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Effects of preservatives and killing methods on morphological features of a forensic fly, *Chrysomya megacephala* (Fabricius, 1794) larva

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Abstract. Preservation of larvae retrieved from cadavers is important in ensuring the quality and integrity of entomological specimens used for the estimation of post-mortem interval (PMI). The process of killing and preserving larvae could distort the larvae leading to inaccurate estimation of PMI. In this study, the effects of killing *Chrysomya megacephala* larvae with hot water at different temperatures and subsequent maintenance in various preservatives were determined. Larvae not killed by hot water but preserved directly were used as control. The types of preservative used were 10% formalin, 70% ethanol and Kahle's solution. The morphological features examined were length, turgidity, curvature and coloration of larvae. Larvae killed in 80ºC hot water have shorter mean length (12.47 ± 2.86mm) compared to those in 60ºC hot water (12.95 ± 2.69mm). Increasing the duration of preservation in all types of preservative caused elongations of larvae treated or untreated with hot water. There were no significant changes in larval turgidity preserved in Kahle's solution compared to other two preservatives and were unaffected by the duration of storage. Larvae preserved in Kahle's solution experienced the least changes in coloration and shape compared to other preserved larvae in 70% ethanol or 10% formalin. Larvae directly immersed alive in 70% ethanol experienced the most changes in curvature, coloration and turgidity. This study suggested that killing larvae with hot water at 80ºC and preservation in Kahle's solution is the optimum method resulting in least changes in morphological features of *Ch. megacephala* larvae.

INTRODUCTION

The determination of maggot length provides invaluable data on time of death or post-mortem interval (PMI) (Gennard, 2007). Determination of PMI is based on identification of maggot species together with estimation of oldest age of larvae found on a corpse (Anderson *et al*., 2000; Grassberger & Reiter, 2002). Therefore, it is important to maintain integrity of maggot specimens collected from a crime scene to be transported to the laboratory for analysis.

The most crucial step of maintaining the integrity of maggot specimens is the killing and preserving stage where these specimens are mostly handled during crime scene investigation (Catts, 1990; Tantawi & Greenberg, 1993; Byrd, 2001). There are many aspects which include the host factors, the types of preservative used, method larvae are killed, the temperature and duration of immersion if larvae are in hot water, duration of larvae stored in preservative before analysis, the species of flies, and even the age of the larvae were known to inflict inaccuracy in determining post-mortem interval (Tantawi & Greenberg, 1993; Adams & Hall, 2003; Day & Wallman, 2008; Midgley & Villet, 2009; Villet *et al*., 2010).

In Malaysia, larvae collected from cadavers during crime investigation were mostly preserved in 70% ethanol (Kavitha *et al.*, 2012; Lee *et al.*, 2004) and Kahle's solution (Kumara *et al.*, 2009) before analysis. The European Association for Forensic Entomology reviewed scientific publications and documented guidelines and standard procedures related to best practice in collection and preservation of entomological samples at crime scene (Amendt *et al.*, 2007). The guidelines stated that 'kill the remaining specimens with very hot (>80ºC), but not boiling water; immersion for 30s is ideal to achieve best preservation' (Adams & Hall, 2003). Killing larvae with hot water is generally recommended as previous studies showed that larvae if directly preserved into preservative experienced various degree of shrinkage.

Tantawi & Greenberg (1993) reported that maggot samples of *Protophormia terraenova* (Robineau-Desvoidy, 1830) and *Calliphora vicina* (Robineau-Desvoidy, 1830) experienced shrinkage when directly immersed into 15 types of killing and preservative solution, while larvae killed in boiling water did not experience any shrinkage. Similar findings were reported by Adams & Hall (2003) whereby they showed that larvae shrunk if introduced alive into preservatives.

