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A novel approach to imaging engorged ticks: Micro-CT scanning of *Ixodes ricinus* fed on blood enriched with gold nanoparticles

RNDr. Thesis

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Annotation

Micro-computed tomography (micro-CT) is an exceptional non-destructive imaging modality, which can be used to visualize both external and internal morphology of arthropods like ticks. The major micro-CT drawback is the soft tissues visualization, which require pre-examination contrasting steps. The commonly employed tick contrasting methods are drying and immersion in a solution containing a high-atomic number element, which both cause major deformation and shrinkage of examined specimens. The aim of this study was to develop a new tick contrasting approach to bypass these limitations. We show that adult *Ixodes ricinus* females engorged *in vitro* on blood enriched with gold nanoparticles with polyethylene glycol coating can be scanned with excellent contrast. By utilizing this technique based on the hematophagous nature of the ticks, we were able to observe midgut lumen, midgut epithelium and rectal sac in their natural state. In addition, we were able to precisely calculate the midgut volume.

Declaration [in Czech]

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I hereby declare that I had a significant contribution to the publication. I was responsible for conceptualization, partial funding acquisition, data curation, investigation, methodology, resources, visualization, writing the manuscript and article submission as the corresponding author.

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A novel approach to imaging engorged ticks: Micro-CT scanning of *Ixodes ricinus* fed on blood enriched with gold nanoparticles

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ABSTRACT

Micro-computed tomography (micro-CT) is an exceptional imaging modality which is limited in visualizing soft biological tissues that need pre-examination contrasting steps, which can cause serious deformation to sizeable specimens like engorged ticks. The aim of this study was to develop a new technique to bypass these limitations and allow the imaging of fed ticks in their natural state. To accomplish this, adult *Ixodes ricinus* females were allowed to engorge *in vitro* on blood supplemented with PEGylated gold nanoparticles (PEG-AuNPs). In total, 73/120 females divided into 6 groups engorged on blood enriched with 0.07–2.16 mg PEG-AuNPs per ml of blood. No toxic effect was observed for any of the tested groups compared to the control group, in which 12/20 females engorged on clear blood. The ticks were scanned on a Bruker micro-CT SkyScan 1276. The mean radiodensity of the examined ticks exceeded 0 Hounsfield Units only in the case of the two groups with the highest concentration. The best contrast was observed in ticks engorged on blood with the highest tested concentration of 2.16 mg/mL PEG-AuNPs. In these ticks, the midgut and rectal sac were clearly visible. Also, the midgut lumen volume was computed from segmented image data. The reduction in midgut volume was documented during the egg development process. According to this pilot study, micro-CT of ticks engorged on blood supplemented with contrasting agents *in vitro* may reveal additional information regarding the engorged ticks' anatomy.

1. Introduction

Several imaging methods that reveal both external and internal tick morphology are available. Histology and electron microscopy require the specimen's mechanical disruption, like sectioning (Lynn et al., 2015) or removing the surrounding tissues (Denardi et al., 2011). Arthropod structure, however, is often affected during the preparation process. Thus, the anatomy may not be observed in its natural state and spatial context can be lost. Alternatively, whole-volume imaging techniques e.g. high-resolution episcopic microscopy (Weninger et al., 2006), microscopic magnetic resonance (Ruffins et al., 2007), optical projection tomography (Sharpe, 2004) or micro-computed tomography (micro-CT) (Starck et al., 2018) can be employed to construct a three-dimensional (3D) visualization of a whole small biological specimen. Among them, micro-CT offers a suitable, quick and easy way to visualize ticks' internal structures and can bypass some other imaging techniques' limitations.

Micro-CT allows arthropod visualization in a non-destructive manner. In brief, when scanning the specimen, a set of two-dimensional (2D) projections are taken from divergent angles using X-rays. The obtained projections are subsequently used to compute a three-dimensional representation of the entire sample's attenuation coefficient's spatial distribution. Reconstructed image datasets can be

Abbreviations: DLS, dynamic light scattering; ELS, electrophoretic light scattering; FBS, fetal bovine serum; HU, Hounsfield Units; Micro-CT, micro-computed tomography; PEG, polyethylene glycol; PEG-AuNPs, PEGylated gold nanoparticles; TEM, transmission electron microscopy.

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analysed, modified and visualized using various imaging software systems. Several detailed reviews concerning micro-CT's the biological application are available (Ritman, 2011; Mizutani and Suzuki, 2012; du Plessis et al., 2017; O'Sullivan et al., 2018). Micro-CT enables high-resolution non-destructive imaging, scanning multiple specimens at the same time and provides precise morphometric analysis by applying software to image datasets. However, visualizing arthropods e. g. engorged ticks might be problematic due to the composition of their bodies. Hard cuticles comprising densely packed microfibrils of chitin embedded in a protein matrix and tracheae filled with air branching through the body provide sufficient natural contrast (Starck et al., 2018). Nevertheless, the inner soft compartments e. g. blood-filled midgut or salivary glands are visualized poorly as a result of their similar and low X-ray attenuation. Therefore, the contrast in these tissues needs to be enhanced prior to scanning.

