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Age estimation of forensically important blowfly, *Chrysomya megacephala* (Diptera: Calliphoridae) pupae using micro-computed tomography imaging

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Abstract. Accurate estimation of the minimum post-mortem interval (minPMI) is important in the investigation of forensic cases. Various thanatological methods are being used to estimate this interval. However, entomology approach is the most reliable method for this minPMI estimation especially when death has occurred over 72 hours and involved insects or other arthropods evidence at the death scene. The current methods of age estimation are daunting and destructive especially when dealing with pupal stage. The aims of this study were to characterize the morphological changes during intra-puparial period of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) and their relation with minPMI estimation by using a high resolution micro-Computed Tomography (micro-CT). Gravid *C. megacephala* were collected from a rural area in Sungai Buloh, Selangor and cultured in the laboratory at 23.83±0.25°C with light: dark hour of 12:12 to initiate oviposition. The resulting larvae were reared until pupal stage. A pupa was collected at first (24 hours), second (48 hours), third (72 hours), and fourth quarter (96 hours) of the intra-puparial period. The pupal samples were placed directly into 70% ethanol for preservation. Micro-CT scanning was employed to acquire microstructural information following pupal sample staining for contrast enhancement. Eight age-informative internal morphological landmarks were mapped from the micro-CT scanning. The present study enhanced the potential value of micro-CT for the estimation of minPMI based on the internal morphological changes of *C. megacephala* pupae. This novel method is a promising tool for improving medico-legal investigations in forensic entomology.

INTRODUCTION

Interpretation of insect and other arthropods evidences in forensic investigation is known as forensic entomology (Hall, 2001). This method is very useful in suspicious death, suicide, homicide, abuse, and neglect cases (Hall, 2001; Richards *et al.*, 2012). Forensic entomological evidence is very useful for estimation of time since death beyond 72 hours compared to the other methods such as algor mortis, livor mortis, rigor mortis, and tache noir. Application of this method had increased dramatically in recent years because of its ability to provide objective estimation of the minimum time since death (Buchan *et al.*, 2001).

Insect evidence was present on the cadaver because of the presence of ammonia-rich compound and moisture of the decomposing body (Tomberlin *et al.*, 2011; Rajagopal *et al.*, 2014). The insect evidences were collected based on the oldest insect specimen on the dead body. The oldest individuals were derived from the eggs laid by the female insect. The most common

insects that regarded as early colonisers are blowflies. Blowflies were found colonising the body within minutes or hours since death (Anderson, 2001; Richards *et al.*, 2012; Martín-Vega *et al.*, 2017b). In Malaysia, the most common blowfly species that was associated with dead body was Calliphoridae specifically *Chrysomya megacephala* (Fabricius, 1794) (Nor Afandy *et al.*, 2001; Noratiny, 2002; Syamsa *et al.*, 2010; Rajagopal *et al.*, 2013; Syamsa *et al.*, 2017). This species is found in various areas including rural, residential, and aquatic areas in Malaysia (Kurahashi *et al.*, 1997; Syamsa *et al.*, 2010). The species spent about 8-9 days as immature (i.e., egg, larva, and pupa) before emerging into adult fly. The most challenging stage for age estimation was the pupal stage (i.e., due to the intra-puparial development that prevent direct observation externally) and this stage take up about 4 days in its life cycles (Sukontason, *et al.*, 2003; Ismail *et al.*, 2007).

Accurate estimation of the age of blowfly pupae in forensic cases is very important when determining the minimum post-mortem interval (minPMI). MinPMI is defined as the minimum period of time since death occurred until the discovery of the cadaver (Villet *et al.*, 2009). Several methods have been established for the age estimation of the blowfly pupae including molecular-based techniques (e.g., PCR and gene sequencing), puparium dissection, and scanning electron microscopy (SEM). However, all of these methods are destructive which can be disadvantageous especially when only limited samples are available. Moreover, molecular-based techniques showed inconsistencies in the haplotypes among different species of flies (Rajagopal *et al.*, 2014). Although molecular-based techniques are promising, however, expertise and specific reagents are required during the process (Wells *et al.*, 2008; Martín-Vega *et al.*, 2017b). A reference data sequences are needed to make sure that this techniques are reliable in the time since death estimation (Harvey *et al.*, 2003) Dissection of puparium is relatively simple, easy, and reliable but there is a potential loss of the most informative tissues during dissection of the puparium (Martín-Vega *et al.*, 2017b). It is no doubt that improvement of spatial resolution has been achieved using the SEM but more works are needed to prove that this method produces solid and reliable data for the age estimation of blowflies' pupae (Richards *et al.*, 2012).

Alternatively, micro-Computed Tomography (micro-CT) imaging has been reported as a potential method to facilitate detailed descriptions of insect anatomy (Friedrich *et al.*, 2008). This non-destructive approach to study the internal morphological changes during metamorphosis of blowfly pupae was first described by Richards *et al.* (2012) using *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae). Following this, a qualitative and quantitative tomographic analysis to a morphological study of the development of the similar species has been reported (Martín-Vega *et al.*, 2017a and Martín-Vega *et al.*, 2017b). However, to the best of our knowledge, no further radiographic investigation on forensically important Malaysian blowfly species has been reported using micro-CT imaging. In the present study, we assessed the value of micro-CT imaging as a potential method for characterizing the morphological changes during intra-puparial period of *C. megacephala* and its relation with minPMI.

MATERIALS AND METHODS

Blowflies culturing and sampling

Adults *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) were collected from rural areas in Sungai Buloh, Selangor, Malaysia (GPS coordinates: 3° 13' 18.74" N, 101° 35' 44.16" E, 46.02 m above sea level). A decomposing chicken liver (~ 100 g) was used as bait to attract the adults *C. megacephala* and they were captured by using an insect net. The collected adult flies were placed into a refrigerator (<4°C) for about five minutes for quick knockdown in order to sort out *C. megacephala* from other species. After the separation process, the colony of *C. megacephala* was established in a plastic cage (53.5 x 38.0 x 28.5 cm). The

flies were reared at 23.83°C (±0.25°C) with light:dark hour of 12: 12 to initiate oviposition. The fly colony was supplied with water and sugar *ad libidum* as food source and fresh chicken liver was supplied as an oviposition medium. After 24 hours, fly eggs were oviposited on the chicken liver. The eggs and chicken liver were transferred into a smaller plastic container (10 x 10 x 15 cm) to allow emergence of first instar. The larvae were supplied with fresh chicken liver as food source and reared until pupation.

