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Chrysomya megacephala (Fabricius, 1794) (Diptera: Calliphoridae) development by landmark-based geometric morphometrics of cephalopharyngeal skeleton: a preliminary assessment for forensic entomology application

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Abstract

Background: Considering the practicality of geometric morphometrics which could discriminate insect species, this application was extended to the analysis of blow fly larval growth based on cephalopharyngeal skeleton. In forensic entomology, cephalopharyngeal skeleton plays a crucial role in species identification but the morphometric information of this part is scarce. In this study, *Chrysomya megacephala* (Fabricius, 1794) was reared in two study replicates in natural conditions and samplings were conducted at fixed daily intervals. Cephalopharyngeal skeletons were removed from larvae and mounted on glass slides. Images were obtained from the specimens; digitized and geometric morphometric analysis on *C. megacephala* cephalopharyngeal skeletons was performed with MorphoJ software based on the ordination of five landmarks. The assessments of this analysis were based on centroid size measurements, visualization on the landmarks displacements, classification of the relative landmarks by using canonical variate analysis, and ontogenetic allometry determination.

Findings: Centroid size was strongly correlated with developmental time ($p < 0.05$) and significantly different between daily intervals ($p < 0.05$). Ontogenetic allometric effect based on multivariate regression on Procrustes coordinates and centroid size was significant ($p < 0.0001$), indicating that shape was influenced by growth (60.3%). Disposition occurred on all landmarks during development and was further discriminated based on age groups.

Conclusions: Other than discriminating between species, geometric morphometrics was found to be practical to visualize larval growth based on cephalopharyngeal skeletons which can be useful in forensic entomology.

Keywords: Forensic entomology, Development, Geometric morphometrics, Centroid size, Allometry, MorphoJ

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Introduction

In forensic entomology, the age of dipterous larvae, found feeding on decomposing human remains, can be used to estimate minimum post mortem interval (mPMI) . The larval age is estimated based on their growth parameter such as larval body length, which can be inferred from the species-specific developmental model (Sharma et al. 2015). However, there were drawbacks when using larval body length because it can be affected by specimens handling (Tantawi and Greenberg 1993; Adams and Hall 2003; Day and Wallman 2008; Richards et al. 2013) and subsequently lead to inaccuracies in mPMI estimation. Therefore, previous researches suggested cephalopharyngeal skeleton as an alternative growth parameter to larval body length because of its measurement consistency and positive allometry with larval body length (Eliza and Zuha 2018; Rabbani and Zuha 2017). In Calliphoridae larvae, cephalopharyngeal skeleton is the invaginating mouthparts in the cephalic region of the larva, consisting of pharyngeal sclerites and mandibles which are used to facilitate food intake (Teskey 1981). These structures provide vital diagnostic features to identify blow fly species of forensic importance (Greenberg and Kunich 2002) but apparently, the morphometric information of cephalopharyngeal skeleton is still lacking.

In recent years, geometric morphometrics (GM) has been increasingly utilized as a multivariate tool to classify insect species based on morphological shape in both mature and immature stages including flies (Canal et al. 2015; Nuñez and Liria 2016; Nuñez-Rodriguez and Liria 2017a; Tatsuta et al. 2018). GM also provides detailed visualization of morphological transformations and morpho-spatial differences in shape and size unique to species by using shape landmark coordinates, thus providing more accurate species discriminations (Viscosi and Cardini 2011; Cooke and Terhune 2015). In interpreting speciation and sexual dimorphism among flies, adult wing morphology is the most frequently utilized body part (Gidaszewski et al. 2009; Schutze et al. 2012; Nuñez-Rodriguez and Liria 2017b). Apart from discriminating species into phenetic groups, GM also covers ontogenetic allometry which can explain how morphological variation attributes directly to growth (Klingenberg 1998). This scope of application in GM could be useful to describe the growth of forensically important insects.