However, the shrinkage of larvae varied with species and dependent on the types of preservatives used (Byrd, 2001). *Calliphora vicina* experienced greater shrinkage compared to *P. terraenovae* when placed alive into preservative (Tantawi & Greenberg, 1993), while *Calliphora vomitora* (Linnaeus, 1758) shrunk more in 10% formalin than in 80% ethanol (Adams & Hall, 2003). However, Day & Wallman (2008) found that *Calliphora augur* (Fabricius, 1775) and *Lucilia cuprina* (Wiedemann, 1830) did not shrink when preserved directly in both Kahle's solution and 10% formalin. They concluded that different species reacted differently to preservatives and might be species specific.

These findings prompted us to study the effects of preservatives and method of killing on *Ch. megacephala* larva which is the commonest and predominant fly colonising human cadavers in in Malaysia (Lee *et al.*, 1984; Omar *et al.*, 1994; Lee *et al.*, 2004).

MATERIALS AND METHODS

Study species

Adult flies of *Ch. megacephala* were collected from Gombak (3.215'N, 101.7'E) using fly trap baited with beef and identified using taxonomic keys provided in Kurahashi *et al.* (1997). Collected flies were transferred into fly cages in laboratory at 26 ± 2ºC and 85 ± 5% relative humidity. Adults were reared until post-feeding third instar larvae and divided into three treatment groups; untreated with hot water by immersing larvae alive in preservatives, treated with 60ºC or 80ºC hot water. Larvae not killed by hot water but preserved directly were served as control. Each treatment group consisted of 30 larvae was further subdivided into 3 subgroups of 10 blowfly larvae each and treated with 10% formalin (Sigma, USA), 70% ethanol (Dulab, UK), and Kahle's solution (Sigma, USA). The blowflies from the same generation (F1) were used until four cycles (F4) in order to obtain four replicates of post-feeding third instar larvae.

Measurement of larval length

The larva lengths of all treatments groups were measured after storage at 1, 3, 7, 10 and 15 days in the preservatives. The measurements of length were obtained using a binocular microscope (Leica Microsystems, USA) fitted with an eye-piece micrometer, at 10x magnification. To avoid bias, single blind was performed when labelling the larvae so that the larval length measurement for all four replicates was unknown by the observer (single blind observation).

Measurement of discoloration, curvature and turgidity

A scoring system was used to determine the subjective determination of discoloration, curvature and turgidity. Scoring for discoloration ranged from 0 to 12 depending on number of larval segments involved in changes from original colour (creamy) into

orange, brown or black, while score for curvature ranged from 0 to 12 depending on numbers of bent larval segments. Scoring for discoloration and curvatures followed study of Adams & Hall (2003) with modification only for scoring of turgidity. The turgidity of larva was measured by touching it with scalpel. The score ranged from 1 to 4; instead of 1 to 5 proposed by Adams & Hall (2003). Score 1 indicated that the body of larvae became brittle when touched with scalpel and giving an empty appearance, score 2 indicated that the body of larvae shape followed the scapel shape, score 3 indicated that the body of larvae shape followed the scalpel shape and changed back to original shape of larvae, while score 4 indicated that the body of larvae did not bounce back after touching and became stiff. Half point (0.5) will be given if the particular larval segment showed intermediate level of discoloration, curvature and turgidity.

Statistical analysis

ANOVA with post hoc test was carried out to determine the effect of all treatment on larval length, while Kruskal-Wallis test (non-parametric test) was used to analyse median score of turgidity, discoloration and curvatures of larvae. The statistical test was performed by using IBM SPPS Statistics version 19.0 software (IBM Corporation, U.S.). The level of significant was at $\alpha < 0.05$.

RESULTS

Killing larvae by immersing directly into preservatives or killing with hot water at 60°C or 80°C all significantly affected mean larval length (d.f. = 2, F= 60.18, $\alpha < 0.0001$). There was significant reduction of mean larval length for larvae placed alive (11.22 ± 2.89mm) in preservatives, compared with larvae killed with hot water (12.73 ± 2.81mm). In addition, larval mean length of larvae killed in 60°C (12.95 ± 2.69mm) was slightly increased compared with larvae killed in 80°C hot water (12.47 ± 2.86mm) ($\alpha < 0.05$).

