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Influence of citronella and chlorpyrifos on *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) infesting rabbit carcasses

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Abstract. Influence of citronella and chlorpyrifos on oviposition and duration for completing life cycles for *Chrysomya megacephala* and *Chrysomya rufifacies* infesting decomposing rabbit carcasses was studied. Male rabbit carcasses (n = 12) were equally divided into control, citronella- and chlorpyrifos–treated groups, and left to decompose for 14 consecutive days. *C. megacephala* was the first necrophagous fly oviposited in all control and citronella-treated carcasses followed by *C. rufifacies*. Although initial oviposition of *C. megacephala* was delayed (4-6 hours) in citronella-treated carcasses ($P < 0.05$), prolongation in completing its life cycle was not observed. Neither delayed initial oviposition nor prolonged life cycle for C. *rufifacies* in citronella-treated carcasses was observed. Oviposition was delayed for chlorpyrifos-treated carcasses (0.42 g/L), and eclosion of eggs remained unsuccessful. The findings deserve consideration because these chemicals are easily accessible and can be used by cunning criminals to confuse forensic entomologists while estimating minimum post-mortem interval.

INTRODUCTION

Forensic entomology remains as one of the most useful tools for estimating minimum post mortem interval (mPMI), especially when dealing with highly decomposed bodies (Anderson, 2010). In general, *Chrysomya megacephala* (Fabricius) is the dominant and first necrophagous species to infest decomposing human corpses (Lee *et al.*, 2004) as well as animal models (Mahat & Jayaprakash, 2013; Mahat *et al.*, 2016) in Peninsular Malaysia followed by *Chrysomya rufifacies* (Macquart). Moreover, these two forensically important necrophagous species are also ubiquitous in many Asian countries (Kurahashi *et al.*, 1997), Hawaii (Goff, 2000) and several regions in Australia (Harvey *et al.*, 2003). Since variations in species compositions and durations for completing life cycles for necrophagous insects attributable to differences in biogeoclimatic regions have been indicated, empirical baseline data established for one biogeoclimatic region may not be applicable for estimating PMI in other regions (Wells & Stevens, 2008). Such aspect accentuates the importance for replicating forensic entomological studies across various biogeoclimatic regions.

Importantly, many commercial formulations, especially those naturally originated from plants (e.g. citronella) have been reported to deter colonisation of insects such

as mosquitoes (Debbown *et al.*, 2007) and ants (Soria *et al.*, 2012). Citronella is the common name for organic preparation of *Citronella nardus* that is commonly used for topical application for preventing mosquito bites (Debbown *et al.*, 2007). Besides, aqueous citronella has been commonly used for general applications on surfaces (Nakahara *et al.*, 2003). It has been reported that citronellal, citronellol and geraniol are the three major chemical compounds in the citronella aqueous preparation that are responsible for its repellent effect towards insects (Debbown *et al.*, 2007; Koba *et al.*, 2009; Solomon *et al.*, 2012). In addition, the repellent effect has been advocated to be concentration-dependent (Solomon *et al.*, 2012). Although toxicity of citronella remains low in mammals, notable toxicities have been indicated to occur in insects (Debbown *et al.*, 2007). Pertinently, review of the literature reveals limited number of specific studies focusing on the repellent effect of citronella preparation towards different species of Diptera. While a study (Charabidze *et al.*, 2009) utilised recently thawed female rat carcasses (which may be too small for sustaining the growth of different necrophagous species), indications on seasonal variations and rainfall during the experiment, as well as the purity of citronella product used were not provided. Moreover, the fact that *C. megacephala* and *C. rufifacies* were not included in their observations (Charabidze *et al.*, 2009), generalisation of the influence of citronella towards these two prevailing necrophagous flies in Malaysia, without suitable empirical data, can be misleading.

It has been indicated that malathion (an organophosphorus insecticide) delays the initial oviposition and prolongs the durations for completing the life cycles of *C. megacephala* and *C. rufifacies* (Mahat *et al.*, 2009). Studies have also revealed that chlorpyrifos (another organophosphorus insecticide) can potentially influence the fecundity, reproductive performance and development of *Musca domestica* (Dad *et al.*, 2011) and *Drosophila melanogaster* (Gupta *et al.*, 2007), leading to delayed oviposition and reduced number of eggs laid as well as failure for the eggs to hatch (Dad *et al.*, 2011). However, specific studies focusing on the influence of chlorpyrifos on *C. megacephala* and *C. rufifacies* have not been reported, so far. Although fatalities due to chlorpyrifos toxicities have not been indicated in the literature, the fact that chlorpyrifos is easily accessible in Malaysia, its possibility to be cunningly used for delaying oviposition to create unlawful and dubious alibi may cause significant confusion in forensic investigation. In this context, it is pertinent to indicate here that any factors that may interrupt insect colonisation as well as developmental patterns may lead to erroneous estimation of mPMI (Sharanowski *et al.*, 2008), resulting from inaccurate interpretation of forensic entomological empirical baseline data.

