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ORIGINAL ARTICLE

Effects of Preservative Concentrations on Larval Cephalopharyngeal Skeleton of *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae), an Alternative Indicator to Larval Body Length for mPMI Estimation

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ABSTRACT

Introduction: In forensic entomology, dipterous larval specimens found feeding on decomposing corpses are usually preserved in a range of 70-95% ethanol before being subjected to minimum postmortem interval (mPMI) assessment. However, larval body size, which is commonly used to infer mPMI, can be affected by preservatives resulting miscalculation of estimation. This study compared the effect of ethanol concentrations on larval body and cephalopharyngeal skeleton, a potential substitute to estimate larval age for mPMI estimation. **Methods:** Experiments were conducted on homogenised colony of *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) third instar larvae reared in similar conditions. They were fixed with hot water (≈80ºC) and preserved in 70% and 90% ethanol. After seven days in preservatives, larvae were measured for total body length from furthest part of the head to the last abdominal segment. Cephalopharyngeal skeleton was subsequently extracted from the body and measured based on morphometric landmarks on the pharyngeal sclerite. Centroid size of the cephalopharyngeal skeleton was also calculated based on the configuration of five morphometric landmarks. **Results:** In all four study replicates, pairwise comparisons with the original size indicated that larval body length was significantly affected by ethanol concentration (p<0.001) whilst only two of the study replicates showed cephalopharyngeal skeleton maintained its size when preserved in different ethanol concentration. **Conclusion:** Possible causes of variations are discussed herein with the results clearly indicated cephalopharyngeal skeleton should be considered as alternative growth parameter for mPMI estimation.

KEYWORDS: morphometry, forensic entomology, preservative, centroid size

INTRODUCTION

In death investigation, necrophagous insects found on human corpses can provide valuable input to the evaluation of minimum postmortem interval (mPMI) or the minimum time elapsed after death. The mPMI estimation is usually derived from the analysis of dipterous insects' age from any developmental stage such as egg, larva or pupa [1, 2]. Particularly on dipterous larvae, information obtained from developmental landmarks of this stage could help to narrow the gap from the time of body discovery to the closest actual time of death due to its feeding activity on decomposing tissue and linear growth on corpses [3]. Therefore, handling of larval specimens is very crucial to reduce any influence that could affect the reliability and validity for the mPMI assessment [4].

Killing methods, preservation techniques and storage procedures of larval specimens have been the subjects of interest in forensic entomology practice since these factors could change larval conditions and lead to erroneous mPMI value [5–7]. In general, dipterous larvae collected from human corpses must be killed with hot water (≈80ºC) to halt their development, expand the size to the maximum length and improve its permeability to preservatives [6]. Furthermore, killing with hot water helps to clean the larva [8] while

avoiding internal putrefaction [9]. This procedure is followed by storing the larvae in various recommended preservatives such as the commonly used 70-95% ethanol [2, 4, 10–12], Kahle's, KAA (KAAD) or XAA solutions [10, 13].

Even though the main purpose of preservation is to maintain the morphological characteristics of larvae from decay, killing method and type of preservatives could alter the morphology and morphometry of the larvae, which later compromised mPMI evaluation. For instance, Tantawi and Greenberg [5] were the first to record *Protophormia terraenovae* (Robineau-Desvoidy, 1830) and *Calliphora vicina* Robineau-Desvoidy, 1830 third instar larvae shrunk in preservatives, such as 70% ethanol, without killing them first in hot water, and the variations were even greater in younger larvae. Subsequent study using *Calliphora vomitoria* (Linnaeus, 1758) larvae showed those killed with boiling water and afterwards preserved in 10% formalin, 80% ethanol and 95% ethanol had higher mean length compared to those directly killed in preservatives [6]. Of the three preservatives, 80% ethanol and 95% ethanol had more consistent effects on larval body length compared to 10% formalin that caused significant shrinkage in larvae. Furthermore, the postmortem length variability could also be depending on immersion time of larvae in hot water and water temperature used for killing [6, 14]. In another study, preservatives could produce conflicting reactions on larvae of different species. For instance, larval body length of third instar *Calliphora augur* (Fabricius, 1775) expanded when preserved for 10 days in a range of 70-100% ethanol, 10% formalin and Kahle's solution but for *Lucilia cuprina* (Wiedemann, 1830), the larval body shrunk when stored in the same preservatives [9]. Even storage temperature could not minimize the effect of preservatives on larval length. In a study using *C. vicina* larvae preserved in 80% ethanol at -25ºC, 6ºC and 24ºC for short (72 hours) and long (365 days) duration, shrinkage occurred in the first and second instar larvae but slight elongation recorded in the third instar larvae [7]. From the study, only post feeding larvae showed consistency of sizes whether in short or long storage duration.

