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## Revealing the larval anatomy of the hoverfly *Sphaerophoria rueppellii* (Wiedemann, 1820) (Diptera, Syrphidae) using micro-computed tomography

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Micro-computed tomography (micro-CT) is an X-ray-based technique that allows visualisation of the internal anatomy of insects *in situ* and does not require dissections. Traditionally, the study of insect anatomy has been mainly based on dissection techniques and microtome sections. However, micro-CT is becoming an increasingly widespread study technique. We report the use of micro-CT scans to study, in detail, the external and internal structures and organs of the third instar larva of the dipteran Syrphidae *Sphaerophoria rueppellii*, a Palaearctic species included in the list of effective agents used in European biocontrol programmes against aphids. Detailed images and videos provided evidence of the external morphological characteristics and an overview of the internal anatomy (musculature, digestive tube, salivary glands, Malpighian tubules, fat body, circulatory system, nervous system, cephalopharyngeal complex, tentorium, and mouth parts). The study evidenced the following: an external lateroventral complex of muscles that form muscular rings that allow the compression of the body segments in a similar functional way as occurs in annelid worms; the existence of mandibles with prosthecae; the nervous system present a conspicuous distinguishable suboesophageal ganglion; the so-called antennomaxillary organs are the antennae; the maxillae are separate structures, with an articulated maxillary palpus; *S. rueppellii* has only one pair of Malpighian tubules instead of the two pairs that were thought to be the general rule for Syrphid larvae; and the evidence of an imaginal disc of genitalia in the posterior part of the body, below the rectum, which in male larvae has a clear correspondence with the morphology of the external male genitalia, which allows to differentiate the sexes in the larval stage. This study constitutes a true anatomical atlas of the third instar larvae of *S. rueppellii*. High-quality rendered images and additional supplementary videos together with a 3D model, suitable for use with mobile devices, are useful tools for future research and teaching aids.

Syrphidae, commonly known as hoverflies or flower flies, are a diverse family of Diptera with 6674 recognised species worldwide<sup>1</sup>; the larvae have several feeding habits, including predation, saprophagy, mycophagy, and herbivory<sup>2,4,5</sup>. Syrphid adults depend on flowers for nectar as an energy source and pollen for egg maturation<sup>6,7</sup>, contributing to the pollination of many plant species, including economically important crops<sup>8</sup>. Larvae are voracious predators of mainly aphid species as well as some other agriculturally important pests, such as thrips, psyllids, and spider mites<sup>1,9</sup>, although some species are also known to be primary parasitoids of ants<sup>10</sup>. The attractive appearance of the adults, which mimic various species of Hymenoptera, and their fascinating biology have made Fredrik Sjöberg's masterfully written novel, which is based on his experiences of catching hoverflies on a remote island in Sweden, a bestseller that has been translated into several languages<sup>11</sup>. The anatomy of Syrphidae larvae has been studied in the context of their phylogeny using larval morphological characters<sup>2</sup>. However, although there are studies on different details of the internal structures of the larvae (i.e.<sup>12–17</sup>), and even a recent publication has used synchrotron-radiation computed tomography to study the larvae of Syrphidae in Eocene forest amber fossils<sup>18</sup>, there are no studies that deal in depth with the complete internal anatomy of the larvae, except the pioneering study by Krüger<sup>17</sup>. In particular, the work of Prof. Madan Lal Bhatia published in

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the first half of the twentieth century, is the most detailed compilation of the anatomy of different aphidophagous syrphid species<sup>19</sup>.

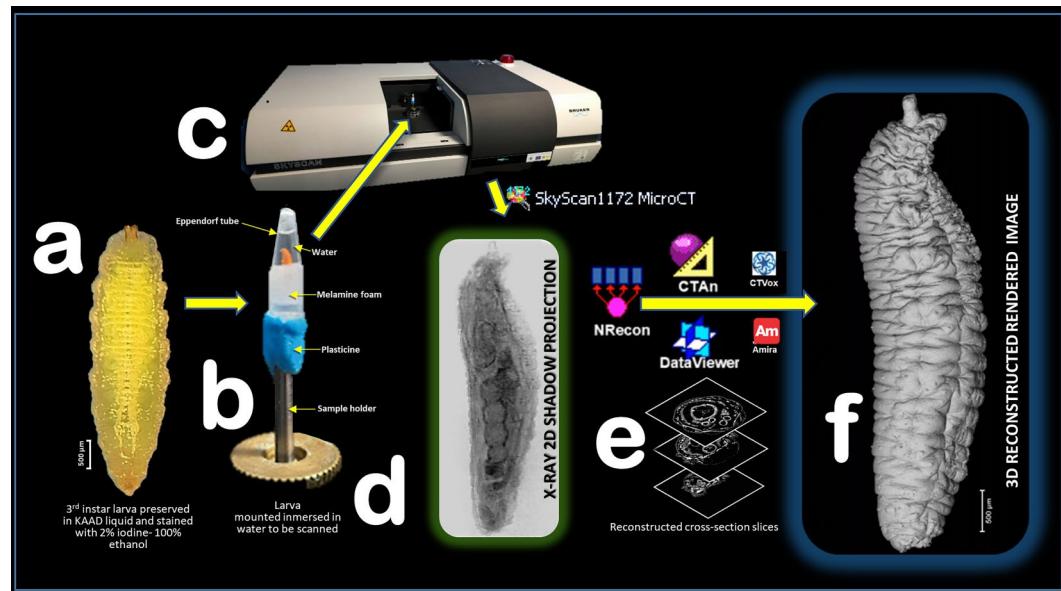
Here, using micro-CT, a detailed study of the entire anatomy is presented. Although dissection techniques are useful, they are also limited because they distort the spatial arrangement of internal structures. Histological techniques have been used to obtain consecutive histological sections (i.e.<sup>20</sup>). Micro-computed tomography (micro-CT), which is based on X-rays, allows visualisation of the internal anatomy *in situ*, without the need to dissect or section the specimen, and therefore without damaging it. Micro-CT results have been validated by comparing them with classical destructive methodologies<sup>21,22</sup>, has been used to study the larval digestive and Malpighian tubules of Oestridae<sup>23</sup>, and detailed methodological papers have been published (i.e.<sup>20,24–26</sup>) of the 3rd larval instar (L3) of *Sphaerophoria rueppellii* (Wiedemann, 1820), a Palaearctic species. The species was virtually unknown until recently, when it became commercially available<sup>27,28</sup> and was included in the list of effective agents used in European biocontrol programmes against aphids<sup>29</sup>. Bathia<sup>19</sup> under the name of *S. flavicauda* Zetterstedt, 1843 (a synonymy of *S. rueppellii*) described superficially and figured the third instar larva, the cephalic skeleton, prothoracic spiracle, and posterior respiratory processes, and has recently been published on the micromorphology of eggs, third larval instars, and puparium<sup>15</sup>. However, until this study, nothing was known about the internal anatomy of the larvae of this species. The use of micro-CT facilitated detailed examination of the external and internal anatomical structures. Improvement in current knowledge, not only in the knowledge of the anatomy of the species but also of the Syrphidae larvae in general, helps to understand the structures in their natural anatomical position, avoiding the deformations that usually occur using standard dissection techniques and/or slide preparation. We also present videos as supplementary information to provide an accurate view of the actual position and internal components of the organs and structures (Supplementary Videos S1–S7) and a 3D model suitable for use with mobile devices (Supplementary 3D model S8). Spinning animations using different rotational axes allow exceptional views of the minutiae of structures from different 3D perspectives. These are useful tools for future research and teaching.

## Materials and methods

The entire process from specimen preparation to rendering the images is schematised in Fig. 1 and Supplementary Video S1.

### Specimen preparation

For the microtomographic study, four laboratory-reared L3 larvae already preserved in KAAD liquid (1 part paraffin, 7–9 parts 95% alcohol, 1-part glacial acetic acid and 1-part dioxane) were submerged in 100% ethanol with 2% iodine for 1 week (Fig. 1a) and stored in a 0.2 ml Eppendorf tube containing water for immediate scanning. It was attached to the specimen holder with plasticine, and larvae were fixed inside the tube with Basotect® [melamine resin foam, created by the Chemical Company BASF], a material that is easy to remove digitally<sup>22</sup> (Fig. 1b).



**Fig. 1.** Scheme of the process of obtaining volume-rendered images: starting from larvae preserved in KADD, stained with iodine (a), mounted for scanning (b), and installed into the microtomograph (c) to obtain X-ray images (d). After software reconstruction and cleaning procedures, cross-sectional slices of images are obtained (e), and finally, by volumetric reconstruction software, high-quality 3D rendered images are obtained (f).

## CT scanning conditions

A SkyScan 1172 desktop high-resolution microtomograph, upgraded to a Hamamatsu L702 (100/250) source and a Ximea 11Mp camera, was used. The scanning parameters were set as follows: isotropic voxel size = 0.68  $\mu\text{m}$ ; Source voltage = 92 kV, Source current = 66  $\mu\text{A}$ , Image rotation step = 0.3°, 360° rotation scan, and no filter. To capture the entire length of the larvae, four connected oversize scans were required. The Tiff images were reconstructed using the recent Bruker micro-CT NRecon software (v.2.0.0.5), which uses the single-distance phase-retrieval algorithm described by Paganin *et al.*<sup>30</sup> and enables the generation of phase-contrast-reconstructed enhanced images of soft tissues.

## Rendered images and supplementary videos

The Bruker micro-CT Skyscan software CTAnalysyer v.1.20.8.0 was used for primary ‘cleaning’ process. Resulting images were reoriented with DataViewer v.1.6.0.0, and CTvox v.3.3.1 was used to obtain 3D-rendered images of Figs. 1f, 4a, and Supplementary Video S2, as well as a vxm file for use in mobile devices (Supplementary 3D model S7). FEI’s Amira software (v. 2019.3)<sup>31,32</sup> was used to segment the structures and obtain 3D-rendered images (Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12). The built-in colour filter “volrenRed.col” was applied to obtain the colour of Amira’s rendered images (Figs. 3, 6, 8, 9, 10b-e), and the built filter “volrenWhite.col” was used to obtain the black/white of Amira’s rendered images. Different anatomical parts were independently segmented to obtain the rendered coloured images shown in Figs. 4b-d, and 5, 6, 7, 8, 9 and 10. To obtain the actual texture of structures in desired colours, after segmentation, each structure was subjected to the following arithmetic operation: A\*(B > 0), where A represents the whole animal and B represents the segmented structure. To colour the buccal cephalic structures in Fig. 2j and the muscles and mouthparts in Figs. 11 and 12, they were manually highlighted in Photoshop. The composition of figures and final editing of the supplementary videos were performed using PowerPoint 2019.