For micro-CT scanning of arthropods, drying and staining with a contrasting agent are commonly used contrast enhancing techniques. However, the processing of invertebrates before scanning often leads to morphological changes in the scanned object. For example, drying by both critical-point drying and hexamethyldisilazane (HMDS) (Nation, 1983) causes specimen shrinkage (Bray et al., 1993; Kääb et al., 1998). Despite the fact that certain efforts can be made to minimize the deformation, dried samples are always very fragile and prone to breaking. During staining, biological samples are typically immersed in an aqueous solution containing a high-atomic-number element (Metscher, 2009a, 2009b; Pauwels et al., 2013). This technique, however, is limited by the used chemicals' diffusion rates and also suffers from contrasting-induced shrinkage (Vickerton et al., 2013). Since engorged ticks are relatively sizeable specimens and are loaded with a large volume of condensed blood, both staining and dehydration are not suitable contrasting approaches. This was demonstrated recently, when a fully engorged *Ixodes ricinus* female was dehydrated in graded ethanol, stained in iodine solution and dried by critical-point drying (Starck et al., 2018).

In this study, we present an innovative contrasting technique for imaging adult *I. ricinus* females exploiting their hematophagous nature that bypasses some traditional arthropod staining limitations and allows quick and easy imaging by micro-CT as close to their natural state as possible. We show that the contrast of midgut lumen, epithelial cells of the midgut and rectal sac can be significantly increased by allowing ticks to imbibe blood supplemented with gold nanoparticles in the artificial *in vitro* feeding system. This novel contrasting approach not only enables us to monitor the above-mentioned body compartments in a preserved native spatial context, but also permits a precise calculation of the midgut lumen volume.

2. Materials and methods

2.1. Tick collection

Questing adult ticks were collected by flagging from vegetation in a mixed forest located in Brno, Czech Republic (49.2502214N, 16.5614825E) in April 2019. Flagging was done by one person using a white denim square 1 m × 1 m attached to a wooden stick as a flag as described before (Tkadlec et al., 2018). According to an available key (Nosek and Sixl, 1972) only *I. ricinus* were collected, and they were not further examined for being *I. inopinatus* (Estrada-Peña et al., 2014). Adults were placed into 50 mL Falcon Tubes with respect to their sex, together with 5 blades of the grass, where they remained in a dark place at 15 °C for a maximum of 21 days until the start of the feeding. Grass was changed twice a week, tubes once a week during this period.

2.2. Preparation of contrast agent for in vitro feeding

PEGylated gold nanoparticles (PEG-AuNPs) used as a contrast agent in blood meals for ticks were prepared in-house as follows. Citrate-stabilized AuNPs were synthesized according to previous studies (Turkevich et al., 1951; Frens, 1973) with several modifications. Briefly, a tenfold molar excess of trisodium citrate was added to the boiling solution of 0.5 mM chloroauric acid while stirring vigorously. The reaction conditions were kept unchanged until the colour of the mixture turned ruby red. The citrate stabilized AuNPs were PEGylated by mixing an aqueous solution of methoxy-PEG-thiol 2 kDa with the colloid solution while stirring (Wang et al., 2013). The ratio of 100 nmol/mg (mPEG--SH/AuNPs) was used. The colloid solution was incubated at room temperature overnight. Subsequently, the PEG-AuNP solution was concentrated using a tangential flow filtration system (TFF KrosFlo KR2i; HF Filter: MIDIKROS P/N: D04-E030-05-N; Repligen, Boston, USA) (Sweeney et al., 2006). Likewise, in this step excess mPEG-thiol was removed. Thereafter, the concentrated colloid solution of PEG-AuNPs was sterilized by filtration (TPP Syringe-Filter P/N: 99722). The final concentration was reached through centrifugation (50 000 × g/90 min). The prepared nanoparticles were characterized using transmission electron microscopy (TEM) and dynamic and electrophoretic light scattering (DLS, ELS - Zetasizer Ultra, Malvern Panalytical, Malvern, UK). The PEG-AuNP's core size was around 13 nm (TEM) (see Supplementary Fig. 1), while the hydrodynamic diameter was 23 nm (DLS). The particles' zeta potential was -2.3 mV (10 mM PB, pH 7.4). The prepared PEG-AuNPs' stability was tested in 80 % fetal bovine serum (FBS). As confirmed on a TEM, the PEG-AuNPs suspended in FBS did not show any signs of aggregation.

2.3. In vitro feeding

An *in vitro* feeding system was established according to Kröber and Guerin (2007) with minor modifications. In brief, blood for the tick feeding was collected from an adult Holstein cow at a local slaughterhouse, immediately treated with heparin (15 U/mL), 5 μg/mL Gentamicin, 100 U/mL Nystatin, 1 mM Adenosine 5'-triphosphate (ATP) and 4 g/mL Glucose and stored at 4 °C until use (all chemicals were purchased at Sigma-Aldrich, Darmstadt, Germany). Cellulose membranes 60–80 μm thin reinforced with silicone were prepared. Glass fibre mosquito netting pieces as mechanical stimuli and feeding chambers were glued to the membranes. No hair nor chemical stimuli were used. A total of 10 females and 10 males were put into each chamber and sealed with cotton wool wrapped in a gauze. The chambers were put into the standard 6-well culture plates (Thermo Fisher Scientific, Roskilde, Denmark) filled with 3.0 mL of bovine blood in each well. The PEG-AuNPs were pipetted into the blood shortly before each of the blood exchanges, which were done approximately every 12 h. The tested concentrations were 0.07; 0.14; 0.27; 0.54; 1.08 and 2.16 mg of PEG-AuNPs per ml of blood. The feeding chambers were placed into a partially covered water bath WNB 29 (Memmert GmbH, Schwabach, Germany) at 37 °C for 10 days. The females that were dead or not attached during the third day of the feeding were removed. Also, some females that were feeding in close proximity to the others were discarded to prevent hindering the others. The numbers of engorged ticks and their weights at the end of the feeding were recorded immediately after detachment.