Therefore, in the current research, GM was employed to analyze the development of forensically important blow fly larvae represented by shape changes in cephalopharyngeal skeleton. *Chrysomya megacephala* larvae were used as sample, as this species was one of the most prevalent sarcosaprophagous species found at death scenes in Malaysia, Thailand, and the rest of the world (Lee et al. 2004; Sukontason 2005; Sukontason et al. 2008; Kumara et al. 2012; Badenhorst and Villet 2018). Additionally, this species also played a significant role in bacterial and parasitic transmissions in humans (Sulaiman et al. 1988; Sulaiman et al. 1989; Sukontason et al. 2000).

The cephalopharyngeal skeleton shape was profiled based on centroid size, i.e., the square root of the sum of squared distances between each landmark and the centroid of the cephalopharyngeal skeleton (Zelditch et al. 2012). We hypothesized that centroid size correlates with developmental time and varied independently between age groups. The ontogenetic allometry, i.e., the relationship between size and shape across different ages, and visualization of landmark dispositions were also performed by using prescribed methods (Klingenberg 2013; Mitteroecker et al. 2013).

Materials and methods

This study was conducted in two replicates in natural conditions, i.e., study replicate 1 from 14 August 2018 to 18 August 2018, and study replicate 2 from 24 September 2018 to 29 September 2018.

Sample preparation and species identification

Chrysomya megacephala eggs were obtained from fresh baits placed in an open area adjacent to Forensic Entomology Laboratory, Forensic Science Program, Universiti Kebangsaan Malaysia, Bangi. Baits consist of approximately 300 g of fresh fish and cow's liver in a 500-ml plastic container and were placed on the ground. They were left exposed and checked hourly for ovipositon activity by a single female *C. megacephala.* The adults were identified based on taxonomic descriptions by Kurahashi et al. (1997) while the subsequent larval species were determined based on Barros-Cordeiro and Pujol-Luz (2010) and Sukontason et al. (2008).

A single batch of eggs oviposited by a female *C. megacephala* was collected carefully by using fine-tip forceps and transferred into a rearing container with approximately 30 g fresh cow's liver as food source. The liver was placed on a 3-cm-thick coarse sawdust and separated by a piece of tissue paper. Eggs were reared overnight at outdoor ambient temperature (23.5–34.0 °C) and relative humidity (RH) (44.0–96.0%).

On the following day, at 0900 hours, newly emerged first instar larvae were transferred evenly into five freshly prepared rearing containers labeled as day 1 to day 5. They were reared at 27.8 ± 2.7 °C and 76.2 ± 7.7% RH (first replicate) and 26.1 ± 1.7 °C and 81.8 ± 8.9% RH (second replicate). Larval sampling was conducted twice at 0900 hours and 1500 hours, per day, based on rearing containers sequence. During each

sampling occasion, a total of 10 larvae were randomly selected and killed in near-boiling water (≈ 80 °C) for 30–40 s (Amendt et al. 2007). Post-feeding larvae were excluded from sampling.

Sample processing

Cephalopharyngeal skeleton was obtained by removing larval internal content and adhering tissue in 10% potassium hydroxide (KOH) (Rabbani and Zuha 2017). The cephalopharyngeal skeleton was subsequently immersed in 10% acetic acid and 70% ethanol for 5 min each. Then it was mounted on a glass slide with Berlese Fluid in lateral position, covered with a 5-mm round coverslip. For the first instar larvae, cephalopharyngeal skeletons were mounted directly on the glass slide without KOH and subsequent treatments because the specimens were too delicate. Cephalopharyngeal skeletons that were not thoroughly cleared or inclined from lateral position were omitted from being used as samples.

Data acquisition and analysis

Images of cephalopharyngeal skeleton were obtained directly after specimen mounting by using a stereomicroscope (Nikon, Japan) fitted with a 12-megapixel USB3.0 CMOS microscope camera (Toupcam, China). The two-dimensional images were then converted to a readable format using tpsUtil (version 1.74) and landmarks were plotted by using tpsDig2 (version 2.31) (download link: http://life.bio.sunysb.edu/morph/). Landmarks were selected based on geometrical shape of the cephalopharyngeal skeleton, i.e., (1) clipeal arc (anterodorsal process/dorsal bridge), (2) dorsal cornu, (3) concavity of pharyngeal sclerite (tentorial phragma/medial incision), (4) lower ventral cornu, and (5) base of parastomal bar (Nuñez and Liria 2016) (Fig. 1). Geometric morphometric analysis of cephalopharyngeal skeleton was carried out by using MorphoJ software (Klingenberg 2011) (download link: http://www.flywings.org.uk/morphoj_page.htm_), which includes visualization of landmark shifts and canonical variate analysis (CVA) to provide graphical ordination of individuals

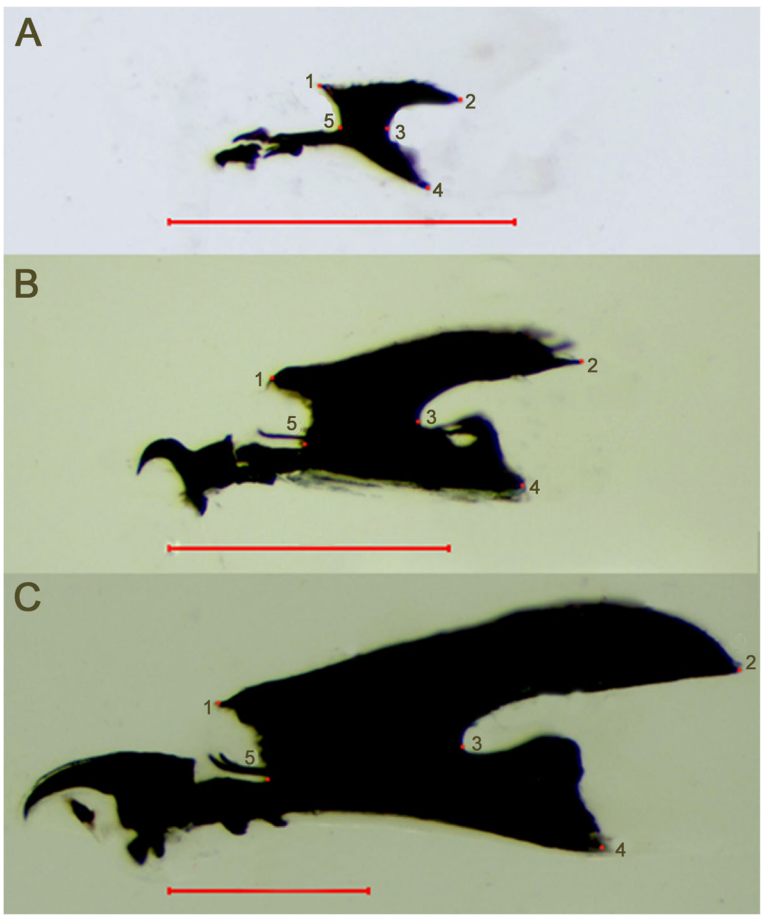

Fig. 1 Geometrical landmarks of *C. megacephala* cephalopharyngeal skeleton in three different instars. **a** First instar. **b** Second instar. **c** Third instar (bar = 0.5 mm). The landmark consist of 1 clipeal arc (anterodorsal process/dorsal bridge), 2 dorsal cornu, 3 concavity of pharyngeal sclerite (tentorial phragma/medial incision), 4 lower ventral cornu, and 5 base of parastomal bar (Nuñez and Liria 2016) (scale bar = 0.5 mm)

Table 1 Descriptive statistics summary of the centroid size of *C. megacephala* cephalopharyngeal skeletons in study replicates 1 and 2

Replicate	Age group (h)	Mean	Standard deviation	Variance	Min	Max	Range	*N*
1	20	0.19487	0.00853	0.000	0.17939	0.20533	0.02594	10
	26	0.20939	0.00517	0.000	0.19895	0.21697	0.01802	9
	44	0.49301	0.02204	0.000	0.45608	0.53149	0.07539	10
	50	0.48572	0.02476	0.001	0.43375	0.51238	0.07863	8
	68	1.03376	0.07173	0.005	0.89706	1.11066	0.21350	10
	74	1.05578	0.03524	0.001	1.00870	1.13109	0.12239	8
	92	1.12013	0.04468	0.001	1.05996	1.19808	0.13812	8
2	20	0.17959	0.01615	0.000	0.15938	0.20099	0.04161	8
	26	0.19601	0.01927	0.000	0.17253	0.22604	0.05351	10
	44	0.49435	0.04156	0.002	0.41849	0.52725	0.10575	7
	50	0.52639	0.01336	0.000	0.51015	0.55445	0.04430	8
	68	0.80697	0.04716	0.002	0.75632	0.88640	0.13009	7
	74	1.11720	0.04639	0.002	1.04375	1.19669	0.15294	10
	92	1.13567	0.03129	0.001	1.07889	1.16663	0.08774	7

and groups in multidimensional space, followed by cross-validation test in discriminant function analysis (DFA) to classify individuals in separate groups. The regression function in MorphoJ was used to determine ontogenetic allometry which is the influence of size changes on the shape. Prior to morphometric analysis, landmark coordinates were inspected for outliers. In SPSS™ Version 21 software, centroid sizes were classified based on the larval sampling intervals (age group) as independent groups and they were also checked for normality. They were subsequently analyzed by using one-way analysis of variance (ANOVA) for independent groups ($\alpha = 0.05$). Pearson correlation test was used to determine the significant relationship between centroid size and developmental time.

Results and discussion

The developmental period of *C. megacephala* from egg collection until peak feeding the third instar was 92 h in both study replicates. Based on the conditions of slit on posterior spiracle which could be used to discern larval instars (Barros-Cordeiro and Pujol-Luz 2010), *C. megacephala* larva was the first instar at 20 and 26 h and developed to

Table 2 *p* values of pairwise comparisons between age group using Games-Howell post hoc analyses after analysis of variance (ANOVA) for independent groups in study replicates 1 and 2

Study replicate	Age group (h)	Age group (h)					
		26	44	50	68	74	92
1	20	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	26		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	44			1.000	< 0.0001	< 0.0001	< 0.0001
	50				< 0.0001	< 0.0001	< 0.0001
	68					0.974	0.079
	74						0.076
2	20	0.470	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	26		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	44			0.507	< 0.0001	< 0.0001	< 0.0001
	50				< 0.0001	< 0.0001	< 0.0001
	68					< 0.0001	< 0.0001
	74						0.95

