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3D MORPHOMETRICS OF *LUCANUS CERVUS CERVUS* (LINNAEUS, 1758) (COLEOPTERA: LUCANIDAE)

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Abstract.— The European stag beetle *Lucanus cervus cervus* is the largest European member of the Lucanidae and is characterised by the extremely enlarged mandibles of the male. Some aspects of the morphology of this species have been extremely well studied, while others have been largely neglected. In the present paper we fill some of these gaps by focusing on 3D measurements based on a μ-CT scan. We show that the largest part of the body is filled by a massive tracheal system (48.8%), followed by the exoskeleton (21.1%). The massive tracheal system covers large parts of the internal anatomy, leaving limited space for other systems such as the nervous system (0.2%) or the digestive system (0.7%), which are very small and delicate compared to other species. We suggest that no significant relative increase in the volume of the tracheal system is possible in a flying insect without potentially rendering another organ system inoperable. The cuticle of *L. cercus cercus* is extremely thick compared to other species, which is most likely due to the fact that the males have extremely enlarged mandibles, which are also used in male-male fights. They can generate strong forces that also result in massive stresses that need to be dissipated by the thick cuticle. We can also show that there are significant differences between 2D and 3D measures of specific distances in highly 3-dimensional structures such as the length of the mandibles. We therefore suggest that such 3D measures be included in future analyses. In summary, we hope to further promote the importance and significance of 3D morphometric measures and suggest that at least the volumes of the studied materials should be included in future CT-based studies of insects.

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Key words.— *Lucanus*, micro-CT, tracheal system, morphometrics

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INTRODUCTION

The stag beetles (Lucanidae) comprise about 1200 species and are found on almost all continents except Antarctica (Gillot 2005). These beetles are characterised by a large and broad head with the forehead, clypeus and upper lip forming a uniform sclerotized surface. Males often have cephalic horns, ridges, carinae or other projections, which are important taxonomic characters and elements of sexual dimorphism. The antennae are 10-segmented, geniculate, with a comb-like club composed of three or four clearly separated lamellar segments. In the males of the subgroups Lucaninae and Syndesinae, the mandibles can reach extreme proportions. The thorax of Lucanidae is strongly sclerotized, with a strongly convex pronotum. The abdomen is flat, five-segmented and the first sternite is partially hidden under the coxae of the hind legs. The copulatory apparatus of the males differs from that of other Scarabaeoidea in that the penis is located in a tubular tegmen in a special sheath inside the abdomen (Stebnicka 1983). In Central Europe, Lucanidae are represented by seven species (Bunalski 1999, 2005, Bunalski *et al.* 2022), which are clearly distinguished from the rest of the Scarabaeoidea by a number of morphological characters of the imagoes. In the case of the Central European species, the size of the beetles ranges from 5 to 83 mm, with some of them showing a considerable range of this feature. The largest of these European species is *Lucanus cervus cervus* (Linnaeus, 1758). In males of this species, the gody length ranges from 25-83 mm and in females between 27-45 mm. The colouration is mostly brown or dark brown, with the head and pronotum usually darker, blackish brown. The upper part of the body is slightly shiny and convex, and the underside is finely hairy. The structure of the mandibles in males is very characteristic. They are usually strongly developed, slightly curved and much longer than the head and pronotum combined. The inner edge of the mandibles has several teeth of varying size, the largest of which are located midway along the length of the mandible and at its tip. In some populations, especially on the southern margins of the range, an amphidont form with much shorter mandibles is more common. The mandibles of females are much smaller than those of males, short and curved towards the middle. Their total length does not exceed the length of the head, and the inner margin has several small, sharp teeth (Bunalski 2012).

Certain aspects of the morphology of *Lucanus cervus cervus* have been relatively well studied. This is particularly true for the extremely enlarged mandibles, where previous studies have addressed variability, cost, biomechanics, sexual dimorphism or their effect on flight performance (e.g. Harvey 2006, Kawano 1997, Romiti *et al.* 2016, Popa *et al.* 2021, Packard 2021, Goyens *et al.* 2014, Goyens 2015). Other studies have described the nervous system (Jawłowski 1936), the thoracic and abdominal musculature (Larsen 1966) or parts of the cephalic muscles (Dorsey 1943). However, other aspects of its morphology remain unknown. In the present paper we aim to fill some of these gaps. We focus on the possibilities of 3-dimensional measurements based on models generated by µ-computed tomography. These models allow the easy study of many features that are difficult or impossible to study using conventional morphological techniques. Specifically, we will look at the volumes and volumetric relationships of the tracheal system, digestive tract, musculature, nervous system and exoskeleton, cuticular thickness and the difference between 2D and 3D measurements of specific distances.

MATERIAL AND METHODS

Sample collection. The present study is based on a male *Lucanus cervus cervus* collected in June 2021. It was obtained within the framework of the project "Monitoring of animal species taking into account special areas of conservation of Natura 2000 habitats, 2020-2022" under the permit No. WOPN-ON.6205.21.2021.AS.

µ-CT scanning. The sample was scanned using a SkyScan 1172 microtomograph from the Museum and Institute of Zoology of the Polish Academy of Sciences. The sample was sedated with ethyl acetate immediately before scanning and mounted in a 50 ml falcon tube. Scanning parameters were as follows: voltage 40 kV, current 250 µA, resolution 2069 × 4000 pixels, image pixel size = 8.74 µm, exposure 2356 ms, frame averaging = 2, random motion = 10, oversize scan = 5.

The scan was reconstructed in NRecon (ver. 1.7.4.2), resulting in an image stack of 7256 images of 4000 × 4000 pixels and a resolution of 8.75 µm per voxel.

Segmentation and measurements of selected systems were performed in Amira ver. 5.3 and Blender ver. 4.0. After segmentation into individual systems (exoskeletal, muscular, tracheal, nervous and digestive), the volumes of each system were measured. The digestive system was traced to the level of the first abdominal segment. The tracheal system was manually divided into the cephalic, prothoracic and pterothoracic + abdominal parts. The 'thickness map' option of Amira was used to measure the thickness of the exoskeleton, musculature and tracheal system. All measurements were made without appendages.