## Results

### External anatomy (Figs. 1f, 2 and Supplementary Videos S1, S2)

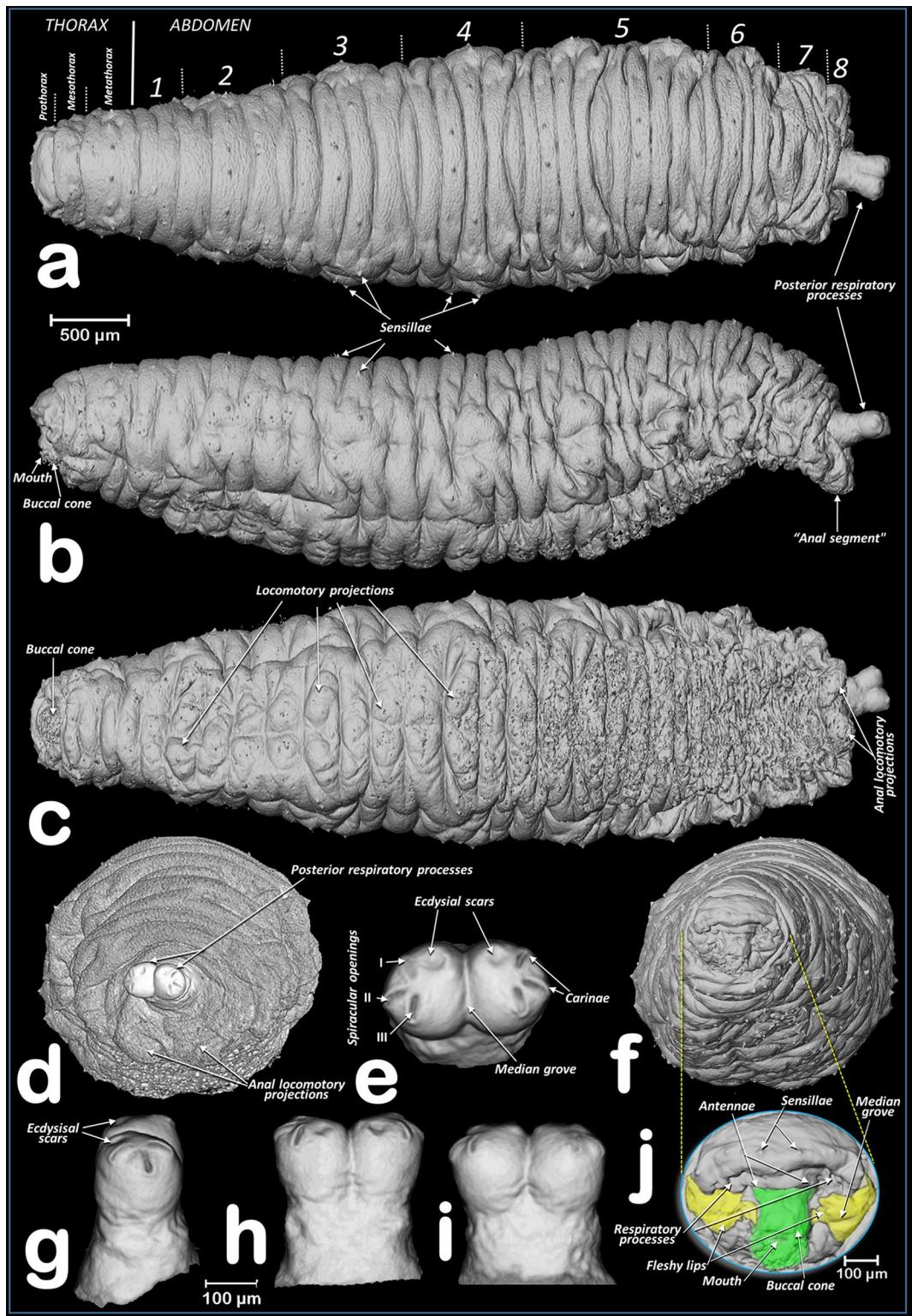
The body has transverse grooves (wrinkles) that give a ringed appearance in greater numbers than the body segments, and sensillae in the form of small protuberances with or without setae are observed on the surface (Fig. 2a-d, f, j). As in normal Syrphidae larvae, their mouthparts are hidden. In *S. rueppellii*, the mouth opens at the end of a mouth cone at the base, where there are a pair of triangular fleshy lips (one on each side). These images show a median groove on the surface (Fig. 2f, j). The short antennae protrude dorsally near the insertion points of the lips and on both sides of the mouth cone. The first apparent segments correspond to the thorax, with the metathorax being as long as the prothorax and mesothorax together (Fig. 2a, b). The abdomen has eight apparent segments (Fig. 2a) that present ventrally locomotory projections in pairs (Fig. 2c); the last pair (anal locomotory projections) is situated on the 8th apparent segment, which syrphidologists refer to as the “anal segment” (it is the result of the fusion of several segments, and Wipfler *et al.*<sup>20</sup> used the term “anal division” to refer to it) (Figs. 2c, d). Posteriorly, two well-sclerotised respiratory processes protrude, fuse along almost their entire length, slightly diverge apically and are separated by a median groove (Fig. 2a-e). In the posterior surface plane of each process, there are three slit-like spiracular openings (delimited by conspicuous carinae) and an ecdysial scar (Fig. 2e, g-i).

Both the antennae and anterior respiratory processes are retractile, and in the studied specimens, only the exit points were barely visible externally (Fig. 2j). Nevertheless, based on microtomography, the rendered images clearly show both the anterior respiratory processes (Figs. 4d 10d, 12) and antennae (Figs. 11a-c, 12). On the apical part of the antennae, there are two sensillae that give a Y-shaped appearance at the tip of each antenna (Fig. 12, and Supplementary Video S7).

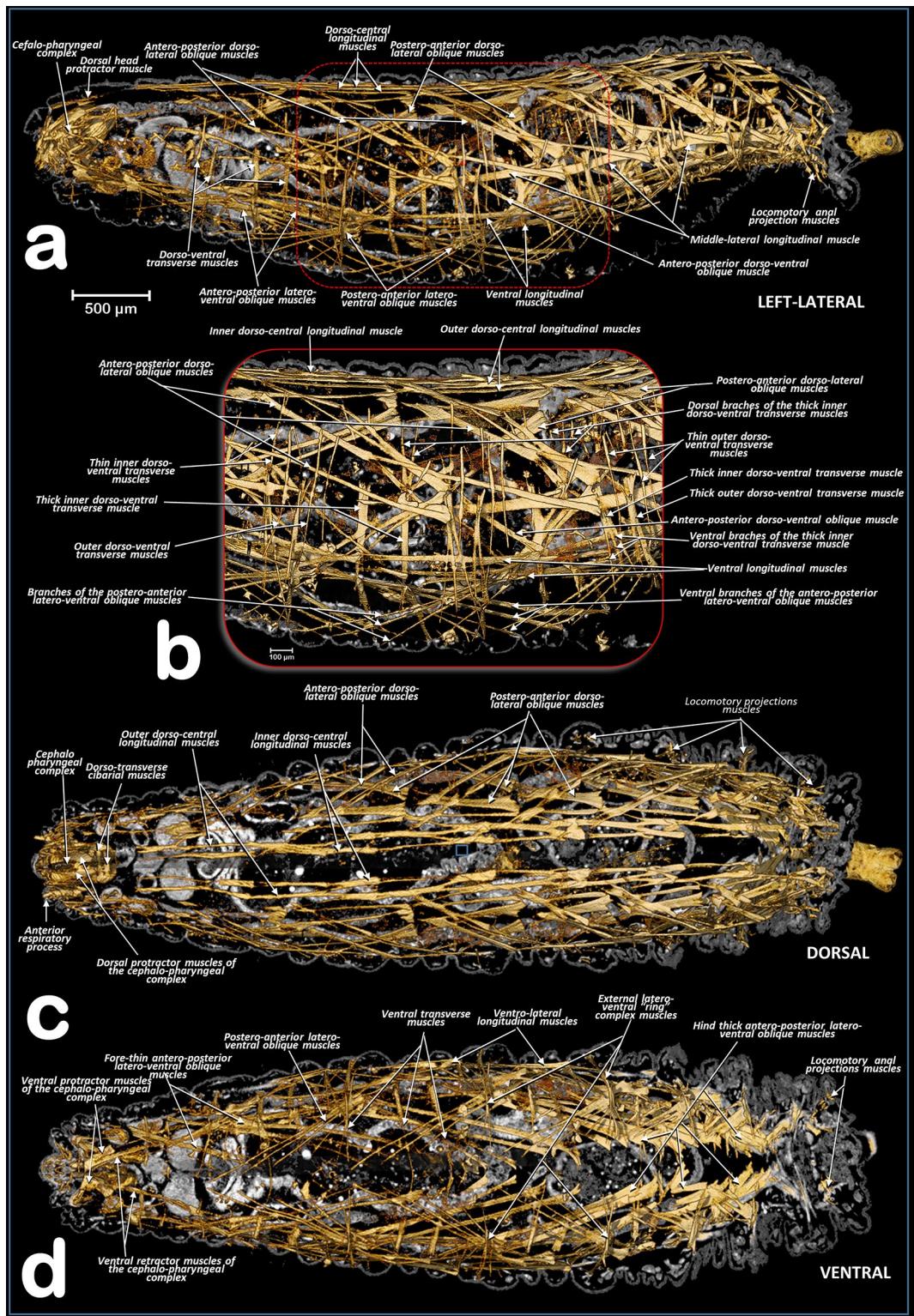
### Internal anatomy (Figs. 3–12 and Supplementary Videos S1-S7)

#### Body wall muscles (Fig. 3 and Supplementary Video S3)

Dorsally and in a medial position, two parallel bands of muscles, the “outer” and “inner” “dorso-central longitudinal muscles”, located on each flank of the dorsal vessel (Figs. 3a-b and 4d) are formed by the successive junctions of muscles of each segment, with a total of four muscle bands (compare Fig. 3c and 4d). In the rest of the body, there are muscle bands organised at three levels: the outermost layer is formed by segmental bands of fine muscles (“ventral transverse muscles”), some external (“outer dorso-ventral transverse muscles”) and some internal (“inner dorso-ventral transverse muscles”) (Fig. 3a-b). The latter are of two types “thin inner dorso-ventral muscles” and “thick inner dorso-ventral muscles”), between which pass oblique intersegmental bands that are inserted between the latero-dorsal and latero-medial zones (Fig. 3b). These oblique intersegmental bands are formed by the “antero-posterior dorso-lateral oblique muscles”, the “antero-posterior dorso-ventral oblique muscles” and the “postero-anterior dorso-lateral oblique muscles”, (Fig. 3c). Internal bands formed in the lateromedial areas of the posterior half of the body are thick “middle-lateral longitudinal muscles”. In the latero-ventral flanks are located “ventral longitudinal muscles”, “antero-posterior latero-ventral oblique muscles” and “postero-anterior latero-ventral oblique muscles” which, before inserting in the ventral area, divide into thin ventral branches giving a webbed appearance, in the same way as the “external latero-ventral muscles” (which come to form a real complex of outer circular muscles “external latero-ventral ‘ring’ complex muscles”). In the ventral view (Fig. 3d) thin “ventral transverse muscles” and thick muscular bands in the posterior third formed by the “hind thick antero-posterior latero-ventral oblique muscles” are observed. Small individual bundles of muscles from the locomotory projections, and especially the locomotory anal projections, are clearly visible (Fig. 3a, c-d).



