Micro-CT unveils the secret life of the coffee berry borer (*Hypothenemus hampei*; Coleoptera, Curculionidae: Scolytinae) inside coffee berries

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Aims

The coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae: Scolytinae) is the most important insect pest of coffee worldwide (Vega *et al.* 2015). The small bark beetle attacks both *Coffea arabica* L. and *C. canephora* Pierre ex. A. Froehner (known as robusta coffee). Damage begins when an adult female enters the coffee berry and oviposits within galleries bored throughout the two seeds inside the berry. Upon hatching, larvae consume the seeds, resulting in a reduction in both yields and quality of the marketable product. Losses caused by the coffee berry borer in Brazil have been estimated at US$215-358 million on a yearly basis (Oliveira *et al.* 2013). The biggest challenge in attempting to control the insect is that it spends most of its life cycle hidden inside the coffee berry. This cryptic life habit also makes it impossible to observe its behaviour under natural conditions (Vega *et al.* 2015, 2017). Various videos are available showing how the coffee berry borer bores into coffee berries (Twkapi 2010; Jimenez Soto 2016; Thodi 2016; Yong 2017), and as an example of the amount of money invested to fight the pest, see Big Island Video News (2014) and NBC News – Papua New Guinea (2017).
To observe the behaviour of the coffee berry borer, Vega *et al.* (2017) developed diet sandwiches, consisting of coffee berry borer artificial diet (Vega *et al.* 2011) enclosed within two 3.8 x 3.8 cm glass plates separated by 1 mm. The use of diet sandwiches have allowed to observe for the first time various types of behaviour, including gallery blocking, grooming of larvae and eggs, and maternal sanitation of the nest. After studying the internal morphology of the coffee berry borer using micro-CT (Alba-Tercedor *et al.*, in preparation), it occurred to us that the technique could be useful to visualize the insect inside coffee berries. To the best of our knowledge, only two studies have involved micro-CT of coffee beans: a study by XIMEA (n.d.) was solely based on demonstrating the power of the technique by using a coffee bean as example, and a study by Pittia *et al.* (2011) was focused on elucidating the microstructural properties of coffee beans. The present study represents the first visualizations of coffee berry borer eggs, larvae, pupae, and adults inside coffee berries.

Materials and Methods

In November 2017, the senior author visited the *Me Linh Coffee Garden* plantation in Vietnam (address: Tổ 20 thôn 4, Tà Nung, tp. Đà Lạt, Lâm Đồng; 11º53'57.39" N, 108º20'51.16" E; 1043 m.a.s.l.) (Fig. 1a, 1b), where robusta plants (Fig. 1c, 1d) were examined and coffee berry borer infested berries were collected (Fig. 1e, 1f). Infested berries were kept at room temperature in the laboratory at the Department of Zoology, University of Granada. Two berries exhibiting internal insect activity through x-rays were selected for subsequent scans (Fig. 2b, 2d). One berry was scanned 13 days after it was collected in the field and it showed a

