X-ray micro-CT visualisation and quantification of calcium accumulation in the anterior Malpighian tubules of Black Soldier Fly larvae (*Hermetia illucens*)

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Introduction

Black Soldier Fly Larvae (BSFL) are very efficient at bioaccumulating minerals from their substrate,

BSFL, have 4 Malpighian tubules (MPs) and the two larger anterior ones are frequently visible on X-ray micro-computed tomography (micro-CT) because their lumens are filled with radio-opaque calcium-rich material.

While studying two groups of BSFL using an unstained X-ray micro-CT technique, it was apparent that the first group (that had been fed a diet relatively high in Calcium) seemed to have more radio-opaque material in their anterior MPs than the second group fed a diet with significantly less calcium in it.

We decided to attempt to quantify the amount of radio opaque material in the respective MPs using a radio-attenuation method akin to that we had used previously to quantify fat content in wasps (Bell et al 2024). We also wished to see if in BSFL, as previously observed in *C. vomitosa* larvae (Bell et al 2021), the MPs retained their calcium concretions throughout the prepupal and pupal stages.

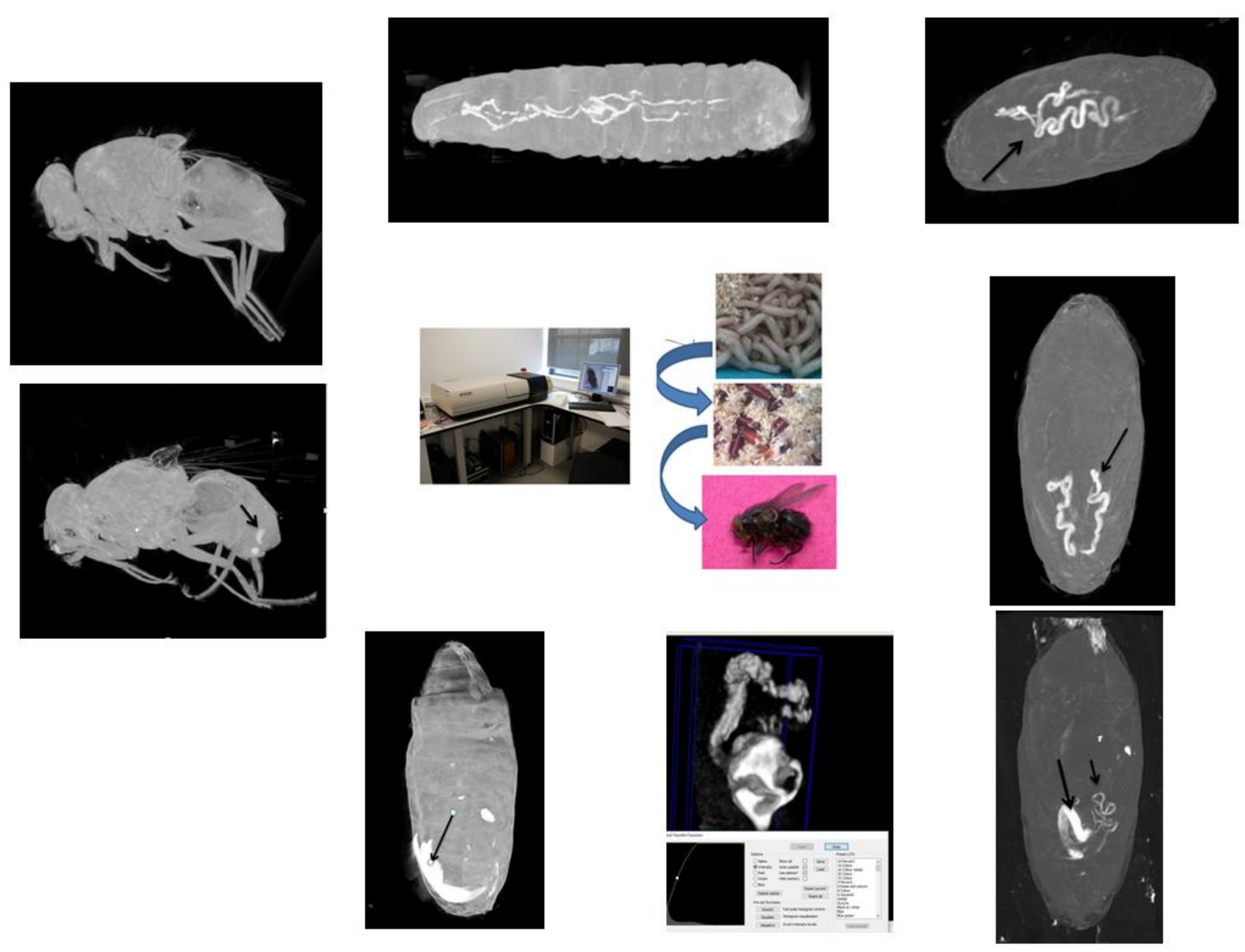