To perform the concentration analysis of each system, they were exported from Amira as 3D models and then imported into Blender, where a uniform distribution of points within the volume of each system was achieved using geometry nodes. This resulted in 160,000 points describing the volume distribution. Their coordinates were exported to a CSV file and then imported into Python using packages such as numpy, matplotlib, pandas and sklearn.neighbours. Gaussian mixture models were calculated to measure density. The analysis parameters were set to h = 0.2 and kernel = gaussian. The visualisation of the 3D models was done in Blender ver. 4.0. This program was chosen for its easy and free accessibility.

RESULTS

Exoskeleton. Fig. 1 gives an overview of the exoskeletal elements of *L. cervus cervus*. The examined male of *L. cervus cervus* has a total length of 71.5 mm. The length of the mandibles is 28% (20.1 mm) of the total body length. The head is 11.5 mm long and 21.4 mm wide. The prothorax is 9.8 mm long and 17.8 mm wide. The rest of the body (pterothorax + abdomen) is 30.1 mm long and 20.9 mm wide. Figure 1 shows the exoskeleton of the studied specimen without legs.

The length of the mandible, the head capsule, the prothorax and the whole animal were measured either in 2D as a direct line (red in Fig. 2, values also given in the description above) or in 3D along the body surface (blue in Fig. 2). For the head capsule, the prothorax and the pterothorax + abdomen this was also done with the width. Fig. 2 shows the measured values for each of these distances and the difference to the 2D measurements.

Exoskeletal thickness. The thickness of the exoskeleton in the studied *L. cervus cervus* specimen varies between 26.01 µm (close to the spatial resolution of the CT scan) and 778 µm (Fig. 3 and Fig. 4).

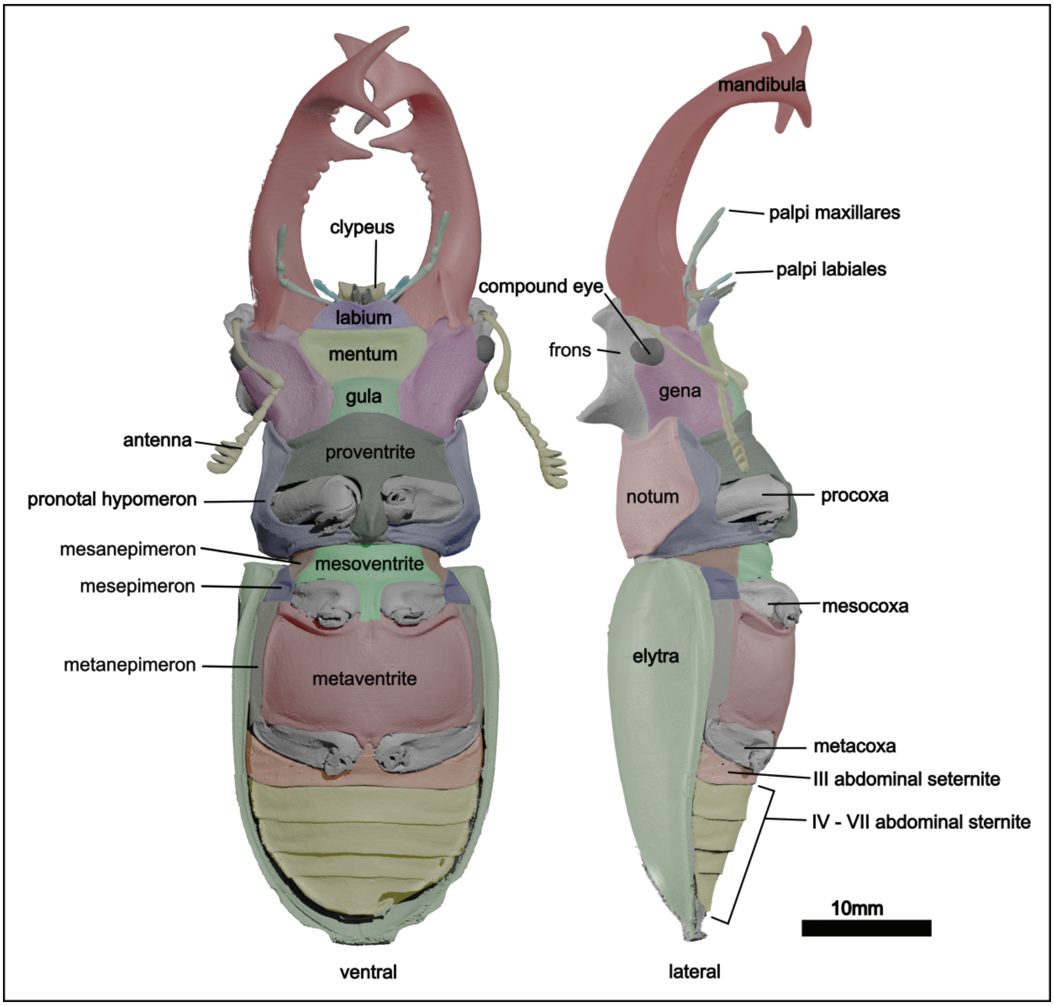
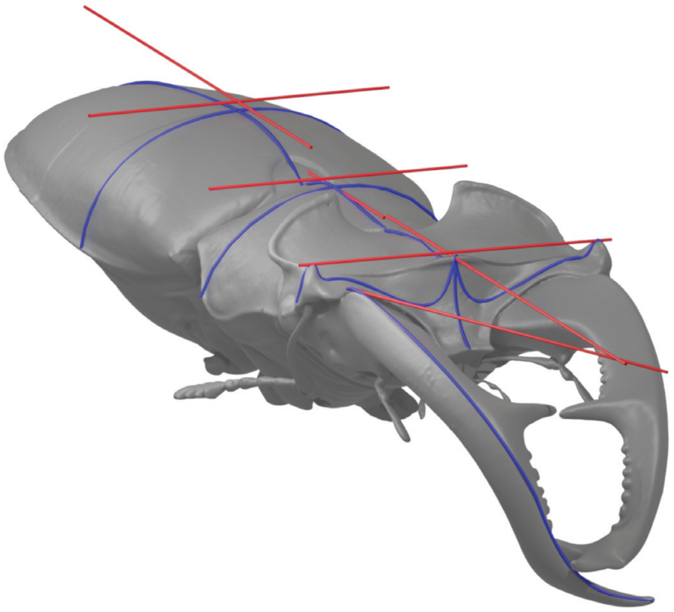


Figure 1. External view of the exoskeleton of *L. cervus cervus*.



