A PRELIMINARY EVALUATION ON BIVARIATE ALLOMETRY IN ACTIVE-FEEDING *Chrysomya megacephala* (FABRICIUS, 1794) (DIPTERA: CALLIPHORIDAE) LARVAE

RAJA MUHAMMAD ZUHA[1,2,3*]

[1]Forensic Science Program, Faculty of Health Sciences, Basement One, Perpustakaan Tun Seri Lanang, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[2]Centre for Insect Systematics, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[3]Fraser's Hill Research Centre, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[*]E-mail: rmzuha@ukm.edu.my

ABSTRACT

The potential use of larval cephalopharyngeal skeleton as an alternative growth indicator in forensic entomology practice was assessed based on its developmental pattern, growth performance, and allometric relationship with the larval body. *Chrysomya megacephala* (Fabricius, 1794), was used as an experimental species and larval development was studied at ambient temperatures and relative humidity. Larval body size was measured from the furthest part of the head to the last abdominal segment. The cephalopharyngeal skeleton was extracted from the body and measured from the tip of the dorsal bridge to the left face of the dorsal cornu. Daily progression of larval body length and cephalopharyngeal skeleton length showed the latter significantly had slower growth rates. The allometry of the larval body and cephalopharyngeal skeleton showed they were correlated only because both variables increased across the three larval instars, suggesting a spurious correlation. Separate bivariate correlations between the two variables showed only the first instar larvae had a significant ($p<0.01$) but weak correlation, $r=0.33$. However, removing the effect of larval instars still produced a statistically significant correlation ($p<0.001$) albeit with moderate strength, $r=0.57$. From this study, there was not enough evidence to support the cephalopharyngeal skeleton as an equal or a better alternative to the larval body as a growth indicator.

Key words: Blowfly, correlation, development, forensic entomology

INTRODUCTION

The main purpose of forensic entomology in the medicolegal investigation is to determine the minimum post-mortem interval (PMI_{min}) or the minimum time elapsed after death by estimating the age of necrophagous insects found breeding and feeding on decaying human bodies. The most common method being employed for this purpose is by assessing dipterous larval age found actively feeding on decaying human tissues. The age of dipterous larvae can be estimated by referring to the growth parameter represented by the larval body length. In routine forensic entomology practice, larvae collected from the human body at the death scene or during autopsy are preserved in 70-95% ethanol (Smith, 1986; Catts & Haskell, 1990; Byrd, 2001; Amendt *et al.*, 2007; Gennard, 2007) before being subjected to PMI_{min} analysis. Methods to kill larvae are also varied such as immersion of larvae in hot water more than 80 °C (Amendt *et al.*, 2007), 70-80 °C hot water (Manlove,

2010), or near-boiling water (Smith, 1986). In some recommendations, larvae can be fixed in Kahle's, KAA (KAAD) or XAA solutions (Catts & Haskell, 1990; Byrd, 2001).

However, the type of preservatives and killing methods that were used to store larvae could affect the size of various calliphorid larvae and thus lead to inaccuracy in their age estimation. For instance, *Protophormia terraenovae* (Robineau-Desvoidy, 1830) larvae shrunk when killed directly in preservatives without killing them first in boiling water (Tantawi & Greenberg, 1993). In another study using *Calliphora vomitoria* (Linnaeus, 1758) and *Lucilia sericata* (Meigen, 1826) larvae stored in 10% formalin, 80% ethanol, and 90% ethanol, changes were not only observed on the size but also the characteristics of preserved larvae such as turgidity, curvature, and discoloration (Adams & Hall, 2003). In *Chrysomya megacephala* (Fabricius, 1794) larvae, minimal changes were detected when preserved in Kahle's solution compared to 70% ethanol and 10% formalin (Rosilawati *et al.*, 2014). Richards *et al.* (2013) concluded that there were variations on larval

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body length of *L. sericata* and *Calliphora vicina* Robineau-Desvoidy, 1830 when preserved in 80% ethanol for a duration up to 365 days but depended on storage temperatures and larval instars.