Because (a) crimes committed by educated and cunning criminals are gaining notoriety, (b) easy accessibility to commercial insect repellents (e.g. citronella) and insecticides (e.g. chlorpyrifos), as well as (c) vast availability of scientific information, the possibility that citronella and chlorpyrifos can be used to confuse forensic entomologists in estimating mPMI could not be ruled out. Furthermore, deaths of persons whom used citronella to prevent insect bites are also likely. Therefore, this present research that was specifically designed to investigate the influence of citronella and chlorpyrifos on the oviposition and developmental patterns of *C. megacephala* and *C. rufifacies* using rabbit carcasses as the animal merits forensic consideration.

MATERIALS AND METHODS

Experimental design

Twelve male rabbit (*Oryctolagous cuniculus*) carcasses were used in this present research. The freshly slaughtered (with the front part of the neck severed partially) rabbit carcasses (about 2 kg) were purchased from a rabbit meat seller in Johor Bahru, and details of the rabbit carcasses used in one replicate experiment are provided in Table 1. All rabbit carcasses were transported in separate sealed plastic bags

Table 1. Details of the rabbit carcasses used in each replicate experiment

No.	Experimental use	Treatment	Labelling
1	Control	Tap water	C
2	Treated 1	Citronella[a]	T1
3	Treated 2	Chlorpyrifos[b]	T2

[a]One volume of the commercially available aqueous citronella preparation was mixed thoroughly with two volumes of tap water. [b]An amount (2 grams) of chlorpyrifos emulsified concentrate (21.2% w/w) was diluted with tap water using a calibrated 1000mL volumetric flask.

and placed at the decomposition site by about 8 a.m. on the first day of decomposition. Among the three rabbit carcasses used in each replicate, one was used as the negative control and the remaining two designated as T1 and T2 were used for administering citronella and chlorpyrifos, respectively. The commercially available ~5% v/v aqueous citronella (AFY haniff Bena & Bekal) preparation and chlorpyrifos emulsified concentrate (Plasbon 250: 21.2%, w/w, Choon Huat Sdn. Bhd.) used in this present research were purchased from a local agricultural supplier and diluted following the suggestion made by manufacturers as detailed below.

For preparing the citronella solution, one volume of the commercially available ~5% v/v aqueous citronella preparation was mixed thoroughly with two volumes of tap water. For preparing the chlorpyrifos treatment (0.42 g/L), an amount (2 grams) of chlorpyrifos emulsified concentrate (21.2% w/w) was diluted in 1000 mL of tap water. As much as possible, all the freshly prepared aqueous citronella and chlorpyrifos preparations (100 mL) were evenly and continuously sprayed throughout the respective carcasses for approximately 50 seconds using separate identical mist sprayers. As for the control carcasses, tap water (100mL) alone was used. The carcasses were sprayed on both sides of the torso, back, head (nose, mouth, slaughtered site), as well as the anus region and were left to decompose for 14 consecutive days. Replicating the same procedure, four replicates (a total of 12 rabbit carcasses) were included in this present research. The decompositions for all of the four replicate experiments were conducted from the 1st – 13th September 2015, 13th – 26th September 2015, 27th September –10th October 2015 and 11th – 24th October 2015, respectively. The citronella and chlorpyrifos preparations were also submitted to the University-Industrial Research Laboratory of Universiti Teknologi Malaysia (UTM) to determine their chemical compositions using Gas Chromatography-Mass Selective Detector (Agilent-HP7820A coupled with Agilent-5977E MSD).

Decomposition site and entomological observation

The decomposition site was within the UTM Johor campus, located in the southernmost part of Peninsular Malaysia (1.5632°N, 103.6452°E) at about 82.60 m above sea level with sandy soil type. Each carcass was placed in direct contact with the grass, exposed to direct sunlight and rain, and randomly separated by a minimum distance of 20 m. For ensuring the independence of these replicates, the decomposition sites for the different replicate experiments were further separated by a minimum distance of 50 m. The carcasses were covered with appropriate sized slotted plastic baskets (basket length: 56 cm; basket width: 45 cm; basket height: 20 cm; slot length: 1.7 cm; slot width: 1.1 cm) with bricks on top to allow the ingress and egress of flies yet preventing scavenger activity.