A total of ten *C. megacephala* pupae (n=10) were randomly selected in each quarter for preservation in 70% of ethanol. The pupae were selected based on 25% of intra-puparial period which was every 24 hours (i.e., one quarter). In general, the pupae spend approximately 96 hours in intra-puparial stage, and the pupae were sampled in all four quarters of intra-puparial development for micro-CT scanning. One pupa (n=1) was selected in each quarter of development which were the first quarter (24 hours), second quarter (48 hours), third quarter (72 hours), and fourth quarter (96 hours).

Sample staining and micro-CT scanning

One pupa was randomly chosen (out of 10 pupae) in each quarter of intra-puparial development for micro-CT scanning (total four samples, n=4). The selected pupa was pierced using an entomological pin (#1) at three places which were the anterior, center, and posterior of the pupa as the piercing allows deeper penetration of the staining solution into the pupal internal structures (Martín-Vega *et al.*, 2017). The pierced pupae were stained for seven days using 0.5 M iodine in aqueous solution (Metscher, 2009; Richards *et al.*, 2012). After completion of staining, the pupae were cleaned with 70% ethanol before placing it into 70% of ethanol for 24 hours.

After 24 hours of ethanol immersion, the representative pupal specimens (n=4) were mounted in a plastic straw by using micropore tape to hold the specimens during scanning. A corn kernel was placed as a marker to indicate the anterior region of the specimen. Pupal specimen of the first quarter was placed at the most anterior and followed by the subsequent quarters (Figure 1). Micro-CT scans of the specimen were performed using Skyscan 1176 High Resolution In-Vivo Micro-CT (Skyscan, Kontich, Belgium), with a 0.5 mm Al filter, a current of 90 mA, a voltage of 40 kV, a rotation of 180°, a rotation step of 0.4° and an exposure time of 500 ms.

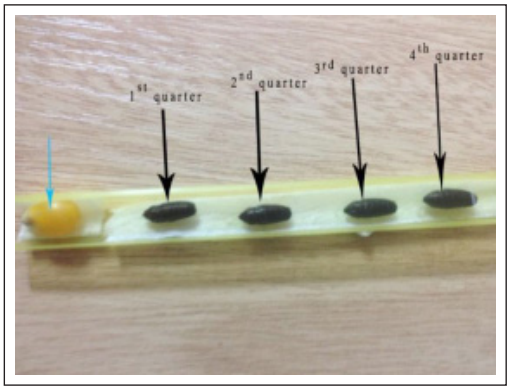

Figure 1. Pupal specimens were placed in a plastic straw in sequence according to the quarter of pupal development. Note the corn kernel at the anterior region of the plastic straw that serves as the position indicator.

Image data reconstructions and image analysis

Each specimen was scanned and reconstructed into three-dimensional (3D) microstructure with voxel size of 18 μm^3 using NRecon software version 1.4.3 (Skyscan, Kontich, Belgium). The microstructure of the specimen was examined using Data Viewer software version 1.4.3 (Skyscan, Kontich, Belgium). The landmarks were labelled by using Microsoft Publisher 2016. The labelling was done by referring to previous study of Richards *et al.* (2012) and Martín-Vega *et al.* (2017) for the identification of the major morphological landmarks in each quarter of pupal development.

3D analysis of pupae

2D visualisation and 2D/3D analysis of the pupae from each quarter of intra-puparial stage were performed using CT Analyser (CTAN) (Skyscan, Kontich, Belgium). The software enables 3D image processes to obtain 3D geometrical analysis.

RESULTS

Eight age-informative internal morphological landmarks of intrapuparial period of *C. megacephala* were identified in micro-CT scanning (Table 1). These landmarks allow the age estimations of 25% to 100% of pupal development. Sagittal and coronal planes were used to visualized these landmarks. Larval-pupal apolysis and pupal adult apolysis were clearly visualised in sagittal plane. Separation of the epidermal cells from the cuticle is term as apolysis (Martín-Vega *et al.*, 2017a). The separation of epidermal cells from larval cuticle (i.e. puparium) is the larval pupal apolysis and the separation of epidermal cells from pupal cuticle is known as pupal-adult apolysis (Martín-Vega *et al.*, 2017a).

First quarter of development (24 hours post-pupation)

Attachment of the epidermal cells to the larval cuticle (i.e., puparium) was observed in most of the regions. This quarter of development showed the prepupa stage as the cephalopharyngeal skeleton filled up the entire anterior region (Martín-Vega *et al.*, 2017a). Generally, the whole body was filled up with undifferentiated tissues. As larval tracheal trunk and larval midgut were visualised (Figure 3B), these two structures seemed went through early histolysis in this quarter of development. The two larval brain hemispheres were visualised.

Second quarter of development (48 hours post-pupation)

Differentiation of the brain can be observed in this quarter. Three body principal which were head, thorax, and abdomen were visualised. Two of the indirect flight muscles which were longitudinal muscle and dorsal ventral muscle filled up the dorsal region but not fully developed (Figure 2 E-F). The longitudinal muscle filled up most of the thorax and a pair of dorsal ventral muscle was present at both sides of the thorax. Adult midgut or archenteron was appeared occupying centre posterior region of thorax. Bilateral adult salivary glands developed replacing the larval salivary glands at both side of posterior adult midgut (Figure 3E). Although the adult structures started to develop in this series, pupal-adult apolysis was still not completed over the entire body.

Third quarter of development (72 hours post-pupation)

The brain has developed into a different region called medulla (Figure 3I). Development of ommatidia at the edges of the eyes was visualised. Long-bottle shaped of adult midgut (Figure 6H) developed on the centre

Table 1. Chronological development of the morphological landmarks in four quartiles during the intra-puparial development (IPP) of *Chrysomya megacephala* (Diptera: Calliphoridae) as visible on micro-CT scanning

Morphological landmark	1st Quarter (25%) IPP	2nd Quarter (50%) IPP	3rd Quarter (75%) IPP	4th Quarter (100%) IPP
Larval-pupal apolysis complete	Incomplete	Complete	Complete	Complete
Cephalopharyngeal skeleton within insect body	Fully within	N/A	N/A	N/A
Head and wings fully everted	None	Visible	Visible	Visible
Pupal-adult apolysis complete	Incomplete	Incomplete	Complete	Complete
Shape of the adult midgut (in sagittal plane)	None	Closed sack (occupying centre body region)	Long-necked bottle	Visible (appear diffuse)
Yellow body	None	None	Visible	None
Rectal pouch swollen	No	No	No	Yes
Medulla visible	No	No	Visible	Visible