Other than effects caused by preservation techniques, the methodological aspect of larval body length measurement was rarely mentioned. The size of a dipterous larva used for age-referencing was one-dimensional, measured by inter-landmark distances from the most distal parts of the head and the last abdominal segment in lateral position (Day & Wallman, 2006; 2008), or width, measured between the ventral and dorsal surfaces at the junction at the fifth and sixth abdominal segments (Day & Wallman, 2006). However, in a situation where larval forms were curved or deviated from the straight position due to preservation effects such as in Rosilawati *et al*. (2014), no specific measuring techniques were reported in the literature. In some approaches, the measurement of larval body length was performed segment-by-segment and perpendicular to intersegmental spines without emphasizing the reliability and validity of this technique (Rosilawati *et al*., 2014; Syamsa *et al*., 2017), which possibly lead to inaccuracy in PMI_{min} estimation (Villet *et al*., 2010).

In forensic entomology guidelines (Amendt *et al*., 2007), the larval mouth part, which refers to the cephalopharyngeal skeleton, was suggested as an alternative to larval body length. The cephalopharyngeal skeletons of dipterous larvae are one of the vital parts used in species and instar determination (Greenberg & Kunich, 2002). This structure invaginates and is enclosed in the cephalic region of the larva. Generally, it can be divided into three segments i.e., tentoropharyngeal sclerite (basal/pharyngeal sclerite), hypopharyngeal sclerite (intermediate sclerite), and mandibles (mouth hooks) (Teskey, 1981). Mouth hooks and the pharyngeal sclerite are connected by the 'H'-shaped intermediate sclerite. The largest section of the cephalopharyngeal skeleton, the pharyngeal sclerite, has four projections i.e., two dorsal and two ventral cornua. Anteriorly, the dorsal cornua are joined by a dorsal bridge. The terms of these parts in cephalopharyngeal skeleton can be homologized with Roberts (1971) and principle morphological terms can be found in Ferrar (1987).

To evaluate cephalopharyngeal skeleton as a potential alternative to larval body length, few studies have been carried out on the morphometry of this structure but the data were not extended to forensic applications (Lawrence, 1979; Ferrar, 1987; Petitt, 1990; Simon *et al*. 2011). Nateeworanart *et al*. (2010) and Chaiwat *et al*. (2012) were possibly the earliest to show cephalopharyngeal skeleton as reference values in forensic entomology by using the necrophagous calliphorids, i.e. *C. megacephala* and *Chrysomya rufifacies* (Macquart, 1843) third instar larvae. Subsequently, Rabbani & Zuha (2017) highlighted

measurement consistencies of *Hypopygiopsis violacea* (Macquart, 1835) cephalopharyngeal skeleton morphometry compared to larval body length when preserved in 70% ethanol for 0, 7, and 14 days. The measurement of cephalopharyngeal skeleton was made based on the inter-landmark distances between mouth hook to dorsal cornu (MH-DC), mouth hook to ventral cornu (MH-VC) and anterior dorsal process (tip of dorsal bridge) to dorsal cornu (ADP-DC). Cephalopharyngeal skeleton length also positively correlated with larval body length as observed from the growth of *Hemipyrellia ligurriens* (Wiedemann, 1830) larvae based on inter-landmark distances between ADP-DC, the anterior dorsal process to ventral cornu (ADP-VC), and dorsal cornu to ventral cornu (DC-VC) (Eliza & Zuha, 2018). Recently, geometric morphometrics analysis has been utilized on cephalopharyngeal skeletons to discriminate species based on the morphological shape of *Chrysomya albiceps* (Wiedemann, 1819), *C. megacephala,* and *Lucilia cuprina* (Wiedemann, 1830) (Nuñez & Liria, 2016). Geometric size information that was derived from the geometric morphometric analysis has been further explored on the ontogenetic properties, i.e., shape changes due to growth based on *C. megacephala* larval development (Sim & Zuha, 2019). Based on both studies (Nuñez & Liria, 2016; Sim & Zuha, 2019), the preliminary functions of geometric morphometric analysis include the ordination of shape based on coordinates of selected landmarks on cephalopharyngeal skeleton which later could produce the landmark-coordinated size known as the centroid size.