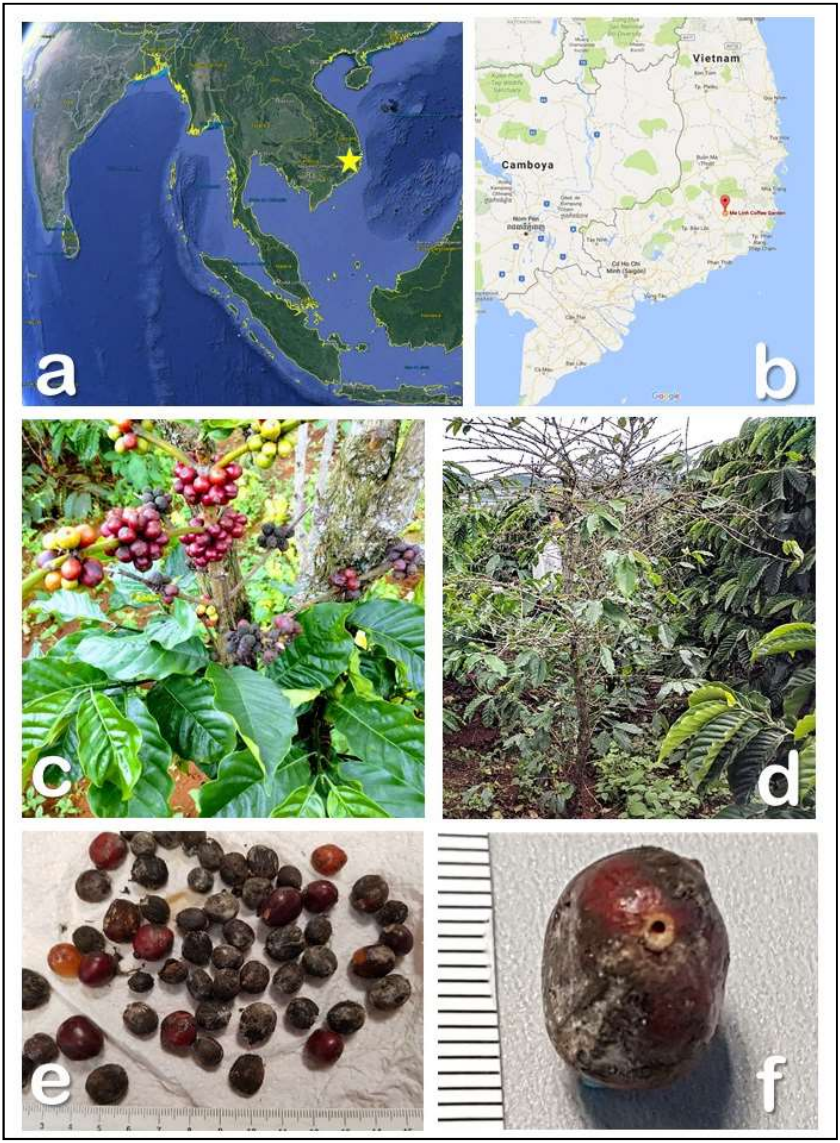

Figure 1: Location of the collection site in Vietnam (**a, b**)**;** coffee plants (**c, d**); infested coffee berries (**e**); detail of a coffee berry showing the entrance hole bored by a colonizing female (**f**) (this is the same berry shown in Figs. 3, 4).