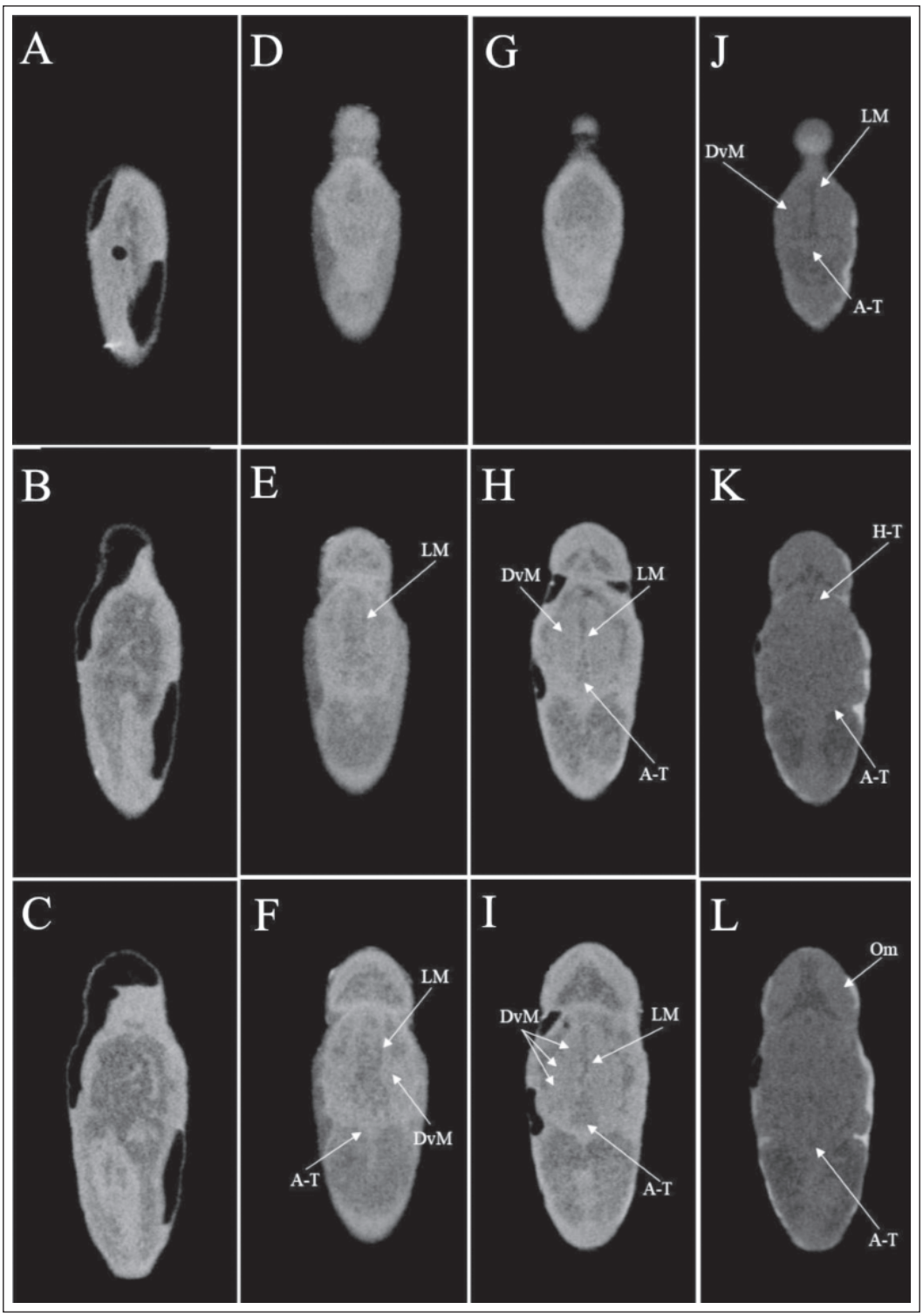

Figure 2. Coronal cross-sectional of dorsal part of pupae from Micro-CT scanning, top of the page is the most dorsal part, A-C, First quarter, D-F, Second quarter, G-I, Third quarter, J-L, Fourth quarter. Abbreviations: DvM=dorso-ventral muscle; LM=longitudinal muscle; H-T=head-thorax division; A-T=abdomen-thorax division; Om=ommatidia.