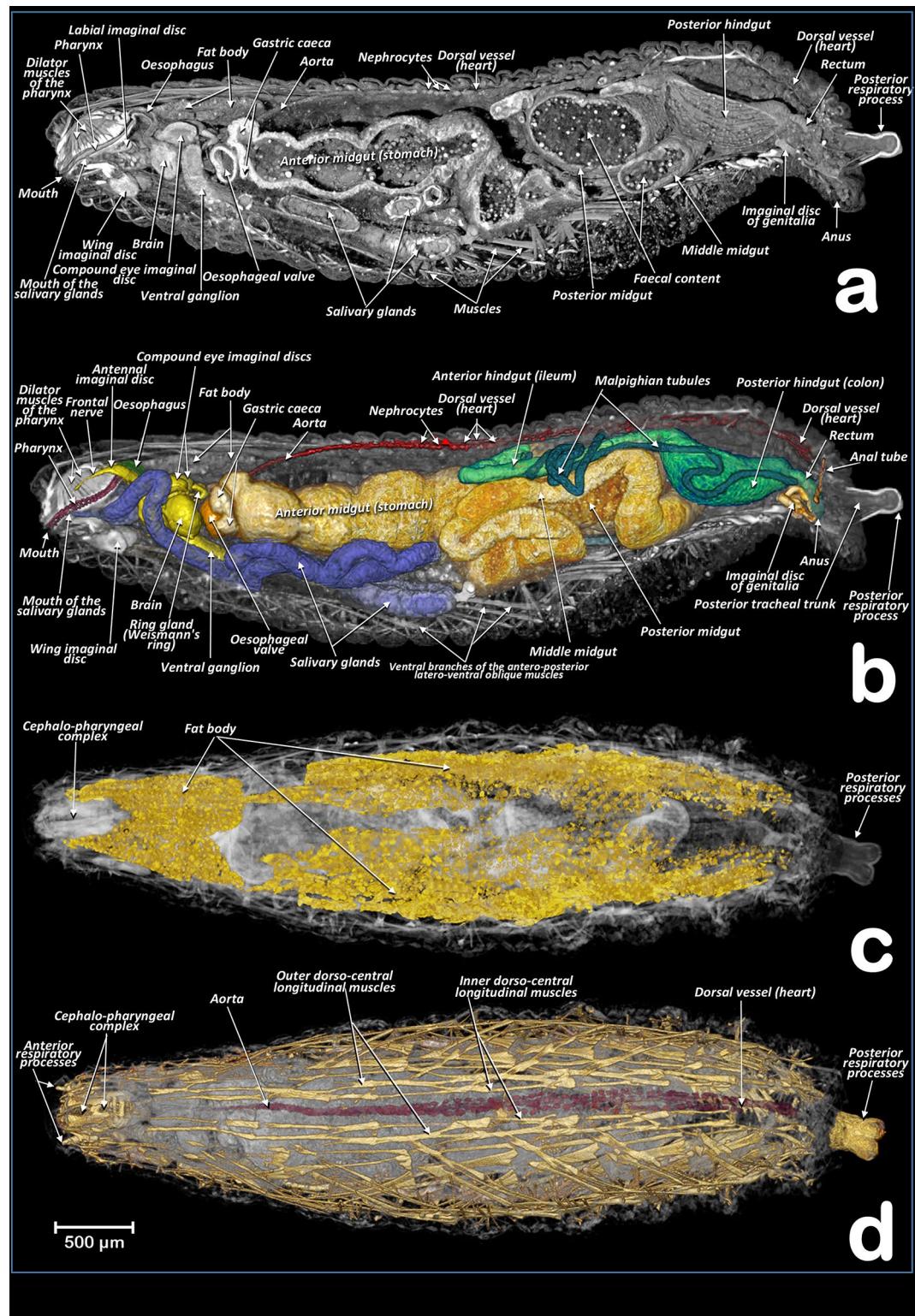
**Fig. 2.** Micro-CT 3D rendered images of the external anatomy of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives (a: dorsal, b: left-lateral, c: ventral, d: posterior, f, j: anterior). Details of the posterior respiratory processes from different perspectives (e: posterior, g: left-lateral, h: dorsal, i: ventral).



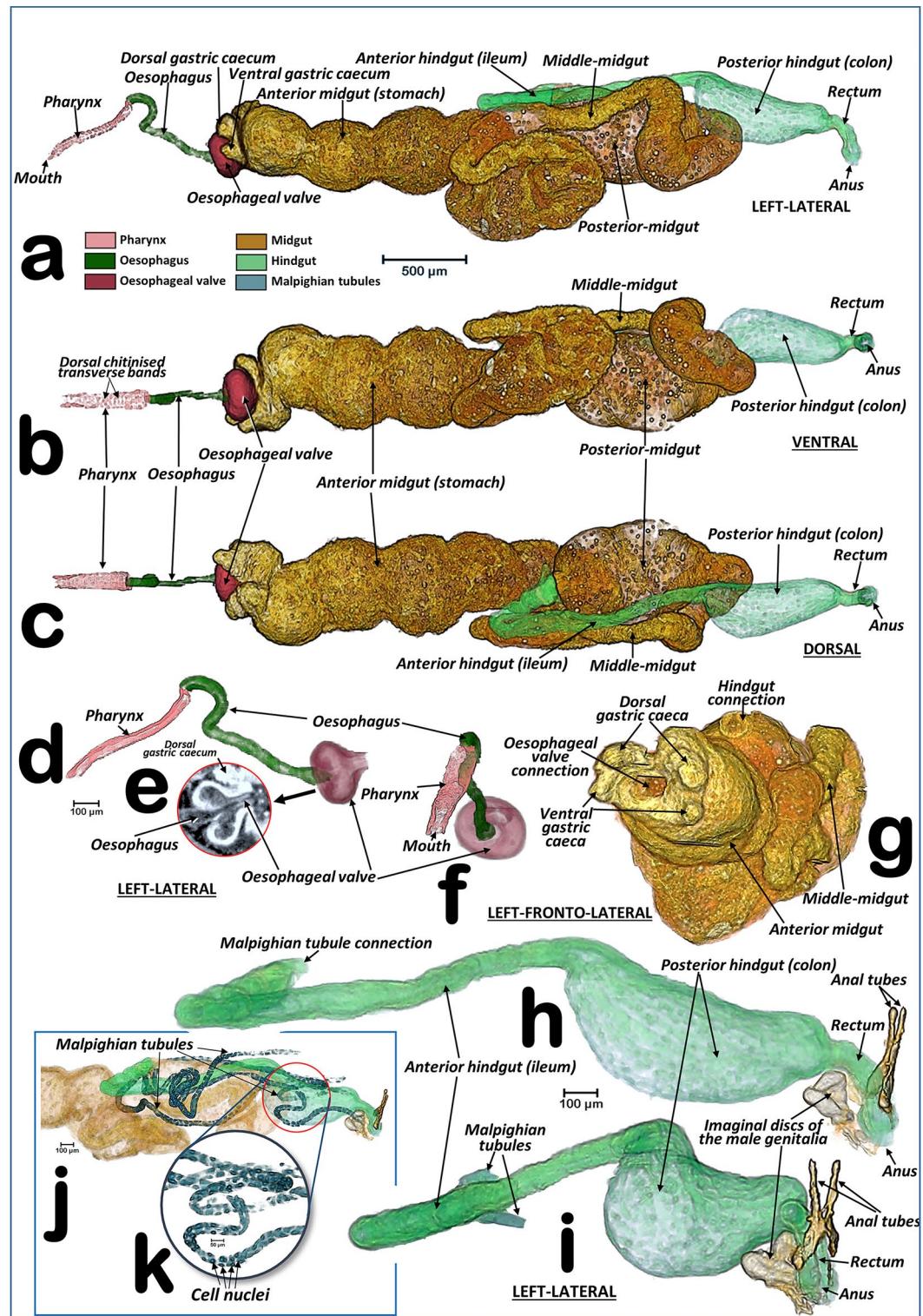
**Fig. 3.** Micro-CT 3D rendered images of the muscles of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives (**a, b**: left-lateral, **c**: dorsal, **d**: ventral).

#### Fat body (Fig. 4c)

The fat body forms an outer layer that occupies the dorsal and mediolateral regions, forming a single conspicuous mass in the anterior region, with two anterior extensions that surround the cephalopharyngeal complex, tapering posteriorly on both sides, and joining two broad bands that leave a central space corresponding to the position occupied by the dorsal vessel.



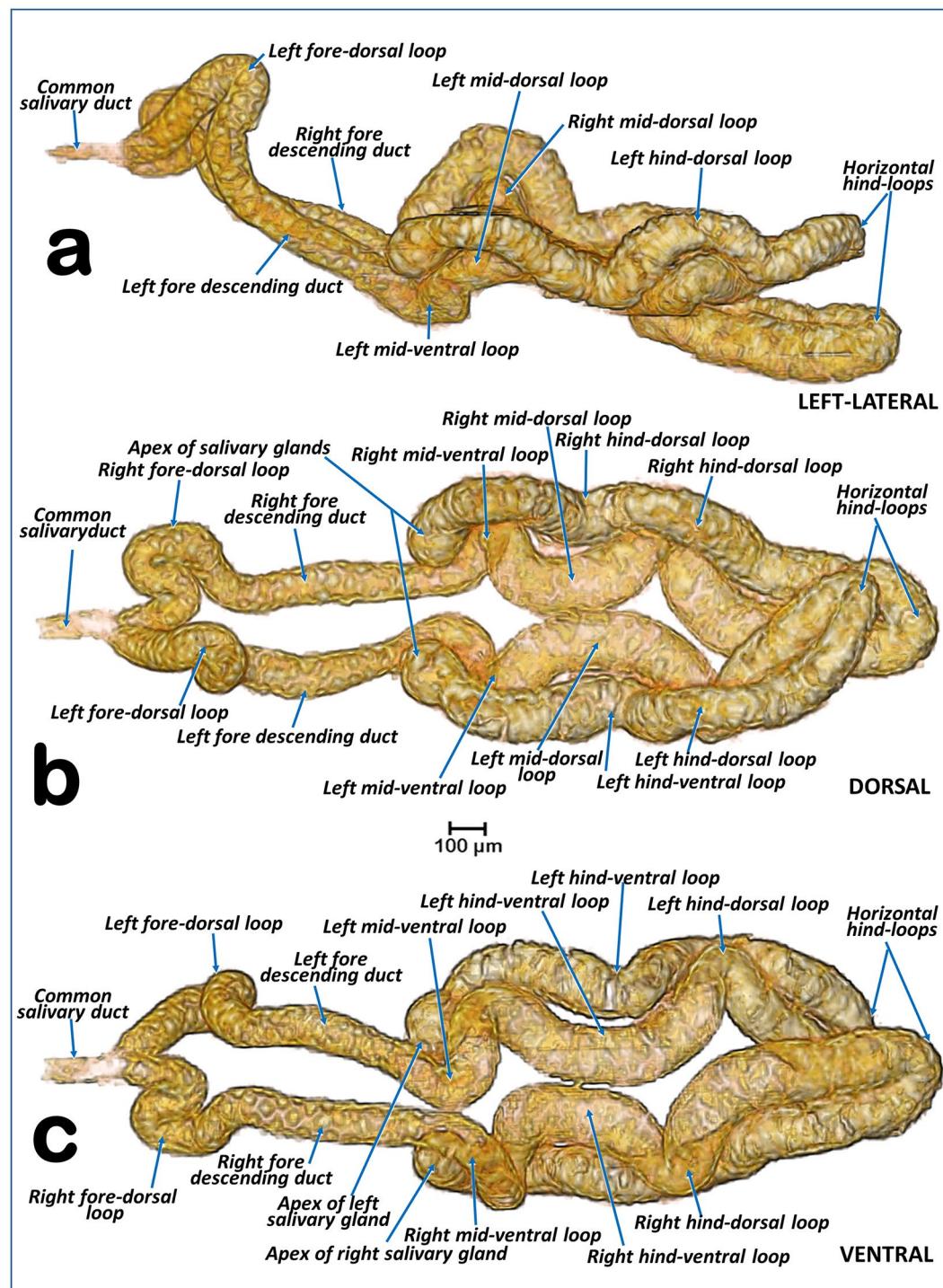
**Fig. 4.** Micro-CT 3D rendered images of the internal anatomy of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives (a,b: left lateral, c,d: dorsal). Sagittal section (a), internal structures (b), fat body (c), muscles, and dorsal vessel (heart) (d).