All findings above clearly indicated that larval body lengths are inconsistent and vulnerable to killing methods and preservation techniques. For this reason, the larval mouthparts, or the cephalopharyngeal skeleton, has been chosen as an alternative to larval body length to estimate age in mPMI assessment [15, 16]. Cephalopharyngeal skeleton or the mouthpart has been suggested in the current forensic entomology guidelines as one of the criteria to age larval instar [4, 17] and its characteristic can be used to confirm dipteran species [1]. This invaginating structure is usually enclosed in the cephalic region of the larva and can be divided into three segments i.e. tentoropharyngeal sclerite (basal/pharyngeal sclerite), hypopharyngeal sclerite (intermediate sclerite) and mandibles (mouth hooks) [18]. Mouth hooks and the pharyngeal sclerite is connected by the 'H'-shaped intermediate sclerite. The largest section of cephalopharyngeal skeleton, the pharyngeal sclerite, has four projections i.e. two dorsal and two ventral cornua. Anteriorly, the dorsal cornua are connected by a dorsal bridge [18].

In earlier studies, cephalopharyngeal skeletons have been used as growth parameter to measure dipterous larval size but they were not intended for forensic purposes [19–21]. Nateeworanart et al. [22] and Chaiwat et al. [23] were possibly the earliest researchers to feature cephalopharyngeal skeleton morphometries for forensic applications by using *Chrysomya megacephala* (Fabricius, 1794) and *Chrysomya rufifacies* (Macquart, 1843) third instar larvae. The measurements of cephalopharyngeal skeleton dimensions from those studies were based on the inter-landmark distances between mouth hook (MH) to dorsal cornu (DC), MH to ventral cornu (VC) and anterior dorsal process (tip of dorsal bridge) (ADP) to dorsal cornu (DC). Subsequent study using *Hypopygiopsis violacea* (Macquart, 1835) larvae looked at the potential of cephalopharyngeal skeleton as an alternative growth parameter to larval body length by observing the reactions of cephalopharyngeal skeleton towards 70% ethanol for 0, 7 and 14 days [15]. Interestingly, cephalopharyngeal skeletons were more consistent and less affected when stored in preservatives for 7 days compared to larval body length. Another research was later carried out on the development of *Hemipyrellia ligurriens* (Wiedemann, 1830) active feeding larvae based on inter-landmark distances established in the pharyngeal sclerite, i.e.

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between ADP-DC, ADP-VC and DC-VC [16]. The allometric relationships recorded between cephalopharyngeal skeleton length and larval body length signified the functionality of cephalopharyngeal skeleton as parameter that can be used to age larvae for mPMI estimation. Recently, the geometric size information that was derived from configuration of morphological landmarks of cephalopharyngeal skeleton was explored [24]. Results from the study showed ontogenetic development of *C. megacephala* active feeding larvae based on morphological shape changes of cephalopharyngeal skeleton from the first until third instar larvae. By employing geometric morphometric analysis, landmark coordinates of the cephalopharyngeal skeleton could be utilized to discriminate species [25].

In addition to larval body length inconsistencies due to killing and preservation techniques, specific guideline to measure larval body length is currently inadequate. Normally, larval body is measured in a single dimension by inter-landmark distances from the most distal parts of the head and the last abdominal segment in lateral position [9, 26], or width, between the ventral and dorsal surfaces at the junction at the fifth and sixth abdominal segments [26]. However, preservatives could transform the highly flexible larval forms to curve or deviate from straight position, making it difficult for measuring [14]. Hence, larval body length had to be measured segment-by-segment and perpendicular to abdominal segments without taking into account the reliability of this technique [14, 27], which possibly lead to inaccuracy in mPMI estimation [3].

Considering the untoward possible outcomes in forensic investigations due to limitations in mPMI calculations as mentioned above, this current study investigated the effect of preservatives on the generalized size parameters based on larval body and cephalopharyngeal skeleton. Third instar larvae of *C. megacephala*, a species of forensic importance in Malaysia, Thailand and the rest of the world [28–31], were used as experimental subjects and preserved in different ethanol concentrations, i.e. 70% and 90%, for a duration of 7 days. Measurement of *C. megacephala* larvae were based on total larval body size, measured from the furthest part of the head and the last abdominal segment in lateral position, and cephalopharyngeal skeleton size, represented by inter-landmark distances between ADP and tip of DC. Centroid size of the cephalopharyngeal skeleton was also introduced as additional size variable based on the configuration of five geometrical landmarks. In geometric morphometric analysis, centroid size is the square root of the sum of squared distances of the landmarks from the centroid or the 'center of gravity' [32].