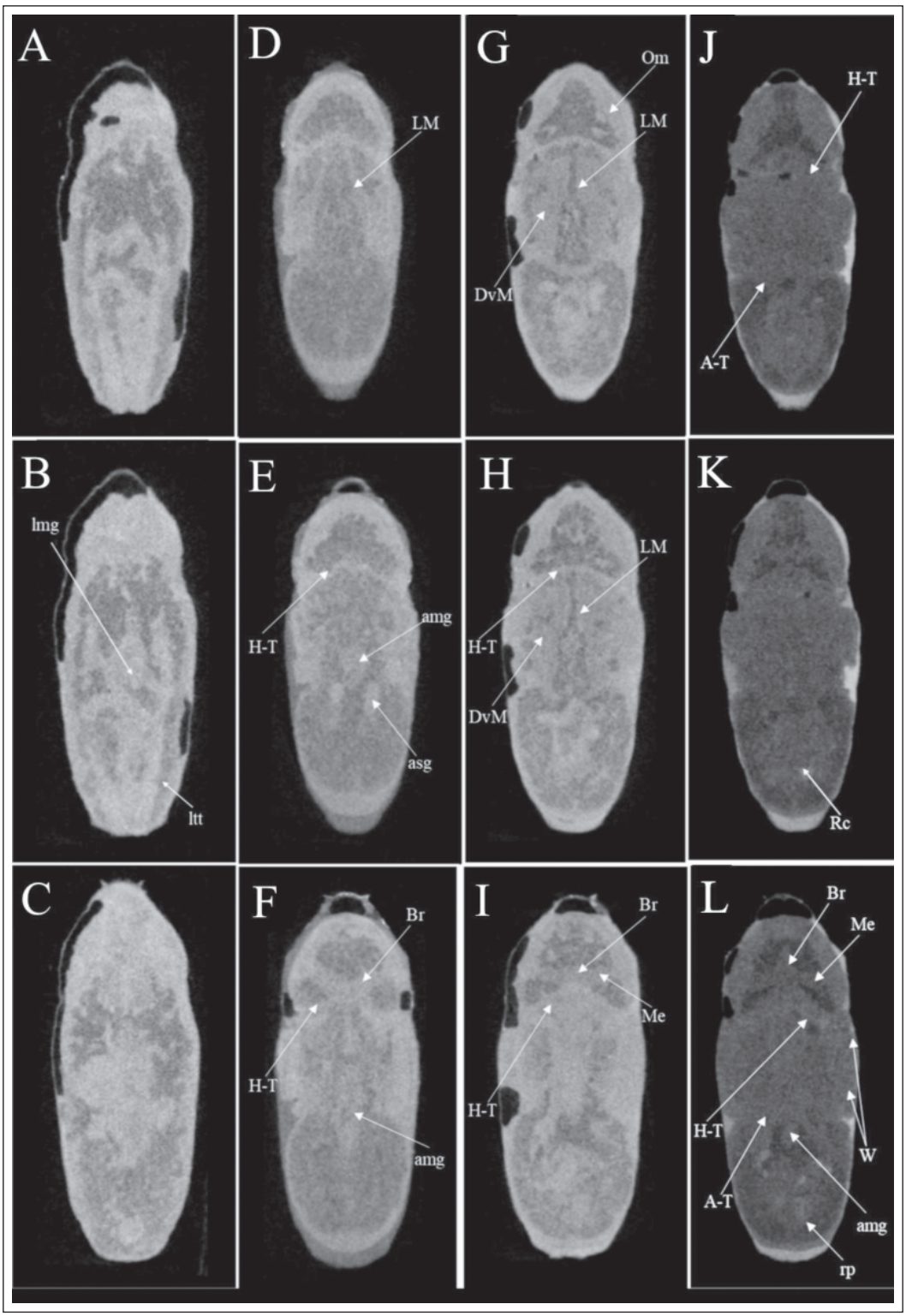

Figure 3. Cross-sectional of medial part of the pupae from micro-CT scanning, top of the page is the most dorsal part. A-C, First quarter, D-F, Second quarter, G-I, Third quarter, J-L, Fourth quarter. Abbreviations not listed previously: lmg=larval midgut.

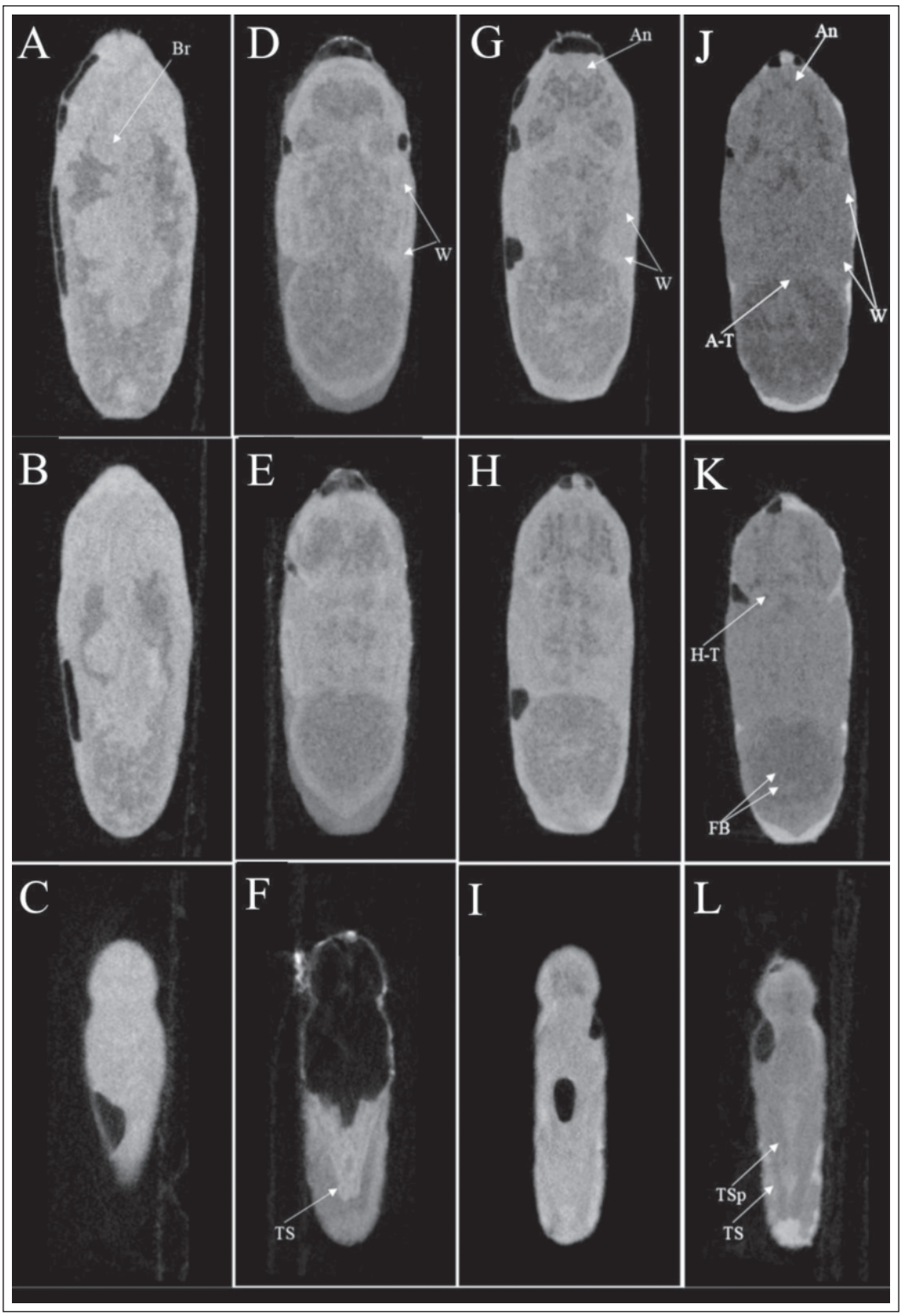

Figure 4. Coronal-cross sectional of ventral part of the pupae from micro CT scanning, top of the page is the most dorsal part. A-C, First quarter, D-F, Second quarter, G-I, Third quarter, J-L, Fourth quarter. Abbreviation that is not previously listed: An=antenna; FB=fat bodies; TS=tarsal segment; TSp=tibial spine.