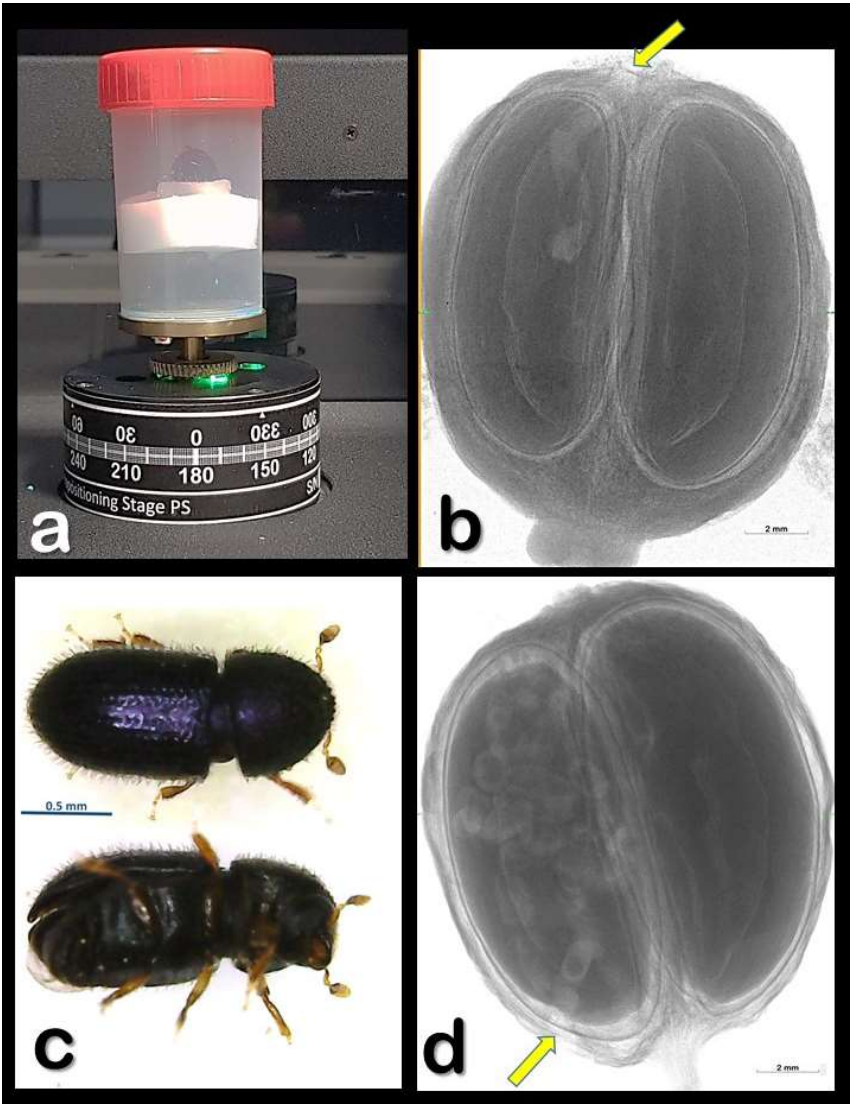

Figure 2: A coffee berry mounted to be scanned in an excavated piece of Basotect® inside a plastic container (**a**)**.** X-ray views of coffee berries visualized with Nrecon software: berry with a simple gallery and a single beetle that has just started the colonization process, scanned 13 days after the berries were collected (**b**); female coffee berry borer in a dorsal (above) and latero-ventral (below) view (**c**); a more complex system of galleries showing many insects scanned 41 days after the berries were harvested. Arrows indicate location of the entrance hole in the disc (**b**) and in a subapical basal position (**d**).