2.4. Micro-CT

After detachment, the engorged females were kept in glass jars in a dark and humid environment at 20 °C. For each group of different PEG-AuNP concentration fed ticks, one specimen was killed and scanned 1, 4, 6, 8, 12, 14, 16 and 24 days after detachment. The ticks were killed by chloroform vapours sealing them into 11 ml glass air-tight vials with a small piece of cotton wool wrapped in a gauze spiked with 20 μl of chloroform for 30 min to prevent their movement during scanning. The dead ticks were immediately transferred into a small scanning bed (diameter 12 mm) and scanned on a Bruker micro-CT SkyScan 1276 (Bruker, Kontich, Belgium) at 40 kV, 100 μA without filter, with

resolution 4032 × 2966 px, pixel size 4.0 μm, step and shoot mode with rotation step 0.06 degrees, 2944 projections. Backward projection datasets of all ticks were reconstructed using Insta-Recon software (Bruker microCT, Kontich, Belgium) with windowing 0 - 0.05 intensity, ring artefact reduction 25 and automatic post-alignment correction.

2.5. Data processing and statistical analysis

Post-processing image dataset and radiodensity analysis adjustment was carried out using CTanalyser software (Bruker microCT, Kontich, Belgium). Three-dimensional (3D) visualization was performed in CTVox (Bruker microCT, Kontich, Belgium). The radiodensity of ticks in Hounsfield Units (HU) was calculated as a mean of all voxels in the entire volume of ticks in each group. Post-scanning radiodensity calibration was performed by scanning cylindrical phantoms (diameter 5 mm) with deionized water, using an identical scan setting as for ticks. The volumes of the whole ticks and the midgut lumen were calculated using CTanalyser according to the manual for image data processing for invertebrates (Alba-Tercedor, 2014). Body weight data were analysed and visualized using GraphPad Prism version 8.3.0, chi-square tests were calculated using an online quantpsy calculator (Preacher, 2011).

3. Results

In total, 140 *I. ricinus* females divided into 7 groups of 20 individuals which were allowed to feed on bovine blood supplied with 0; 0.07; 0.14; 0.27; 0.54; 1.08 and 2.16 mg of PEG-AuNPs per ml of blood. The numbers of engorged females, average body weights and radiodensity values of one randomly selected specimen from each group are summarized in Table 1. The numbers of engorged females among these groups did not differ significantly ($p > 0.99$), neither did their average body weights ($p > 0.70$). Individual tick's body weights are shown in Fig. 1.

Transaxial views of individual, randomly selected ticks from each group are shown in Fig. 2. A dose dependent progressive increase in the contrast in the 3D volume rendered ticks was apparent. Radiodensity values exceeding 0 HU were detected in the females engorged on blood supplemented with 1.08 and 2.16 mg of PEG-AuNPs per ml of blood (Table 1). Only ticks fed on the blood with the highest PEG-AuNPs concentration were further examined due there being the best contrast and no nanoparticle impact on body weights and engorged female survival. In female ticks scanned 1–8 days after detachment, PEG-AuNPs present in the midgut were not distributed evenly and patterns of sedimented, highly contrasted spots were apparent inside the midgut lumen (Supplementary Fig. 3). In ticks scanned 12 and 24 days after detachment, the midgut lumen was homogenously filled with the contrast agent (Fig. 3). Apart from the highly contrasted midgut, the rectal sac was also clearly visible. In addition, increased contrast in the midgut due to progressive blood meal digestion was also noted in the specimen 24 days post detachment.

Table 1
Body weights and radiodensities of examined ticks.

Concentration of PEG-AuNPs in blood [mg/mL]	Weight [mg]				Radiodensity [HU]	
	n	Mean	SD	SEM	Mean	SEM
C-	12	233.25	55.74	16.09	−55	0.0130
0.07	13	213.92	66.20	18.36	−12	0.0120
0.14	11	228.00	68.33	20.60	−14	0.0112
0.27	10	200.90	48.96	15.48	−19	0.0112
0.54	13	232.30	64.40	17.86	−17	0.0117
1.08	13	218.23	61.69	17.11	79	0.0213
2.16	13	219.07	68.87	19.10	618	0.0280

PEG-AuNPs: PEGylated gold nanoparticles.
SD: standard deviation.
SEM: standard error of mean.
HU: Hounsfield units.
C-: negative control.

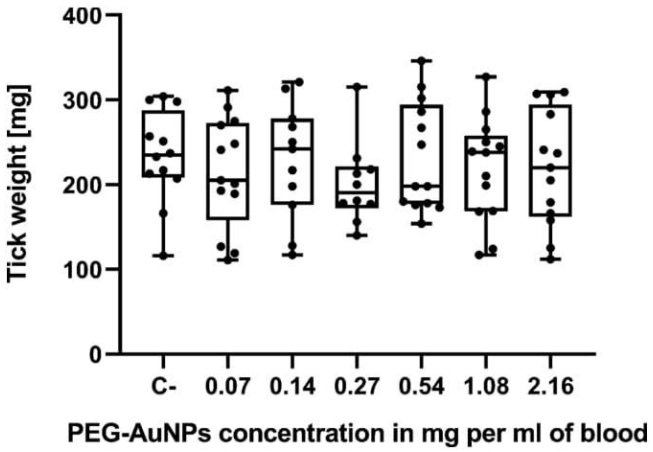

Fig. 1. Body weights of *Ixodes ricinus* females fed *in vitro* on blood enriched with PEG-AuNPs.