	Length along the surface (blue) [mm]	in relation to body length [%]	Length as straight line in 2D (red) [mm]	in relation to body length [%]	Width along the surface (blue) [mm]	Width as strait line in 2D (red) [mm]
Mandibles	31.9	35.7	20.1	28.1	-	-
Head capsule	15.5	17.3	11.5	16	30.2	21.4
Prothorax	10.1	11.3	9.8	13.7	23.6	17.8
Pterothorax + abdomen	31.8	35.6	30.1	42.1	28.6	20.9
Body length (head - abdomen)	89.3	-	71.5	-	-	-

Figure 2. 2D and 3D measurements of specific distances in *L. cervus cervus*. Blue lines: length measured along the body surface (3D); red lines: length measured as a straight line (2D).

The head has the thickest cuticle both on average (217.33 μm) and in maximum number. The most massive exoskeleton is found at the articulations of the mandibles, where it reaches 778 μm. Other massive sites include characteristic ridges along the head capsule. The prothoracic cuticle is on average slightly less thick than the cephalic one (205.68 μm). The prothorax has a more evenly distributed elemental thickness than the head capsule, with the upper part (notum) being thicker than the sternum. The pterothorax and abdomen have the lowest cuticle thickness, both in maximum (572 μm) and average (163.93 μm). The highest values are recorded at the wing base and veins, where several layers of cuticle overlap. The edges of the sternites adjacent to the posterior part of the midlegs are also thickened.

Musculature of the head and thorax (Fig. 5, 6). Dorsey (1943) described the head musculature of *Lucanus placidus*. That of *L. cervus cervus* is almost identical. The head musculature is dominated by the mandibular muscles. The cephalic musculature has a total volume of 263.3 mm^3. The vast majority of the cephalic muscles are covered by the mandibular abductor (242.9 mm^3), which is significantly larger and more voluminous than all the other head muscles combined. The second largest cephalic muscle is the mandibular adductor (16.7 mm^3). The thoracic musculature was described in detail by Larsen (1966). It has a total volume of 812.3 mm^3. The abdominal musculature was not reconstructed and examined in the current analyses.

Alimentary canal. The entire digestive system is small and delicate (Fig. 7). The anterior part of the digestive system has thin walls and is difficult to see. It is only in the midgut that a larger diameter can be observed. In the abdomen it forms two small loops at the level of the hind legs. The hindgut is extremely thin and difficult to follow.

Table. 1. Thickness and volume analyses of the exoskeleton of the studied male *L. cervus cervus*.

	Max thickness (μm)	Min thickness (μm)	Volume (mm^3)	Weighted average thickness (μm)
Head with mandibles	778	26.01	290.58	217.33
Prothorax without legs	634	26.27	139.62	205.68
Pterothorax and abdomen without legs	572	26.18	429.00	136.93

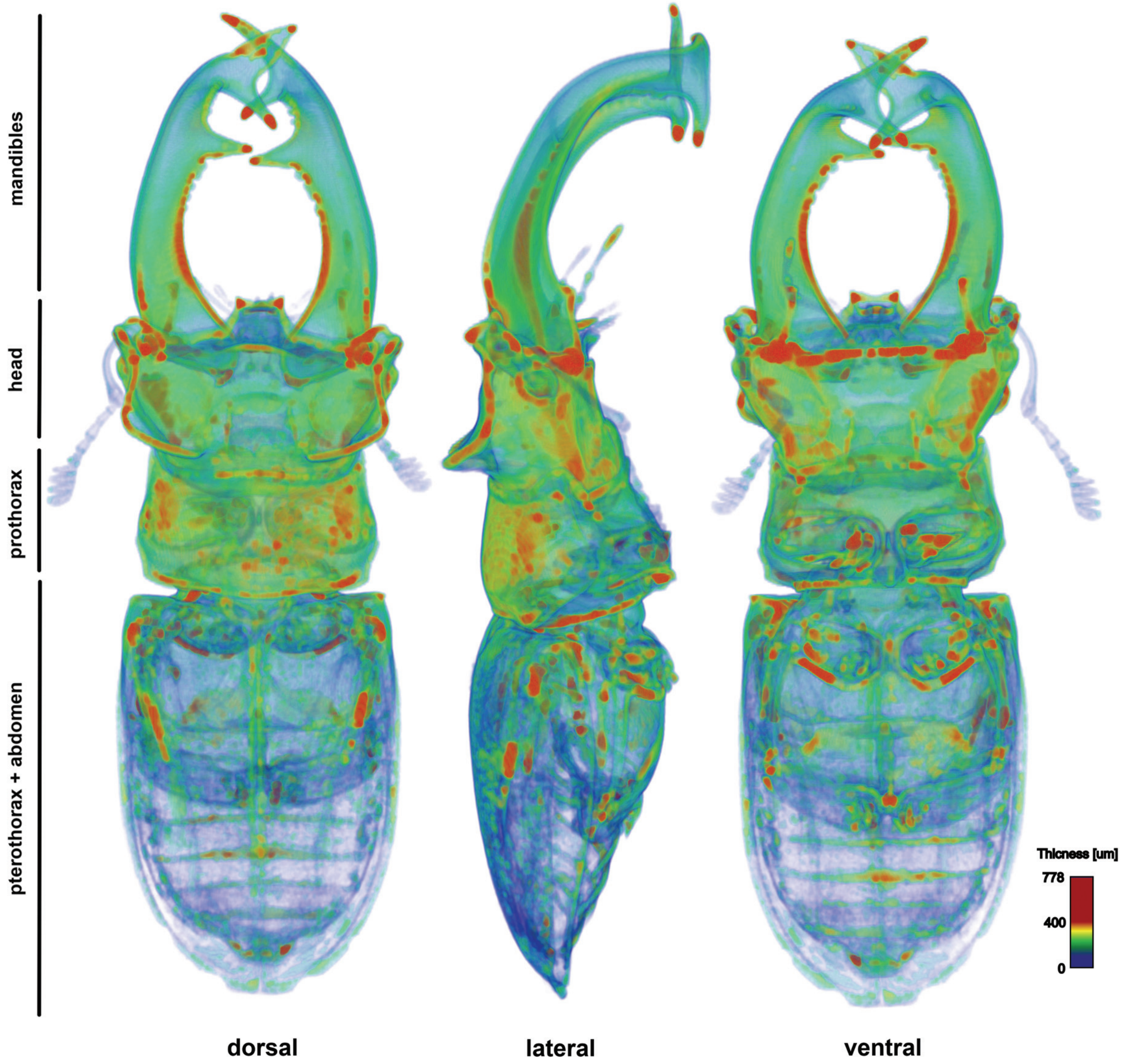


Figure 3. Visualisation of the exoskeleton thickness distribution of the studied *L. cervus cervus* male.