Following the method described by previous researchers (Mahat *et al.*, 2014), entomological observations were made. All carcasses were observed at every 2-hour interval between 8 a.m. to 6 p.m. until the observation of second instar larvae, and then at every 4-hour interval until the onset of the pupation period. Considering the possibility of underestimation of PMI whenever entomological observation was restricted to only once daily during the pupation period (Mahat *et al.*, 2009), observation interval during such period was made twice a day around 10 a.m. and 4 p.m. The entomological observations made included the discovery of eggs, larvae and their instars, prepupae, pupae, empty pupal cases and emerging tenerals.

The ambient and carcass surface temperatures as well as rainfall data were recorded *in situ* using a data logger (KKI Instruments, Malaysia), a calibrated digital thermometer (ETI Thermometers, United Kingdom) and a rain gauge (ClimeMet, United Kingdom), respectively. The carcass surface temperature was recorded by placing the digital thermometer probe directly on the surface of the carcass, while for recording the ambient temperature, the data were recorded at every 2- hour interval using a data logger placed at the decomposition site. Representative specimens of larvae with observed differences in appearances as well as sizes were sampled whenever applicable during each visit. Their instar larvae were ascertained by observing the number of spiracular slits in the posterior spiracles (Gennard, 2007).

All larvae collected were killed using hot water (80-90ºC) and preserved in 80% alcohol using appropriate containers. In this present research, the completion of a particular instar stage was decided if at least one of the ten larvae sampled (10%) had reached the next instar stage, as recommended by Clarkson *et al.* (2004). Whenever the wandering larvae/prepupae were observed, specimens of such larvae were collected and reared in rearing cups, while the soil surrounding the decomposing carcass (within 1 m radius) was examined for the presence of prepupae and pupae. The rearing process utilized in this present research was that suggested by Tantawi *et al.* (1996) with a minor modification, whereby soil collected from the decomposition site was used to replace the sawdust. For ensuring that the prepupae and pupae reared developed at similar ambient condition with that left surrounding the carcasses, each rearing plastic cup (with a small punctured hole at its bottom to minimise water retention) was placed at the same decomposition site. The rearing cups that contained sufficient amount of soil (about 3 cm in depth) for burying the prepupae/pupae were secured using cotton gauze and rubber band and observed twice a day (around 10 a.m. and 4 p.m.) until the emergence of tenerals. To prevent overcrowding, each rearing cup contained no more than 10 prepupae/pupae. The taxonomic identification of the adults as well as the larvae of *C. megacephala* and *C. rufifacies* was made following the keys provided by previous researchers (Kurahashi *et al.*, 1997; Omar, 2002; Sukontason *et al.*, 2004; 2008).

Statistical analysis.

Statistical analyses were performed using the IBM SPSS version 22.0 and the normality of data was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The data analysed included the ambient temperature and insect development (duration for the initial oviposition to occur as well as the completion of life cycles) for *C. megacephala* and *C. rufifacies*. Analysis of Variance (ANOVA) was used for comparing the ambient temperature data among the four replicate experiments. Because the insect development data violated the assumption of normality, statistical inference using the non-parametric Mann-Whitney U test was conducted. For inferring significance of the findings, the level of significance (α) was set at 0.05.

RESULTS AND DISCUSSION

Chemical compositions of the aqueous citronella and chlorpyrifos preparations

It is pertinent to mention here that although the general effect of citronella aqueous preparation in delaying oviposition and development of necrophagous insects have been indicated in the literature (Charabidze *et al.*, 2009), the chemical constituents of such preparations and discussions on their possible influences on *C. megacephala* and *C. rufifacies* remain unreported, so far. Moreover, specific studies focusing on the influence of chlorpyrifos on oviposition and development of necrophagous insects remain lacking, too. Therefore, identification and quantitation of chemical constituents in the citronella and chlorpyrifos preparations used in this present research may prove useful for providing better discussion on its potential effects on *C. megacephala* and *C. rufifacies*. While fenchyl acetate (23.89%) was found as the major constituent in the ~5% v/v

citronella aqueous preparation used in this research, citronellol (5.2%), citronellal (4.9%), eucalyptol (4.7%) and geraniol (3.0%) were also present, and they have been reported for having repellent and/or fumigant effects towards insects (Debbown *et al.*, 2007; Sfara *et al.*, 2009, Gilles *et al.*, 2010). Other constituents with unknown repellent and/or fumigant properties found included fenchol, carbonic acid, benzeneeathanol, δ-cadinene, lauric acid, β-selinene, α-pinene, fenchyl acetate, longipinene, dodecenyl succinic anhydride, benzothiazole, bicycloheptane, 6-methylene, 5-(1-Bromo-1-methyl-ethyl)-2-methyl-cyclohexanol, 1, 4-Dimethyladamantane and germacyclopentane.