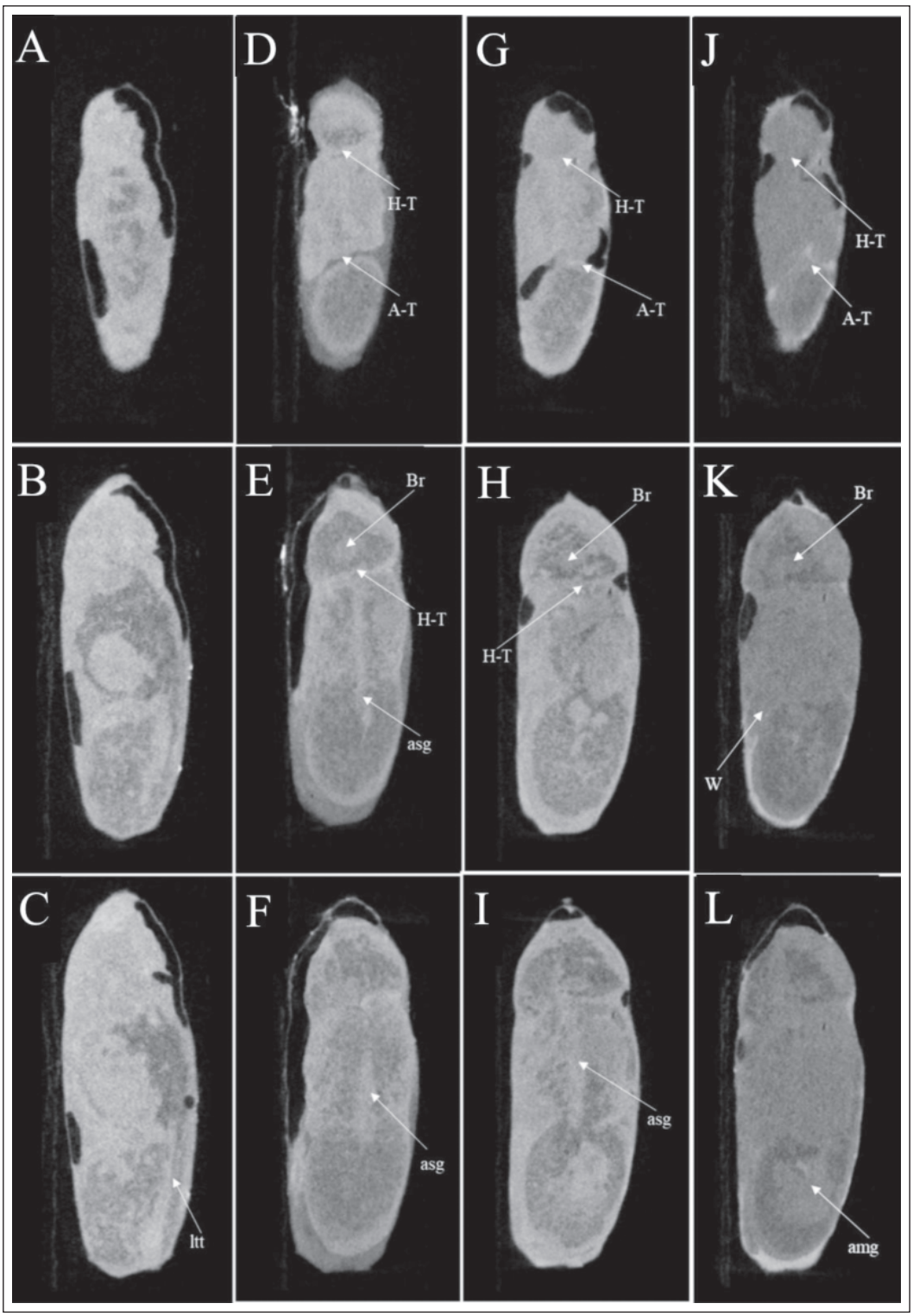

Figure 5. Sagittal cross-sectional of ventral part of the pupae from micro-CT scanning, top of the page is the most lateral part. A-C, First quarter, D-F, Second quarter, G-I, Third quarter, J-L, Fourth quarter.