Fig 1. Unstained micro-CT scans of *Calliphora vomitosa* in its larval, pupal and adult stages. The calcium-rich radio- opaque material that accumulated in the two anterior Malpighian tubules (MTs) during the larval stage remains there throughout most of the pupal stage. Once the MTs reconnect with bowel lumen, the material passes into the hind gut and is passed with the rest of the meconium at the time of ecdysis. (Bell et al 2021)

Methodology

X-ray micro- CT was used to visualise calcium-rich deposits in the Malpighian tubules of Black Soldier Fly larvae (*Hermetia illucens*) reared on substrates differing in calcium content. Ten Larvae in each group were either fed poultry feed waste (6.0–8.5 g/kg Ca) or wheat bran (0.7–1.0 g/kg Ca) for eight days before scanning unstained specimens using a Skyscan 1072 system and reconstructed using NRECON.

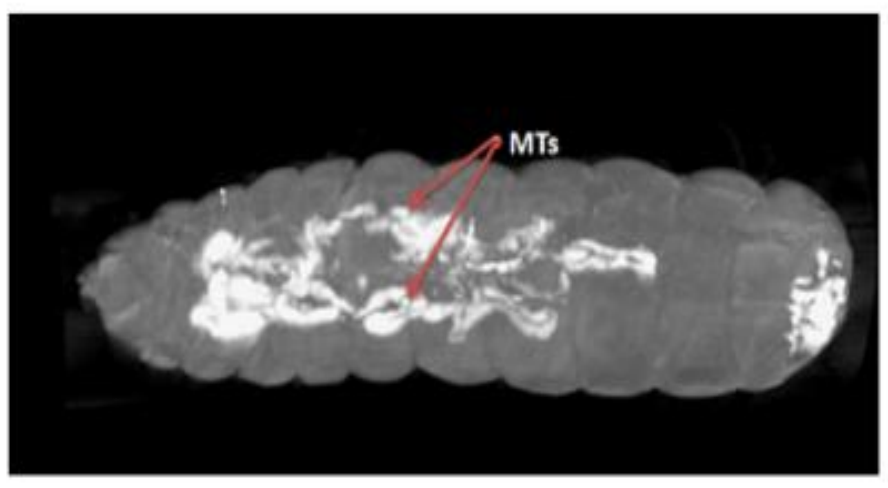
The most densely radio opaque material, interpreted as calcium-rich concretions, were detectable in the anterior Malpighian tubules. To quantify this material In Tomomask we arbitrarily identified the 16-bit voxels with the highest range of X-ray attenuation values.

We also studied other wheat bran fed BSFLs when they a) entered the prepupal phase (n=10) b) were in the mid pupal stage (n=10) or c) within 24 hrs of emerging as young adult flies (N=10).