Considering the potential of the cephalopharyngeal skeleton as an alternative growth parameter for PMI_{min} estimation, this current study investigated the development of *C. megacephala*, a blowfly species of forensic importance in Malaysia, Thailand, and in other tropical and temperate-climate regions (Sukontason *et al*., 2001; Lee *et al*., 2004; Thevan *et al*., 2010; Badenhorst & Villet, 2018). The morphometry of *C. megacephala* larvae was presented based on total larval body size, measured from the furthest part of the head and the last abdominal segment in the lateral position, and cephalopharyngeal skeleton size, represented by inter-landmark distances between the anterior tip of the dorsal bridge and to the tip of left dorsal cornu. Data obtained from this study were used to investigate the growth of *C. megacephala* based on both variables of interest, and allometry in *C. megacephala* by taking into account that other insect limbs or organs (i.e., cephalopharyngeal skeleton) can be used as a proxy for insect body size (i.e., larval body size) (Bai *et al*., 2016).

MATERIALS AND METHODS

This study was conducted in six replicates from

15 August 2018 to 7 November 2018. Each study replicate consists of the following steps:

Sample preparation

Fly eggs were obtained from baits placed in an open area adjacent to Forensic Science Entomology Laboratory, Universiti Kebangsaan Malaysia, Bangi (2.93ºN 101.78ºE, 47 m alt.). Baits, consisting of approximately 400 g raw yellow stripe scads, *Selaroides leptolepis* (Cuvier, 1833), Indian mackerel, *Rastrelliger kanagurta* (Cuvier, 1816), and 400 g raw cow's liver, were mixed inside a black plastic container. The baits were left exposed on the ground during daytime from 0900 h to 1500 h and checked hourly for the presence of adult female *C. megacephala*. In local natural surroundings, *C. megacephala* has been observed to be the commonest and earliest blow fly species to feed and oviposit on decaying organic matter. Getting the eggs mixed with other fly species was unlikely to occur since the arrival and oviposition of *C. megacephala* were closely monitored. A single

batch of blow fly eggs oviposited by a single female adult were then carefully collected by using fine-tip forceps and transferred into a 200 mL rearing container with 70 g fresh cow's liver on 2 cm layer of sawdust. Rearing of eggs was conducted in the same vicinity where baits were placed.

On the next day, at 0830 h, groups of 10 newly emerged first instar larvae were transferred into separate, freshly prepared rearing containers. The rearing containers and amount of food were similar to the ones used during egg-rearing. During each sampling occasion at 0900 h and 1500 h daily, a rearing container consisting of 10 larvae was withdrawn and the larvae were killed by using hot water (~80 °C) for 60 s (Adams & Hall, 2003). Post feeding larvae from the remaining containers were excluded from the sample, but they were reared until the adult stage to facilitate species identification. Ambient temperatures and relative humidity during rearing from eggs until adult emergence in each study replicate are shown in Table 1.

Table 1. Mean ± standard deviation (SD), minimum and maximum values of ambient temperatures, and relative humidity in each study replicate

Study Replicate	Ambient Temperature (ºC)			Relative Humidity (%)		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max
1st 15 − 22 August 2018	27.8±2.7	22.0	34.0	76.2±7.7	60.5	87.5
2nd 25 September − 2 October 2018	26.1±1.7	22.0	29.5	81.8±8.9	57.0	92.5
3rd 2 − 14 October 2018	26.4±1.7	21.5	31.5	86.9±7.5	68.5	96.0
4th 9 − 18 October 2018	25.2±1.2	21.5	28.5	89.5±5.2	74.5	97.5
5th 31 October − 7 November 2018	26.0±1.7	22.0	30.0	88.7±7.7	71.0	97.0
6th 31 October − 7 November 2018	26.0±1.7	22.0	30.0	88.7±7.7	71.0	97.0