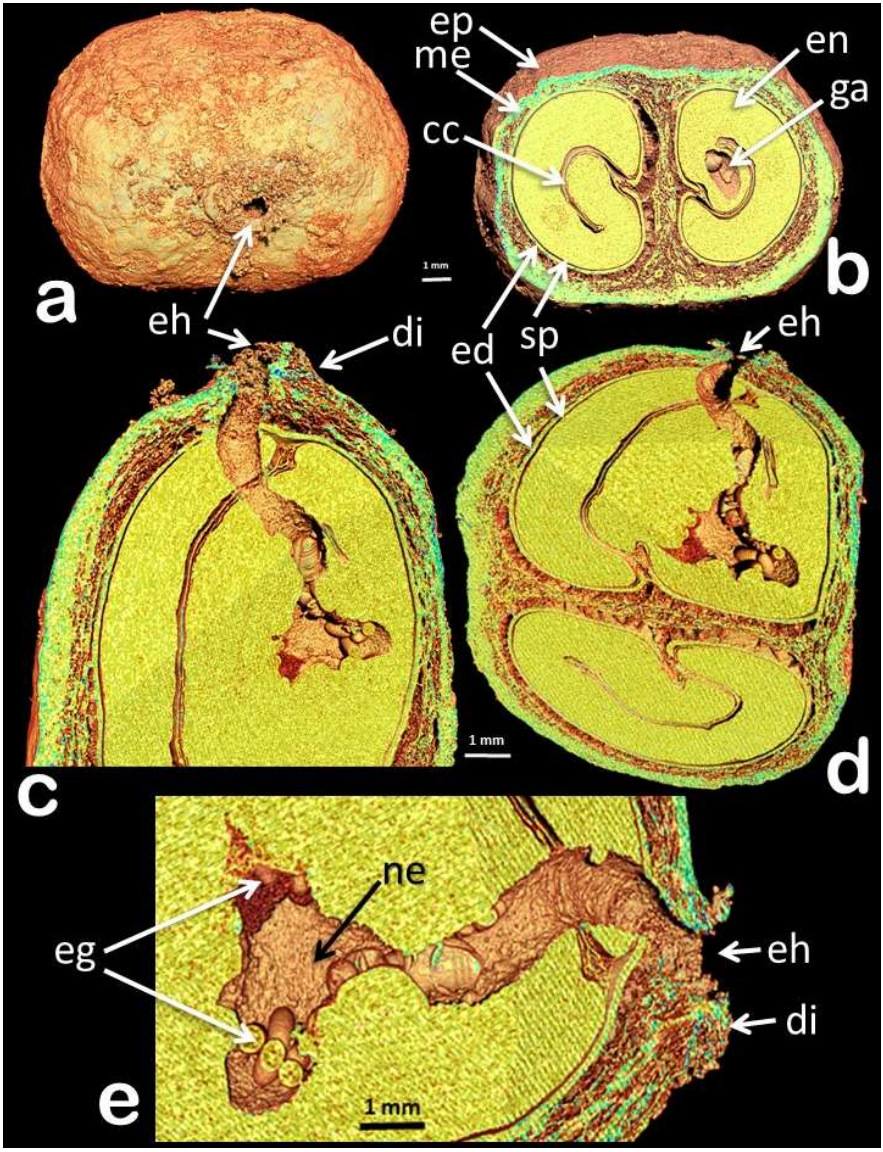

Figure 3: CTVox's volume renderings images of a coffee berry borer-infested coffee berry: apical view showing the entrance hole (**a**)**;** transversal cut view of the distal part (**b**); lateral cut (**c**); view from the basal part with three different cut planes enhancing the whole gallery and nest (**d**); details of the gallery, nest and a female adult with the eggs (**e**). Abbreviations: **cc**=center cut; **di**=disc; **ed** =endocarp (parchment); **eg**=eggs; **eh**=entrance hole; **en**=endosperm; **ep=**epicarp (outer skin); **ga**=gallery; **me**=mesocarp (mucilage); **ne**=nest; **sp**=spermoderm (silverskin).

