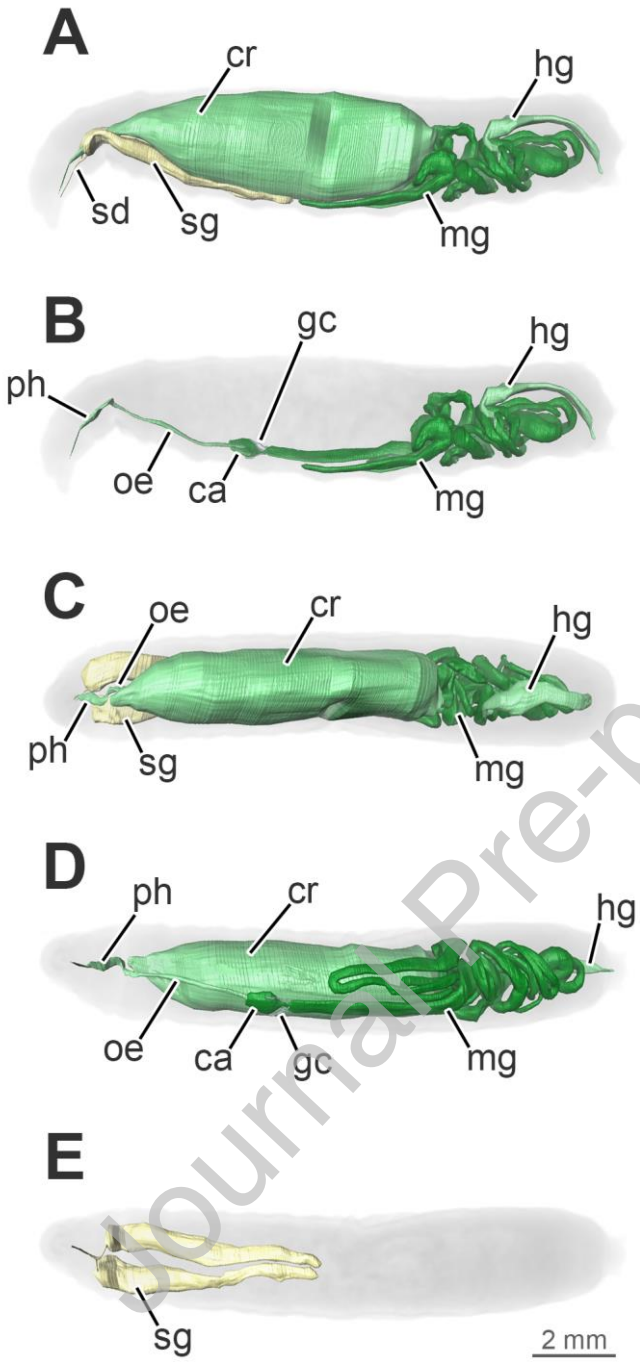

Fig. 4. *Lucilia sericata*, false coloured 3D surface models of a third-instar larva showing different organs of the digestive system. (A) lateral view showing the entire digestive system. (B) lateral view hiding the crop. (C) dorsal view. (D) ventral view showing the alimentary canal. (E) ventral view showing the salivary glands. Abbreviations: ca, cardia (shown in dark green); cr, crop (light green); gc, gastric caeca (blue); hg, hindgut (light green); mg, midgut (dark green); oe oesophagus (light green); ph, pharynx (light green); sd, salivary duct (yellow); sg, salivary gland (yellow).

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract