

## Introduction

X-ray micro-CT is an established method for studying insects<sup>1</sup>. Often, internal soft tissues have similar X-ray attenuation values, causing investigators to resort to using contrast enhancing agents (CEAs) such as iodine in the form of either Lugol's iodine or Iodine dissolved in 100% ethanol to enhance tissue definition<sup>2</sup>. The results obtained can be excellent<sup>3</sup> but there are often downsides.

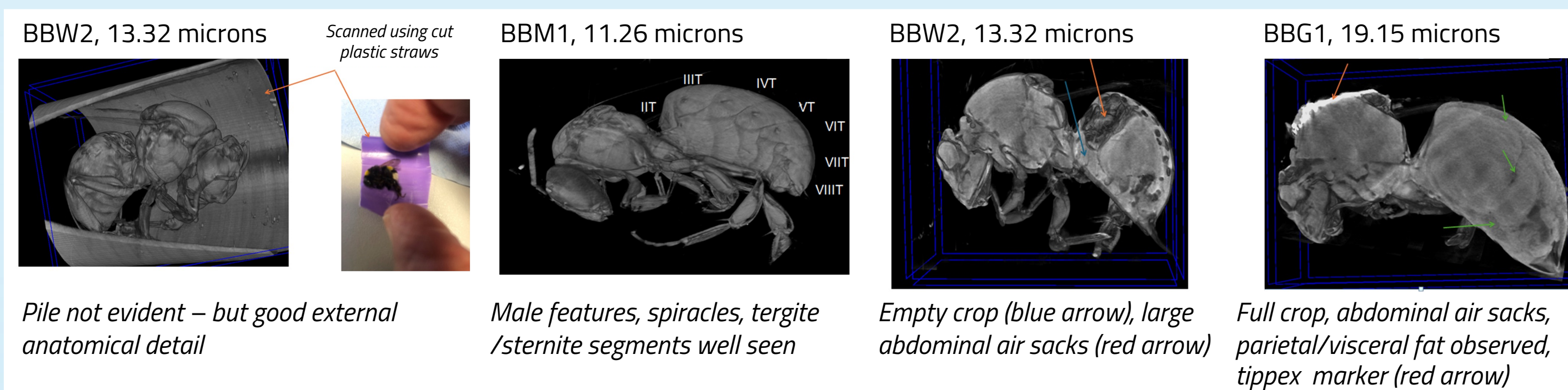
Iodine sublimation has previously been reported to enhance micro-CT image clarity in mammalian tissue<sup>4</sup>. Here, we show that exposing bumble bees (*Bombus terrestris*) to sublimated Iodine (from 5 - 100 days) can enhance image tissue definition in whole-body insects.

## Methodology

We collected 36 bees from commercial colonies (14 workers, 10 gynes, 10 males, and 2 queens), and 4 bees from the wild (2 spring foraging queens and 2 workers), stored them all at -80 °C. We then thawed and scanned the bees using a benchtop Skyscan 1072 X-ray scanner at isotropic interpixel distances varying from 9 – 20 µm. 6 individuals were also scanned at 2–7 µm using a Neoscan80. Following the first scan each bee was placed in air-tight plastic screw top bottles at room temperature either with (N=35) or without (N=3) their containing Iodine crystals. The bees were rescanned after varying periods between 5 days and 106 days. Scans were reconstructed using *NRECON* and viewed using *Disect* and *Tomomask*.