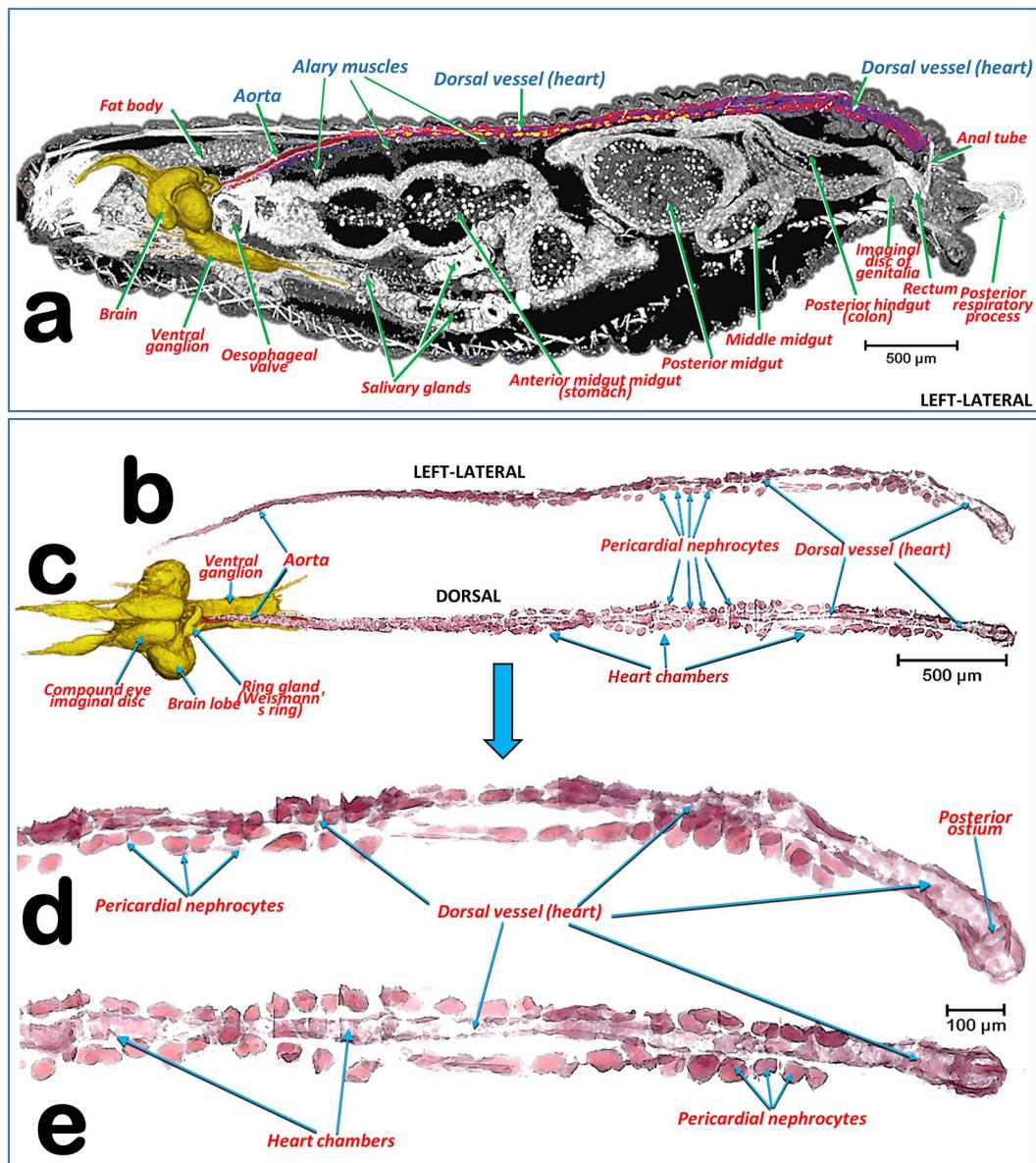
**Fig. 5.** Micro-CT 3D rendered images of the digestive system of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives: left-lateral (**a, d, e, h, j, k**), ventral (**b**), dorsal (**c**), left-frontal (**f, g**), and left-posterior (**i**). Detail of the foregut (stomodaeum) (**d, f**) and a virtual 29 µm slice of the oesophageal (stomodaean) valve (**e**). Midgut foreshortened view (**g**). Details of the hindgut (**h–k**) and Malpighian tubules (**j, k**). **Fig. 5.**

The digestive system and associated structures (Figs. 4a, b, 5, 6, 7a, 8a, 10a, b, d, 11, and Supplementary Videos S1, S2, S4, S5)

The buccal opening, situated at the tip of the buccal cone, continues with a preoral cavity (atrium), surrounded by mouth parts (Fig. 10a), which gives way to the pharynx, which, crossing the cephalopharyngeal complex, is of an oblong section (Fig. 11d), with chitinized transverse rings (Fig. 5b), and on its dorsal external face are inserted strong dilator (cibarial) muscles (Figs. 4a, b, 8a, 10a, b, d, 11, 12a). The pharynx gives way to a narrower oesophagus of circular section that bends obliquely backwards and passes through the brain through a brain foramen oesophageal passage (Fig. 8e,f), connecting with the midgut through an oesophageal valve of the spherical external aspect (Figs. 4a, b, 5a-f, 7a, 8a). At the point of connection, the midgut extends anteriorly



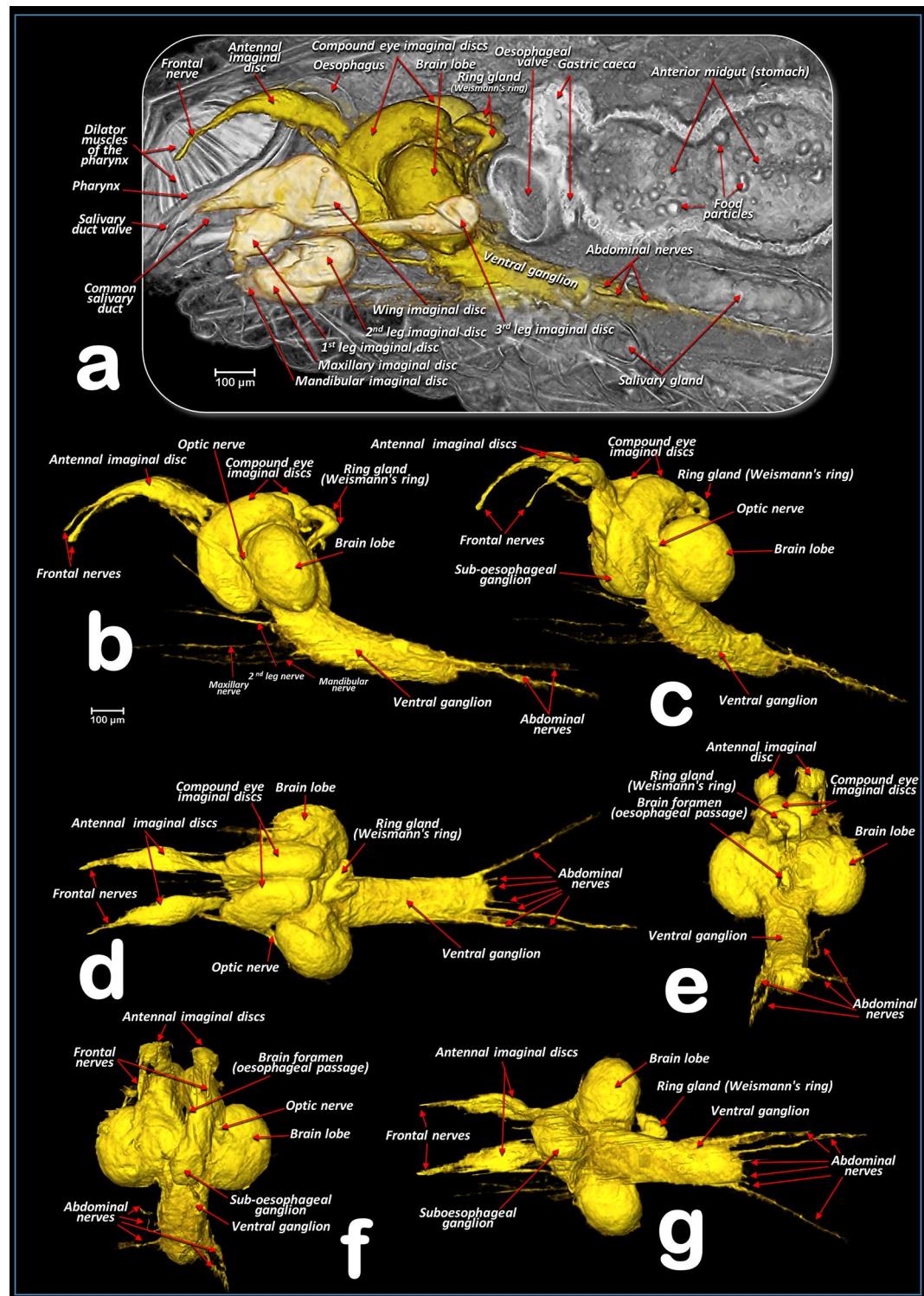
**Fig. 6.** Micro-CT 3D rendered images of the salivary glands of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives: left lateral (a), dorsal (b), and ventral (c).



**Fig. 7.** Micro-CT 3D rendered images of the circulatory system of the third instar larva of the hoverfly *Sphaerophoria rueppellii*. General location with respect to the remaining internal structures in the left sagittal view in a 109  $\mu\text{m}$  virtual section on which the circulatory and anterior nervous systems are superimposed (a). Circulatory system in the left lateral (b, d) and dorsal views (c, e).

into four short finger-shaped gastric caeca (two dorsal larger and two ventral shorter) (Figs. 4b, 5a-c, g). Three parts are distinguished in the midgut: a) the anterior midgut with three successive spherical chambers at the end of which a wide posteriorly directed dilated cavity is bent ventrally and widens; b) the middle-midgut, which starts from the last cavity of the anterior midgut and continues in a narrow tube that bends anteriorly and runs dorsally for a length equal to that of this final wide cavity of the anterior midgut, bending and continuing in a posterior direction; and c) the posterior-midgut, a wide anteriorly oriented cavity formed when the middle-midgut bends ventrally and widens (Figs. 4a, b, 5a-c, 7a, 8a). After the dilated posterior midgut, the digestive tract narrows into a tube that the Malpighian tubules enter. From there, the hindgut begins, in which a narrow anterior hindgut (ileum) is distinguished, which turns and runs posteriorly until it communicates with a dilated cavity, the posterior hindgut (colon), which narrows into a short rectum that leads to the exterior through the anus (Figs. 4a, b, 5a-c, h,i), situated in a ventral position immediately posterior and flanked by the anal locomotory projections (Figs. 2c, 4a, b).