The types of preservatives significantly affected the mean larval length. The mean larva length of larvae preserved in 10% formalin (12.49 ± 2.90mm) was significantly different (d.f. = 2, F = 4.31, $\alpha < 0.05$) compared with larvae preserved in either 70% ethanol or Kahle's solution. Larvae preserved in Kahle's solution and 70% ethanol elicited slight reduction in larva length but were not statistically significant (12.04 ± 2.81mm vs. 12.12 ± 2.99 mm, respectively) ($\alpha > 0.05$).

The impact of methods used to kill larvae contributed more changes in mean larval length than types of preservatives used. By placing live larvae directly into preservatives, a decrease of 1.73 ± 0.13mm and 1.25 ± 0.45mm in length was observed compared with larvae killed in 60°C and 80°C hot water, respectively, while preserving larvae in 10% formalin increased the length by only 0.45 ± 0.09mm and 0.37 ± 0.10mm, compared with larvae preserved in Kahle's solution and 70% ethanol, respectively.

Storage duration significantly affected mean larval length regardless of types of preservative or killing methods. Analysis indicated that time factor alone significantly affected larval mean length, (d.f = 4, F= 2.97, $\alpha < 0.05$) with no interaction of mean effects between types of preservatives or killing methods with the durations used to store the larvae (d.f. = 16, F= 0.04, $\alpha > 0.05$). All treatment groups showed similar pattern whereby mean length increased with prolonged storage duration (Table 1).

Both types of preservatives and killing method significantly affected the larval coloration ($\alpha < 0.0001$), curvature ($\alpha < 0.001$) and turgidity ($\alpha < 0.0001$) of the maggot, while storage duration significantly affected coloration ($\alpha < 0.0001$) and turgidity ($\alpha < 0.01$), but not the curvature ($\alpha > 0.05$).

All treatment groups showed median score with minimum of 2 to 4 larval segments curvature and their turgidity with score of 3 except for larvae directly placed into 70% ethanol with scored from 2 to 1 after 7 days of storage (Table 2).

Table 1. Mean larval length (mm ± S.D.) of *Ch. megacephala* larvae treated and untreated with hot water for 15 days of storage

Preservative	Killing method	Duration of larvae in preservative (day)/larval length (mm)				
		Day 1	Day 3	Day 7	Day 10	Day 15
10% Formalin	Alive	11.50 ± 2.78	11.82 ± 2.99	11.96 ± 3.03	12.11 ± 3.01	12.43 ± 3.25
	60°C hot water	12.59 ± 2.65	12.66 ± 2.55	12.73 ± 2.56	12.79 ± 2.57	12.94 ± 2.68
	80°C hot water	12.50 ± 3.05	12.50 ± 3.21	12.69 ± 3.00	12.91 ± 3.11	13.15 ± 3.01
Kahle's solution	Alive	10.37 ± 2.46	10.45 ± 2.53	10.56 ± 2.61	10.73 ± 2.52	11.07 ± 2.65
	60°C hot water	12.85 ± 2.59	12.91 ± 2.62	12.93 ± 2.73	13.20 ± 2.58	13.46 ± 2.71
	80°C hot water	12.18 ± 2.79	12.27 ± 2.59	12.27 ± 2.63	12.55 ± 2.69	12.80 ± 2.80
70% ethanol	Alive	10.77 ± 2.90	10.80 ± 2.95	10.97 ± 3.01	11.18 ± 3.00	11.57 ± 2.97
	60°C hot water	12.87 ± 2.97	12.90 ± 2.96	13.18 ± 2.96	12.96 ± 2.68	13.24 ± 2.89
	80°C hot water	11.98 ± 2.82	12.00 ± 2.86	12.17 ± 2.86	12.40 ± 2.88	12.75 ± 2.81