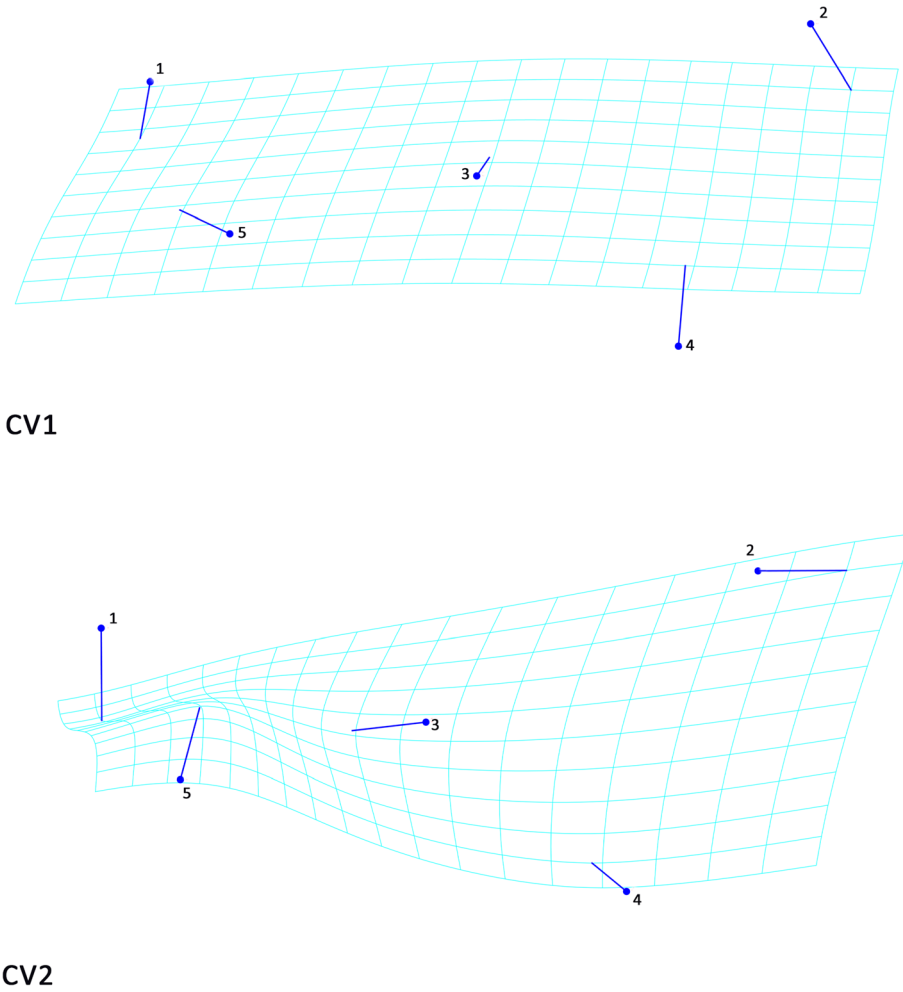

Fig. 2 Shape changes associated with CV1 and CV2 on deformed grids in positive directions (scale factor 10.0). The "lollipop" diagram with dots indicate the average starting shape and the lines are the movement of landmark to the target shape

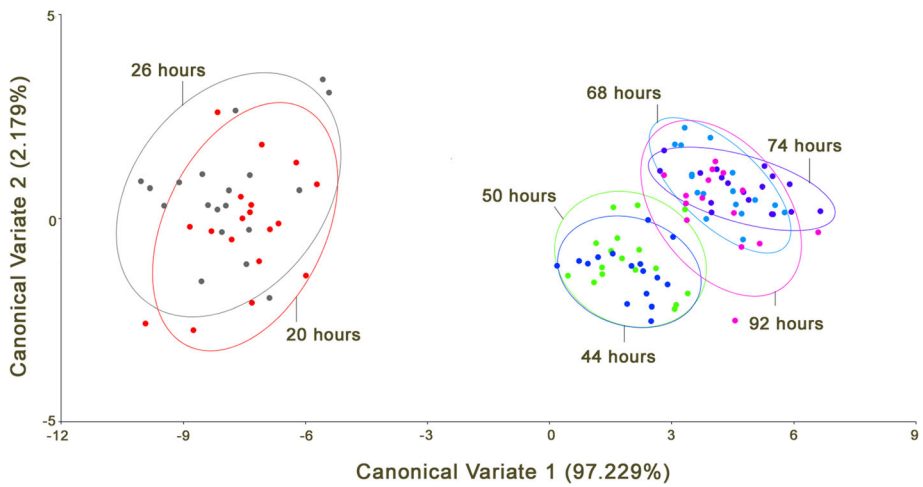

Fig. 3 Scatter plot along CV1 (97.229%) and CV2 (2.179%) axes shows the variation in cephalopharyngeal skeleton shapes grouped by equal frequency ellipse ($p = 0.9$). The coordinates are clustered based on the larval-age group sample