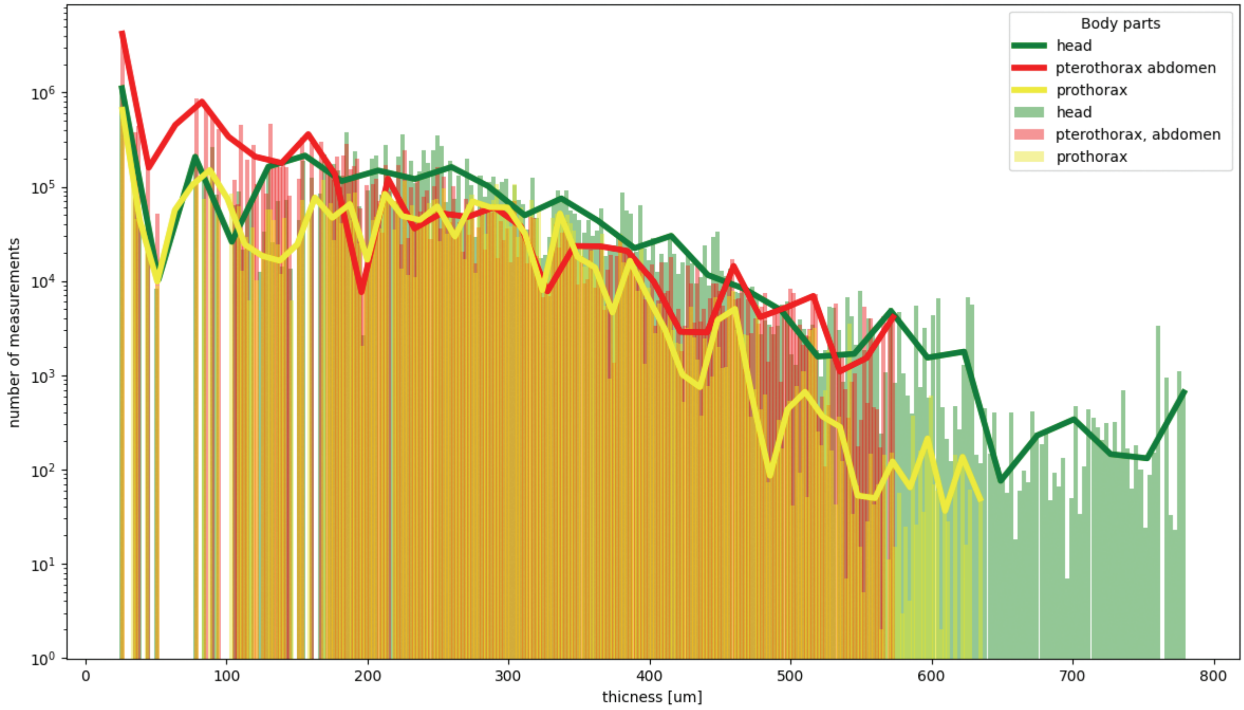


Figure 4. Distribution of exoskeleton thickness. The solid lines show the approximate distribution of thickness sampled at 50 points. A difference in the maximum thickness of the elements can be observed in different body segments.

Nervous system. The entire nervous system is thin and delicate (Fig. 5, 7). The supraoesophageal ganglion is very thin and hardly widens in its central part, but remains the same diameter between the two compound eyes. The innervation of the antennae initially follows the nerves towards the compound eyes, then it separates halfway and changes direction laterally anteriorly. From the subesophageal ganglion, nerve bundles branch anteriorly towards the mouthparts, innervating their components. The thoracic and abdominal ganglia are clearly visible and well developed. The abdominal ganglia are the smallest. We were only able to trace the nervous system up to the ganglia; peripheral nerves were not considered. The entire nervous system has a volume of 7.7 mm^3 (details below).

Tracheal system. The tracheal system of *L. cervus cervus* is massively developed and consists of numerous air sacs (Fig. 8). These air sacs tightly surround all internal organs and fill almost the entire mandible. Several internal organs, such as the muscles or the supraoesophageal ganglion, are tightly squeezed between these tracheal structures. In Figure 8A, the tracheal system appears as a massive balloon-like sac, but it is actually composed of numerous small cavities or air sacs, as shown in Figure 8B, where each bubble represents one of these sacs. This dense system of air sacs makes it almost impossible to trace individual tracheae. Figure 8A shows parts of the trachea from the lower jaw to the metathorax. In total, the tracheal system occupies 1976.6 μm^3 or 48.6% of the total body volume (see below). It is noteworthy that very small amounts of haemolymph were observed in the examined individual. The density (Gaussian mixture) of the tracheal system is highest between the head and the prothorax and in the abdomen (Fig. 8C).

Volumetrics. Table 2 shows the volume of the whole body, the tracheal system, the musculature, the exoskeleton, the nervous system and the digestive system for the whole animal, the head, the prothorax, the pterothorax and the abdomen (except for the musculature where the abdomen is missing).

Of all the systems, the respiratory system is the largest, occupying 1976.6 mm^3 or 48.6% of the whole body. Within the head, it occupies 53.1%. The second largest structure is the exoskeleton (858.1 mm^3 or 21.1% of the total body). Again, the head shows relatively higher values than the prothorax, the pterothorax or the whole animal. The nervous system has a volume of 7.7 mm^3 (0.2% of the body volume). Most of it is located in the head (5.0 mm^3). The digestive system has a volume of 29.1 mm^3 (0.7% of the whole body) and is mainly located in the abdomen (17.6 mm^3 or 60.5%).