Table 2. Median score for discoloration (D), curvature (C) and turgidity (T) for all treatment groups

Preservative	Killing method	Duration of larvae in preservatives (days)														
		Day 1			Day 3			Day 7			Day 10			Day 15		
		D	C	T	D	C	T	D	C	T	D	C	T	D	C	T
10% Formalin	Alive	6	3	3	12	3	3	12	3	3	12	3	3	12	3	3
	60°C hot water	4	4	3	5	4	3	5	4	3	5	4	3	5	4	3
	80°C hot water	8	4	3	12	4	3	12	4.5	3	12	4.5	3	12	4.5	3
Kahle's solution	Alive	2	3	3	2.5	3	3	4	3	3	4	3	3	4	3	3
	60°C hot water	3	3	3	3	3	4	3	3	3	3	3	3	4	3	3
	80°C hot water	4	3	3	4	3	3	5	3	3	5	3	3	5	3	3
70% ethanol	Alive	8	2.5	3	12	3	2	12	3	2	12	3	1	12	3	1
	60°C hot water	4	4	3	4.5	4	3	5	4	3	5	4	3	5	4	3
	80°C hot water	6	3	3	7.5	4	2.5	8	4	2	10	4	2	10	4	2

DISCUSSION

The purpose of preserving maggots is to arrest the growth so that they can be identified and the body length measured to estimate the PMI. Maggot body length provides the best resolution in determining PMI rather than body weight and width (Midgley & Villet, 2009; Richard *et al.*, 2013). This study focused on the effects of killing *Ch. megacephala* post-feeding third instar larvae with hot water or direct introduction of larvae into preservatives. In addition, morphological changes of *Ch. megacephala* when preserved in three types of commonly used preservatives were also studied.

Our result showed maggots shrunk when they were directly preserved in all preservatives tested compared to those killed with hot water. This finding was similar to studies conducted by Tantawi & Greenberg (1993) and Adams & Hall (2003). They reported their tested larvae when immersed in preservatives experienced a degree of shrinkage due to dehydration. The internal fluids were forced to leak out and move into higher solute concentration, causing them to shrink. Killing maggot in 80°C hot water caused the least changes in larval length, turgidity, colour and curvature. In fact, if they were then immersed in Kahle's solution, their morphological features were better preserved than larvae killed with 60°C hot water.