Table 3 Canonical variate analysis (CVA) of *C. megacephala* cephalopharyngeal skeleton shape given by the Mahalanobis distances (gray boxes) and Procrustes distances (clear boxes). Significant differences are represented by asterisks, i.e., ***$p < 0.0001$, **$p < 0.01$, *$p < 0.05$, while the p value for non-significant differences is indicated in the bracket (permutation 10,000 rounds in MorphoJ)

Age-group	20	26	44	50	68	74	92
20	-	0.0386 (P=0.3827)	0.1924***	0.1890***	0.2658***	0.2796***	0.2580***
26	1.2207 (P=0.2697)	-	0.2125***	0.2108***	0.2839***	0.2986***	0.2775***
44	9.5345***	10.0145***	-	0.0198 (P=0.5071)	0.0886***	0.1065***	0.0862***
50	9.5229***	10.0468***	0.8199 (P=0.2418)	-	0.0923***	0.1074***	0.0854***
68	11.7312***	12.1014***	2.9225***	3.1292***	-	0.0382 **	0.0423*
74	12.2430***	12.6227***	3.2893***	3.4575***	0.8833 (P=0.0649)	-	0.0255 (P=0.0713)
92	11.7174***	12.1209***	2.6743***	2.7782***	1.0716*	0.7707 (P=0.1878)	-

the second instar at 44 and 50 h before progressing to the third instar.

Descriptive statistics of cephalopharyngeal skeleton centroid size in both study replicates are summarized in Table 1. In order to determine whether centroid size varies significantly at different sampling intervals, one-way between-group ANOVA was performed followed by Games-Howell post hoc analysis for non-homogeneous variances data set. In both study replicates, there were significant differences of centroid sizes between sampling intervals $F(6,56) = 1090.996$, $p < 0.05$, $\eta^2 = 0.991$ (large effect size) (study replicate 1) and $F(6,50) = 1219.740$, $p < 0.05$, $\eta^2 = 0.993$ (large effect size) (study replicate 2). Pairwise comparison of centroid sizes between sampling intervals indicated the shape mostly transformed during early developmental stages (Table 2). In replicate 1, differences were detected between the 44- and 50-h group, 68- and 74-h group, 68- and 92-h group, and 74 and 92-h group. In replicate 2, differences were detected between 20- and 26-h group, 44- and 50-h group, and 74- and 92-h group.

Correlations between centroid size and cephalopharyngeal skeleton developmental time were determined by using Pearson's correlation test. Results indicate a strong significant and positive correlation between centroid size and developmental time with the correlation coefficient, r, ranged from 0.964 (study replicate 1) to 0.973 (study replicate 2) ($p < 0.05$). On the ontogenetic allometric effect, regression analysis on the Procrustes coordinates on centroid size among age groups revealed a significant relationship between cephalopharyngeal skeleton shape variation and size (permutation 10,000 rounds in MorphoJ: $p < 0.0001$). This effect accounted for 60.3% of the total shape variation. We also performed regression test by using the Procrustes coordinates on log centroid size, resulting $p < 0.0001$ with improvement in the effect of the total shape variation of 65.7%.

Table 4 Percentage of correctly classified specimens by cross-validation test in discriminant function analysis (DFA). Significant differences are represented by asterisks, i.e., ***$p < 0.0001$ (permutation 10,000 rounds in MorphoJ). Numbers in brackets represent the number of correctly classified over the total number of specimens

True assignment to age group	20	26	44	50	68	74	92
20		61.11 (11/18)	100.0*** (18/18)	100.0*** (18/18)	100.0*** (18/18)	100.0*** (18/18)	100.0*** (18/18)
26	47.36 (9/19)		100.0*** (19/19)	100.0*** (19/19)	100.0*** (19/19)	100.0*** (19/19)	100.0*** (19/19)
44	100.0*** (17/17)	100.0*** (17/17)		58.82 (10/17)	94.12*** (16/17)	94.12*** (16/17)	88.24*** (15/17)
50	100.0*** (16/16)	100.0*** (16/16)	56.25 (9/16)		100.0*** (16/16)	100.0*** (16/16)	100.0*** (16/16)
68	100.0*** (17/17)	100.0*** (17/17)	100.0*** (17/17)	100.0*** (17/17)		52.94 (9/17)	64.71 (11/17)
74	100.0*** (18/18)	100.0*** (18/18)	100.0*** (18/18)	100.0*** (18/18)	72.22 (13/18)		72.22 (13/18)
92	100.0*** (15/15)	100.0*** (15/15)	86.67*** (13/15)	93.33*** (14/15)	60.0 (9/15)	53.33 (8/15)	