By considering the effect of preservatives on larval morphometry, we hypothesized that cephalopharyngeal skeleton sizes would not react similarly as larval body length in 70% and 90% ethanol after 7 days of storage. Results from this study will be useful to further demonstrate the potential of cephalopharyngeal skeleton as alternative growth parameter to larval body length.

MATERIALS AND METHODS

This study was conducted in four rounds from 7 May 2018 to 19 May 2018 (study replicate 1), 16 May 2018 to 29 May 2018 (study replicate 2), 26 May 2018 to 2 June 2018 (study replicate 3) and 24 July 2018 to 5 August 2018 (study replicate 4). In each study replicate, the methodology is described below.

i) Baiting process

An approximately 400g raw fish - yellow stripe scads, *Selaroides leptolepis* Cuvier, 1833 and Indian Mackerel, *Rastrelliger kanagurta* (Cuvier, 1817), and 400g raw cow's liver were used as baits and placed inside a black plastic container. During each study replicate, a baited trap was left exposed on the ground for 8 hours during daytime from 0900 to 1700 hours in an open area adjacent to the Forensic Entomology Laboratory, Forensic Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Bangi (2.93ºN, 101.78ºE). In local warm natural surroundings, *C. megacephala* has been observed to be among the earliest to arrive to feed and oviposit on the decaying organic materials especially human corpses both indoors and outdoors [29, 33]. A single batch of calliphorid eggs presumably *C. megacephala* was carefully transferred into a plastic rearing container with 70 g fresh cow's liver on 2 cm sawdust layered at the bottom. The eggs were reared at ambient temperature and relative humidity overnight (Table 1).

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Table 1 Mean ± standard deviation (SD), minimum and maximum values of ambient temperatures and relative humidity during larval rearing from egg stage until third instar larval stage in each study replicate

Study replicate	Ambient temperature (ºC)			Ambient relative humidity (%)		
	Mean±SD	Min	Max	Mean±SD	Min	Max
1	29.0±2.3	19	34.5	87.5±7.1	69.2	96.4
2	28.7±2.3	21	34	86.6±7.2	70.3	94.7
3	26.2±4.5	15	29	79.2±5.2	60.3	87.2
4	28.5±3.4	20	37	79.5±8	70.5	87.3

ii) Sample preparation

At 0900 hours on the following day, first instar larvae that newly emerged from (i) were transferred evenly into three separate rearing containers. They were used to represent: i. the control (fresh larva), ii. treatment 1 (preserved in 70% ethanol for 7 days) and treatment 2 (preserved in 90% ethanol for 7 days). To ensure sample homogeneity, each rearing container consists of 20 first instar larvae (except study replicate 1, n = 30) and supplied with 70 g fresh cow's liver from the same animal source and reared in similar environments (Table 1). The remaining larvae that were not used for sampling were reared in a separate container until adult stage to facilitate species identification. After 48 hours, larvae from all rearing containers which were by then already in third instar (based on the number of slits on the posterior spiracles) were killed by immersion in hot water (80ºC) for 60 seconds [4, 6]. To ensure the accuracy of the temperature, Fluke 51 II handheld digital probe thermometer with type K thermocouple was immersed in 1.5L boiling water. Larvae from day 0 rearing container were subjected to measurement immediately as in (iii) whilst larvae from remaining two containers were transferred to universal glass vials containing 20 ml 70% ethanol and 20 ml 90% ethanol, respectively. All larvae in 70% and 90% ethanol were preserved at room temperature and relative humidity for 7 days (Table 2). The type of vials and amount of preservatives used in this study were decided based on forensic specimens received by the Forensic Entomology Laboratory, UKM.

Table 2 Mean ± standard deviation (SD), minimum and maximum values of ambient temperatures and relative humidity larval storage in 70% and 90% ethanol for 7 days

Study replicate	Ambient temperature (ºC)			Ambient relative humidity (%)		
	Mean±SD	Min	Max	Mean±SD	Min	Max
1	21.3±2.3	19.0	23.5	66.9±8.4	56.5	84.0
2	21.6±4.5	19.0	25.0	70.9±8.9	58.5	85.5
3	21.6±4.5	19.0	25.0	70.9±8.9	58.5	85.5
4	22.0±3.4	16.5	25.0	73.6±7.4	57.0	85.5

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iii) Data acquisition

After 7 days of storage, all larvae from 70% and 90% ethanol were withdrawn from respective vials and each larval body length were measured based on lateral body segments i.e. furthest part of the head and the last abdominal segment [9] by using Nikon SMZ745T stereomicroscope fitted with Toupcam 12-megapixel industrial digital camera. After measuring larval body length, cephalopharyngeal skeleton was removed from the larva and immersed in 10% KOH for 15 minutes. The internal tissues surrounding cephalopharyngeal skeleton were carefully removed in 10% KOH and subsequently transferred into 10% acetic acid for 10 minutes. Cephalopharyngeal skeleton were then soaked in 70% ethanol for 20 minutes before mounting onto a glass slide in lateral position using Berlese fluid and covered by a 6 mm round coverslip. The current clearing technique to mount only the cephalopharyngeal skeleton parts on glass slides was modified from [34, 35].