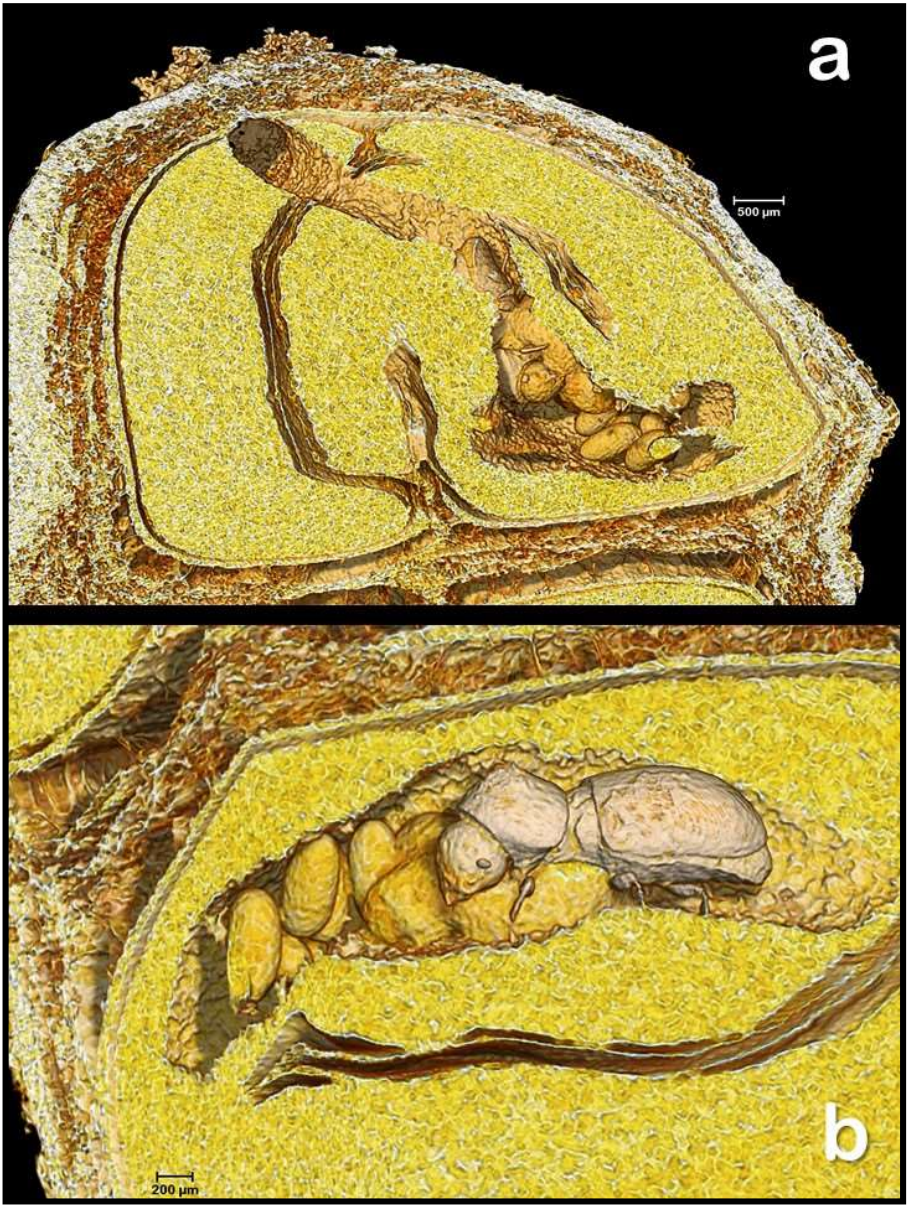

Figure 4: Amira's volume rendering of the gallery and an adult female in the nest with the eggs (**a**), and detail (**b**)**.** Female appears to be grooming an egg. In order to be able to see the whole adult it was separately segmented and separate volume renderings were superimposed.