The relationship between centroid size and age in ontogenetic allometry through multivariate regression analysis has been found reliable to explain biological shape changes across different ages including humans (Bulygina et al. 2006; Rodríguez-Mendoza et al. 2011; Mitteroecker et al. 2013; Murta-Fonseca and Fernandes 2016). Based on the results, the centroid size of *C. megacephala* cephalopharyngeal skeleton was positively correlated with developmental time, indicating the shape transformation occurred along larval progression from the first to the third instar. The transformation of cephalopharyngeal skeleton could be presented in growth trajectory to estimate larval age for PMI_{min} analysis based on centroid size. In contrast with its body length, the measurement based on cephalopharyngeal skeleton morphometry was more reliable and consistent to be used as growth parameter in forensic entomology (Rabbani and Zuha 2017; Eliza and Zuha 2018). In addition, these changes explained by the ontogenetic allometry in *C. megacephala* larva were represented by the cephalopharyngeal skeleton. By using regression analysis of the Procrustes coordinates and centroid size/log-transformed centroid size, the association between shape and size across different age groups was significant with moderate to high effect. This allometric effect has been reported in other organisms and the relationships vary and were usually high during ontogenesis (Rocha et al. 2005; Scalici et al. 2010; Strelin et al. 2018). However, for future studies, we recommend using an equal amount of sample size between groups and to reassess the GM landmarks.

Subsequently, CVA was employed to describe shape variations between age groups by maximizing the effect of separation (Cooke and Terhune 2015). Landmark coordinates of cephalopharyngeal skeletons in study replicates 1 and 2 were pooled and further analyzed on thin-plate spline transformation grid and "lollipop" diagram (scale factor 10.0). Figure 2 displays shape changes along CV1 (97.2%) axis with landmark 2 (dorsal cornu) and 4 (ventral cornu) displaying the most variation, followed by landmark 1 (anterodorsal process) and 5 (base of parastomal bar). Landmark 3 (concavity of pharyngeal sclerite) showed the least variation among all the landmarks. Along CV2 (2.2%), landmark 1, 3, and 5 dispositions were amplified.

Shape conformation for cephalopharyngeal skeletons in study replicates 1 and 2 scattered along the first two canonical variate axes (CV1 and CV2) (Fig. 3). The scatter plot from CV1 and CV2 shows that the cephalopharyngeal skeletons of *C. megacephala* at 20- and 26-h age group was clearly isolated from those at 44- to 92-h group. Mahalanobis and Procrustes distances by pairwise comparisons of all age groups showed significant differences between daily intervals (permutation 10,000 rounds in MorphoJ: $p < 0.0001$) (Table 3). However, there were no significant differences detected between groups sampled on the same day such as group 20 and 26 h (day 1), group 44 and 50 h (day 2), group 68 and 74 h (day 3), and group 74 and 92 h (day 4). There were also no significant differences between 68 and 74 h (day 3) with 92 h (day 4). Cross-validation test in DFA revealed high percentages of correctly classified specimens in all sampling intervals (86.7–100.0%; $p < 0.0001$) except for pairs of 20–26, 44–50, and 68–92 age groups, which corresponded to non-significant results in Mahalanobis and Procrustes distances output (Table 4). Due to the allometric effect, we reran cross-validation test on the groups without the effect of size on the morphological changes by using residuals from multivariate regression analysis (Klingenberg 2016). The result showed similar classifications as in Table 4.