In the anterior half of the body, there is a pair of thick tubular salivary glands (Figs. 4a, b, 6, 7a, 8a, 10a, and Supplementary Video S5). Situated ventrally, they join anteriorly in a common salivary duct that leads to the ventral side of the anterior part of the pharynx (Figs. 4a, b, 8a). The mouth of the salivary glands (Fig. 4a) is passed through a salivary duct valve (Fig. 8a). From the distal area (point of junction with the common salivary



**Fig. 8.** Micro-CT 3D-rendered images of the larval nervous system of the third instar larva of the hoverfly *Sphaerophoria rueppellii*. General location with respect to the fore internal structures in 189 µm left sagittal view virtually sliced on which the nervous and imaginal disks are superimposed (**a**). Different perspective views: left lateral (**b**), ventral-lateral (**c**), dorsal (**d**), posterior (**e**), anterior (**f**), and ventral (**g**).

duct), each tube is directed posteriorly in several loops (symmetrical on both sides) until half the length of the body, where they bend horizontally towards the anterior half, so that the proximal area of each tube is situated in the anterior ventral third (Fig. 4b). A detailed study of the different parts of the salivary glands is presented in Fig. 6 and Supplementary Video S5, in which the rendered images show in detail the different loops and even the cells of the glandular tubules.

A pair of Malpighian tubules extend into the posterior third of the body, leading into the digestive tract at the point marking the separation of the midgut and hindgut (Figs. 4b, 5j, k), and Supplementary Videos S1, S2, and S4). The rendered images show the cell nuclei (Fig. 5k).

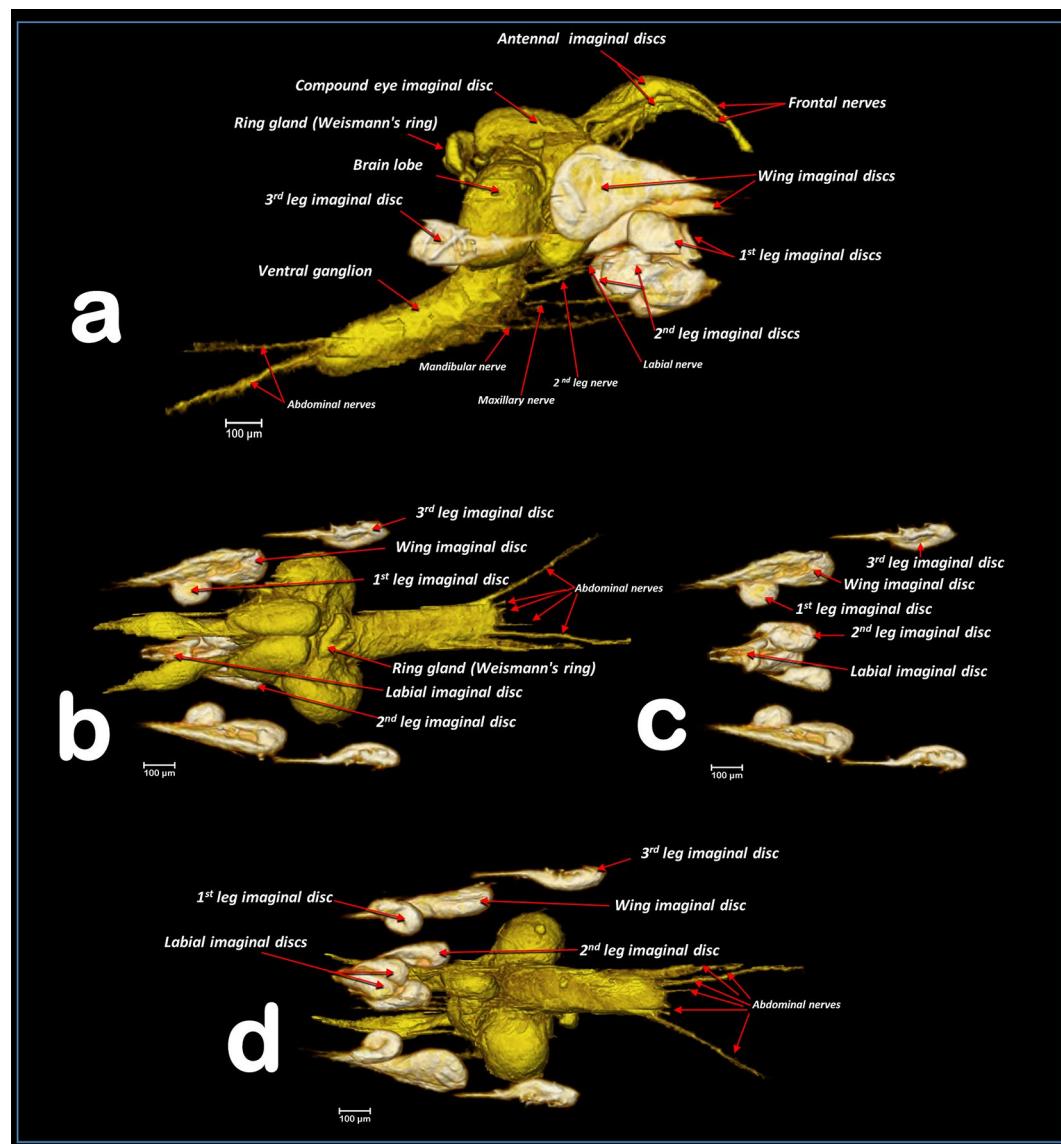
On the posterior part of the hindgut before the anus, a pair of anal tubes positioned vertically led to both sides of the rectum (Figs. 4b, 5h-j, 7a, and Supplementary Videos S1, S4).

#### *Imaginal discs (Figs. 4a,b, 5h-j, 7a, 8a, 9, 10a, and Supplementary Videos S1, S4 and S6)*

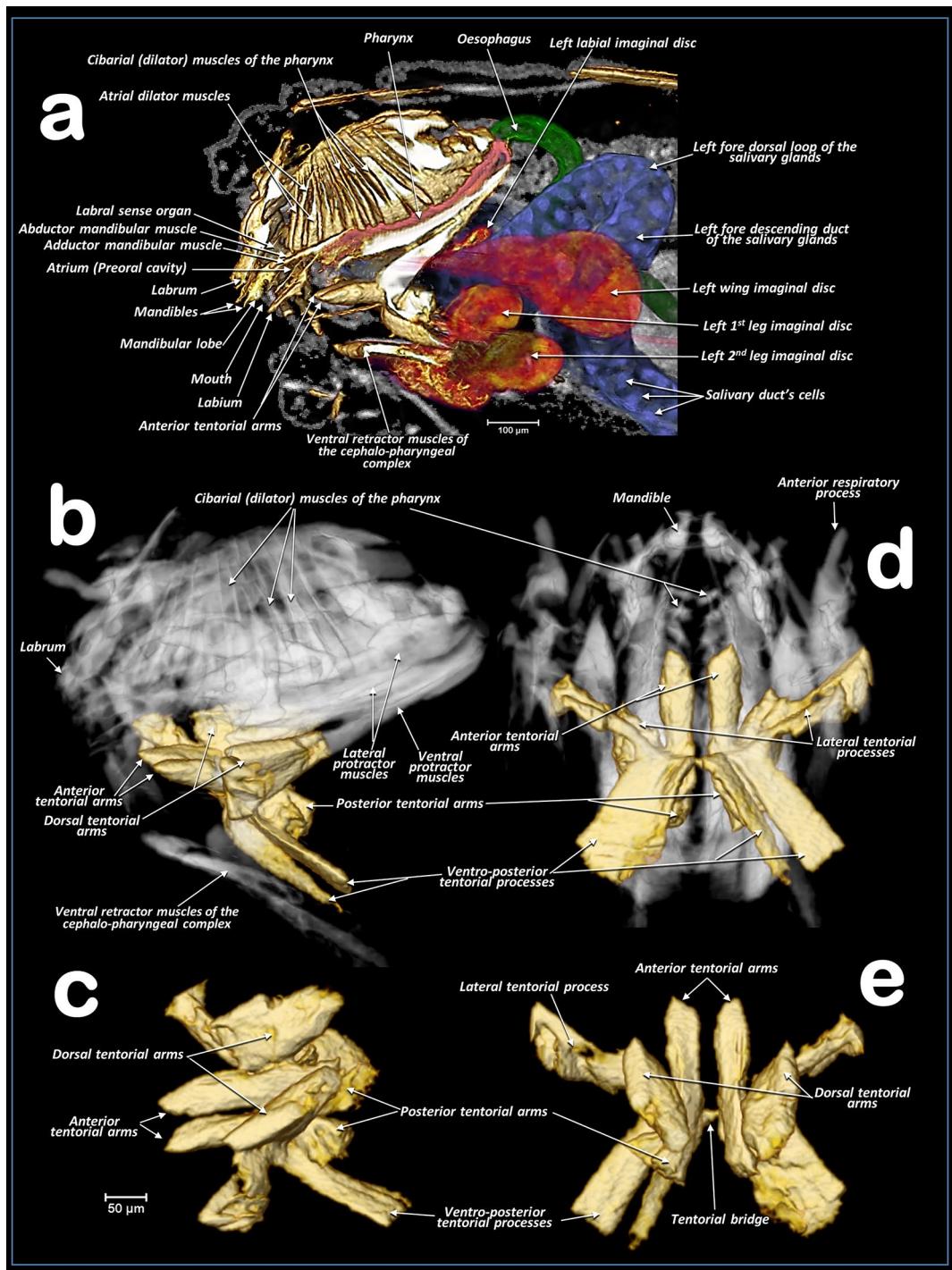
In the anterior part of the body, on both sides of the central nervous system and ventrally to it, there are some ampulla-shaped structures that correspond laterally to: wing imaginal discs, legs imaginal discs (1st to 3rd), and in the most ventral position the labial imaginal discs. In addition, both the compound eye and antennal imaginal discs are directly connected to the brain. Moreover, ventral to the rectum, the imaginal discs of genitalia have been observed.

#### *Central nervous system (Figs. 4a, b, 7a, c, 8, 9, and Supplementary Video S1, S2, S6)*

Consists of a bilobed brain. Each lobe is connected fronto-dorsally to a compound eye imaginal disc with an optic nerve, and two nerves emerge from the brain. These nerves thicken into antennal imaginal discs that pass through the cephalopharyngeal complex and thin out to a frontal nerve each at the level of the pharyngeal dilator muscles. Ventrally, the brain connects with a conspicuous subesophageal ganglion. A brain foramen oesophageal passage is observed (Fig. 8c, f) resulting from massive periesophageal connectives connecting with



**Fig. 9.** Micro-CT 3D rendered images of the larval nervous system and imaginal discs of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives: right-lateral (a), dorsal (b, c), and ventral (d).