Larval body length and cephalopharyngeal skeleton length acquisition

The lengths of every larval body and cephalopharyngeal skeleton were obtained pairwise. Larvae were processed based on methods prescribed in Amendt *et al*. (2007) and Rabbani and Zuha (2017) with modifications. Larval body length was measured with SMZ745T stereomicroscope (Nikon, Japan) fitted with a 12-megapixel digital camera (Toupcam, China), based on lateral body segments i.e., the furthest part of the head and the last abdominal segment in lateral position (Day & Wallman, 2006) (Figure 1A). Subsequently, each larva was dissected by removing the anterior segment of the body which contains the cephalopharyngeal skeleton, and immersed in 10% potassium hydroxide (KOH) for 15 min. The internal tissues surrounding the cephalopharyngeal skeleton

were carefully removed and the cephalopharyngeal skeleton was immersed in 10% acetic acid for 10 min. Cephalopharyngeal skeletons were then washed in 70% ethanol for 20 min before they were mounted onto a glass slide in lateral position by using Berlese fluid and covered with a 6 mm round coverslip. Cephalopharyngeal skeleton morphometrics was obtained immediately after mounting procedure based on inter-landmark distances of the left face pharyngeal sclerite from the anterior tip of the dorsal bridge (d brg) to dorsal cornua (d corn) by using ToupView software (Nateeworanart *et al*., 2010; Nuñez & Liria, 2016; Rabbani & Zuha, 2017) (Figure 1B-D). For both larval body and cephalopharyngeal skeleton length, test-retest reliability assessment was conducted before the measurement using randomly selected samples by two researchers (*n*=10), *r*>0.95.
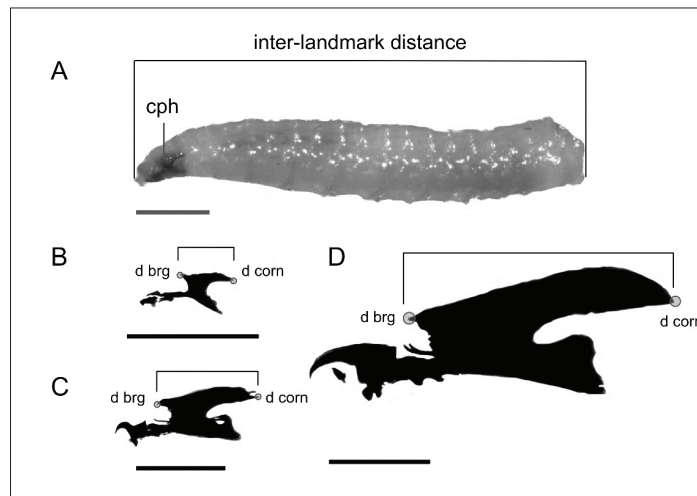
Fig. 1. Morphometrical landmarks of *C. megacephala* **larval body and cephalopharyngeal skeleton at three different instars**, (A) Inter-landmark distance from the anterior part of the head and the last abdominal segment from the lateral position, with the cephalopharyngeal skeleton (cph) is in the first three segments of the larval body anteriorly; Cephalopharyngeal skeleton of the (B) first instar larva; (C) second instar larva; and (D) third instar larva. Inter-landmark distances of cephalopharyngeal skeletons were measured from the tip of the dorsal bridge (d brg) to dorsal cornu (d corn) from the left face (*Bar*=0.5 mm).

Statistical analysis

The development of *C. megacephala* larvae which are based on mean larval body length and mean cephalopharyngeal skeleton length was analyzed in Microsoft Excel software for each study replicate. Larval age cohorts were treated as independent groups and presented in the bar chart (error bar = ± 95% CI) because of the uneven sampling intervals (6 h and 18 h periods alternately). To demonstrate the growth performance between larval body and cephalopharyngeal skeleton, multiple comparisons of means of the two variables were conducted by using one-way between-groups ANOVA (Welch's *F*) in SPSS 22.0 and follow up analysis using Games-Howell (a=0.05), after the assumptions of normal distribution were passed for all age groups but the homogeneity of variances was violated (Levene's *F* test, *p*<0.001). In those tests, samples from study replicate 1, 2, and 4 comprising of the same larval instar which developed within the 6 h period were compared pairwise, e.g., larval body length of the 18 h group against larval body length of the 24 h group. This was because the developmental patterns were relatively more gradual and similar than those in study replicates 3, 5, and 6. Finally, the bivariate allometry or the correlation between cephalopharyngeal skeleton length (x_1) and larval body length (y) were determined by using Pearson's coefficient correlation. In this analysis, x_1 and y were pooled across replicates and they fulfilled the assumptions of statistical normality. Larval instar (x_2) was used as an additional variable to be controlled for in the partial bivariate correlation test. Transitional stages from the first to second instar, and from the