Measurements of cephalopharyngeal skeleton by using same stereomicroscopic technique as larval body length were conducted immediately after mounting process based on inter landmark distances between anterodorsal process (tip of dorsal bridge) to dorsal cornu (ADP-DC) [15, 22, 25] (Figure 1). Test-retest reliability assessment was conducted prior to the measurement using randomly selected samples (n=10) by two researchers, $r>0.95$ ($p<0.05$). The same two-dimensional images of cephalopharyngeal skeleton 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/). The accuracy of plots were assessed visually from the Procrustes superimposition and centroid sizes were obtained from the MorphoJ software [36] (download link: http://www.flywings.org.uk/morphoj_page.htm_).
Landmarks were selected based on two-dimensional geometrical shape of cephalopharyngeal skeleton, i.e.: 1) base of parastomal bar; 2) clipeal arc (anterodorsal process/tip of dorsal bridge); 3) dorsal cornu; 4) concavity of pharyngeal sclerite (tentorial phragma/medial incision); and 5) lower ventral cornu [24, 25].

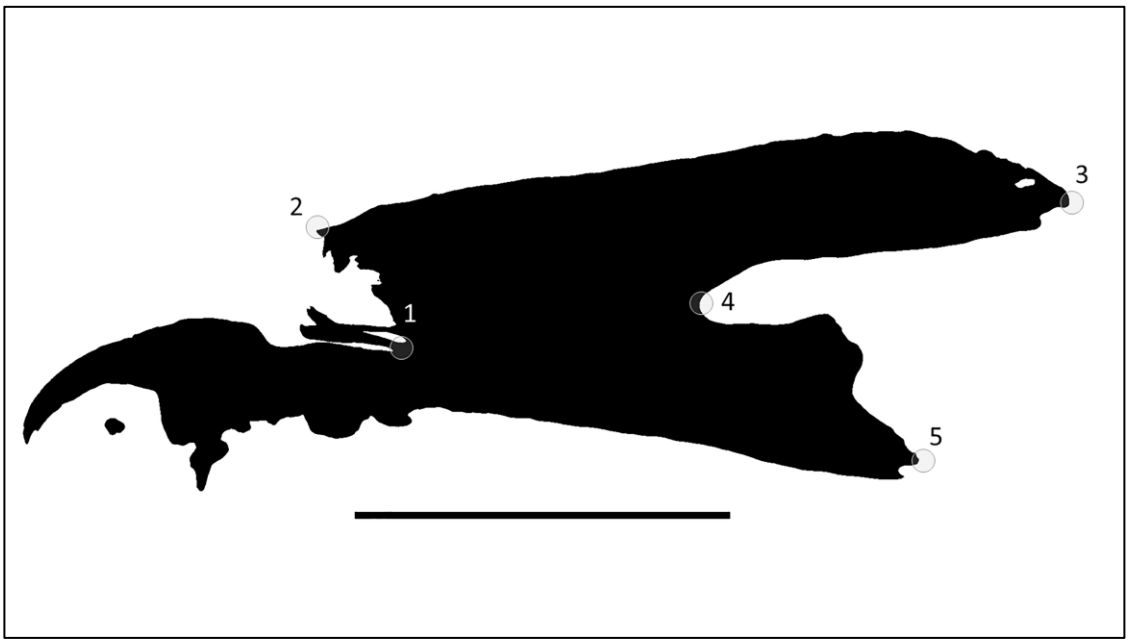

Figure 1 Morphometrical landmarks of C. *megacephala* cephalopharyngeal skeleton from third instar larva after 48 hours. Inter landmark distance of cephalopharyngeal skeleton was measured from landmark 2 (clipeal arc/anterodorsal process/dorsal bridge) to landmark 3 (dorsal cornu). The geometrical landmarks for centroid size calculation consist of 1) base of parastomal bar; 2) clipeal arc (anterodorsal process/tip of dorsal bridge); 3) dorsal cornu; 4) concavity of pharyngeal sclerite (tentorial phragma/medial incision); and 5) lower ventral cornu (Bar = 0.5 mm).