**Fig. 10.** Micro-CT 3D rendered images of the cephalopharyngeal complex of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives (**a**, **b**: left-lateral, **c**: dorsal, and **d**: ventral). Sagittal section view (**a**). Progressive transparency to isolate the tentorium (**b-e**).

a large ventral ganglion, of which the rendered images show different abdominal nerves directed posteriorly and forward depart, mandibular, maxillary, labial, and leg nerves (Figs. 8b, d-g, 9a, b-d). In the mid-dorsal position, behind the compound eyes imaginal discs appears the ring gland (Weismann's ring) (Figs. 8a, b, d-e, 9a, b).

#### Dorsal vessel (heart) (Figs. 4a, b, d, 7, and Supplementary Videos S1,S2)

Located immediately below the body wall in the dorsal position. It runs from the posterior part of the body, where it is widened, and there is a posterior ostium slit-shaped (Fig. 7d), narrowing very much (Fig. 7b, c), and heart chambers (Fig. 7c, e). It is directed towards the anterior region, bending ventrally in the anterior third to form the aorta (Figs. 4a, b, 7a, b), which passes through the ring gland (Weismann's ring) (Fig. 7a, c) and ends

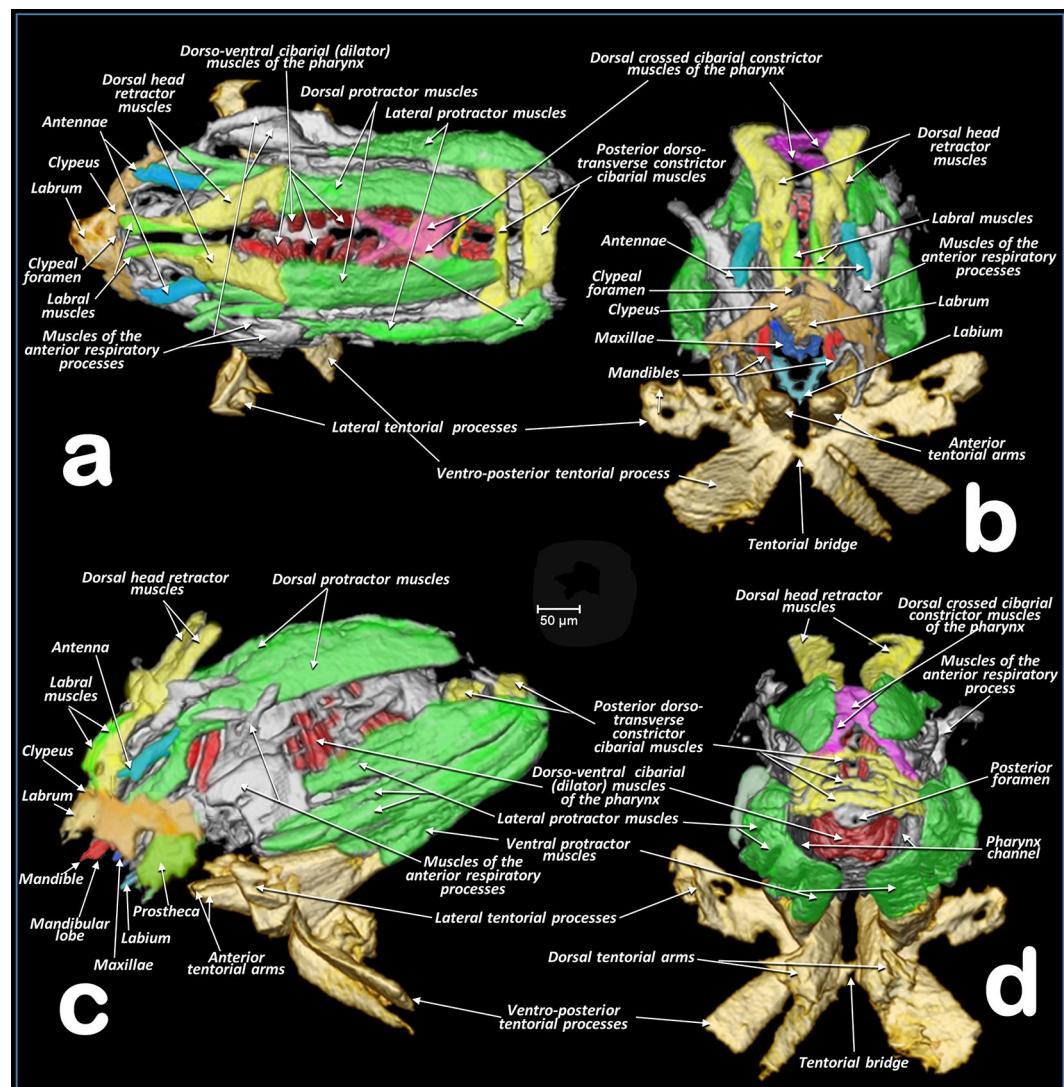
anteriorly between the brain lobes. The rendered images also show the alary muscles attached to the dorsal vessel walls with the narrow end directed ventrally (Fig. 7a). On both sides of the dorsal vessel are spherical pericardial nephrocytes in the form of small spheres, which together have a rosary appearance (Figs. 4a, b, 7).

*Tentorium* (Figs. 10, 11, and Supplementary Video S7).

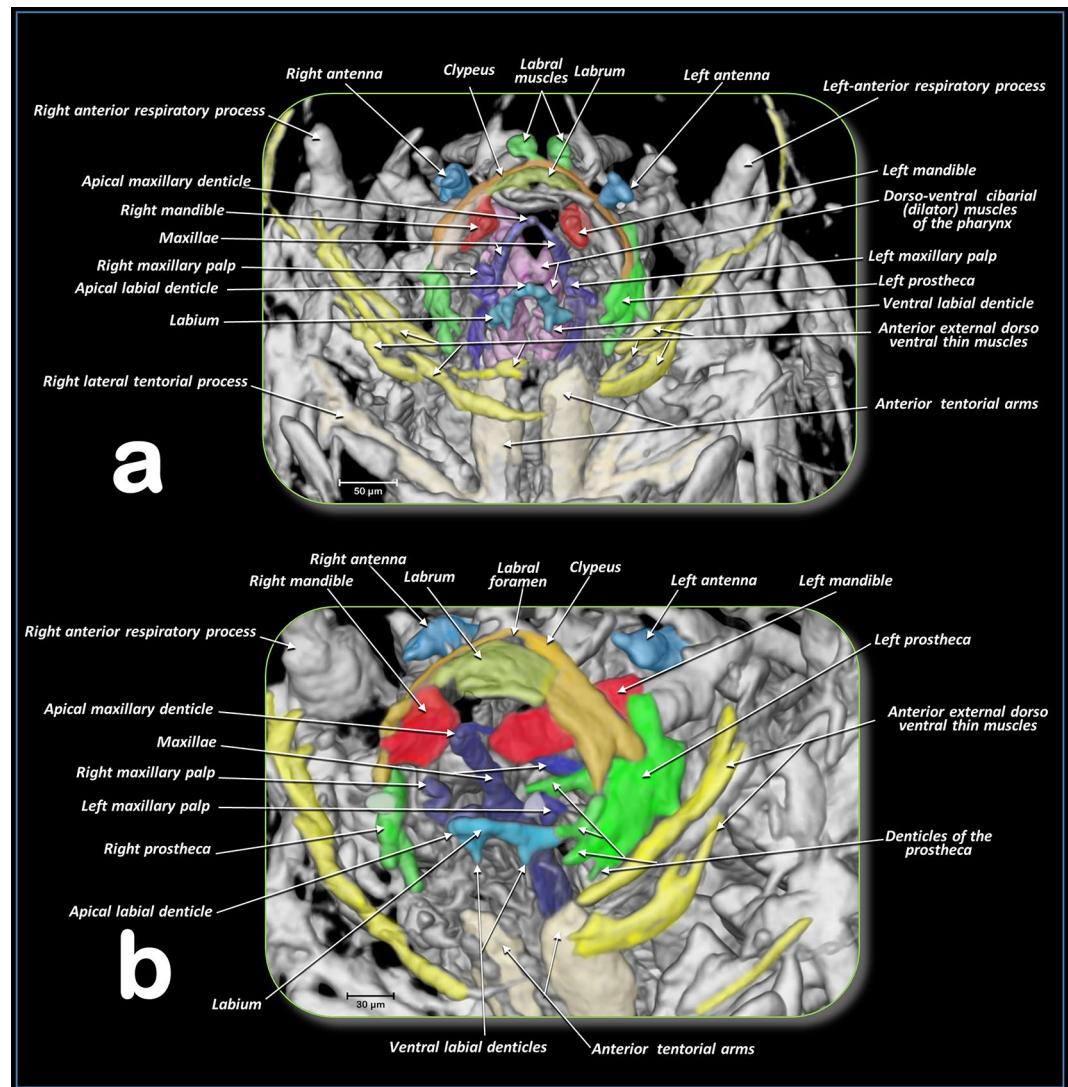
The tentorium consists of two structures joined by a tentorial bridge that gives it the appearance of the typical H-shaped tentorium of insects. There are two anterior tentorial arms directed forward and ending in a slightly tapering point, two short, massive dorsal tentorial arms, two short and thick posterior tentorial arms, a pair of lateral tentorial processes directed obliquely forward, and four long ventro-posterior tentorial processes directed obliquely to the ventral side.

*Cephalopharyngeal complex and mouth parts* (Figs. 3a, c, d, 4, 8a, 10–12, and Supplementary Video S1, S2, S7)

The highly mobile structure called the cephalopharyngeal complex allows the mouthparts to project outward and exhibits a well-developed musculature. In Fig. 11, the longitudinal musculature is highlighted in green ("dorsal protractor muscles", "lateral protractor muscles", "ventral protractor muscles", and in the anterior dorsal part the "labral muscles"). The posterior transverse musculature is highlighted in yellow ("posterior dorso-transverse constrictor cibarial muscles") and in the anterior dorsal part the "dorsal head retractor muscles". Posteriorly, and below the dorsal protractor muscles, are in purple the dorsal crossed cibarial constrictor muscles. of the pharynx and in red (except in Fig. 1 that are highlighted in purple) the "dorso-ventral cibarial dilator muscles of the pharynx", which allow the cibarium to function as a suction pump. The muscles of the anterior respiratory processes are inserted in the anterior flanks (Figs. 10d, 11).



**Fig. 11.** Micro-CT 3D rendered images of the cephalopharyngeal complex of the third instar larva of the hoverfly *Sphaerophoria rueppellii* from different perspectives (a: dorsal, b: frontal, c: left-lateral, and d: posterior).



**Fig. 12.** Micro-CT 3D rendered images of the internal anatomy of the anterior region of the third instar larva of the hoverfly *Sphaerophoria rueppellii* showing mouthparts and other structures in the ventral (a) and left-latero-ventral (b) views.