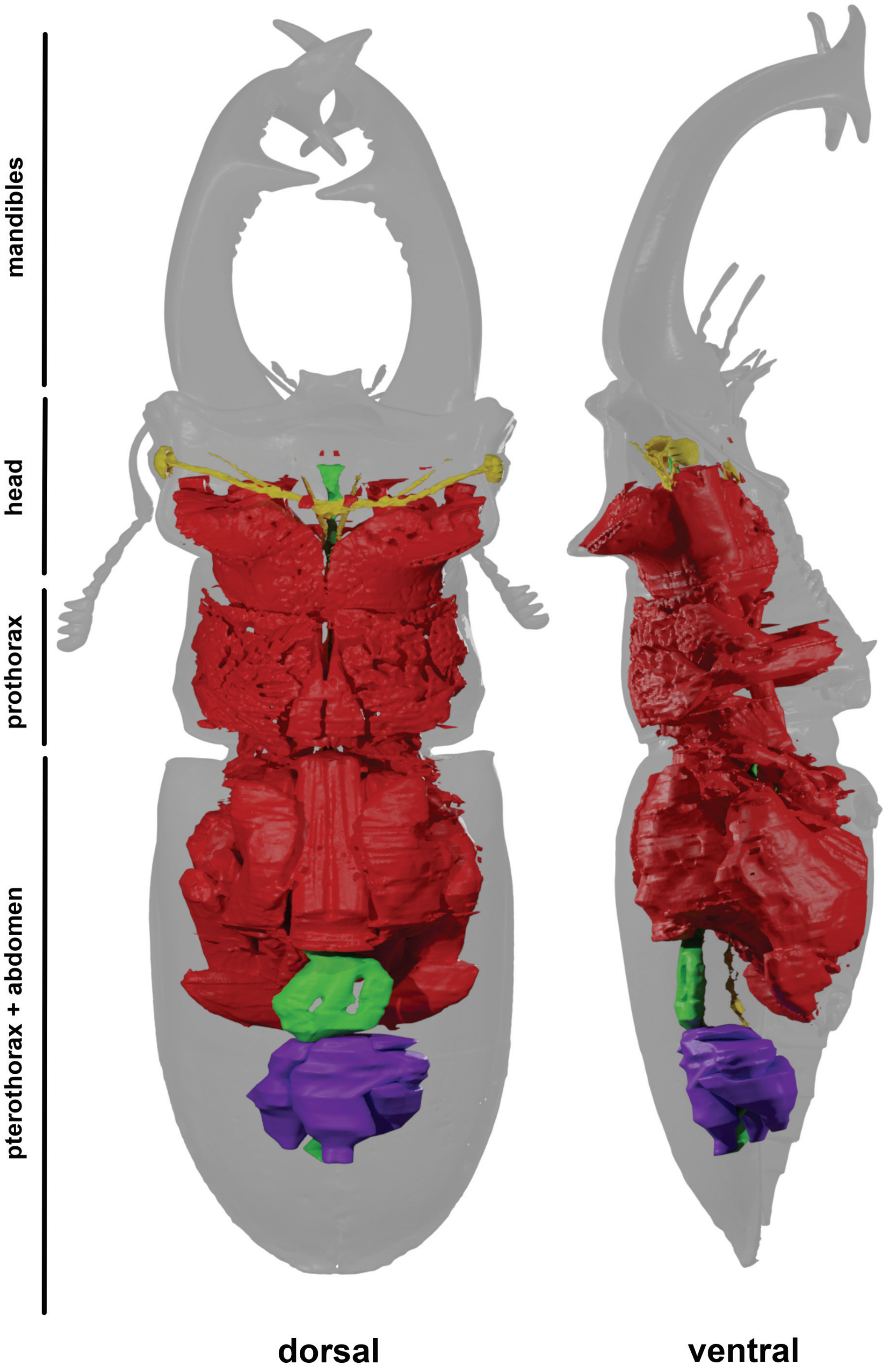


Figure 5. Visualisation of the muscular system (red) of *L. cervus cervus* against the background of the nervous system (yellow) and digestive system (green), other unspecified tissues in purple.