Each preservative has its limits and benefits. Normally, 70% ethanol is the

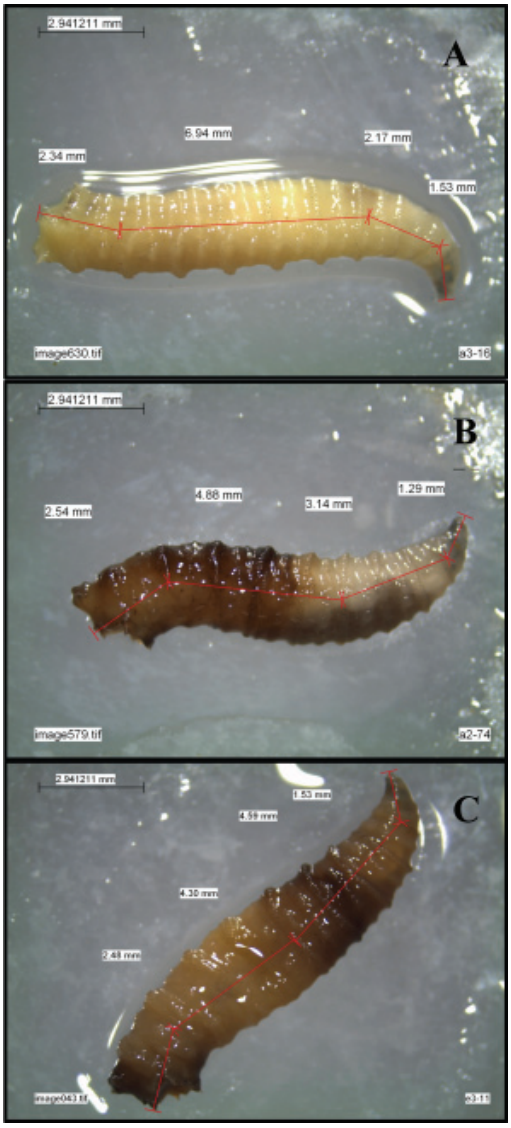

Figure 1. Preserved *Ch. megacephala* larva in various types of preservative. Larva preserved in Kahle's solution **(A)**, 10% formalin (**B**), and 70% ethanol (**C**). Scale bar = 2.94 mm

favourable choice to preserve maggots collected from a corpse (Amendt *et al.*, 2007) as it is easily available and safe to use. It is widely used to preserve soft body insects such as mite, moth, fleas and louse (Schauff, 2004). Kahle's solution has also been used. Gennard (2007) preferred to preserved maggot in Kahle's solution, because this solution was able to maintain the original flexibility. In addition, preserved samples in Kahle's solution were less curated than those in 70% ethanol.

Kahle's solution consists of 4 parts of glacial acetic acid; 12 parts formaldehyde, 30 parts of 95% ethanol and 60 parts of water (Byrd, 2001). Based on our findings, Kahle's solution is able to preserve maggots better than 70% ethanol and 10% formalin by maintaining the maggot's rigidity and prevented it from discoloration. The proportion of acetic acid in Kahle's solution governed the penetration of 70% ethanol into maggot. However, the composition of formaldehyde in Kahle's solution was able to cross-link proteins in maggot, making it unusable for DNA extraction (Krogmann & Holstein, 2010).

Therefore, it is recommended that 70% ethanol should be used for preservation if maggot sample is later subjected to DNA analysis. However, care needs to be taken if 70% ethanol is used as our finding showed that larvae preserved in 70% ethanol become brittle after one week of storage. In addition, it is easy to evaporate (Gennard, 2007; Notton, 2010). Notton (2010) suggested routine topping up 70% ethanol according to the needs of different collections to maintain the concentration.

Another reason accounting for larvae preserved in 70% ethanol becoming brittle may be due to the size of the container used in our study. The bijou bottle used is able to hold 3ml of 70% ethanol. In the course of the study, the daily opening and closing the bottle would have caused changes in the ethanol concentration due to evaporation, which may affect the preservation. The effects of container size and on preservation should be evaluated.

10% formalin is widely used to preserve large tissue specimen for microscopic observation in histopathology (Melissa *et al.*, 2006), amphibian larvae, invertebrates (John, 1999), and vertebrates such as fish (Patrick & Stan, 1996). Although this solution excellently preserved the maggot up to six months (Linville *et al.*, 2004), it is not advisable to use this solution to preserve

maggot, as formaldehyde is known to cause DNA degradation (Nakamura *et al*., 1990). In addition, 10% formalin can harden preserving specimen, by penetrating tissue and reaching the inner layer of the cells and cross-link the proteins, thus increasing the strength and rigidity of preserved sample (Thavarajah *et al*., 2012). Owing to this, in forensic entomology, a hardened maggot with disruption at molecular level will affect analysis in species identification, mitochondrial DNA and short tandem repeat loci of maggot crop contents (Linville *et al*., 2004).

In conclusion, professionals involved in forensic investigation such as police officers, entomologists and forensic pathologists must be aware of the impact of preserving maggots in different types of preservative, as preservatives are known to affect the maggot morphological features. Based on our study, it is advisable that personnel involved in collecting larvae specimens in a crime scene to bring hot water in a thermos flask and thermometer to ensure killing the larvae at 80°C before placing them into a desired preservative. The choice of preservative is dependent on types of test to be carried out subsequently.

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