The volumes of the whole body and the PEG-AuNPs containing midgut for the female scanned 12 days post detachment (Fig. 3B) were 133 and 48 mm^3, respectively, and 97 and 27 mm^3 for the female scanned 24 days after detachment (Fig. 3C). Therefore, midgut volume decreased from approximately 36.1 % to 27.8 % of the tick's total volume during this period.

4. Discussion

In this study, we show that our novel approach in micro-CT imaging engorged ticks with oral administration of a nanogold-based contrast agent may provide useful information about the internal morphology of ticks. We report that ticks fed artificially on bovine blood supplied with PEG-AuNPs are able to fully engorge even on a relatively high concentration of 2.16 mg of PEG-AuNPs per ml of blood. This allows post-detachment visualization of the midgut and the rectal sac in their natural state unaffected by conventional contrasting procedures and exact calculation of the midgut lumen volume. This novel approach may be utilized to observe the midgut at a specific point during or after feeding, to monitor midgut dynamics in time, to visualize the midgut's pathologic alterations, to gain additional information regarding the mode of action of acaricides or similar substances added into the bloodmeal during *in vitro* feeding or to track microorganisms by conjugating gold nanoparticles with microorganism-specific ligands.

To date, micro-CT has been used in tick imaging very sporadically. For the first time, Dunlop et al. (2016) employed micro-CT to reconstruct a female *I. succineus* from a Baltic amber fossil to investigate its relations to modern ticks. Starck et al. (2018) scanned fasting, semi-engorged and fully engorged *I. ricinus* females to investigate the morphological adaptations that allow engorgement during feeding. Recently, micro-CT was used to investigate the mechanisms of blood acquisition and salivation in *I. ricinus* (Vancová et al., 2020). The latter two studies visualized ticks' internal structures stained by standard approaches like immersion in a contrasting agent and drying. Meanwhile, we used a new staining concept based on feeding ticks with blood supplemented with a contrasting agent.

Using this approach, we were able to significantly increase the contrast in the midgut lumen, the midgut epithelium and the rectal sac (Fig. 3). Interestingly, PEG-AuNPs were not dispersed homogenously in midgut content during the first 8 days after detachment and the largest conglomerations sedimented down at the bottom of the midgut diverticula. These conglomerations of highly radiodense particles created artifacts, such as beam hardening and scattering (see Supplementary Fig. 3). In contrast, PEG-AuNPs were distributed evenly in the midgut in

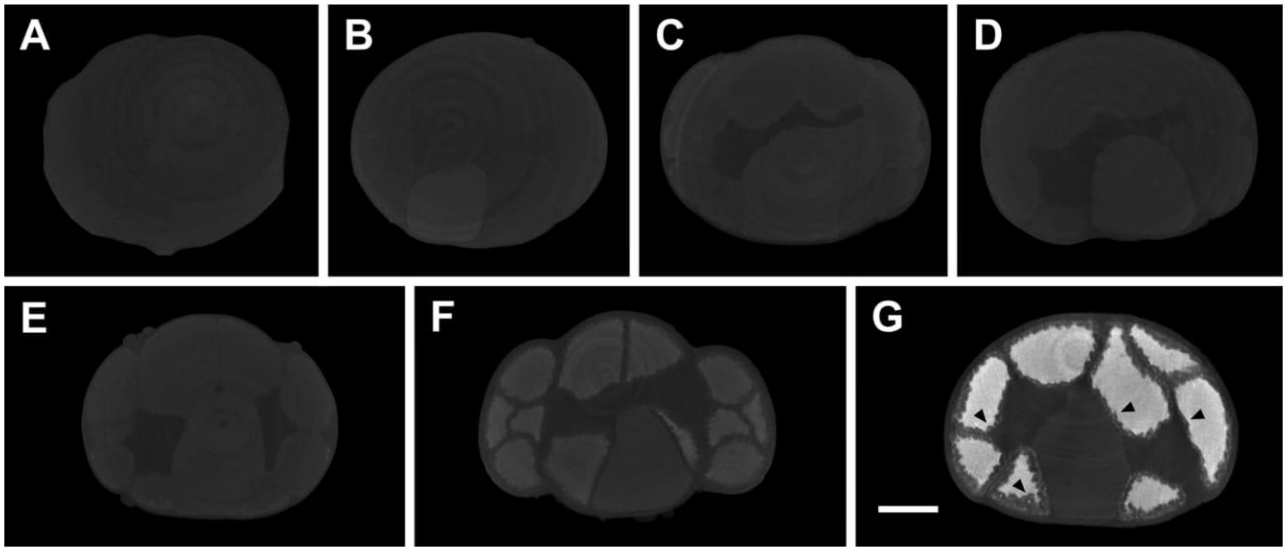

Fig. 2. Transaxial views of engorged *Ixodes ricinus* females fed on blood with 0 (**A**); 0.07 (**B**); 0.14 (**C**); 0.27 (**D**); 0.54 (**E**); 1.08 (**F**) and 2.16 (**G**) mg of PEG-AuNPs per ml of blood. Black arrowheads: epithelial cells of the midgut. Scale bar = 1 mm.