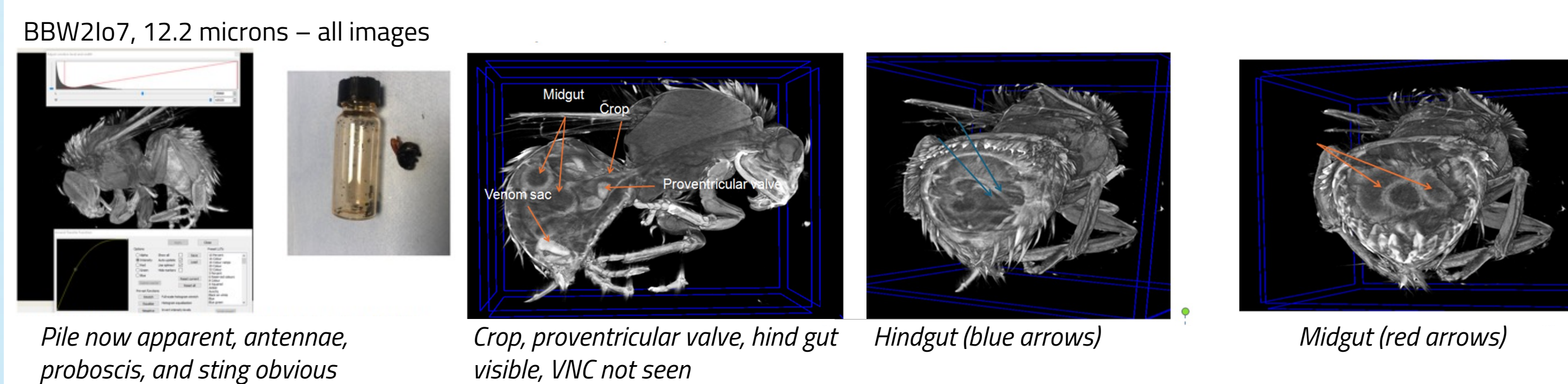
## Results – Scans using Skyscan 1072

Before exposure to iodine vapour (Fig 1) most external bee features (where there was good X-ray contrast between the surrounding low attenuation air and the chitinous exoskeleton) were well seen except for the