Results 1:

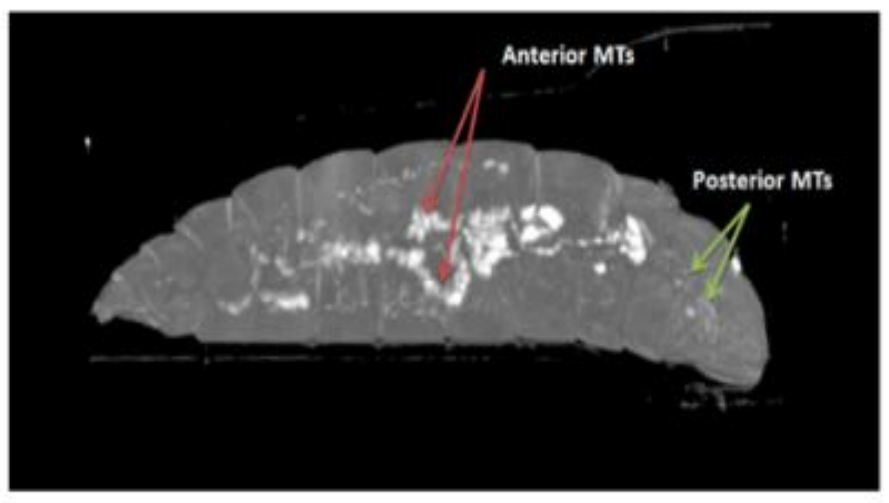
Larvae reared on poultry feed waste consistently showed greater accumulation of calcium-dense material in their two anterior MPs when compared to those reared on wheat bran.

High calcium poultry feed waste
3-D MIP view



Anterior 2 MTs full of radio-opaque concretions. Posterior MTs not seen.

Low calcium wheat bran
3-D MIP view



Anterior 2 MTs contain less radio-opaque material but 2 posterior MTs faintly outlined (green arrow)

	volume calcium containing voxels in mm³		volume calcium containing voxels in mm3		
File name			File name		
BSFL1	0.00642		Emma1	0.002365	
BSFL2	0.004940		Emma2	0.000702	
BSFL3	0.008220		Emma3	0.001002	
BSFL4	0.011800		Emma4	0.000676	
BSFL5	0.003180		Emma5	0.001301	
BSFL6	0.005830		Emma6	0.001975	
BSFL9	0.007150		Emma7	0.004440	
BSFL10	0.010500		Emma8	0.001032	
BSFL11	0.002830		Emma9	0.000544	
BSFL12	0.004440		Emma10	0.000270	
mean	0.006531			0.001431	
SD	0.002964327			0.001239116	
p value	0.000299				

Fig 2 Micro-CT scans of BSFL reared on either poultry feed waste or wheat bran showing considerably more calcium-rich material in the anterior 2 MTs of the former. Curiously the two posterior MTs are faintly visualised in several of the wheat bran fed larvae (compare with fig 4).

Calcium-rich high attenuation voxels were quantified in micro-CT and confirmed significantly higher calcium-associated volumes in larvae fed poultry feed waste (mean ± SD: 0.00653 ± 0.00296 mm 3) compared to wheat bran (0.00143 ± 0.00124 mm 3 ; $p \leq 0.001$)

Results 2:

All 10 of the BSFL that had entered the prepupa stage and similarly all 10 of the pupae X-rayed no longer had any obvious radio opaque concretions remaining in their MPs. Instead, obvious radio opaque material was now seen in the exoskeleton and pupal cases.