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iv) Statistical analysis

Descriptive and inferential statistics were conducted on the morphometric data of larval body length, cephalopharyngeal skeleton length and centroid size based on independent groups i.e., control, treatment 1 (70% ethanol) and treatment 2 (90% ethanol). To test the effect of preservatives on the larval body and cephalopharyngeal skeleton, we performed one-way ANOVA with Tukey HSD/Dunnett T3 post hoc test on the normally distributed samples and homogenous variance ($\alpha = 0.05$) in SPSS Ver.22 [37]. Statistical procedures were conducted separately considering different source of samples were used in each study replicate.

v) Species confirmation

Species identification was conducted based on larval body, cephalopharyngeal skeleton characteristics and remaining third instar larvae and adults reared from calliphorid eggs in (i). Species confirmation was based on taxonomic keys in Kurahashi et al. [38] and Greenberg and Kunich [1]. All specimens that were used as experimental species in this study were identified as *C. megacephala*.

RESULTS

Preliminary assessment on the data sets across four study replicates recorded normal distribution with homogeneity of variance in Levene's Test. One-way ANOVA for independent groups was employed for each independent study replicate.

Generally, preservation of larvae in 70% and 90% ethanol for 7 days caused significant changes on larval body length from its original size after hot water-killed, $F(2,87)=22.757$, $p<0.001$, $\eta^2 = 0.524$ (large effect size) (study replicate 1); $F(2,57)=15.725$, $p<0.001$, $\eta^2 = 0.595$ (large effect size) (study replicate 2); $F(2,57)=2.641$, $p<0.05$, $\eta^2 = 0.115$ (medium effect size) (study replicate 3); and $F(2,57)=18.852$, $p<0.001$, $\eta^2 = 0.428$ (large effect size) (study replicate 4) (Figure 2). Pairwise comparisons using Tukey HSD test ($\alpha=0.05$) showed larvae in 70% ethanol (15.131±0.950 mm) and 90% ethanol (15.711±0.724 mm) were significantly lower from its original size (16.591±0.844 mm) (study replicate 1). Similar results were obtained in study replicate 2 whereby larvae in 70% ethanol (14.090±0.945 mm) and 90% ethanol (13.826±0.944 mm) were significantly lower from its original size (15.477±0.977 mm). In study replicate 3 and 4, the differences were only significant between larvae in 90% ethanol and control group with size extended from 13.128±0.921 mm to 13.839±0.932 mm and shortened from 15.287±1.176 mm to 13.346±0.702 mm, respectively.

For cephalopharyngeal skeleton size, represented by ADP-DC inter landmark distances, only the first and second study replicates showed significant differences among those preserved in 70% and 90% ethanol, $F(2,87) = 16.095$, $p<0.001$, $\eta^2 = 0.369$ (large effect size) (study replicate 1); $F(2,57) = 8.655$, $p<0.05$, $\eta^2 = 0.303$ (large effect size) (Figure 3). Follow up analysis using Tukey HSD test ($\alpha=0.05$) displayed cephalopharyngeal skeleton in 70% ethanol (1.133±0.048 mm) and 90% ethanol (1.103±0.041 mm) were longer than its original condition (1.071±0.037 mm) in study replicate 1. However, in study replicate 2, cephalopharyngeal skeleton in 70% ethanol had significantly increased length (1.111±0.050 mm) than the control group (1.047±0.043 mm). In study replicate 3 and 4, cephalopharyngeal skeleton sizes were not significantly affected by the preservatives, $F(2,57) = 0.812$, $p=0.449$, $\eta^2 = 0.031$ (small effect size) (study replicate 3); $F(2,57) = 1.703$, $p=0.191$, $\eta^2 = 0.057$ (small effect size).

Cephalopharyngeal skeleton was also compared by its centroid size (Figure 4). From the results, only the first and second study replicate detected significant differences among groups; $F(2,87) = 4.269$, $p<0.05$, $\eta^2 = 0.097$ (small effect size) (study replicate 1); and $F(2,57) = 7.428$, $p<0.01$, $\eta^2 = 0.260$ (large effect size). In the first study replicate, significant differences were detected between those in 70% ethanol (1.631±0.057 mm) and 90% ethanol (1.671±0.062 mm) using Dunnett T3 test ($\alpha=0.05$) but not with the control group (1.654±0.037 mm). In the second study replicate, however, only cephalopharyngeal skeleton in 70% ethanol (1.559±0.030 mm) had significantly lower centroid size than control group (1.609±0.041 mm). In study replicate 3 and 4, cephalopharyngeal skeleton centroid sizes were not significantly affected by the preservatives, $F(2,57) = 2.096$, $p=0.132$, $\eta^2 = 0.071$ (small effect size) (study replicate 3); $F(2,57) = 0.601$, $p=0.552$, $\eta^2 = 0.021$ (small effect size).