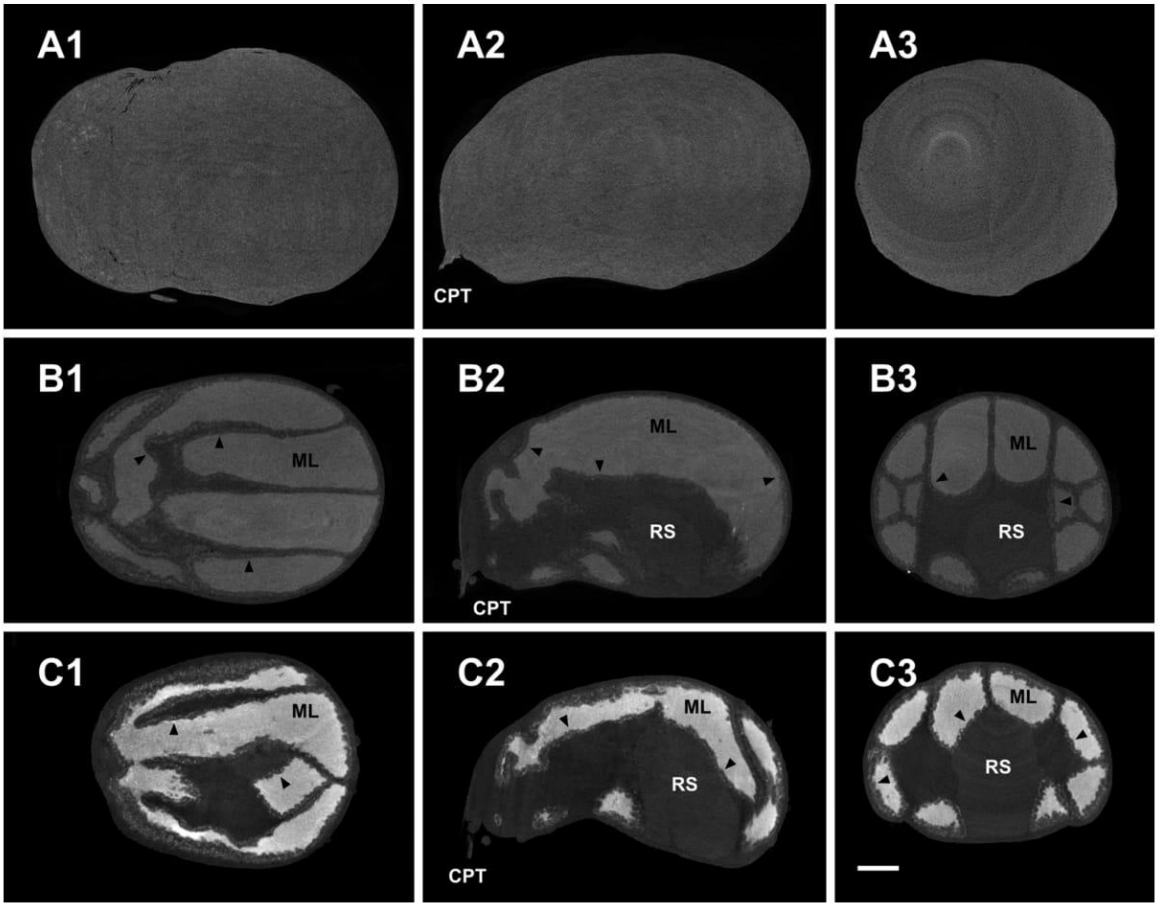

Fig. 3. Coronal (**1**) sagittal (**2**) and transaxial (**3**) views of engorged *Ixodes ricinus* females fed on blood with 0 (**A**) and 2.16 (**B, C**) mg of PEG-AuNPs per ml of blood. Scanned 12 (**B**) and 24 (**C**) days post detachment. ML, midgut lumen; CPT, capitulum; RS, rectal sac. Black arrowheads: epithelial cells of the midgut. Scale bar = 1 mm.

females scanned 12 days and later after detachment and no artifacts were observed (Figs. 2G and 3 B, C). A possible explanation for this phenomenon may be that gold nanoparticles were attached on blood cells when imbibed, progressively released by hemolysins in the course of extracellular blood cells lysis and eventually dispersed uniformly in the midgut lumen by the external muscle layer's peristaltic contractions (Sonenshine and Anderson, 2014). AuNPs could also potentially bind to some proteins in blood (Ajdari et al., 2017). Still, any explanation is very

speculative at this stage and further investigation on nanoparticle kinetics in arthropods is needed.

Morphological changes in internal organs after a blood meal were apparent in time, especially between 12 and 24 days post detachment (Fig. 3, Supplementary Video 1, 2). Changes in the proportions of the midgut and whole tick volume between 12 and 24 days post detachment were most likely associated with blood digestion and the egg development process. Although the ovaries with eggs were not visible at any stage after detachment, an enlarged space between dorsal and ventral midgut diverticula in the specimen scanned 24 days post detachment indicates the presence of the ovaries in this area. Furthermore, increased radiodensity of the midgut content at day 24 post detachment can be explained by the progressive digestion process, when the total volume of the midgut decreased, but the amount of PEG-AuNPs remained the same.

The fact that PEG-AuNPs remained mostly in the alimentary system (see 3D models of the females' segmented midguts depicted in Fig. 3B,C in Supplementary Videos 1, 2, respectively) and did not penetrate into the hemolymph and further into the tick may contribute to the absence of a significant toxic effect on the numbers of engorged ticks or their body weights (Fig. 1). To minimize the toxicity of the used gold nanoparticles in the first place, we used in-house synthesized AuNPs covered in PEG, a coating commonly used to lower the cytotoxicity of nanoparticles (Khlebtsov and Dykman, 2011; Fratoddi et al., 2015). Although the biocompatibility of AuNPs is somewhat controversial (Connor et al., 2005; Hainfeld et al., 2006; Khlebtsov and Dykman, 2011; Fratoddi et al., 2015), from our results it seems that PEG-AuNPs may be safe to use in tick research. Furthermore, oviposition, hatchability and viability of tick larvae could not be statistically evaluated due to the fact that most of the specimens were killed for the scanning. However, we did not observe any obvious differences in laying eggs between females engorged on the highest PEG-AuNPs concentration and females in the control group.