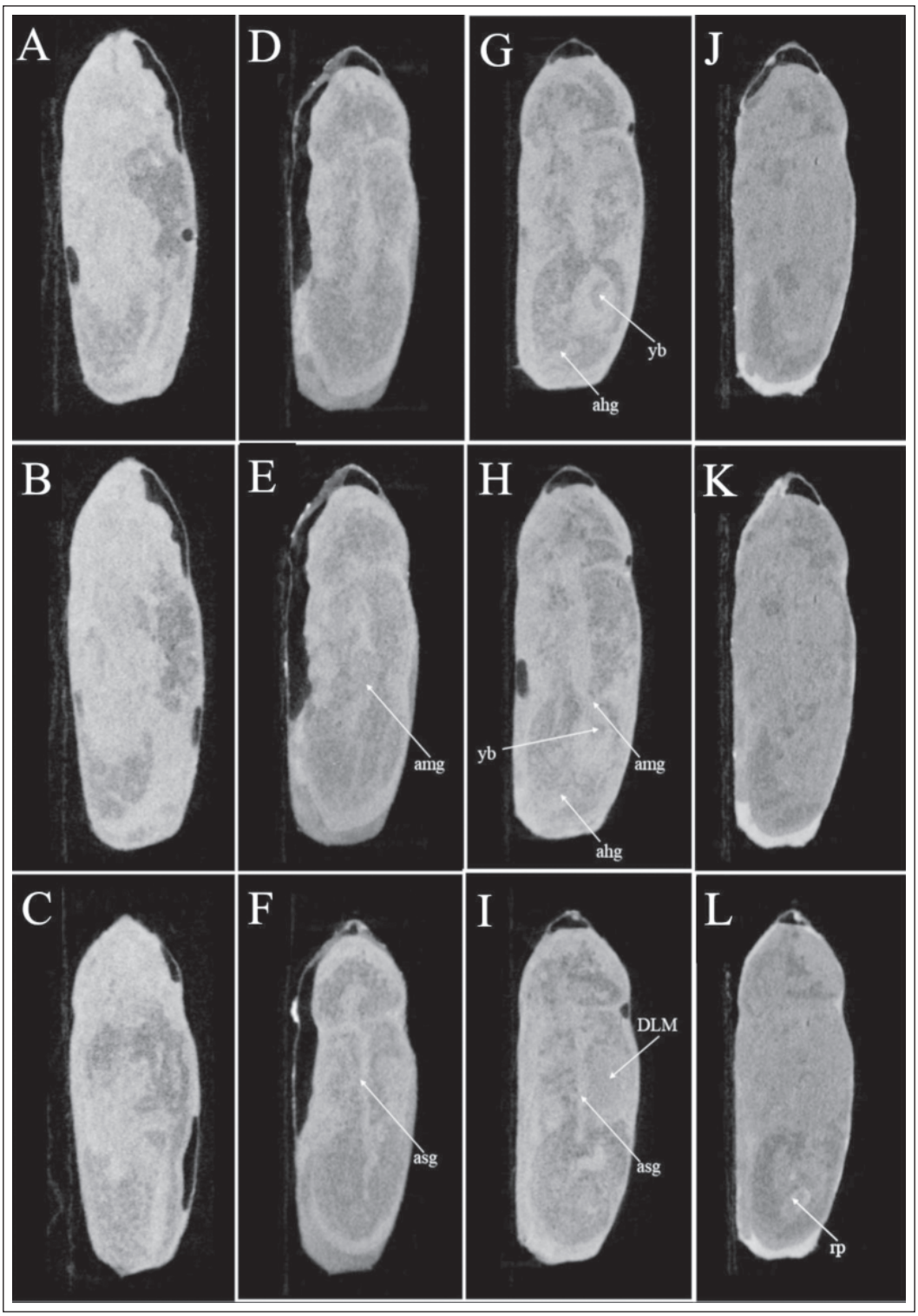

Figure 6. Sagittal cross-sectional of ventral part of the pupae from micro-CT scanning, top of the page is the most medial part. A-C, first quarter, D-F, second quarter, G-I, third quarter, J-L, fourth quarter, Abbreviations that is not listed previously: DLM=dorso-longitudinal muscle; yb=yellow body; ahg=adult hindgut.

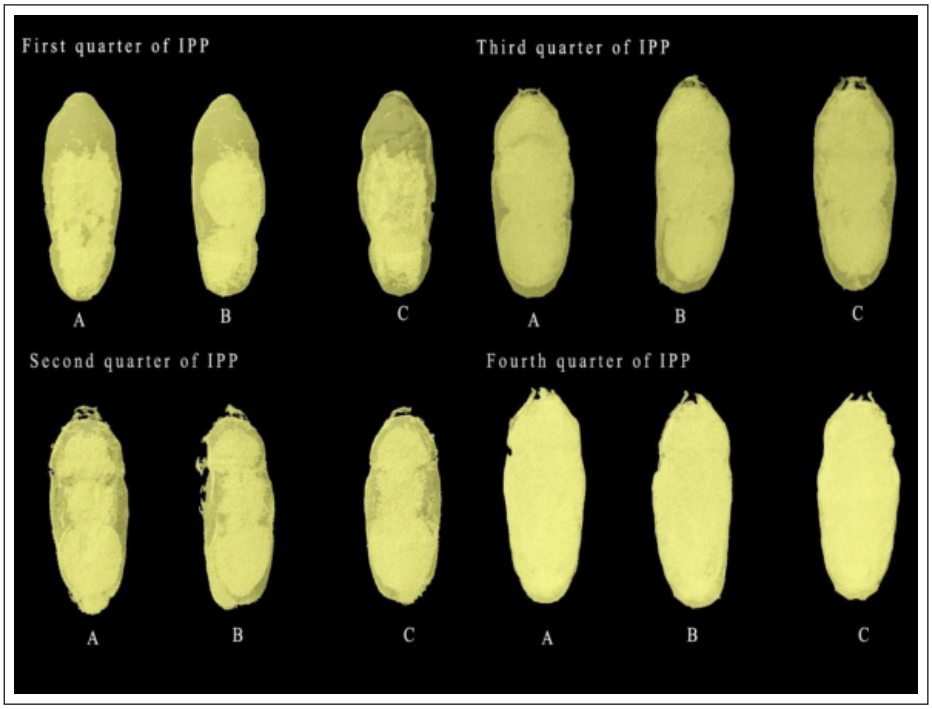

Figure 7. 3D surface models of *Chrysomya megacephala* pupa at different times of puparation; A: dorsal view; B: lateral view; C: ventral view, IPP=intra-puparial period.

further posterior of the thorax. A 'banana-shaped' dark grey yellow body as visualised inside the adult midgut (Figure 6H). This marks the digestion of the larval midgut. Tubular-shaped of adult salivary gland was well developed and clearly visualised in sagittal plane (Figure 5I). Division between head, thorax and abdomen were well divided. A well-developed of the flight muscle were seen but not in full size. This development marked the pharate adult stage.

Fourth quarter of development (96 hours post-pupation)

Three body divisions were distinguishable in this quarter (Figure 4 J-K). The most important features appeared were the fat bodies in the abdominal areas. Rectum and rectal pouch were presented in this series (Figure 3K-L; Figure 6L). Medulla region of the brain was visualised similar to the third quarter. Ommatidia at the edge of the eyes was fully developed. Helicoidal section of midgut was visualised at the ventral side of the pharate adult. However, due to the lack of contrast, the structure appeared as a diffuse structure. Musculature seems to develop in full size but the lack of contrast causes the muscles cannot be differentiated between each other in the pharate adult.

3D models of the pupae

3D model of each quarter of development in Figure 7 showed an embryonic tissue development inside the pupal cuticle. The development of the head, thorax and abdomen was clearly visible in each quarter of development. However, the model did not show any other features that can be helpful in differentiating each quarter of the development other than the growth of internal tissue.

DISCUSSIONS

Internal morphological changes in each quarter of intra-puparial development

This study was performed to characterize the internal morphological changes of *C. megacephala* pupae using Micro-CT imaging. This method allowed a non-destructive assessment of internal morphological changes of *C. megacephala* pupae. As most

of the studies in Malaysia focused on the insect succession and species identification at larval stages (Rajagopal, 2012), the fly pupal stage were not given much attention in research. Invasive procedures such as pupal dissection or histological techniques were mainly performed to assess the internal morphological changes of the pupae due to its opaque barrel-shaped puparium (Richards *et al.*, 2012; Martín-Vega *et al.*, 2017b). These procedures are not only invasive but also destructive, consequently, other analyses on the pupae could not be done, especially in the case of limited samples. Therefore, application of micro-CT imaging is reliable as an alternative method before other supplementary analyses will be carried out. The high-resolution micro-CT allows x-ray beam to penetrate the opaque barrel-shaped puparium without destroying the pupae.