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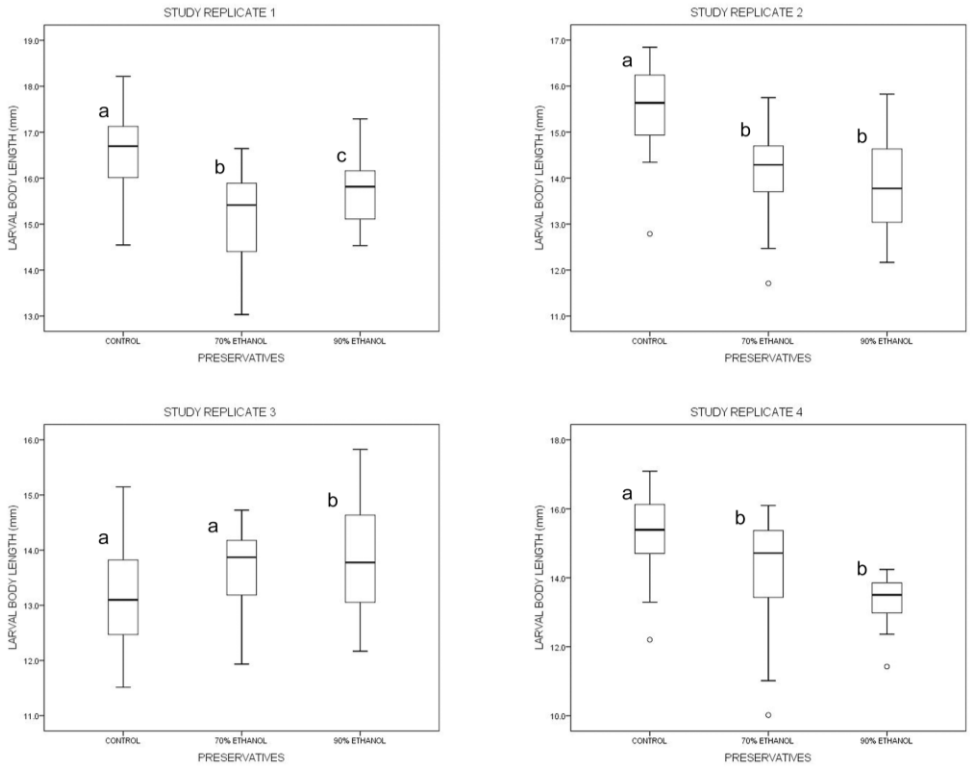

Figure 2 Box-plot comparing larval body length of of C. *megacephala* measured from the furthest part of the head to the last abdominal segment in day 0 (control), and in 70% and 90% ethanol after 7 days of storage. Significant differences between groups ($p<0.05$) are indicated by different letters

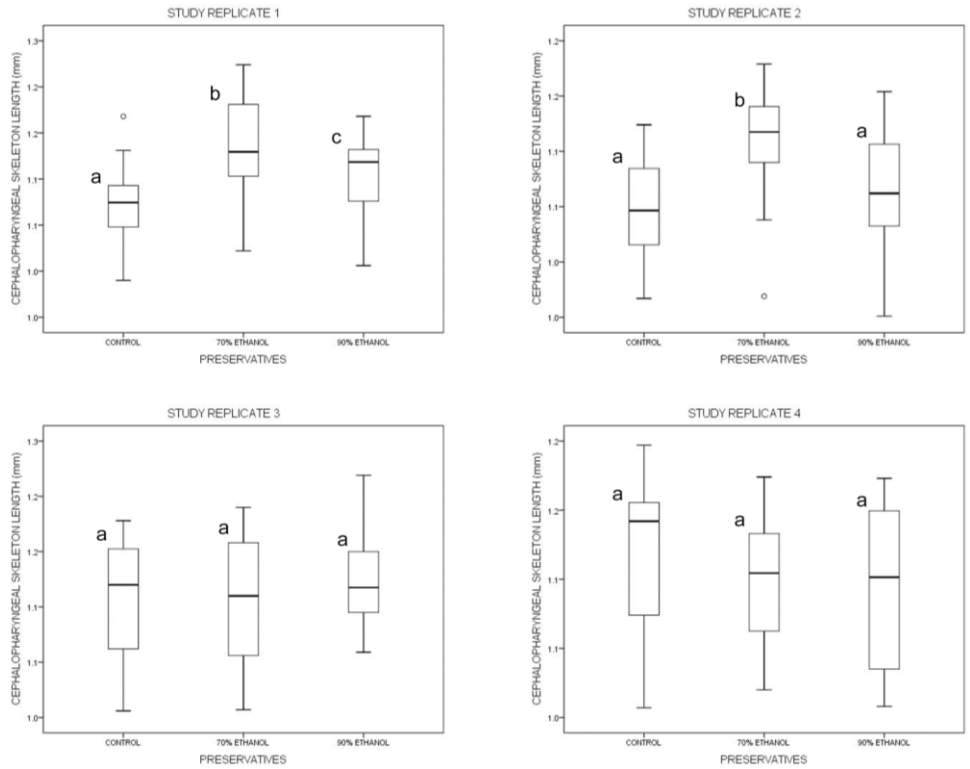

Figure 3 Box-plot comparing cephalopharyngeal skeleton length of *C. megacephala* measured from anterodorsal process to dorsal cornu in day 0 (control), and in 70% and 90% ethanol after 7 days of storage. Significant differences between groups ($p<0.05$) are indicated by different letters.