Customarily, small biological specimens such as arthropods were contrasted by immersion in a solution containing a high-atomic-number element or by drying, both of which are inconvenient for engorged ticks. Although general contrasting guidelines can be followed, every new type of sample needs individual, optimized treatment for the best results. To achieve this, numerous contrasting solutions containing e. g. osmium tetroxide (OsO_4), phosphotungstic acid (PTA) or iodine are available. Even though these elements can satisfactorily enrich contrast in certain samples, they vary substantially in the penetration speed, toxicity and affinity. While OsO_4 preferably binds to lipids and cytoplasmic membranes and should therefore stain all cells, it penetrates slowly into the sample, does not effectively stain samples stored in ethanol and is highly toxic (Johnson et al., 2006; Bentley et al., 2007; Ribi et al., 2008; Metscher, 2009a; Descamps et al., 2014). On the contrary, PTA is safer to use, contrasts tissues fixed by ethanol and can effectively visualize structures rich in collagen or other proteins, but is characterized by moderate diffusion speed (Metscher, 2009b; Descamps et al., 2014; Nierenberger et al., 2015). Iodine contrasting solutions are safe to use, versatile, robust and penetrate quickly, but tend to overstain mineralized tissues (Metscher, 2009a, 2009b). Importantly, staining by immersion can induce artefacts like shrinkage or deformation (Vickerton et al., 2013), which should be especially considered in the case of relatively sizeable and soft specimens like engorged ticks. The same also applies for drying by both critical-point drying and applying HMDS (Nation, 1983; Bray et al., 1993; Kääb et al., 1998). Recently, a study combining these techniques was published; a fully engorged *I. ricinus* female was stained in an iodine solution and dried by critical-point drying (Starck et al., 2018). This approach, however, led to deformation of the tick when the cuticle was pressed into the midgut diverticula. Initially, we tried to modify this concept, but we only obtained unsatisfying results (see Supplementary Fig. 2). Therefore, instead of following these conventional staining methods, relying on applying chemicals to the specimens, we investigated the opposite contrasting direction based on the hematophagous nature of the ticks. We abandoned our early idea to feed ticks on laboratory rodents injected with a blood-pool contrasting agent, because its vascular residence times (Nebuloni et al., 2013) may not satisfactorily cover the entire feeding time of hard ticks like *I. ricinus*. Instead, we employed *in vitro* feeding, which gave us total control over the concentration of the contrasting agent administered to the blood during the whole course of feeding.

In our study, however, PEG-AuNPs remained mostly in the midgut and the rectal sac and did not penetrate further into the tick (Fig. 3B, C). It is possible that other types of AuNPs (modified in size and surface coating) would pass into the hemolymph and contrast additional organs *e. g.* salivary glands or ovaries for it is known that size, surface charge, coating and AuNPs' shape affect their penetration into various tissues in mammals (Hainfeld et al., 2006; Hu et al., 2006; Longmire et al., 2008; Perrault et al., 2009). Similarly, different staining patterns may also be observed for other nanoparticles not based on gold, if blood supplemented with these nanoparticles were imbibed by ticks. In any case, imaging the anatomy of ticks adds to the growing number of AuNPs' utilizations and further broadens its largely unexploited versatility.

5. Conclusions

In this study, we describe a novel *in vivo* technique to stain engorged *I. ricinus* adults using gold nanoparticles for micro-CT imaging. By allowing the ticks to imbibe blood enriched with PEG-AuNPs, we achieved sufficient contrast to observe midgut lumen, midgut epithelium and rectal sac in their natural state unaffected by the shrinkage or deformation caused by traditional contrasting methods. To the best of our knowledge, this is the first study using AuNPs in imaging blood-feeding arachnids.

CRediT authorship contribution statement

Jaroslav Ondruš: Conceptualization, Funding acquisition, Data curation, Investigation, Methodology, Resources, Visualization, Writing - original draft. **František Hubatka:** Investigation, Resources. **Pavel Kulich:** Investigation. **Nikola Odehnalová:** Investigation. **Vratislav Harabiš:** Data curation, Formal analysis, Visualization. **Branislav Hesko:** Formal analysis. **Oldřich Sychra:** Resources. **Pavel Široký:** Resources. **Jaroslav Turánek:** Funding acquisition, Project administration, Supervision, Writing - review & editing. **Adam Novobilský:** Conceptualization, Investigation, Methodology, Formal analysis, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101559.

References

Ajdari, N., Vyas, C., Bogan, S.L., Lwaleed, B.A., Cousins, B.G., 2017. Gold nanoparticle interactions in human blood: a model evaluation. Nanomed. Nanotechnol. Biol. Med. 13, 1531–1542. https://doi.org/10.1016/j.nano.2017.01.019.

Alba-Tercedor, J., 2014. From the sample preparation to the volume rendering images of small animals: a step by step example of a procedure to carry out the micro-CT study of the leafhopper insect *Homalodisca vitripennis* (Hemiptera: Cicadellidae). In: Proceedings of the Bruker Micro-CT User Meeting 2014. Oostende, Belgium, May 5-8th, 2014, pp. 260–288.

Bentley, M.D., Jorgensen, S.M., Lerman, L.O., Ritman, E.L., Romero, J.C., 2007. Visualization of three-dimensional nephron structure with microcomputed tomography. Anat. Rec. 290, 277–283. https://doi.org/10.1002/ar.20422.