In the present study, the geometric morphometric analysis produced a visual classification of *C. megacephala* cephalopharyngeal skeletons based on their age groups. CVA was used to display differences among groups that are relative to within-group variation based on multivariate data (Webster and David Sheets 2010). This technique was previously employed in distinguishing insect species and sex, including forensically important blow flies which were attributed by the wing landmarks (Nuñez-Rodiguez and Liria 2017a; Nuñez and Liria 2017b; Sontigun et al. 2017). In adults, a few species of *Chrysomya* Robineau-Desvoidy 1830, *Lucilia* Robineau-Desvoidy 1830, and *Hemipyrellia* Townsend 1918 were correctly classified and could be further explained by within genus phenetic relationships (Sontigun et al. 2017) while in the larval stages, Nuñez and Liria (2016) successfully differentiated *C. megacephala*, *Chrysomya albiceps* (Wiedemann 1819), and *Lucilia cuprina* (Wiedemann 1830) by using a similar approach.

Landmark displacements based on the "lollipop" diagram (Fig. 2) served as a visual aid to explain the growth of cephalopharyngeal skeletons. Through visual assessment of these five landmarks on *C. megacephala* larval development, transformation occurred at all landmarks with the least change on landmark 3 (the concavity of pharyngeal sclerite or medial incision). These changes were consistent with taxonomic descriptions for all three instars (Barros-Cordeiro and Pujol-Luz 2010; Szpila et al. 2013) whereby the deformation of cephalopharyngeal skeleton shape shifted inward based on the selected landmarks.

Landmarks used for cephalopharyngeal skeleton shape description in the present study were limited to five landmarks instead of the eight used by Nuñez and Liria (2016), because of some clearly undeveloped structures in the first instar larvae such as apical hook, union between hypostomal sclerite and the mouth

hook, and dorsal apodeme of mouth hook. Furthermore, the selection of landmarks for GM analysis adhered to the criteria that they can be found repeatedly and not difficult to locate (Bookstein 1991; Zelditch et al. 2012) while at the same time needs to adequately cover the morphology of the subject. A matter of concern that demands further investigation is the coplanarity of landmarks on cephalopharyngeal skeleton, as the actual three-dimensional shape could have been distorted when projected as a two-dimensional image. To minimize this effect, we removed any obscure landmarks when using a two-dimensional image of cephalopharyngeal skeleton as a sample. Images were also taken in a similar plane by using fixed focal length and lighting. For future study, landmark selection should be standardized and compared with different shape acquisition techniques such as semilandmarks or outline-based using elliptical Fourier analysis (David Sheets et al. 2006; Gunz and Mitteroecker 2013; Changbunjong et al. 2016; Santillán-Guayasamín et al. 2017) or 3D morphometrics (Bai and Yang 2014). It is also possible to tilt the cephalopharyngeal skeleton form dorsally or ventrally to explore shape variation as object symmetry (Klingenberg 2002), i.e., the alternate view to asymmetrical lateral shape as in the current study.

Conclusions

GM analysis on *C. megacephala* cephalopharyngeal skeleton can be useful to discriminate larval age group and aid growth visualization based on landmark displacements. Correlation between centroid size of *C. megacephala* cephalopharyngeal skeleton and developmental time indicated that it can be used as a growth parameter which could be applicable for mPMI estimation. Given the results from the present study, GM analysis on cephalopharyngeal skeleton shape variation merit further exploratory investigations, especially in comparing different forensically important fly species and by using different environmental settings such as rearing temperatures and food sources as these could also influence the biological shape (Dujardin 2008; Gobbi et al. 2013).

Abbreviations

CVA: Canonical variate analysis; DFA: Discriminant function analysis; GM: Geometric morphometrics; RH: Relative humidity

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Authors' contributions

LXS and RMZ conducted the research and organized the structure of the manuscript. Both authors read and approved the final manuscript.

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Please contact the author for data requests.

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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