Chemical analysis of the chlorpyrifos preparation revealed the presence of 9 chemical constituents *viz*. solvents (1,2,3-trimethylbenzenes, 2-ethyl-*m*-xylene, 2,5-dimethyl-*p*-xylene, 5-ethyl-*m*-xylene, 1,2-diethylbenzene, and 1-methylnaphthalene), the degradation product of chlorpyrifos (3,5,6-trichloro-2-pyridinol), as well as the active compound of chlorpyrifos (14.1%) and naphthalene (5.9%). While chlorpyrifos has been undoubtedly indicated to be an insecticide that acts directly on insects (Giesy & Solomon, 2014), the repellent effect of naphthalene has also been reported in the literature (Daisy *et al.*, 2002).

Temperature and rainfall at the decomposition site

The data on ambient temperature and total daily rainfall are presented in Tables 2 and 3, respectively. It was found that the daily mean ambient temperature ranged between 26.11–30.67°C, 27.48–29.42°C, 26.16–29.74°C and 26.16–30.17°C during the replicate experiments 1, 2, 3 and 4, respectively (Table 2). The differences in mean ambient temperatures among the four replicates were not statistically significant (ANOVA, $P > 0.05$). Heavy rains (> 9.0 mm) were recorded on days-4 and 9; day-2; day-12; and days-5, 9 and 12 of decomposition during the replicate experiments 1, 2, 3 and 4, respectively (Table 3). The data on carcass surface temperature remained similar for the control, citronella and chlorpyrifos-treated carcasses, and the pattern prevailed throughout the four replicate experiments. Because there were no significant variations in the ambient temperature (ANOVA, $P > 0.05$), as well as similarity observed in the carcass surface temperature during the four replicate experiments, it can be construed that both the *C. megacephala* and *C. rufifacies* were at least developed at about the same environmental conditions, enabling suitable comparisons to be made. However, the fact that there are still many factors that can influence oviposition other than similar ambient and surface temperatures such as necrobiome, wound size and smell of blood, further studies exploring these factors may prove useful.

Influence of citronella and chlorpyrifos on initial oviposition of *C. megacephala* and *C. rufifacies*

In general, *C. megacephala* was found as the first blowfly species infesting both the control and citronella-treated rabbit carcasses followed by *C. rufifacies*. Oviposition by *C. megacephala* in all the control carcasses was observed within 4-6 hours of decomposition, while observation of the same species was found significantly delayed (at 10 hours) in all the citronella-treated carcasses (Table 4, Mann-Whitney U, $P < 0.05$). Such a significant delay in the oviposition by *C. megacephala* in all the citronella-treated carcasses may be attributable to the presence of insect repellent compounds (*viz*. citronellol, citronellal, eucalyptol and geraniol) found in the citronella preparation used in this research (Debbown *et al.*, 2007; Koba *et al.*, 2009; Sfara *et al.*, 2009; Solomon *et al.*, 2012). Although the repellent effect of citronella preparations towards several species of necrophagous insects prevailing in France has been indicated (Charabidze *et al.*, 2009), this present study remains as the first ever record on the influence of this aqueous preparation towards *C. megacephala* and *C. rufifacies*, ubiquitous species in tropical countries like Malaysia. In addition, this present research provided the chemical compositions of the aqueous citronella preparation used, attempting to explain its insect repellent properties. Despite a few hours delay in oviposition of *C. megacephala*

Table 2. Daily ambient temperature at the decomposition site during the 4 replicate experiments