second to third instar were excluded from study samples because of the high variability of larval body and cephalopharyngeal skeleton sizes.

Species identification

Species identification was conducted based on Calliphoridae identification keys of the third instar larvae (Greenberg & Kunich, 2002) and adults (Kurahashi *et al*., 1997) that emerged from the remaining larvae colony. All specimens collected in this study were identified as *C. megacephala*.

RESULTS

Active-feeding duration of *C. megacephala* larvae and size changes

Larval body and cephalopharyngeal skeleton length measurements were gradually increased with time but varied across study replicates (Figure 2). Based on larval body length, *C. megacephala* larvae reached their peak of the active-feeding stage at 90 h (study replicates 1, 2, & 4), 66 h (study replicates 5 & 6), and 48 h (study replicate 3). In study replicate 3, mean larval body length decreased from 13.10±0.49 mm (48 h) to 12.85±0.84 mm (66 h) within an 18 h period but dispersed from food source after 66 h as post-feeding larvae. The first instar larval duration was observed lasted approximately 18 to 24 h and the second instar larvae were detected around 42 to 48 h (study replicates 1, 2, & 4).

The cephalopharyngeal skeleton displayed a relatively similar growth pattern to larval body length. While the larval body length continued to

progress after 42 h (study replicate 3, 5, & 6) and 66 h (study replicate 1, 2, & 4), the cephalopharyngeal skeleton showed a slower developmental rate until the length measurements became almost consistent. During the second instar phase within 42 to 48 h of sampling occasions, the cephalopharyngeal skeleton developmental rate also moved slower compared to larval body length. It is important to note that the pace of the cephalopharyngeal skeleton was noticeably higher during the transitional phase of larval instar 1 to larval instar 2 (study replicate 3, 4, & 6) and larval instar 2 to 3 (study replicate 3).

On the growth performance between larval body and cephalopharyngeal skeleton in selected study replicates (study replicates 1, 2, & 4), one-way between-groups ANOVA showed statistically significant changes of the two variables between age groups ($p<0.001$) (Table 2). Follow-up analyses to compare the means between age groups of interest using Games-Howell post hoc tests are summarized in Table 3. The results suggest that within 6 h of the developmental period, the samples sufficiently indicated developmental of larval body length occurred without any significant changes in cephalopharyngeal skeleton length. For instance, when comparing first instar larvae of 18 h and 24 h, the differences in larval body length were statistically significant ($p<0.001$) in contrast to cephalopharyngeal skeleton length ($p=0.528$). Even so, such differing patterns were observed throughout study replicates 1 and 2 but rather consistent in study replicate 4, which changes only noticeable in larval body length across age groups.

Table 2. One-way between groups ANOVA results using *Welch's F* to determine statistically significant differences between age groups of *C. megacephala* in study replicate 1, 2, and 4 for the larval body (LB) and cephalopharyngeal skeleton (CS) mean length. Omega squared (ω^2) is the indicator of the effect size