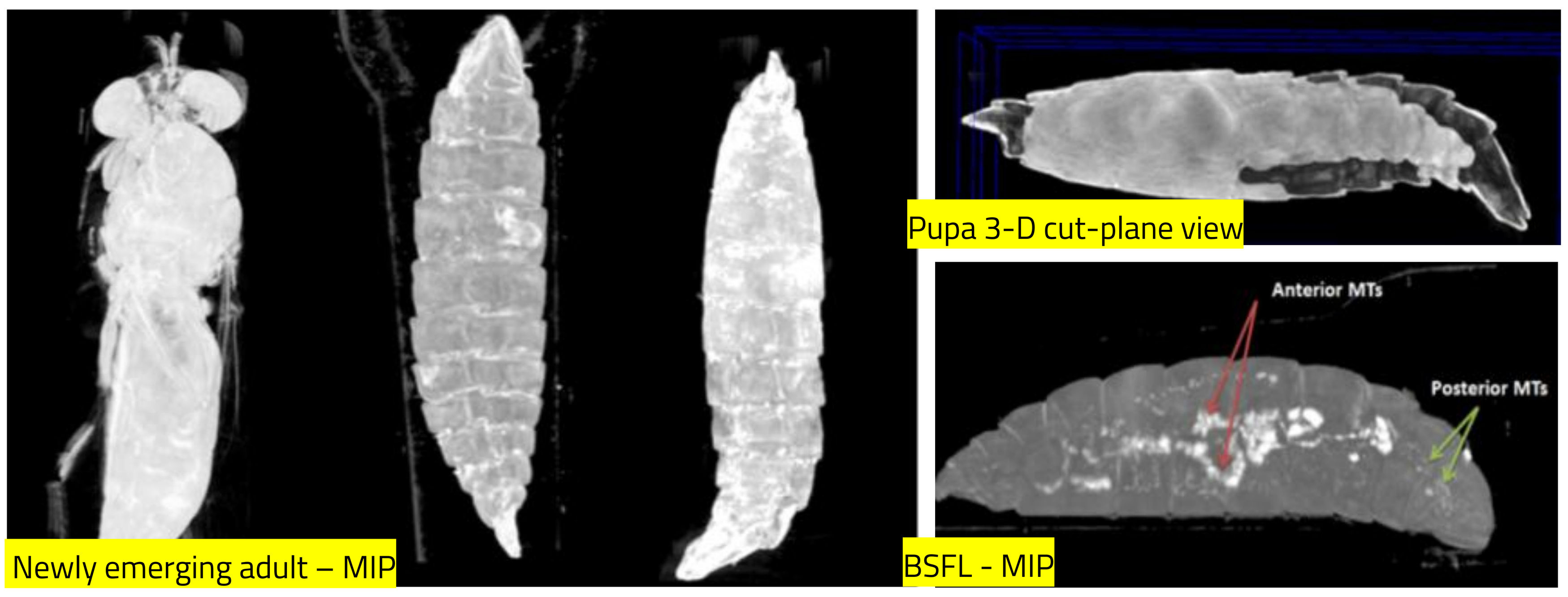


Fig 3 Unstained micro-CT scans of BSFL, prepupae, pupae and newly emerging adult flies.
The calcium-rich radio- opaque material accumulated in the two anterior Malpighian tubules (MTs) during the larval stage disappears. Radio-opaque material is instead deposited in the exoskeleton and pupal case. The adult fly lacks any such radio-opacity.

Conclusion

These findings demonstrate that micro-CT of unstained specimens enables non-destructive visualisation and quantification of mineral bioaccumulation in BSFL. Substrate calcium concentration directly influences mineral deposition patterns and is spatially localised within the anterior Malpighian tubules. Unlike the situation in Blow flies, in BSF the calcium-rich MP concretions are reabsorbed during the prepupal stage and deposited in the pupal case (see fig 3): a situation similar to that described in *Ephydra hians* (Herbst and Bradley 1989). Since no such material was evident in the newly emerging adults, we presume the calcium in the pupal cases remained there after ecdysis as suggested by the studies of Rubio et al 2021.

By using an orally administered iodinated radiocontrast agent, we were able to confirm that the small amounts of radio opaque material seen in the most posterior parts of some of the wheat bran fed BSFLs were indeed within the two posterior cryptonephric MPs. The nature of this material remains to be determined.