pile making the bees appear bald. Internal anatomical detail was limited, and fat stores were only seen in large gynes.



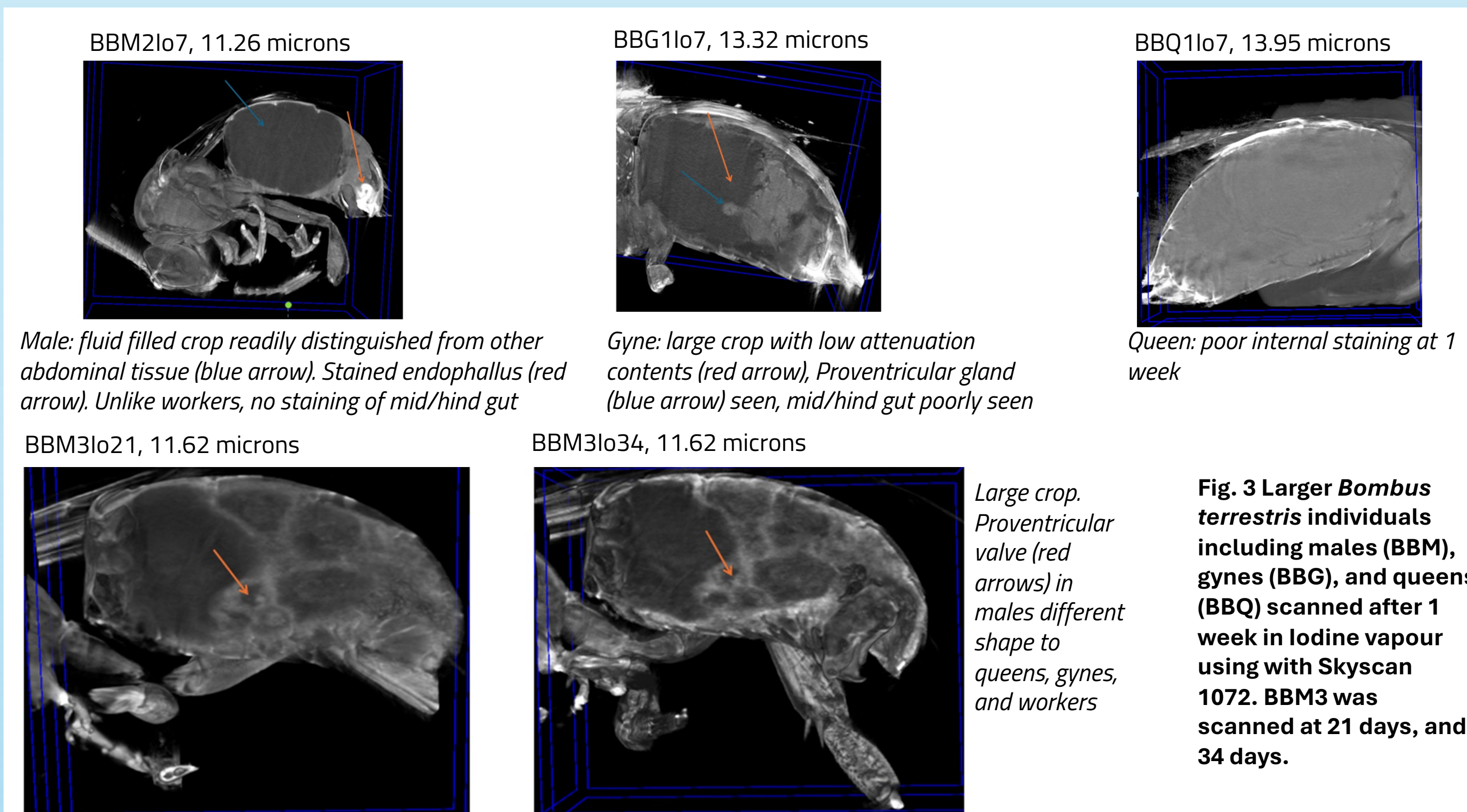
**Fig. 1 Unstained (i.e., no iodine) *Bombus terrestris* worker (BBW), male (BBM), and gyne (BBG) scanned with Skyscan 1072.**