Study replicate	Variable	*n*	*Welch's F*	df1	df2	*p*-value	Effect size, ω^2
1	LB	69	701.953	6	25.889	<0.001	0.910
	CS	69	1726.284	6	26.211	<0.001	0.962
2	LB	65	489.220	6	24.703	<0.001	0.876
	CS	65	772.397	6	25.264	<0.001	0.918
4	LB	68	1090.906	6	25.847	<0.001	0.940
	CS	68	2963.218	6	25.534	<0.001	0.978
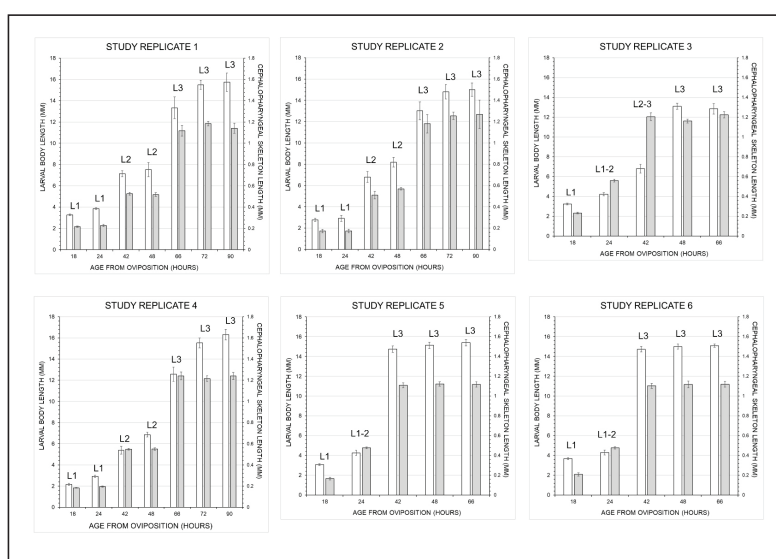
Fig. 2. Mean length of *C. megacephala* larval body (white bar) and cephalopharyngeal skeleton (grey bar) based on age groups from six study replicates and labeled by larval instars, i.e., first instar (L1), second instar (L2), third instar (L3), first-second instar transition (L1-2) and second to third instar transition (L2-3) (*Error bar*=**±95% CI).**

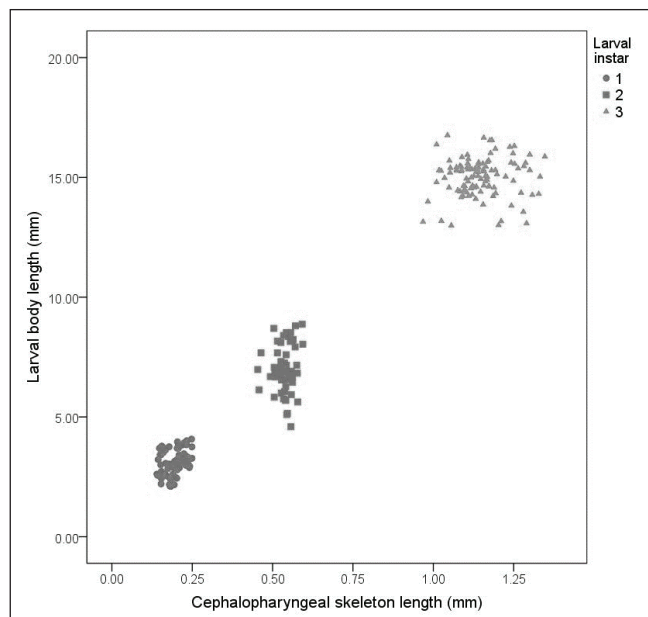## Larval body length and cephalopharyngeal skeleton allometry

The allometric relationships between larval body length and cephalopharyngeal skeleton length were explained by using bivariate correlation across all three larval instars. Correlations between larval body length and cephalopharyngeal skeleton length were statistically significant and strong, $r(247) = 0.98$, $p<0.01$, two-tailed. However, assessment of the scatter plot (Figure 4) showing the three distinct clusters suggested that larval body (y) and cephalopharyngeal skeleton (x_1) were possibly increased with the larval instars (x_2). The scatter plot likely indicates that the correlation between larval body and cephalopharyngeal skeleton was spurious, or the increment of both variables could be attributed to larval instars. Three distinct clusters of scores were observed i.e., first instar larvae have low scores, second instar larvae have intermediate scores, and the third instar have high scores on both larval body and cephalopharyngeal skeleton length.