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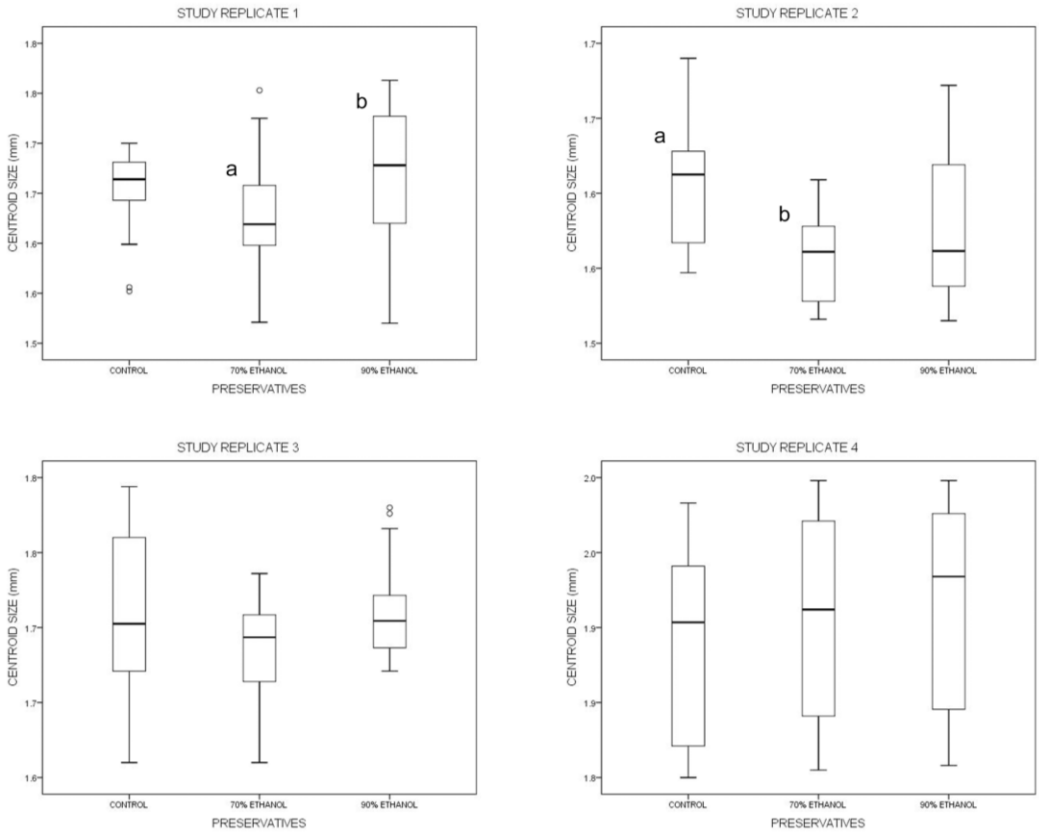

Figure 4 Box-plot comparing cephalopharyngeal skeleton centroid size of *C. megacephala* in day 0 (control), and in 70% and 90% ethanol after 7 days of storage. Significant differences between groups (p<0.05) are indicated by different letters

DISCUSSION

Results from current study indicate that preservatives affected cephalopharyngeal skeleton of *C. megacephala* lesser than its body. After 7 days, cephalopharyngeal skeletons were significantly expanded in the first (70% and 90% ethanol) and second study replicate (70% ethanol) in contrast to the third and fourth and study replicate. It was possible that measurement technique could lead to inconsistencies of the results as cephalopharyngeal skeleton is a three-dimensional shape, but its length was configured based on two-dimensional image. In this case, planarity could be the main issue that affected inter-landmark distances measurement as landmark position could be influenced by structure depth and image quality. The effect was minimized by using images taken from similar plane at fixed focal length and lighting [24] and to avoid cephalopharyngeal skeleton from moving after fixation, it was slightly pressed to the depth closest to the glass slide to maintain its lateral position.