The clypeus appears as a narrow band with a median clypeal foramen, to which is attached in the middle part a trapezoidal-shaped labrum, with the anterior part narrower than the posterior part (Figs. 11a-c, 12b). Internally, just below the clypeus at its junction with the labrum is the labral sense organ (Fig. 10a). The mandibles in the lateral view are tapered at the end, with a subterminal mandibular lobe in the ventral part, visible abductor and adductor muscles (Figs. 10a, 11c), and at their base are articulated prosthecae with distal sharp denticles (Figs. 11c, 12). The maxillae join to form an arch with an apical denticle in the weld zone, and an articulated maxillary palpus protrudes on each side. Similarly, the labium is arc-shaped, with a conspicuous apical labial denticle and one ventral labial denticle on each side (Figs. 11b, 12).

## Discussion

The external anatomical details that appear in the rendered micro-CT images are equivalent to those previously obtained by SEM for *S. rueppellii* by Orengo et al.<sup>15</sup>, with surface sculpture and precise distribution of the thoracic and abdominal sensillae; even the fleshy lips at the base and close to the buccal cone (first time described here) show a conspicuous median groove. Moreover, the rendered images of the posterior respiratory processes obtained in this study were more detailed than those obtained by SEM. The rendered images of the transparent specimen showing the distribution of fat body were almost identical to the figure in Orengo et al.’s paper on a preserved specimen.

The body musculature of some dipteran larvae, as the house fly, or the apple-<sup>33</sup> maggot were studied by Hewitt<sup>33</sup> and Snodgrass<sup>34</sup>, respectively. Particularly *Drosophila melanogaster* Meigen, 1830 larvae, has been well studied<sup>20,35–40</sup>, and Diesner et al.<sup>41</sup> recently published a study of the musculature of the abdominal segments of *Ceratitis*, comparing it with *Drosophila* larvae and relating it to the jumping ability of *Ceratitis* larvae. With

respect to the Syrphidae, the body musculature of *Volucella zonaria* (Poda, 1761) larvae was studied at the end of the nineteenth century by Künckel d'Herculais<sup>42</sup>, although his drawings, too perfect, are not very realistic. Similarly, Andries<sup>43</sup>, at the beginning of the twentieth century, published a cross-sectional diagram of the muscles of the body of a larva of the genus *Microdon* Meigen, 1803. *Microdon* species and *V. zonaria* larvae live in association with nests of Hymenoptera species, ants, and wasps, respectively<sup>44,45</sup>, with different biology and behaviours from those of *S. rueppellii*; therefore, it is not expected that they share similarities regarding larval musculature. However, surprisingly, the work of Hartley in 1959<sup>46</sup> on the saprophagous syrphid larvae of *Eristalis* Latreille, 1804 is noteworthy for its special detail and real appearance, similar to what we obtained using micro-CT. As summarised in Table 1, the names of the different muscles vary from one author to another. Even authors such as Wipfler et al.<sup>20</sup>, Bate<sup>36</sup> or Anderson<sup>47</sup> used a numerical correlative system to refer to the different muscles. Furthermore, the muscular arrangement observed in the larvae of *S. rueppelli* is not the same as those already described in *Drosophila* larvae and other dipteran larvae. Therefore, we have chosen to name the different muscles according to their anatomical position and function. To the best of our knowledge, this study represents the first detailed study of the musculature of a Syrphinae larva that searches for and captures prey. The images show the complexity of muscles that permit movements in every direction, which is similar to that observed in many annelid worms. This similarity to the movements of annelids was recently highlighted in a study of *D. melanogaster* larvae by Wipfler et al.<sup>20</sup>, noting that "it comprises only longitudinal and dorsoventral muscles, whereas ring muscles of the body wall are lacking, as in other adult or immature insects". Despite not having circular musculature in the body wall, in this study, we found an external lateroventral complex of muscles that conform to functional muscle rings allowing compression of the body segments, which produces

Names used in this study	Other used terms		
	Other Syrphidae species	<i>Drosophila</i>	Other Dipteron (House fly /apple-maggot)
<b>Antero-posterior dorso-lateral oblique muscles</b>		Dorsal external oblique(part.); Dorso-lateral external longitudinal <sup>39</sup> (part.); Pleural internal oblique; Oblique lateral external <sup>35</sup> ; Dorsal oblique muscles <sup>40</sup>	External dorso lateral oblique <sup>33</sup> / Dorsales externi mediales <sup>34</sup>
<b>Antero-posterior dorso-ventral oblique muscles</b>	Posterior oblique muscles <sup>42</sup>	Dorso-lateral internal longitudinal <sup>39</sup>	Dorsales interni laterales <sup>34</sup>
<b>Antero-posterior latero-ventral oblique muscles</b>		Ventro-lateral external oblique <sup>39</sup> ; Ventral external oblique (L-M) <sup>39</sup> , Ventral oblique <sup>40</sup>	Ventral oblique/Ventro-lateral oblique <sup>33,34</sup>
<b>External latero-ventral "ring" complex muscles</b>			
<b>Fore-thin antero-posterior ventral transverse muscles</b>			
<b>Hind thick antero-posterior latero-ventral oblique muscles</b>			
<b>Inner dorso-central longitudinal muscles</b>	M. recti dorsalis <sup>42</sup> ; Longitudinal segmental muscles <sup>42</sup> (part.); Longitudinal segmental muscles <sup>32</sup> (part.); Dorso longitudinal oblique muscles <sup>5,46</sup> (part.);		
<b>Locomotory anal projection muscles</b>			
<b>Locomotory projections muscles</b>	Proleg retractor muscles <sup>42,46</sup> (part.)		
<b>Middle-lateral longitudinal muscles</b>	Longitudinal segmental muscles <sup>5,46</sup> (part.); Longitudinal intersegmental	Longitudinal lateral internal <sup>35</sup> ; Longitudinal lateral <sup>40</sup>	
<b>Outer dorso-central longitudinal muscles</b>	M. recti dorsalis <sup>43</sup> ; Longitudinal segmental muscles <sup>42</sup> (part.); Dorso longitudinal oblique muscles <sup>5,46</sup> (part.);	Longitudinal dorsal intermediate <sup>35</sup>	
<b>Outer dorso-ventral transverse muscles</b>			
<b>Postero-anterior dorso-lateral oblique muscles</b>	Dorso longitudinal oblique muscles <sup>42</sup> ; Posterior oblique muscles <sup>5,46</sup> (part.)	Dorsal internal oblique (L-M) <sup>39</sup> ; Oblique lateral intermediate <sup>35</sup>	Internal dorso-lateral oblique recti <sup>33</sup> / Dorsales intermediales <sup>34</sup>
<b>Postero-anterior latero-ventral oblique muscles</b>	Anterior oblique muscles <sup>42</sup> (part.)	Ventral external/lateral oblique (M-L) <sup>39</sup> (part.); Ventral external oblique(L-M) <sup>39</sup> (part.); Oblique ventral external <sup>35</sup> ; Oblique ventral intermediate <sup>35</sup>	Ventro-lateral oblique <sup>33</sup>
<b>Thick inner dorso-ventral transverse muscles</b>	Inner vertical muscles <sup>5,42,46</sup> (part.)	Pleural external transverse <sup>39,40</sup>	Lateral <sup>33</sup> / Laterales externi <sup>34</sup>
<b>Thick outer dorso-ventral transverse muscles</b>	Outer vertical muscles <sup>42</sup>	Dorso-lateral oblique external <sup>39</sup>	
<b>Thin inner dorso-ventral transverse muscles</b>		Pleural internal transverse <sup>39</sup>	
<b>Thin outer dorso-ventral transverse muscles</b>		Transverse lateral external <sup>35</sup>	
<b>Vento-lateral longitudinal (Ventral longitudinal) muscles</b>	Longitudinal intersegmental muscles <sup>42</sup> ;	Ventral internal longitudinal <sup>39,35</sup> ; Ventral longitudinal <sup>40</sup>	Longitudinal ventro-lateral <sup>33</sup> / Ventrales interni laterales <sup>34</sup>
<b>Ventral transverse muscles</b>		Ventral transversal <sup>40</sup>	
<b>Ventral branches of the antero-posterior latero-ventral oblique muscles</b>	Posterior oblique muscles <sup>5,46</sup> (part.)	Ventral external oblique(L-M) <sup>39</sup> (part.)	
<b>Ventral branches of the postero-anterior latero-ventral oblique muscles</b>	Anterior oblique muscles <sup>5,46</sup> (part.)	Ventral external oblique(L-M) <sup>39</sup> (part.); Ventral oblique muscles <sup>40</sup>	

**Table 1.** Body wall muscle names used in this study and alternative terms used for the same muscles are listed with references.

the typical elongation and pecking movements of larvae to search for and capture prey. Thus, as the general cavity is filled with haemolymph, the contraction of these ring muscles would perform a function similar to that performed by the external circular muscle layer of the annelids, allowing the length of the retracted segments to be restored by a simple hydraulic principle. The most striking feature is the large development of muscles in the posterior ventral half of the body, which we have called "hind thick antero-posterior latero-ventral oblique muscles" which, when contracted, facilitate lifting of the larva until it is strongly held by the anal locomotory projections. This movement is performed during the active hunting-predation movements in a manner similar to that of other carnivorous syrphid larvae<sup>48</sup>. In this context, the cephalopharyngeal complex plays an important role, prolapsing externally and moving in all directions because of the strong dorsal, lateral, and ventral musculature and the existence of both protracting and retracting muscles. The strong mandibles ending in a sharp hook and with huge prosthecae help to bite and hold prey tightly. What we have interpreted as prosthecae, so far, is a separate structure with different names (i.e.: maxillary outer tray—"ausenladen der maxillen"—<sup>17</sup>, of triangular sclerites<sup>13,16,19</sup>, labial sclerites<sup>12,49</sup>, lateral lips<sup>15</sup>, hooks<sup>50</sup>). However, as shown in the micro-CT images, these are pieces with strong teeth at the end that articulate basally and laterally to the mandibles and clearly serve to complement their function. Similar prosthecae are found in other Hexapoda<sup>51</sup>, such as Diplura, and immature ontogenetic stages in Ephemeroptera<sup>52,53</sup>, Odonata<sup>54</sup>, and some Neoptera<sup>55</sup>, including some dipteran families<sup>3,4,56</sup>. Moreover, the larvae of *S. rueppellii* show the same asymmetry as that observed in Ephemeroptera, with the left prostheca larger than the right. Both the maxillae and labium form a pointed arch with denticles that help pierce the body of the prey, as previously observed<sup>5</sup>.

Following the publication of Rotheray<sup>57</sup>, evidence conflicts have arisen that have resulted in debates about the origin of the mandible and antennomaxillary organs<sup>58–60</sup>. These uncertainties represent a significant obstacle to their progress<sup>61</sup> and have undoubtedly led to larvae being considered challenging to analyse and discouraged further<sup>55,62</sup>. In contrast, our results shed light on these uncertainties and highlight possible clarifications. First, the antennae are clearly separated from the maxillae. The distal part of the antennae appeared to be cleft with sensilla at their ends, which was also observed in the SEM images of the antennomaxillary organ of *S. rueppellii* by Orengo et al.<sup>15</sup>, and which would correspond to what have been called dorsal and ventral organs<sup>5</sup>, Hartley<sup>12</sup> described it for *Eristalis* as an antennomaxillary organ (first described as "antenno-maxillary complex" by Keilin<sup>63</sup>, and figured for several species by Bathia<sup>19</sup>). Similarly, Roberts<sup>5,14</sup> illustrated it from a larva of the genus *Eumerus* Meigen, 1822, and the ventral part was considered to correspond to the maxillary palp. However, our results clearly show that the maxillae are separated and have articulated maxillary palps. Therefore, in the larvae of *S. rueppellii*, the so-far considered antennomaxillary organ corresponds in reality to the antennae, which, as in other insects, have mechanoreceptor and chemoreceptor (gustatory and olfactory) sensillae in the distal zone (i.e<sup>54,65</sup>).