In bees rescanned after 1 week in iodine vapour (Fig. 2) pile became apparent, and where there was an air/chitin interface, Iodine staining worked well (e.g., detail seen on antennae, proboscis, sting recess (in females), and phallus (in males)). In contrast, internal staining of some structures were variable after 1 week (e.g., flight muscles, crop, proventricular valve, midgut, hind gut, ovarioles and Ventral Nerve Cord (VNC)). The trachea and air sacs were seen equally well before and after 1–3 weeks in Iodine.



**Fig. 2 *Bombus terrestris* worker (BBW) scanned after 1 week in iodine vapour (Io7) with Skyscan 1072 at 12.2 microns.**

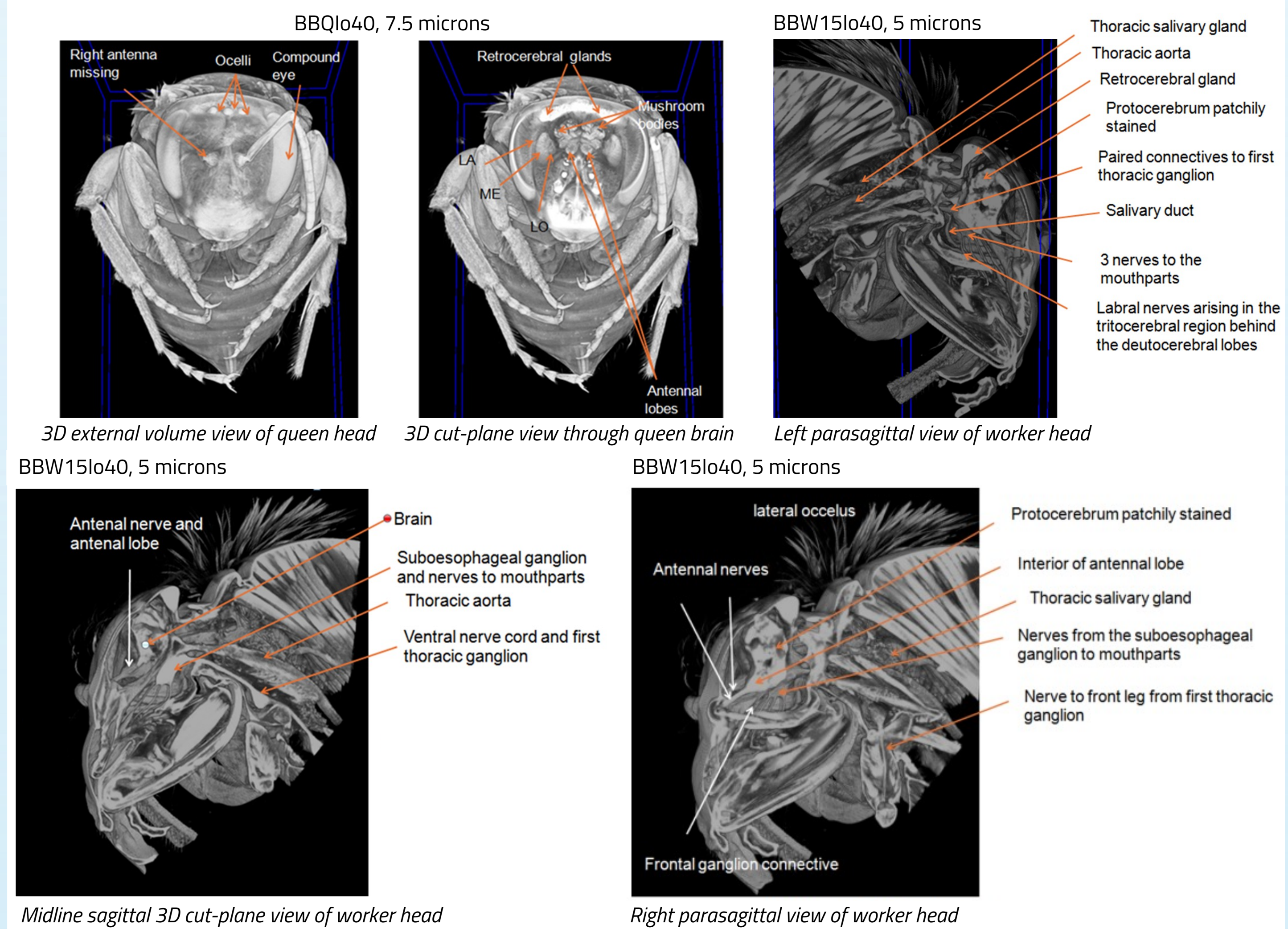
In the larger males, gynes and queen bees (Fig. 3) little evidence of internal staining was obtained at 7 days and most required periods of several weeks for optimal staining. Little extra staining was obtained after 50 days, and several bees scanned after this showed evidence of desiccation. The 3 bees left in the air-tight bottles but not exposed to iodine rapidly showed signs of internal decomposition and became covered by fungal hyphae (images not shown). Specimens exposed to iodine showed no such features.