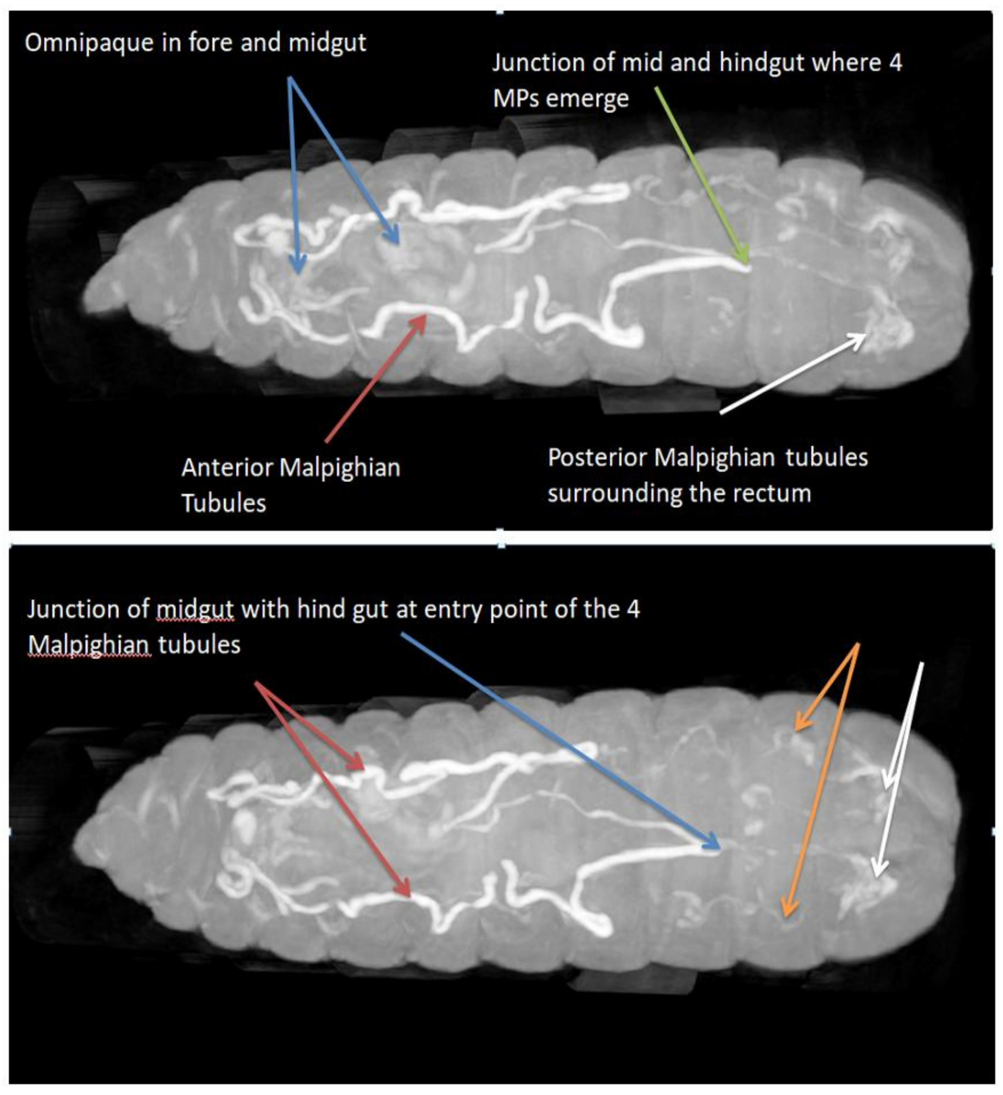


Fig 4 Micro-CT scan of a BSFL fed wheat bran 'laced' with an iodinated radiocontrast agent (Omnipaque) for 2 days prior to being culled. The two posterior MTs are now well visualised.

References

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