To assess the spurious correlation, the relation between larval body and cephalopharyngeal skeleton length was determined separately by larval instars. Only the first instar group was statistically significant with a weak correlation, $r(86) = 0.30$, $p<0.01$, two-tailed, whilst the second and third instar were not statistically significant (Table 3). The partial correlation was also calculated for larval body and cephalopharyngeal skeleton length whilst controlling for larval instars, which produced statistically significant results but lower correlation coefficient, $r(247) = 0.57$, $p<0.01$, two-tailed.

Table 3. Multiple comparisons of larval body (LB) and cephalopharyngeal skeleton (CS) mean length of *C. megacephala* and standard deviation (SD) between age groups within 6-hour developmental period using Games-Howell post hoc tests (homogeneity of variances were not assumed, a=0.05).

Study Replicate	Larval Instar	h	LB Length (mm) Mean	SD	CS Length (mm) Mean	SD	h	LB Length (mm) Mean	SD	CS Length (mm) Mean	SD	Games-Howell post hoc p-value and effect size, Cohen's d LB	d	CS	d
R1	1	18	3.277	0.126	0.217	0.009	24	3.857	0.125	0.227	0.015	<0.001	4.621	0.528	-
	2	42	7.132	0.45	0.525	0.022	48	7.513	1.03	0.518	0.027	0.936	-	0.995	-
	3	66	13.333	1.647	1.118	0.081	72	15.493	0.688	1.185	0.034	<0.05	1.711	0.271	-
R2	1	18	2.782	0.217	0.172	0.023	24	2.913	0.462	0.173	0.027	0.982	-	1.000	-
	2	42	6.802	0.831	0.51	0.058	48	8.197	0.654	0.569	0.015	<0.05	1.866	0.099	-
	3	66	13.023	1.346	1.18	0.14	72	14.811	1.082	1.255	0.555	0.055	-	0.697	-
R4	1	18	2.166	0.169	0.183	0.006	24	2.918	0.172	0.196	0.001	<0.001	4.410	0.986	-
	2	42	5.385	0.599	0.549	0.013	48	6.86	0.328	0.55	0.017	<0.001	3.057	1.000	-
	3	66	12.577	1.033	1.241	0.058	72	15.529	0.769	1.216	0.043	<0.001	3.242	0.696	-

Fig. 3. The 'spurious' correlation between larval body and cephalopharyngeal skeleton. The bivariate scatter plot clearly shows three separate groups of scores. When the bivariate correlations were analyzed separately, only the first instar group was statistically significant ($p<0.01$) but weak correlation, $r=0.33$.

DISCUSSION

Cephalopharyngeal skeleton was mainly utilized to diagnose dipterous larval species at different instars (Sukontason *et al*., 2008; Bunchu *et al*., 2012) with limited information about its morphometry (Nateeworanart *et al*., 2010; Chaiwat *et al*., 2012). The current study shows cephalopharyngeal skeleton and larval body almost had similar growth patterns but when comparing developmental performance between the two variables, the cephalopharyngeal skeleton could not keep pace with the larval body. Pairwise comparisons of selected age groups showed that the cephalopharyngeal skeleton remained nearly at consistent length despite the elongation of larval body length within a 6 h developmental period. However, the larval body did not significantly change throughout all age groups and the outcomes of this test must be carefully evaluated since the sample size for each test group was considerably small, unequal, and increase the risk of committing Type I error. On whether cephalopharyngeal skeleton morphometry can signify maturation in larvae, it depends on further research to include data from post-feeding larvae as the current study only utilized data from active feeding larvae. In the case of the larval body as a growth indicator, it would certainly have a better advantage than the cephalopharyngeal skeleton as the size reduces during the post-feeding phase and dispersal which could also be useful for referencing PMI_{min} values (Gomes *et al*., 2006; Arnott & Turner, 2008; Robinson *et al*., 2018).