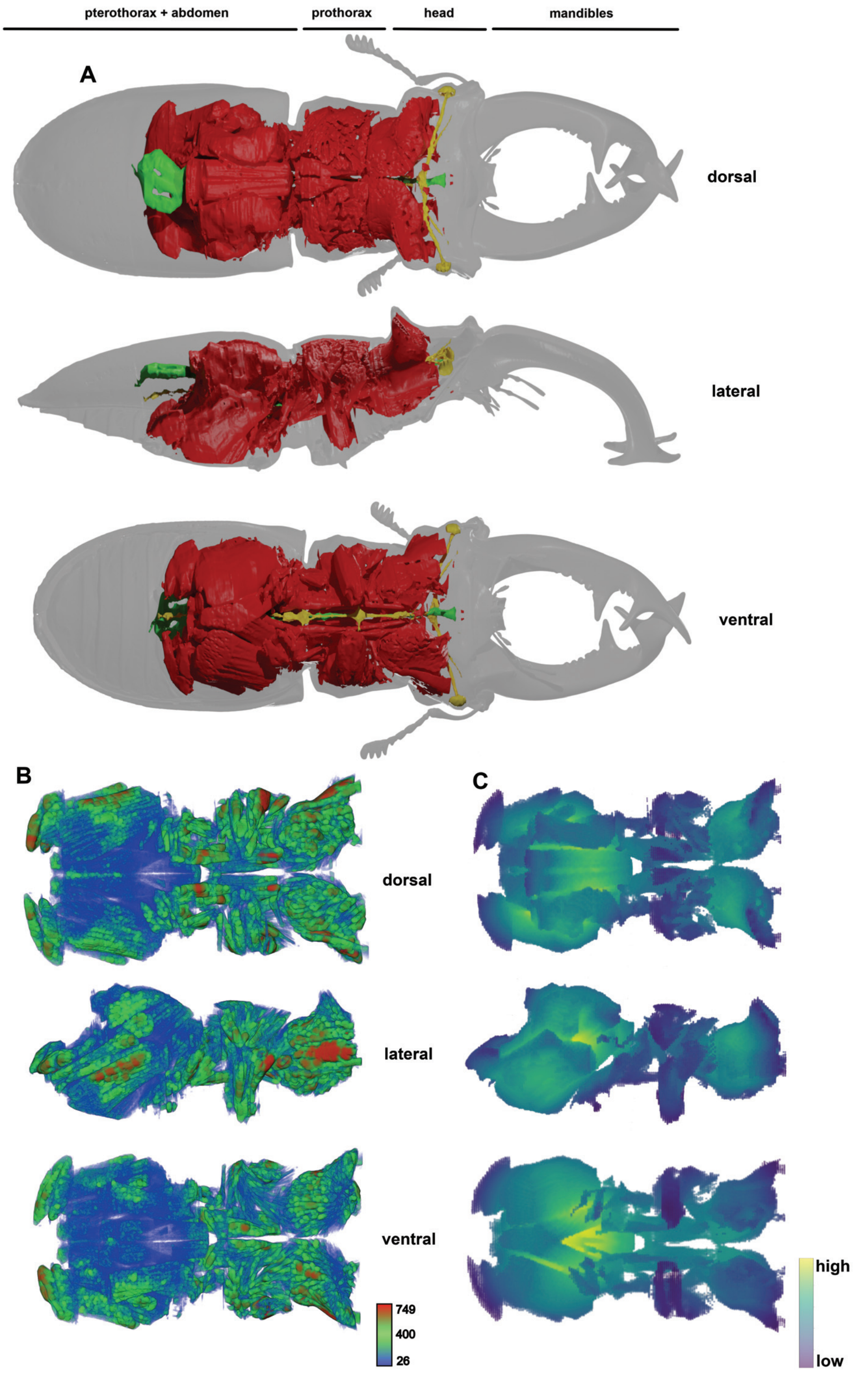


Figure 6. Visualisation of the muscular system of *L. cervus cervus*. (A) General view of the muscles found in the head and thorax; (B) Thickness of the muscles. It reaches up to 743 µm in the head; (C) Density of the musculature obtained using Gaussian mixture models.

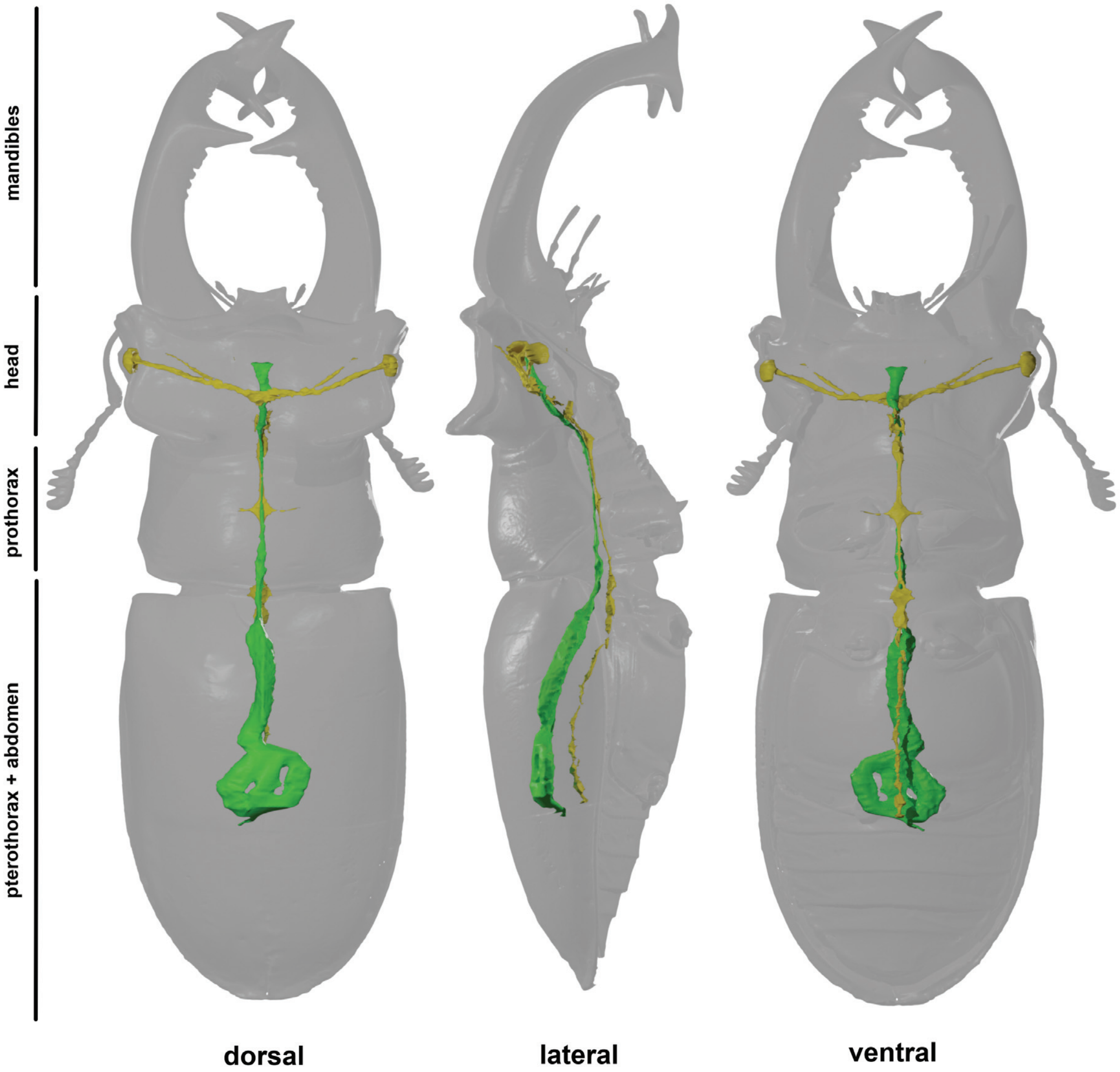


Figure 7. The digestive (green) and nervous (yellow) systems of *L. cervus cervus*.

Table 2. Volumetrics of *L. cervus cervus*.

	Animal		Head		Prothorax		Pterothorax	
	mm^3	%	mm^3	%	mm^3	%	mm^3	%
Total	4066.9	100	1194.2	100	632.1	100	1501.5	100
Tracheal system	1976.6	48.6	633.9	53.1	312.7	49.5	582.5	38.8
Musculature	–	–	263.3	22.0	179.0	28.3	633.3	42.2
Exosceleton	858.1	21.1	290.6	24.3	139.6	22.1	276	18.4
Nervous system	7.7	0.2	5.0	0.4	0.5	0.1	2.2	0.2
Digestive system	29.1	0.7	1.4	0.1	0.4	0.1	9.7	0.7