Current study also introduced the use of centroid size to measure cephalopharyngeal skeleton dimensions, based on the configurations of five landmarks instead of using a one-dimensional measurement in an inter-landmark distance. It could improve reliability of measurements by providing the square root of the summed squared distances between landmarks and their centroid and can detect changes in any directions by representing the overall estimation of size [39]. However, there were slight inconsistencies of results in cephalopharyngeal skeleton size based on inter landmark distances and the centroid size. For instance, pairwise comparisons in study replicate 1 detected significant difference of cephalopharyngeal skeletons length between those preserved in 70% and 90% ethanol with the control group. When centroid size measurement was employed, pairwise comparisons showed no significant different between samples preserved in 70% and 90% ethanol with the control subjects. In the second study replicate, changes were detected between larvae in 70% with control, equivalent to those results from using cephalopharyngeal skeleton inter landmark distances. The measurement and analysis were repeated, and we discovered the significant differences between groups were maintained

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but in terms of its reliability and validity as growth parameter to estimate mPMI, we suggest to conduct further investigation on the variations between using inter landmark distances and centroid size, before standardizing the geometrical landmarks to obtain centroid size for better estimation of cephalopharyngeal skeleton size [24, 25, 39].

Regarding larval body sizes, all study replicates showed they were significantly affected by preservation in 70% and 90% ethanol stored for a duration of 7 days. These changes were based on the comparison of mean larval body length measured immediately after hot water killed for 60 seconds, with those from same third instar cohort preserved for 7 days in 70% and 90% ethanol after hot water killed. The effect of shrinkage of larvae when preserved in 70% to 90% ethanol could be attributed to the killing method by immersion in hot water as it could remove waxy substances on the larval surface [9] and subsequently increased its permeability [6]. Previous study by Tantawi and Greenberg [5] showed that killing the larva with hot water extended larval body size to its maximum length, but current study indicated the size was not retained after preservation in 70 and 90% ethanol.

From the current study, irregularities were also detected in study replicate 3 and 4 whereby the effects of preservatives only happened when the larvae preserved in 90% ethanol. The fluctuating effect of preservatives on body length was also observed in *C. augur* preserved in 80% ethanol for 10 days which it elongated during the first to feeding third instar but shrunk in the post-feeding third instar larvae [9]. Meanwhile, Adham et al. [40] discovered that *C. marginalis* (Wiedemann, 1830) larval body length significantly increased when killed with hot water and preserved in 70%. Interestingly, the size remained constant for the duration of 15 days. As observed by Tantawi and Greenberg [5], preservatives also reacted differently on larvae from different age groups, especially during the third instar. Another point to consider is that the effects of preservatives on larvae might be a gradual process. The reaction to preservatives could be significant in the early period of preservation but the size could be slowly increased or decreased depending on the species [41], larval instars or storage temperatures [7]. In the current study, since *C. megacephala* larvae used in each study replicate derived from different maternal source, it was possible that there were slight age differences due to the influence of variations during rearing such as temperatures and relative humidity. Further study should be conducted to improve current understanding on the effect of preservative concentrations on larvae by using experimental subjects from different larval age, instars, and the transitions between larval instars.

At present, only a limited number of studies have been conducted to investigate the utilization of cephalopharyngeal skeleton in mPMI estimation apart from its main purpose as taxonomic characters for species identification. Previously, a study on *H. violacea* cephalopharyngeal skeleton, represented by the inter landmark distances of ADP-DC was not affected when stored in 70% ethanol for 14 days. Cephalopharyngeal skeleton size was also unaltered when killed with hot water, compared to those directly preserved in 70% ethanol [42]. Furthermore, the cephalopharyngeal skeleton could be a valid and reliable source of reference to estimate larval age because of its allometric growth to the body and the morphometric landmarks could be well established in oppose to larval body [15, 16, 24]. For this reason, cephalopharyngeal skeleton could be considered as an alternative or a complementary parameter to larval body length to aid measurement for mPMI estimation.

CONCLUSION

Current study showed result inconsistencies between test repetitions, but this was possibly due to variations of sample backgrounds and measuring techniques employed in this study. Only study replicate 3 and 4 proved cephalopharyngeal skeleton unaffected by both 70% and 90% ethanol whilst as hypothesized, larval body length was substantially changed by ethanol concentrations. The utilization of dipterous larval cephalopharyngeal skeleton as alternative growth parameter to larval body length for mPMI estimation is still subjected to different experimental settings on various forensic species. Nevertheless, in view of the positive allometry of cephalopharyngeal skeleton and larval body size, combined with its ability to retain its size in different preservative concentrations, cephalopharyngeal skeleton could be considered as a potential larval growth indicator when estimating mPMI.

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Conflict of Interest

Authors declare none.

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Authors' Contribution

Raja M. Zuha conceptualized the research design. Sharifah Shakilah Abdullah and Raja M. Zuha conducted the research but final draft of manuscript was prepared by Raja M. Zuha. Sharifah has already withdrawn from this project by the time this manuscript sent for publication.

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