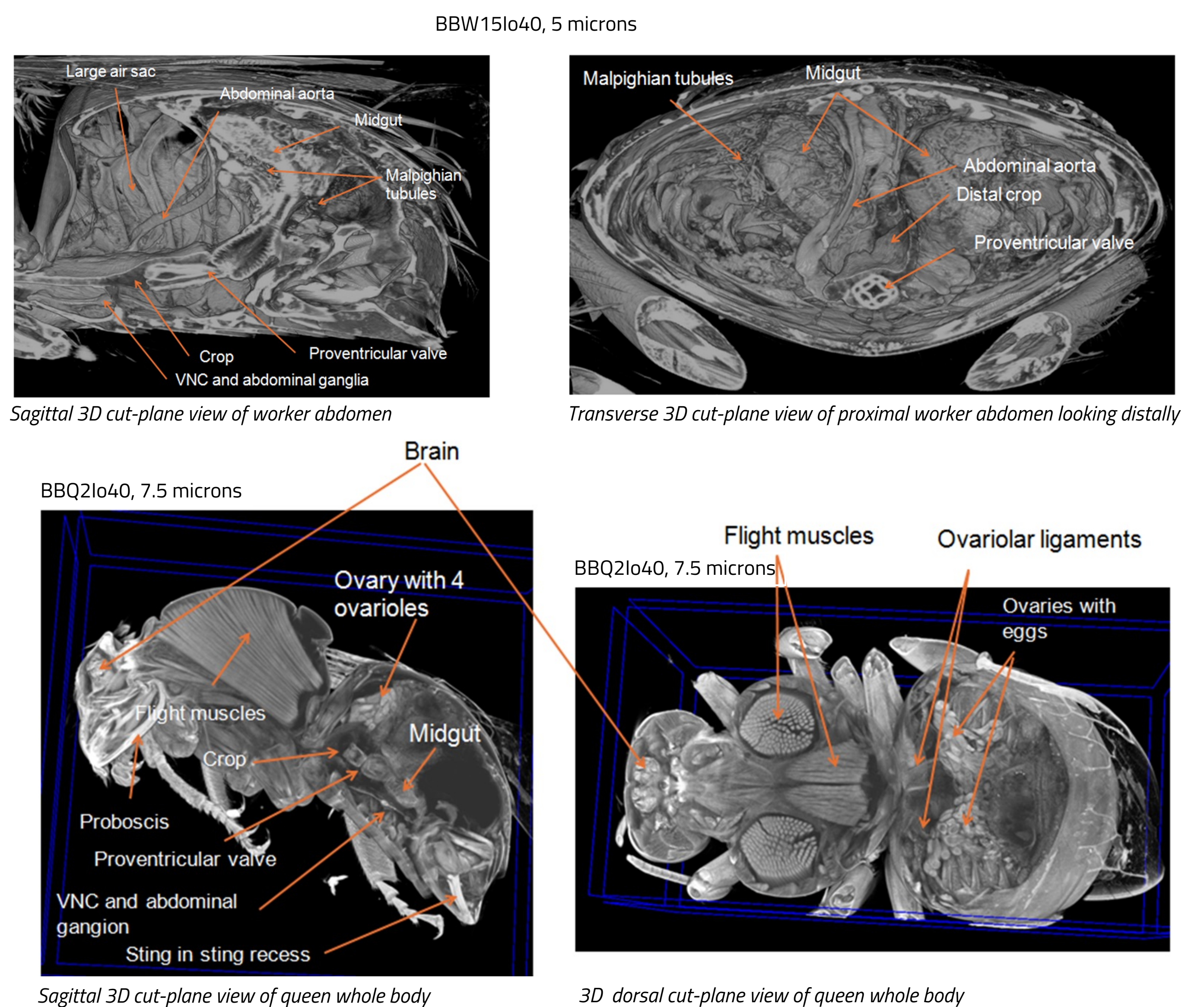
**Fig. 3 Larger *Bombus terrestris* individuals including males (BBM), gynes (BBG), and queens (BBQ) scanned after 1 week in iodine vapour using with Skyscan 1072. BBM3 was scanned at 21 days, and 34 days.**

## Results continued – Scans using Neoscan 80

The best images were seen on the on the higher resolution NeoScan80 (Fig. 4 – 5). On this machine, and after 40 days in Iodine vapour, features of the head, glands, and brain were seen in good detail (Fig. 4). Likewise, features of the thorax (including individual flight muscle fibres) and abdomen (including entire digestive and reproductive systems) were seen well (Fig. 5).



**Fig. 4 Head scans of *Bombus terrestris* queens (BBQ) and workers (BBW) scanned after 40 days in Iodine vapour using Neoscan 80.**



**Fig. 5 Whole-body, and abdomen scans of *Bombus terrestris* including males, gynes, and queens scanned after 40 days in Iodine vapour using Neoscan 80.**

## Conclusion

We have shown that sublimated iodine penetrates and stains biological tissues and is a useful adjunct to performing micro-CT in insects. We conjecture that sublimated iodine penetrates the internal tissues by direct passage through the exoskeleton and by diffusion through the spiracles, tracheal vessels and air sacs. Optimal staining is achieved more quickly in smaller individuals and would presumably be even quicker in much smaller insects such as *Drosophila*.

The method, while slow, has the advantage of preserving tracheal structure. The proventricular valve (the thickest and most muscular structure in the GI tract) proved a valuable marker between the crop and mid gut. We were able to confirm the previously reported<sup>6</sup> marked differences between the shape of the proventricular valve of the male and female bees. The size and lengths of the midgut and hindgut was very similar to that described almost a 100 years ago in a related American *Bombus* species<sup>7</sup>.

Iodine exposed specimens showed remarkably little sign of desiccation or bacterial decomposition over the study period. Theoretically, quicker internal staining could be achieved with live anaesthetised bees because the iodine vapour would enter the tracheal system by active ventilation as well as passive diffusion.

## References

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