From the current study, it is worth mentioning that substantial variations of developmental rate existed between study replicates because of different temperature exposures from natural environments. For example, *C. megacephala* larvae in study replicate 3, with rearing temperature ranged 21.5-31.5 °C (mean temperature, 26.4±1.7 °C) had the shortest duration to reach maximum size, i.e., 48 h, followed by those from study replicate 5 and 6 (66 h), both reared at similar conditions of 22.0-30.0℃ (mean temperature, 26.0±1.7 °C). Sample from study replicates 1, 2, and 4 recorded the slowest development i.e., 90 h, with the highest temperature and range for larval rearing was recorded in study replicate 1, 22.0-34.0 °C (mean temperature, 27.8±2.7 °C). Previous studies also reported various effects of fluctuating temperature on the developmental rate of calliphorid larvae. *Aldrichina grahami* (Aldrich, 1930) and *P. terranovae* larvae developed slower at natural fluctuating temperatures than constant temperatures (Clarkson *et al*., 2004; Chen *et al*. 2019). This effect of fluctuating temperature was also depending on the range of fluctuation (Warren & Anderson, 2013) and could be species-specific as observed by Niederegger *et al*. (2010) or interacted with larval density (Dadour *et al*., 2001). However, in the current study, developmental comparison between study replicate was not inferred because laboratory regulations permitted sampling only twice daily during working hours, i.e., at 0900 and 1500 h. This technique created a large window period and risked excluding active-feeding larvae with maximum sizes. Subsequently, active-feeding third instar larvae with the largest body length might have not been collected beyond the sampling period and could have already progressed to the post-feeding phase before the next 18 h in subsequent sampling occasions.

To assess whether cephalopharyngeal skeleton development is associated with the larval body, a simple bivariate analysis with a noncausal relationship was performed as the two variables existed simultaneously, and they were used to measure the same subject. The scatter plot and bivariate correlation analyses based on larval instars indicated that the relation between the two variables was spurious and strongly attributed to the larval instars. However, when the effect of larval instars was removed, the relation between larval body and cephalopharyngeal skeleton was still statistically significant but the correlation coefficient was reduced to moderate strength. It is conceivable that as larval instar progressed, the development caused an increase in larval body and cephalopharyngeal skeleton lengths. This positive and statistically significant correlation between the two variables even with the effect larval instars removed warranted further studies on elucidating the bivariate allometry between larval body and cephalopharyngeal skeleton. It is important in a future study to improvise experimental design by having equal sampling intervals and increased sampling frequency.

If the bivariate allometry between larval body and cephalopharyngeal could be well-proven in future experiments, it will be important in forensic entomology practice especially when the effect of rearing procedure and specimen processing on the larval body is the common source of error for PMI_{min} estimation (Tantawi & Greenberg, 1993; Adams & Hall, 2003; Day & Wallman, 2008; Richards *et al*., 2013). Since forensic entomology practice mainly deals with specimens stored in preservatives, such as in 70-95% ethanol (Amendt *et al*., 2007), it is imperative to observe variations of size caused by the effect of preservatives on the larval body and how it differs from cephalopharyngeal skeleton. Furthermore, the rigid sclerites of the cephalopharyngeal skeleton could potentially provide a better estimation of PMI_{min} than highly flexible larval body length which was prone to physical distortions due to killing procedure and preservation effects (Tantawi & Greenberg, 1993; Adams & Hall, 2003; Rosilawati *et al*., 2014; Abdullah & Zuha, 2020; Zuha, 2021). Even so, the prospect of using cephalopharyngeal skeleton as an alternative growth indicator to larval body length relies on the sample representation across different ages and instars, including differing outcomes when using other species than *C. megacephala* as experimental subjects.

CONCLUSION

The assessment of the cephalopharyngeal skeleton as an alternative growth indicator to the larval body in *C. megacephala* was based on its developmental pattern, growth performance, and allometric relation with the larval body. Although two variables showed almost similar developmental patterns, the cephalopharyngeal skeleton had a slower growth rate than the larval body during the active feeding phase in each larval instar. The allometry between larval body and cephalopharyngeal skeleton in active-feeding *C. megacephala* larvae must be interpreted and viewed with caution as preliminary investigation displayed spurious correlation between the two variables in the presence of larval instars. However, removing the effect of larval instars still produced a statistically significant correlation albeit the lower correlation coefficient. The results suggest that the noncausal model in the relationship between larval body and cephalopharyngeal skeleton was partly explained by larval instars.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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