Bray, D.F., Bagu, J., Koegler, P., 1993. Comparison of hexamethyldisilazane (HMDS), Peldri II, and critical-point drying methods for scanning electron microscopy of biological specimens. Microsc. Res. Tech. 26, 489–495. https://doi.org/10.1002/jemt.1070260603.

Connor, E.E., Mwamuka, J., Gole, A., Murphy, C.J., Wyatt, M.D., 2005. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 1, 325–327. https://doi.org/10.1002/smll.200400093.

Denardi, S.E., Furquim, K.C.S., Bechara, G.H., Camargo-Mathias, M.I., 2011. Salivary glands of *Amblyomma cajennense* (Acari: ixodidae): a histological and an ultrastructural overview. Exp. Appl. Acarol. 54, 177–189. https://doi.org/10.1007/s10493-010-9421-5.

Descamps, E., Sochacka, A., De Kegel, B., Van Loo, D., Van Hoorebeke, L., Adriaens, D., 2014. Soft tissue discrimination with contrast agents using micro-CT scanning. Belgian J. Zool. 144, 20–40. https://doi.org/10.26496/bjz.2014.63.

du Plessis, A., Broeckhoven, C., Guelpa, A., le Roux, S.G., 2017. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. Gigascience 6, 1–11. https://doi.org/10.1093/gigascience/gix027.

Dunlop, J.A., Apanaskevich, D.A., Lehmann, J., Hoffmann, R., Fusseis, F., Ehlke, M., Zachow, S., Xiao, X., 2016. Microtomography of the Baltic amber tick *Ixodes succineus* reveals affinities with the modern Asian disease vector *Ixodes ovatus*. BMC Evol. Biol. 16, 203. https://doi.org/10.1186/s12862-016-0777-y.

Estrada-Peña, A., Nava, S., Petney, T., 2014. Description of all the stages of *Ixodes inopinatus*n. sp. (Acari: Ixodidae). Ticks Tick. Dis. 5, 734–743. https://doi.org/10.1016/j.ttbdis.2014.05.003.

Fratoddi, I., Venditti, I., Cametti, C., Russo, M.V., 2015. How toxic are gold nanoparticles? The state-of-the-art. Nano Res. 8, 1771–1799. https://doi.org/10.1007/s12274-014-0697-3.

Frens, G., 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nat. Phys. Sci. 241, 20–22. https://doi.org/10.1038/physci241020a0.

Hainfeld, J.F., Slatkin, D.N., Focella, T.M., Smilowitz, H.M., 2006. Gold nanoparticles: a new X-ray contrast agent. Br. J. Radiol. 79, 248–253. https://doi.org/10.1259/bjr/13169882.

Hu, M., Chen, J., Li, Z.-Y., Au, L., Hartland, G.V., Li, X., Marquez, M., Xia, Y., 2006. Gold nanostructures: engineering their plasmonic properties for biomedical applications. Chem. Soc. Rev. 35, 1084–1094. https://doi.org/10.1039/b517615h.

Johnson, J.T., Hansen, M.S., Wu, I., Healy, L.J., Johnson, C.R., Jones, G.M., Capecchi, M. R., Keller, C., 2006. Virtual histology of transgenic mouse embryos for high-throughput phenotyping. PLoS Genet. 2, e61. https://doi.org/10.1371/journal.pgen.0020061.

Kääb, M.J., Nötzli, H.P., Clark, J., Gwyn, ap I., 1998. Dimensional changes of articular cartilage during immersion-freezing and freeze-substitution for scanning electron microscopy. Scanning Microsc. 12, 465–474.

Khlebtsov, N., Dykman, L., 2011. Biodistribution and toxicity of engineered gold nanoparticles: a review of *in vitro* and *in vivo* studies. Chem. Soc. Rev. 40, 1647–1671. https://doi.org/10.1039/c0cs00018c.

Kröber, T., Guerin, P.M., 2007. An *in vitro* feeding assay to test acaricides for control of hard ticks. Pest Manag. Sci. 63, 17–22. https://doi.org/10.1002/ps.1293.

Longmire, M., Choyke, P.L., Kobayashi, H., 2008. Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. Nanomedicine 3, 703–717. https://doi.org/10.2217/17435889.3.5.703.

Lynn, G.E., Oliver, J.D., Nelson, C.M., Felsheim, R.F., Kurtti, T.J., Munderloh, U.G., 2015. Tissue distribution of the *Ehrlichia muris*-like agent in a tick vector. PLoS One 10, e0122007. https://doi.org/10.1371/journal.pone.0122007.

Metscher, B.D., 2009a. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. BMC Physiol. 9, 11. https://doi.org/10.1186/1472-6793-9-11.

Metscher, B.D., 2009b. MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions. Dev. Dyn. 238, 632–640. https://doi.org/10.1002/dvdy.21857.

Mizutani, R., Suzuki, Y., 2012. X-ray microtomography in biology. Micron 43, 104–115. https://doi.org/10.1016/j.micron.2011.10.002.

Nation, J.L., 1983. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. Stain Technol. 58, 347–351. https://doi.org/10.3109/10520298309066811.

Nebuloni, L., Kuhn, G.A., Müller, R., 2013. A comparative analysis of water-soluble and blood-pool contrast agents for *in vivo* vascular imaging with micro-CT. Acad. Radiol. 20, 1247–1255. https://doi.org/10.1016/j.acra.2013.06.003.