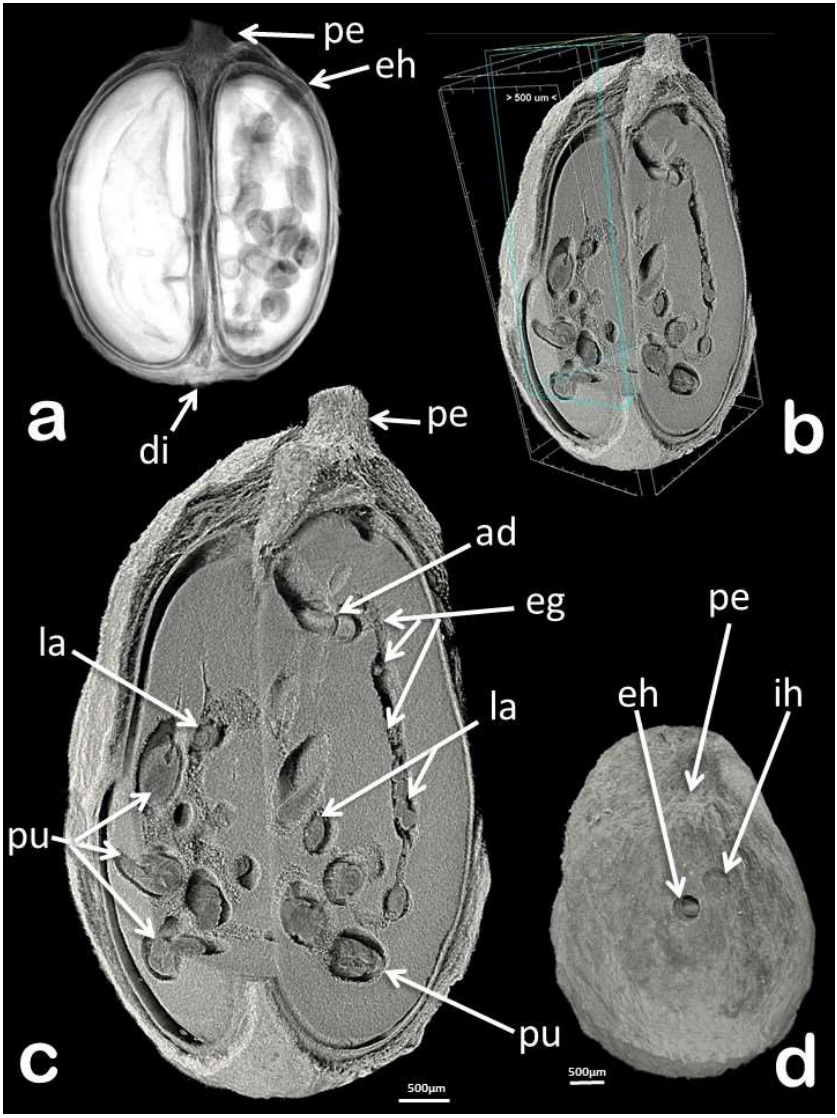

Figure 5: CTVox's images showing galleries and cavities containing different developmental stages of the coffee berry borer: attenuation image (**a**)**;** volume rendering reconstructions showing the cutting shape (turquoise) and clipping box (white) used to obtain the image shown in figure "c" (**b**); detail of galleries and cavities containing different developmental stages of the insect (**c**); and basal subapical view of the coffee berry showing an adult female inside the entrance hole (**eh**) and an initiated, non-completed hole (**ih**) (**d**). Abbreviations: **ad**=adult female; **di**=disc (*style remnant*); **eg**: eggs; **la**= larva; **pe**=pedicel; **pu**= pupae.

single female inside a simple gallery (Figs. 2b, 3, 4) and another, scanned 41 days after it was collected exhibited many insects as well as galleries (Figs. 2d, 5). Samples were mounted in a piece of BASOTECT® (melamine resin foam, BASF), inside a plastic container (Fig. 2a). The Basotect's material has a very low density, and is very transparent to X-rays, which allows to remove it during the segmentation procedure (Alba-Tercedor and Alba-Alejandre, 2017). Several drops of ethyl acetate were added to the melamine foam. Thus, after closing the container, ethyl acetate vapours killed the insects. Scans started 30 minutes later, ensuring that the insects did not move during the scans.

A SkyScan 1172 high-resolution microtomograph, upgraded to have a Hamamatsu 100/250 source and a SHT 11Mp camera was used. The scanning parameters were as follows: (1) For coffee berry in Figs. 2b, 3, and 4, isotropic voxel size = 6.7µm per pixel; voltage=48KV, current=124µA. Image rotation step=0.2º, 360º of rotation scan, an Al 0.5 mm filter, resulting two connected scans; (2) For coffee berry in Figs. 2d, and 5, isotropic voxel size = 3.25 µm per pixel; voltage=69KV, current=59µA. Image rotation step=0.3º, 360º of rotation scan, no filter, resulting two connected scans. The most recent versions of the Bruker micro-CT's Skyscan software (NRecon, DataViewer, CTAnalyser) were used for primary reconstructions and the "cleaning" process to obtain the datasets of "slices". Volume renderings of Fig. 5 were obtained with Amira's software v. 6.4.0 (Amira 2016). The free Skyscan's software CTVox was used for volume rendering reconstructions images in Figures 3 and 5. For a more detailed explanation of the process see Alba-Tercedor (2014).

Results and Discussion

This is the first time that coffee berry borers in different developmental stages have been observed under natural conditions, i.e., inside the coffee berry, thus providing yet another example of the versatility of uses of micro-CT in biological studies.

It is quite common for the coffee berry borer to colonize the berry through the disc, which is the small circular area located at the apical end of the berry (Figs. 1f, 2b, 3a, 3c, 3d, 3e). Nevertheless, penetration holes are occasionally seen in other areas of the berry, such as the entrance hole depicted in Figs. 2d and 5d. The study has also allowed visualizing two attempts of entrance into a single berry (Fig. 5d, "eh" and "ih"), one of which was abandoned (Fig. 5d, "ih") and an entrance hole leading to galleries in the seed (Fig. 5a, 5b, 5c).

It is particularly noteworthy that Fig. 3e and Fig. 4 show a female grooming an egg, a type of behaviour also observed by Vega et al. (2017) when using diet sandwiches. Egg grooming is a sign of subsocial behaviour.

The use of micro-CT has also allowed observing that in one berry (Fig. 5), the eggs appear to have been laid in the galleries and not in a defined nest as depicted in Fig. 4.

Conclusions

Micro-CT has proven to be an extremely valuable technique to unveil the hidden biology of the coffee berry borer inside coffee berries, thus allowing visualization of the internal structure of the berry, the seeds, and the galleries built by the colonizing female within the seeds. The technique also allows visualizing different developmental stages of the insect inside the berry, as well as one important type of behavior, i.e., egg grooming. The technique could also be useful to study bark beetles than inhabit branches, including bark beetles that feed on fungi (i.e., ambrosia beetles), as well as other important insect pests that develop inside fruits.

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