Both similarities and differences were noted in internal morphological changes of *C. megacephala* when compared with previous studies on *Ca. vicina* and *Lucilia sericata* (Diptera: Calliphoridae) (Richards *et al.*, 2012; Martín-Vega *et al.*, 2017b). Fly puparial development rate were consistent in each quarter of development. Furthermore, a clear distinction in the changes of internal morphological landmarks were visualised in each quarter of development. The first landmark visualised in the present study were the two indirect flight muscles namely longitudinal muscle and dorso-ventral muscle. These muscles were observed to be developed during the second quarter and filled up the entire length of the thorax. This musculature continued to develop in the third quarter and making it distinguishable from the second quarter. There was only a pair of dorso-ventral muscles seen in the second quarter and more pairs appeared in the third quarter. Due to the lack of contrast in the fourth quarter, the musculature development could not be distinguished from the third quarter. However, the muscles did seem bulkier than the third quarter. Development of musculature does help in the separation of third and fourth quarter because the muscles were weakly developed in the third quarter but almost all muscles were well developed in the fourth quarter (Richards *et al.*, 2012). Similarly, the present study shows indirect flight muscles started to developed after 24 hours of development as reported by Martín-Vega *et al.* (2017a).

Next, the three principal of body division is an informative evidence to separate each of age group (Richards *et al.*, 2012). In the present study, these divisions were started to visualise in the second quarter. First quarter did not show any division as pupal epidermal cells were attached to the larval cuticle, indicating a pre-pupal stage. In fact, most features observed in the first quarter were originated from the third instar stage where larval-pupal apolysis were not yet completed. The only structure that was deeply remodelled from larval stage was the brain. The brain was not being replaced by any adult structure during the pupal development. Separation between the first quarter and the later quarters (i.e., second, third, and fourth quarters) was relatively easy because of the obvious changes that can be seen on the brain development.

Furthermore, visualization of the midgut shape is another major landmark that can be used to differentiate each quarter during pupal development. Adult midgut was seen in the second, third, and fourth quarters and only the larval midgut was seen in the first quarter. This structure was best visualized on the sagittal plane. The larval midgut was replaced by the adult midgut in the second quarter. Larval midgut can be recognized as undifferentiated tissues at the centre of pupal thorax region whereas adult midgut can be differentiated along each quarter of pupal development and it was gradually moving further away from pupal thorax region. In the second quarter, closed-sacked shaped adult midgut was visualised occupying the centre of posterior thorax region. As the midgut moved further to the posterior half of the abdomen in the third quarter, long-necked bottle-shaped midgut was visualised. Digestion of larval midgut in the third quarter was marked by the presence of yellow body in the adult midgut. Parts of adult midgut helicoidal structure were seen in the fourth quarter but due to the lack of contrast, the midgut shape determination was not possible.

minPMI estimation using internal morphological changes in pupal stages

The present study shows a promising result in improving estimation of minPMI based on internal morphological changes of blowfly pupae. The ability of micro-CT scanning to map the changes of internal morphological changes in each pupal stage with simple staining without sample destruction was a key hallmark of this method. This high-resolution scanning has shown its capability to differentiate the changes in the first and second quarter of pupa development which is limited in conventional histology (Richards *et al.*, 2012). Furthermore, results from our study showed advanced development in the second quarter compared to the first quarter. Similar observation was also observed in the fourth quarter when compared with the third quarter.

First feature used in the estimation of minPMI was the development of indirect flight muscles which were the longitudinal muscle and dorso-ventral muscle. Muscles development were not seen in the first quarter as prepupa possessed third instar characters. This finding was similar as in Martín-Vega *et al.* (2017a), where no muscles development was recorded in early stages of *Ca. vicina* intra-puparial period. Muscles were seen underdeveloped in second and third quarters with only a pair of both muscles seen in the second quarter. A few more pair of muscles arose in the third quarter which enable the third quarter to be distinguished from the second quarter. The musculature development in the fourth quarter could not be analysed in the current study due to over staining of the sample. However, the muscles seemed bulkier compared to the third quarter of development and filled up most of the thorax region. Similarity was seen when compared to Richards *et al.* (2012) where the thoracic musculature in the fourth quarter of *Ca. vicina* were fully developed and occupied almost the entire region of the thorax.

Brain development in intra-puparial period was another significant landmark that could be used in minPMI estimation. The development of the brain can be observed from the first to fourth quarters of intra-puparial period. The brain was deeply remodelled throughout the development especially from the second quarter to the fourth quarter. Two hemispheres were seen in the first quarter and remodelled into a different shape in the second quarter. The brain was divided into different regions starting from the third quarter. A pair of medulla were seen in the third quarter and fourth quarter. The brain development in the third and fourth quarters were indistinguishable because of the similar appearances and the lack of contrast in this study.

Furthermore, the midgut development was another significant landmark that could be useful in the estimation of minPMI. Note that the adult midgut was not a structure originated from the third instar larva. The progression of adult midgut development can be used as an indicator for the pupal age estimation. The shape changes of the adult midgut were the feature that helps in the differentiation of each quarter of pupal development. The first quarter was easily distinguished from the other quarters (i.e., second, third, and fourth quarters) as the adult midgut had not yet been developed to replace the larval midgut. The long-necked bottle-shaped midgut with yellow body in the third quarter showed an advanced development when compared to the second quarter which was presented with a sacked-shape midgut. Diffused appearance of the adult midgut in the fourth quarter was not helpful in the differentiation between the fourth and the third quarter but the appearances of the fat body at the abdominal region in the fourth quarter can be useful in distinguishing these two quarters of pupal development. This feature was also found in the fourth quarter of *Ca. vicina* pupae (Richards *et al.*, 2012). The yellow body was not seen in the fourth quarter indicated that the larval midgut was fully digested.

Our study showed some limitations with the sample size and numbers of distinguishable morphological landmarks. Hence, further investigation using more pupae samples with 10% interval intra-puparial period development as reported by Martín-Vega *et al.* (2017b) is suggested

to acquire more accurate and precise age-informative internal morphological landmarks. Moreover, different types of staining solution such as (e.g. Lugol's iodine and phosphotungstic acid) could be further investigated in insect samples analysis to improve the spatial and contrast resolution for morphological change analysis. Appropriate selection of staining solution does show better contrast and spatial resolution (Metscher, 2009: Swart *et al*., 2016). Suitable fixation and duration of sample staining should be taken into consideration for better contrast enhancement that result to improve the samples analysis.