Nierenberger, M., Rémond, Y., Ahzi, S., Choquet, P., 2015. Assessing the three-dimensional collagen network in soft tissues using contrast agents and high resolution micro-CT: application to porcine iliac veins. C. R. Biol. 338, 425–433. https://doi.org/10.1016/j.crvi.2015.04.009.

Nosek, J., Sixl, W., 1972. Central-European ticks (Ixodoidea). Mitt. Abt. Zool. Landesmus Joanneum 1, 61–92.

O'Sullivan, J.D.B., Behnsen, J., Starborg, T., MacDonald, A.S., Phythian-Adams, A.T., Else, K.J., Cruickshank, S.M., Withers, P.J., 2018. X-ray micro-computed tomography (μCT): an emerging opportunity in parasite imaging. Parasitology 145, 848–854. https://doi.org/10.1017/S0031182017002074.

Pauwels, E., Van Loo, D., Cornillie, P., Brabant, L., Van Hoorebeke, L., 2013. An exploratory study of contrast agents for soft tissue visualization by means of high resolution X-ray computed tomography imaging. J. Microsc. 250, 21–31. https://doi.org/10.1111/jmi.12013.

Perrault, S.D., Walkey, C., Jennings, T., Fischer, H.C., Chan, W.C.W., 2009. Mediating tumor targeting efficiency of nanoparticles through design. Nano Lett. 9, 1909–1915. https://doi.org/10.1021/nl900031y.

Preacher, K.J., 2011. Calculation for the Chi-square Test: an Interactive Calculation Tool for Chi-square Tests of Goodness of Fit and Independence [WWW Document]. URL http://www.quantpsy.org/chisq/chisq.htm (Accessed 11.10.19).

Ribi, W., Senden, T.J., Sakellariou, A., Limaye, A., Zhang, S., 2008. Imaging honey bee brain anatomy with micro-X-ray-computed tomography. J. Neurosci. Methods 171, 93–97. https://doi.org/10.1016/j.jneumeth.2008.02.010.

Ritman, E.L., 2011. Current status of developments and applications of micro-CT. Annu. Rev. Biomed. Eng. 13, 531–552. https://doi.org/10.1146/annurev-bioeng-071910-124717.

Ruffins, S.W., Martin, M., Keough, L., Truong, S., Fraser, S.E., Jacobs, R.E., Lansford, R., 2007. Digital three-dimensional atlas of quail development using high-resolution MRI. Sci. World J. 7, 592–604. https://doi.org/10.1100/tsw.2007.125.

Sharpe, J., 2004. Optical projection tomography. Annu. Rev. Biomed. Eng. 6, 209–228. https://doi.org/10.1146/annurev.bioeng.6.040803.140210.

Sonenshine, D.E., Anderson, J.M., 2014. Mouthparts and digestive system. In: Sonenshine, D.E., Roe, M.R. (Eds.), Biology of Ticks. Oxford University Press, New York, pp. 122–162.

Starck, J.M., Mehnert, L., Biging, A., Bjarsch, J., Franz-Guess, S., Kleeberger, D., Hörnig, M., 2018. Morphological responses to feeding in ticks (*Ixodes ricinus*). Zool. Lett. 4, 20. https://doi.org/10.1186/s40851-018-0104-0.

Sweeney, S.F., Woehrle, G.H., Hutchison, J.E., 2006. Rapid purification and size separation of gold nanoparticles via diafiltration. J. Am. Chem. Soc. 128, 3190–3197. https://doi.org/10.1021/ja0558241.

Tkadlec, E., Václavík, T., Kubelová, M., Široký, P., 2018. Negative spatial covariation in abundance of two European ticks: diverging niche preferences or biotic interaction? Ecol. Entomol. 43, 804–812. https://doi.org/10.1111/een.12668.

Turkevich, J., Stevenson, P.C., Hillier, J., 1951. A study of the nucleation and growth processes in the synthesis of colloidal gold. Faraday Discuss. 11, 55–75. https://doi.org/10.1039/DF9511100055.

Vancová, M., Bílý, T., Šimo, L., Touš, J., Horodyský, P., Ružek, D., Novobilský, A., Salát, J., Strnad, M., Sonenshine, D.E., Grubhoffer, L., Nebesářová, J., 2020. Three-dimensional reconstruction of the feeding apparatus of the tick *Ixodes ricinus* (Acari: Ixodidae): a new insight into the mechanism of blood-feeding. Sci. Rep. 10, 165. https://doi.org/10.1038/s41598-019-56811-2.

Vickerton, P., Jarvis, J., Jeffery, N., 2013. Concentration-dependent specimen shrinkage in iodine-enhanced microCT. J. Anat. 223, 185–193. https://doi.org/10.1111/joa.12068.

Wang, W., Wei, Q.-Q., Wang, J., Wang, B.-C., Zhang, S., Yuan, Z., 2013. Role of thiol-containing polyethylene glycol (thiol-PEG) in the modification process of gold nanoparticles (AuNPs): Stabilizer or coagulant? J. Colloid Interface Sci. 404, 223–229. https://doi.org/10.1016/j.jcis.2013.04.020.

Weninger, W.J., Geyer, S.H., Mohun, T.J., Rasskin-Gutman, D., Matsui, T., Ribeiro, I., Costa, L., da, F., Izpisúa-Belmonte, J.C., Müller, G.B., 2006. High-resolution episcopic microscopy: a rapid technique for high detailed 3D analysis of gene activity in the context of tissue architecture and morphology. Anat. Embryol. 211, 213–221. https://doi.org/10.1007/s00429-005-0073-x.