Within the cephalopharyngeal complex, the observed structure of the cibarial pump, with the atrial and pharyngeal dilator muscles, is comparable to that observed in *Eristalis*<sup>12,66</sup> and *Eumerus*<sup>14</sup> larvae, even though what they call the pharyngeal constrictor muscles correspond to the dorsal cibarial constrictor muscles of the pharynx that we observe in *S. rueppellii*.

In insects, the alimentary canal, from the mouth to the anus, consists of three main parts: foregut (stomodaeum), midgut (mesenteron), and hindgut (proctodeum)<sup>67–69</sup>. The stomodaeum includes the pharynx and oesophagus. The pharynx is much wider and has strong dilator muscles inserted in the dorsal chitinised bands of the dorsal external face. Because it is an oblong section, it can easily be dilated by the action of the muscles, thereby facilitating the entry of liquid food from the prey. The oesophagus flows into the midgut through an oesophageal valve (known in other insects as cardia, stomodaeal or cardiac valve<sup>67</sup>). The thickening of this structure has led to it being erroneously called proventriculus (i.e<sup>17</sup>), but it is not a true proventriculus as it is not involved in the crushing or cutting of food; its function is to continuously secrete the peritrophic membrane (see<sup>5</sup>). There has been some controversy as to whether the thickening belongs to the foregut or midgut<sup>17,43</sup>. Observing anatomical aspects in a longitudinal section, our study (which corresponds closely to what was figured in detail in other species, i.e<sup>17,19</sup>) shows that this thickened structure is clearly an evagination prolongation of the midgut that invaginates slightly anteriorly at the point where it is crossed by the oesophagus, in whose final tract it forms the stomodaeal valve. This finding agrees with the functional observations of Gilbert<sup>5</sup>, who concluded that given that the peritrophic membrane is thought to be produced by midgut epithelial cells<sup>69</sup>, the central part of the cardia is clearly the foregut, while the thickened outer part is of midgut origin.

Syrphinae larvae do not defaecate during their larval stage until they are about to pupate, when they expel a pasty liquid known as meconium<sup>70</sup>. This would explain the complexity of the midgut and hindgut. The three contiguous spherical cavities of the anterior midgut (stomach) are responsible for the first digestion of prey. Thanks to enzymes produced in the gastric caeca<sup>65,70,71</sup>, food which after this first digestion would pass through the middle midgut into a large pouch, the dilated posterior midgut, where the larva will accumulate waste, which will pass into the hindgut and accumulate in the mid posterior midgut, forming a mixture of excrement with high bacterial content that will form the meconium that is expelled before pupation<sup>70</sup>. In fact, by transparency, it has been observed that in live syrphid larvae, 24–48 h before pupation the hindgut appears darker<sup>72</sup>.

The insertion of Malpighian tubules marks the separation between the midgut and hindgut<sup>67</sup>. Until now, syrphid larvae were considered to have four tubules<sup>17,19,46</sup>, which either end separately or are grouped in pairs and end in the digestive tract at two points<sup>5</sup>, but *S. rueppellii* larvae clearly show only one pair of single, no bifurcating tubes.

Connected laterally to the rectum, just before the anus, are the anal tubes. Krüger<sup>17</sup> described them from the saprophagous larvae of *Syritta pipiens* (Linnaeus, 1758) and commented: "I found a ring of small anal tubes about one-sixth the length of the animal. By applying light pressure, I could also press these anal tubes out of the living animal". Similarly, Bathia<sup>19</sup> described and identified four tubes in the predatory larvae of *Platycheirus scutatus* (Meigen, 1822) and pointed out that "anal tubes are usually retained within the body of the larva, but

when a larva is deprived of food and left on a wet surface the anal tubes can be evaginated through the anus". There is a consensus that they play an important osmoregulatory role, and for water abstraction<sup>5</sup>. Has been reported from twelve extrusible papillae in some species of the genus *Volucella* (Geoffrey, 1762) and *Milesia* Latreille, 1804<sup>73</sup>, as being very small, with a minimum of four short tubes in the sirphines<sup>5</sup>. Our results show a single pair of anal tubes on each side of the rectum; whether they are really two single structures or whether each of them contains several extrusible papillae, we have not been able to clarify at this stage.

Without entering into the controversy regarding the formation of reproductive imaginal discs<sup>5</sup>, our results showed a thickened structure below the rectum with a median weld, which is continued by a duct leading to and below the anus. This corresponds to the position of the genitalia imaginal disc reported by Bathia in predatory syrphid larvae<sup>19</sup>. In fact, its configuration and shape coincide with the external genitalia of the male *S. rueppellii* figured by Orenco et al.<sup>74</sup>. This finding is of great importance because, from now on, this anatomical feature would allow the differentiation of larval sexes, which was not possible until now.

The nervous system shows the general organisation of many dipteran larvae (i.e.<sup>75,76</sup>), with two highly concentrated cerebral lobes and the abdominal ganglia concentrated in a large ganglionic mass. In the syrphids, at the end of the nineteenth century, Künckel d'Herculais<sup>42</sup> published drawings of the general morphology of the larval nervous system of *Volucella*. Bathia<sup>19</sup> described and made schematic drawings of the nervous system of larval L3 of *Platycheirus scutatus*. Possibly the most detailed study and figures are those from an *Eristalis* larva by Hartley<sup>46</sup>(see<sup>5</sup>), which are broadly similar to what we observe in rendered microtomographic images. However, in *Eristalis* described that the subesophageal, thoracic, and abdominal ganglia merge into a single ventral thoracic nerve mass, whereas in *S. rueppellii*, we observed a suboesophageal ganglion clearly separated from the large ventral ganglion. In addition, as part of the stomatogastric system, the rendered images clearly showed the ring gland (Weismann's gland) surrounding the aorta just above the brain, which is formed by fused aortic bodies, cardiac bodies, and prothoracic glands<sup>65,69</sup>.

The circulatory system fits the typical configuration of insects, with a contractile dorsal vessel acting as a heart and dilated chambers connected with heart valves and the lateral ostia to permit the haemolymph to enter and be pumped forward through the anterior aorta<sup>65,67-69</sup>. Gilbert reports that syrphid larvae have four pairs of large ostia, and Bathia reported two pairs, one situated anteriorly at the level of the second abdominal segment and the other close to the end of the heart. However, although our microtomographic results show a configuration of the circulatory system identical to that described and figured by Bathia<sup>19</sup>, we were not able to provide evidence other than the posterior ostia.

## Conclusions

This study constitutes a true anatomical atlas of the predatory third instar larvae of the syrphid *Sphaerophoria rueppellii*. The tracheal system, of which some tracheal trunks are slightly visible in the present study (Fig. 4b), is excluded from this study, which we have left for a later work similar to our previous publication of the coffee borer beetle<sup>77</sup>.

The main novelties of this study are: 1.- micro-CT evidenced an external latero-ventral complex of muscles that form muscular rings that allow the compression of the body segments in a similar functional way as occurs in annelid worms; 2.-the existence of mandibles with prosthecae presenting strong distal denticulations (so far considered them a separate structure with different names (maxillary outer tray, triangular sclerites, labial sclerites, and lateral lips); 3.- The nervous system presents a conspicuous distinguishable suboesophageal ganglion; 4.- the so-called antennomaxillary organs are actually the antennae which at their distal end have two small lobes bearing sensillae; 5.-the maxillae are separate structures, joined together to form an arch and each with an articulated maxillary palpus at its basal external part; 6.- *S. rueppellii* has only one pair of Malpighian tubules instead of the two pairs that were thought to be the general rule for Syrphid larvae; 7.-the evidence of an imaginal disc of genitalia in the posterior part of the body, below the rectum, which in male larvae has a complete correspondence with the morphology of the external genitalia of the male, will allow from now on to differentiate the sexes in the larval stage.

We have included supplementary videos and a 3D model suitable for use with mobile devices. Both will be useful tools for future research and teaching.

## Supplementary information

**Supplementary video S1.** General processes and anatomy: This animated video begins with a schematic explanation of the process of sample preparation, scanning, and reconstruction to obtain the rendered images and continues with spinning animations of the general external and internal anatomical structures and organs.

**Supplementary video S2.** Animated virtual dissection video.

**Supplementary video S3.** Spinning animation video showing body muscles.

**Supplementary video S4.** Spinning animations from different perspectives of the digestive system, with a general view and details of different tracts.

**Supplementary video S5.** Spinning animation of the salivary glands.

**Supplementary video S6.** Spinning animations of the nervous system and closely related imaginal discs.

**Supplementary video S7.** Animated videos showing the details of the cephalopharyngeal complex, muscles, tentorial, and mouthparts.

**Supplementary 3D model S8.**- A vxm file with a 3D model of the larva for use in mobile devices using the CTvox software of Bruker microct.

## Data availability

The datasets generated and analysed during the study are available from J.A.-T. upon reasonable request, and always within the framework of a collaborative scientific project.

Received: 17 August 2024; Accepted: 18 October 2024

Published online: 21 November 2024

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## Acknowledgements

Special mention to Francis Gilbert, University of Nottingham, UK, for kindly sharing the draft manuscript he is preparing for a comprehensive review of the current knowledge of Syrphidae worldwide. The senior author thank the staff of Bruker SkyScan in Kontich (Belgium) for their effectiveness, fast support, constant improvements to the software, and implementation of the requested new features. In this respect, we are especially indebted to Alexander Sasov (who despite being now at NeoScan, [www.neoscan.com](http://www.neoscan.com), has not hesitated to continue providing unconditional support), Stephan Boons, Xuan Liu, Phil Salmon, and Vladimir Kharitonov. To BioNostrum Pest Control (Alicante, Spain <https://www.bionostrum.com>) for providing the syrphid larvae. This study benefited from funds from: AGROALNEXT programme (AGROALNEXT/2022/052-BIOPESTNEW) supported by the Spanish MCIN with funding from the NextGeneration EU (PRTR-C17.I1) and by Generalitat Valenciana, Spain.

## Author contributions

Conceived and designed the study: J.A.-T. and MªA.M.-G.. Sample preparation, micro-CT scans, software treatment to obtain rendered images, plates of figures, Supplementary Videos, and Supplementary 3D model: J.A.-T. Analysis and interpretation of results: J.A.-T. and MªA.M.-G., wrote the paper: J.A.-T., and MªA.M.-G.

## Funding

University of Granada Spain, and AGROALNEXT programme, AGROALNEXT/2022/052-BIOPESTNEW, Ministerio de Ciencia e Innovación, NextGeneration EU (PRTR-C17.II), Generalitat Valenciana, Spain

## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-77013-5>.

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