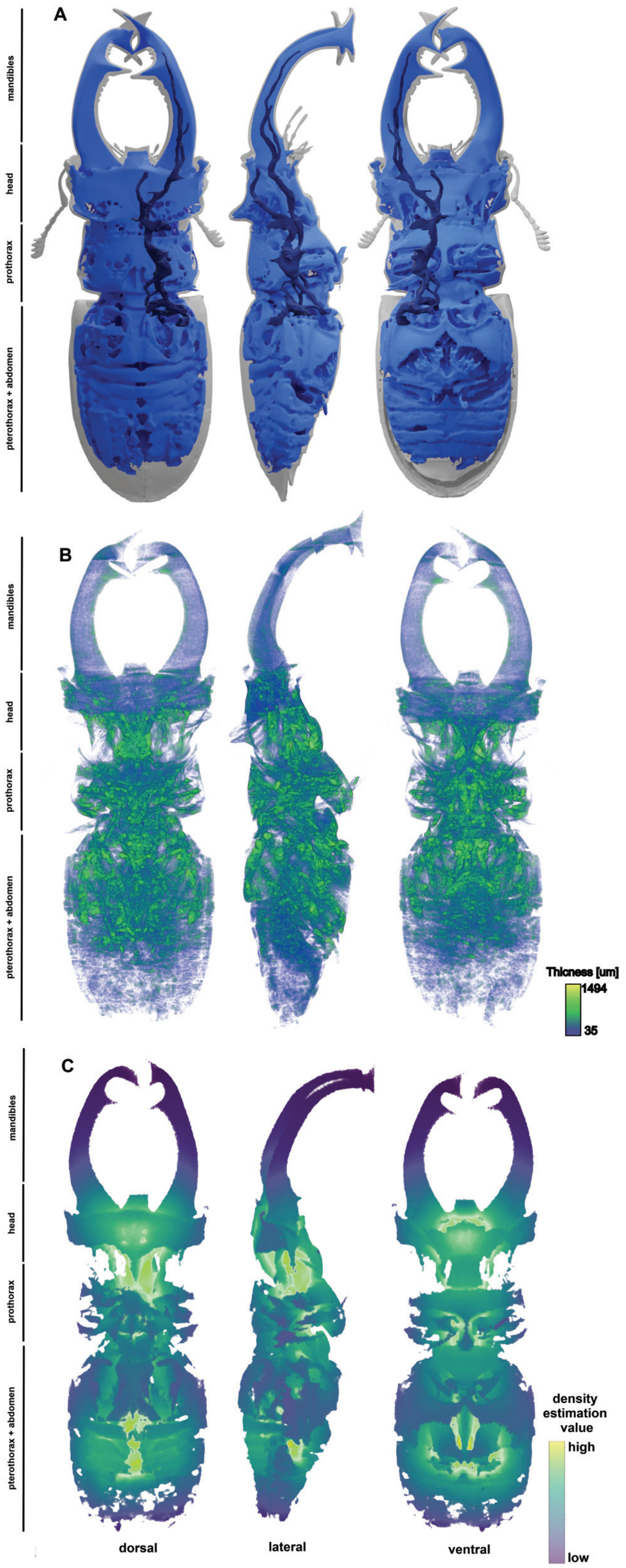


Figure 8. Tracheal system of *L. cervus cervus*. (A) Overview of the tracheal system (blue) with one trachea shown (dark blue); (B) Estimation of the thickness of the tracheal system; (C) Density of the tracheal system obtained using Gaussian Mixture Models.

DISCUSSION

Volumetrics. The most surprising result of our analyses is the extremely massive and voluminous tracheal system of the studied beetle (see supplementary material). In total, the respiratory system occupies 48.8% of the entire body (excluding the exoskeleton) or 66.1% of the space inside the exoskeleton. Inside the head, this value rises to 53.1%. The musculature is most massive in the pterothorax, where it occupies 42.2% (as opposed to 28.3% in the prothorax and 22.0% in the head capsule). This correlates with the massive presence of flight muscles in this part of the body. Because of these muscles, the tracheal system has less space and occupies ‚only' 38.8% of the ptherothoracic space (prothorax 49.5%). As a result, other organ systems such as the nervous or digestive systems are severely compressed and occupy minimal space in the head and thorax (between 0.1 and 0.7% of the body space). The cephalic supra- and suboesophageal ganglia are extremely small and delicate, even compared to blind cave beetles (e.g. Luo *et al.* 2018), which in contrast to *L. cervus cervus* lack optical lobes. The extent to which the nervous system is compressed in the head of *L. cervus cervus* can be seen by comparing it with the eyed beetle *Tenebrio molitor* (Ras *et al.* 2018, 2022). In this species, the cephalic nervous system occupies 2.1% of the head volume, more than five times the volume of *L. cervus cervus* (0.4%). The same applies to several muscles, some of which do not have a straight course but are squeezed into the available space between the tracheae.