The present study suggested that micro-CT imaging was useful for characterising the internal morphological changes and mapping the age-informative internal morphological landmarks of *C. megacephala* pupae. Three important features for minPMI estimation include muscles, brain and midgut development were significantly mapped by the high-resolution Micro-CT scanning. Micro-CT imaging is a potential and alternative method for pupal sample analysis in forensic entomology, especially when dealing with cases involving blowfly pupae. Therefore, this valuable technique could improve the precision of minPMI, hence provide better justice outcome in medico-legal investigations.

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REFERENCES

Anderson, G.S. (2001). Insect succession on carrion and its relationship to determining time of death. *Forensic Entomology: The Utility of Arthropods in Legal Investigation*, Byrd, J.H. & Castner, J.L. (editors) 1st edition. USA: CRC Press LLC, pp. 143-175.

Buchan, M.J. & Anderson, G.S. (2001). Time since death: A review of current satatus of methods used in the later postmortem interval. *Canadian Society of Forensic Science Journa*l **34**(1): 1-22.

Friedrich, F. & Beutel, R.G. (2008). Micro-computed tomography and renaissance of insect morphology. *Developmental in X-ray Tomography IV*, Stock, S.R. (editor) proceeding of SPIE **7078**: 1-6

Hall, R.D. (2001). Perception and status of forensic entomology. *Forensic Entomology: The Utility of Arthropods in Legal Investigation*, Bryd, J.H. & Castner, J.L. (editors) 1st edition. USA: CRC Press LLC, pp.1-15.

Harvey, M.C., Mansell, M.W., Villet, M.H. & Dadour, I.R. (2003). Molecular identification of some forensically important blow flies of southern Africa and Australia. *Journal of Medical Veterinary Entomology* **17**(4): 363-369.

Ismail, M.I., Osman, K., Ambia, K.M., Ghazali, A.R., Mohamed, J., Omar, B., King, O.H., Hassan, H. & Elias, E. (2007). Accelerating *Chrysomya megacephala* maggot growth for forensic entomology cases. *Malaysian Journal of Health Sciences* **5**(1): 17-26.

Kurahashi, H., Benjaphong, N. & Omar, B. (1997). Blow flies (insecta: diptera: calliphoridae) of Malaysia and Singapore. *Raffles Bulletin of Zoology* **45**(5 SUPPL.): 88-91.

Martín-Vega, D., Simonsen, T.J. & Hall, M.J.R. (2017a). Looking into the puparium: Micro-CT visualization of the internal morphological changes during metamorphosis of the blow fly, Calliphora vicina, with the first quantitative analysis of organ development in cyclorrhaphous dipterans. *Journal of Morphology* **278**(5): 629-651.

Martín-Vega, D., Simonsen, T.J., Wicklein, M. & Hall, M.J.R. (2017b). Age estimation during the blow fly intra-puparial period: a qualitative and quantitative approach using micro-computed tomography'. *International Journal of Legal Medicine* **131**: 1429-1448.

Metscher, B.D. (2009). MicroCT for developmental biology:A versatile tool for high-contrast 3D imaging at histological resolutions. *Developmental Dynamics* **283**(3): 632-640.

Nor Afandy, H., Baharudin, O., Firdaus, A.M., M.S., Ahmad, M., Halim, A.M., Feng, S.S. & Norhayati, M. (2001). A review of forensic specimens sent to forensic entomology laboratory Universiti Kebangsaan Malaysia for the year 2001. *Tropical Biomedicine* **20**(1): 27-31.

Noratiny, I., Azwandi, A. & Abu Hassan, A. (2002). Case study of forensically important fly species in Penang. *Proceeding of the 4th IMT-GT UNINET Conference*, pp.235–236. Universiti Sains Malaysia.

Rajagopal, K., Nazni, W.A., Tan, T.C., Lee, H.L., Isa, M.N.M. & Azirun, M.S. (2012). Molecular identification of blow flies recorded from human cadaver during crime scene investigation in Malaysia. *Malaysian Journal of Pathology* **34**(2): 127-132.

Rajagopal, K., Nazni, W.A., Tan, T.C., Lee, H.L. & Azirun, M.S. (2013). Review of forensically important entomological specimens collected from human cadavers in Malaysia, 2005-2010. *Journal of Forensic and Legal Medicine* **20**(5): 480-482.

Rajagopal, K., Tian, T., Jeffery, J. & Sofian-azirun, M. (2014). Forensic entomology in Malaysia/: knowledge and practices. *Asian Biomedicine* **8**(5): 603-607.

Richards, C.S., Simonsen, T.J., Abel, R.L., Hall, M.J.R., Schwyn, D.A. & Wicklein, M. (2012). Virtual forensic entomology/: Improving estimates of minimum post-mortem interval with 3D micro-computed tomography. *Forensic Science International* **220**: 251-264.

Sukontason, K.L., Sukontason, K., Piangjai, S.B.N., Chaiwong, T.V.R.C., Kuntalue, B., Thijuk, N. & Olson, J.K. (2003). Larval morphology of *Chrysomya megacephala* (Fabricius) (diptera: calliphoridae) using scanning electron microscopy. *Journal of Vector Ecology* **28**: 47-52.

Swart, P., Wicklein, M., Sykes, D., Ahmed, F. & Krapp, H.G. (2016). A quantitative comparison of micro-CT prparations in Dipteran flies. *Scientific Report* **6** (September): 1-12.

Syamsa, R.A., Ahmad, F.M., Marwi, M.A., Zuha, R.M. & Omar, B. (2010) An analysis of forensic entomological specimens by Universiti Kebangsaan Malaysia. *The Medical Journal of Malaysia* **65**(3): 192-195.

Syamsa, R.A., Baharudin, O., Ahmad Firdaus, M.S. & Shahrom, A.W. (2017). Comparative fly species composition on indoor and outdoor forensic cases in Malaysia. *Journal of Forensic and Legal Medicine*, 41-46.

Tomberlin, J.K., Benbow, M.E., Tarone, A.M. & Mohr, R.M. (2011). Basic research in evolution and ecology enhances forensics. *Trends in Ecology & Evolution* **26**(2): 53-55.

Villet, M.H., Richards, C.S. & Midgley, J.M. (2009). Contemporary precision, bias and accuracy of minimum post-mortem intervals estimated using developmental of carrion feeding insects. *Current Concepts in Forensic Entomology*, Amendt, J., Goff, M., Campobasso, C. & Grassberger, M. (editors). Dordrecht: Springer, pp.109-137.

Wells, J.D. & Stevens, J.R. (2008). Application of DNA-based methods in forensic entomology. *Annual Review of Entomology* **53**: 103-120.