So far, *L. cervus cervus* has the relatively largest tracheal system ever studied, although very few studies provide the volume for both the tracheal system and the whole body. In other beetles, it represents 2–3% of the body volume (Kaiser *et al.* 2007, Raś *et al.* 2018, 2022, Alba-Telcador *et al.* 2019). In males of the grasshopper *Schistocerca americana*, it covers about 40%, while females with eggs have significantly lower values (Lease, Wolf and Harrison 2006). In the longhorned beetle *Batocera rufomaculata*, which has an approximate maximum length of 75 mm, the largest measured tracheal volume is approximately 1.7 ml, corresponding to 1700 mm^3 (Urca *et al.* 2024). The studied specimen of *L. cervus cervus* has a body length of 71 mm and a tracheal volume of 1976.6 mm^3. Considering that the mandibles cover almost one third of the body length of *L. cervus cervus*, it can be expected that *B. rufomaculata* has a higher body volume than *L. cervus cervus*. In conclusion, the volume of the tracheal system in relation to the body volume of *B. rufomaculata* must be considerably lower than that of *L. cervus cervus*. This contrasts with hypotheses that the tracheal system increases in relative size with increasing body size both within (e.g. Urca *et al.* 2024) and between (Kaiser *et al.* 2007) species. This increased investment in the respiratory system is also thought to limit maximum body size in insects (e.g. Klok and Harrison 2008). One possible explanation for the observed volumes in these two large beetles is the presence of airsacs in *L. cervus cervus* and other scarabaeid beetles and their absence in *B. rufomaculata*. Airsacs are massive extensions of the respiratory system that can take several different forms: (1) taenidial sacs in the form of dilated tracheae and taenidia, (2) reticular sacs with taenidia but of irregular shape, and (3) punctate sacs of irregular shape without taenidia (Faucheux and Sellier, 1971, Faucheux, 1972, Harrison *et al.* 2023). In *Drosophila melanogaster*, these sacs are formed by cell proliferation and morphogenesis from a tubular outgrowth called the air sac primordium (ASP) (Guha and Kornberg 2005). They are massively present in *L. cervus cervus*, in *Schistocerca*, where the tracheal volume covers 40% of the total body (Harrison *et al.* 2013), but also in various other insect groups (Harrison *et al.* 2023). In Figure 8A, it appears that the tracheal system of *L. cervus cervus* is, at least in some areas, one large sac, whereas in reality there are numerous individual airsacs (Figure 8B, see supplementary material) that are demarcated from each other. Airsacs are absent in most Cerambycidae, the group to which *Batocera rufomaculata* belongs (Miller 1966). Instead, Cerambycidae rely on a system of extremely widened tracheae between the flight muscles to provide oxygen during flight (Miller 1966). Air sacs are thought to play an important role in insect respiration, but also in the evolution of gigantism (Harrison *et al.* 2023 and references therein). There are several hypotheses for the purpose of air sacs, including altering the function of the tracheal system (Wigglesworth 1963, Robertson 1962, Harrison *et al.* 2023), reducing weight and thus increasing flight ability (Wigglesworth 1963), reducing water loss during respiration (Lighton 1996, Duncan and Byrne 2000, White *et al.* 2007), insulating (Verdu *et al.* 2012) or damping (Harrison *et al.* 2023). At the same time, we observed little space for haemolymph in *L. cervus cervus*, which could lead to problems with nutrient and other transport and a reduction in CO_2 buffering space. These are some of the potential disadvantages of massive air sacs (reviewed in Harrison *et al.* 2023). In addition, the presence of the massive air sacs severely limits the space available for other organ systems as described above, and we suggest that a significant increase in tracheal volume compared to *L. cervus cervus* would not be possible without eventually rendering another organ system inoperable. We therefore speculate that the tracheal system of *L. cervus cervus* is close to its maximum volume in relation to body size. However, our comparison with *Batocera rufomaculata* shows that such massive air sacs are not a prerequisite for a beetle to reach a large body size and to be able to fly. In summary, we can confirm Harrison *et al.* (2023) that the benefits and potential costs of airsacs are poorly understood and need much more scientific attention.

Cuticular thickness. The exoskeleton is the second largest organ system studied in *L. cervus cervus* after the tracheal system, occupying approximately 21% of the total body mass. It reaches a maximum thickness of 778 µm in the articulatory sockets of the mandibles and the reinforcing sulcus on the mentum connecting these sockets. The minimum thickness of 26 µm is found in the head appendages (antennae, palpi, abdominal tergites and partially elytra). However, this minimum thickness is close to the resolution of the CT scan and we cannot exclude that it is thinner in other areas. The cuticle of *L. cervus cervus* is much thicker than most other measured values in insects: reported maximum values in other species include 80 µm in the gula of the beetle *Pachnoda marginata* (Barbakadze *et al.* 2006), 45 µm in the thorax of the honey bee (Casey *et al.* 2022) or 133.7 µm in the mosquito *Anophleles funestus* (Aberle *et al.* 2017, Wood *et al.* 2010). However, there are few studies that have examined cuticle thickness for the whole body, as we did for *L. cervus cervus*, but focused on specific parts. It is therefore possible that the cuticle of the studied animals is considerably thicker in places that were not included in the respective analyses.

This massive exoskeleton in *L. cervus cervus* may be explained by the large body size of the animal, but also by the massive mandibles of the males of this species. These mandibles are used in *L. cervus cervus* to fight other males. They have to withstand high material stress caused by the force of the massive adductor muscles (volume 242.9 mm^3) attached to them. Compared to other Lucanidae, the mandibles of *L. cervus* have the highest von-Mises stress (Goyens *et al.* 2016).

Many studies on cuticular thickness focus on the relationship between the different cuticular layers, such as endo- and exocuticle (Vincent and Wgst 2004, Moussian 2010, Muthukrishnan 2022). In the present analyses, we did not consider these layers and measured the thickness of the exoskeleton in general, as the layers were not distinguishable in the CT scan performed. However, there are staining approaches that allow semi-automated separation of these layers in insects (Stamm and Dirks 2022). It is therefore possible to measure the thickness of each layer over the entire animal using the approach presented here.

2D vs 3D measurements. Our study confirms the strong differences between 2D and 3D measurements (e.g. Galli *et al.* 2007; Cardini and Chiapelli 2020;

Wasiljew *et al.* 2020). In our data we found very heterogeneous results. While the length of the pterothorax and abdomen differs by only 5.6% between the 2D and 3D measurements, this difference increases to 58.7% for the mandible (Fig. 2). Our results thus confirm that the more three-dimensional the structure, the greater the difference. Given the strong reported difference between the 2D and 3D measures, and the fact that the mandible of *L. cervus cervus* has been used in several comparative studies in 2D (Tatsuda, 2001, 2004, Harvey and Gange, 2006, Romiti *et al.* 2016), we emphasise the importance of including the third dimension in future studies. As we can show here, 3D models based on µ-CT (Faulwetter *et al.* 2013) are an excellent tool to measure distances on the surface of the animal. The same can be achieved with specialised 3D scanners (Ströbel, Schmelzle *et al.* 2018).

Conclusion. *L. cervus cervus* is one of the best studied beetles in many respects. This is especially true for its mandible (e.g. Harvey 2006, Kawano 1997, Romiti *et al.* 2016, Popa *et al.* 2021, Packard 2021, Goyens *et al.* 2014, 2016). However, in the present paper we can show that there are still several parts or systems of the *L. cervus cervus* body that are hardly addressed.

The present study is based on data derived from 3D models generated from µ-CT scans. We can show that they are a very valuable tool to study and compare different morphometric approaches, such as the volumes of different body systems, the 3D length or the thickness of the cuticle over the whole body. In recent decades, the application of µ-CT and other 3D methods has become a standard tool in comparative entomology (Friedrich and Beutel 2008; Wipfler *et al.* 2016). Nevertheless, many morphological studies based on these approaches do not fully ex-ploit the morphometric potential of the generated 3D models, but rather use them for imaging and illustration purposes only. With this paper, we hope to raise awareness and demonstrate the enormous potential of including morphometric measurements, or at least providing the corresponding raw data, such as the volumes of individual materials. We believe that this would not only increase the value of the study, but would also eventually lead to a pool of data for broad comparative studies.

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