Day	Daily ambient temperature (°C)							
	Replicate experiment 1 (1st – 13th Sept 2015)		Replicate experiment 2 (13th – 26th Sept 2015)		Replicate experiment 3 (27th Sept –10th Oct 2015)		Replicate experiment 4 11th – 24th October 2015	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
1	30.67 ± 2.42	27.6 – 34.5	28.68 ± 3.36	25.8 – 36.1	28.65 ± 2.99	25.1 – 33.9	28.80 ± 3.21	25.0 – 34.2
2	28.17 ± 2.54	25.3 – 32.3	27.64 ± 3.49	25.5 – 35.5	27.48 ± 1.93	25.0 – 31.2	27.80 ± 2.17	25.7 – 31.6
3	26.11 ± 2.51	22.1 – 31.3	27.70 ± 2.67	25.0 – 33.5	28.20 ± 3.68	24.5 – 33.9	27.87 ± 3.08	25.2 – 34.4
4	26.69 ± 1.76	25.4 – 31.6	28.43 ± 3.26	25.4 – 33.9	27.89 ± 3.29	25.1 – 34.7	28.05 ± 2.52	25.1 – 32.4
5	28.70 ± 3.00	25.7 – 33.9	28.10 ± 2.91	25.0 – 33.2	28 .87 ± 3.42	24.7 – 34.4	26.16 ± 2.53	24.3 – 32.7
6	29.00 ± 3.59	25.5 – 33.6	28.03 ± 2.60	25.1 – 32.4	27.90 ± 3.51	24.7 – 34.3	28.90 ± 4.69	24.2 – 37.1
7	29.95 ± 4.75	25.5 – 38.6	29.42 ± 3.93	25.3 – 37.0	28.43 ± 3.23	24.6 – 33.6	29.74 ± 4.26	24.8 – 37.4
8	28.68 ± 3.36	25.8 – 36.1	28.65 ± 2.99	25.1 – 33.9	28.80 ± 3.21	25.0 – 34.2	30.17 ± 4.63	25.5 – 40.1
9	27.64 ± 3.49	25.5 – 35.5	27.48 ± 1.93	25.0 – 31.2	27.80 ± 2.17	25.7 – 31.6	28. 53 ± 3.59	25.3 – 36.5
10	27.70 ± 2.67	25.0 – 33.5	28.20 ± 3.68	24.5 – 33.9	27.87 ± 3.08	25.2 – 34.4	28.40 ± 3.69	25.1 – 36.6
11	28.43 ± 3.26	25.4 – 33.9	27.89 ± 3.29	25.1 – 34.7	28.05 ± 2.52	25.1 – 32.4	27.17 ± 2.17	25.3 – 31.8
12	28.10 ± 2.91	25.0 – 33.2	28 .87 ± 3.42	24.7 – 34.4	26.16 ± 2.53	24.3 – 32.7	28.70 ± 3.75	25.0 – 35.1
13	28.03 ± 2.60	25.1 – 32.4	27.90 ± 3.51	24.7 – 34.3	28.90 ± 4.69	24.2 – 37.1	27.60 ± 2.48	25.0 – 33.0
14	29.42 ± 3.93	25.3 – 37.0	28.43 ± 3.23	24.6 – 33.6	29.74 ± 4.26	24.8 – 37.4	28.60 ± 3.75	24.5 – 34.8

The ambient temperature was recorded at every 2-hour interval throughout the experiment using a data logger. ANOVA revealed no significant differences ($P > 0.05$) in the ambient temperature among the four replicate experiments. For determining the significant differences among groups, the level of significance of 0.05 was used.

Table 3. Daily occurrence of rain (symbol) and total daily rainfall (mm) during the course of 14 days of entomological observation

Replicate Experiment	Day of decomposition													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.0 ☼	4.6 ●	4.0 ●	14.0 ★	0.0 ☼	0.0 ☼	0.0 ☼	0.4 ●	37.0 ● ★	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼
2	0.4 ●	37.0 ● ★	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	2.0 ★	0.8 ★	0.0 ☼	6.8 ★	0.0 ☼	0.0 ☼	0.0 ☼
3	2.0 ★	0.8 ★	0.0 ☼	6.8 ★	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	0.0 ☼	2.2 ★	48.6 ☁	0.0 ☼	0.0 ☼
4	0.0 ☼	0.0 ☼	0.0 ☼	2.2 ★	48.6 ☁	0.0 ☼	0.0 ☼	0.0 ☼	14.0 ☁	0.0 ☼	0.0 ☼	9.4 ★	1.2 ●	0.0 ☼

Legend:

Symbol	Meaning
☼	No rain recorded
●	Rain in the afternoon
☁	Intermittent rain in the afternoon
★	Rain in the night

in citronella-treated carcasses, oviposition still occurred on the first day of decomposition, in concurrence with the previous studies reported about oviposition of *C. megacephala* on corpses (Lee *et al.*, 2004) and animal models (Mahat *et al.*, 2009; 2014) devoid of such treatments in Malaysia.

For *C. rufifacies*, the oviposition was observed during the first visit at about 8 a.m. on day-2 of decomposition in both the control and citronella-treated carcasses (Table 5, Mann-Whitney U, $P > 0.05$). Because the effectiveness of citronella for repelling insects is exerted only at its vaporized state (Debbown *et al.*, 2007) and since the longevity of this volatile plant preparation can be impeded by rapid evaporation process (Debbown *et al.*, 2007; Maia & Moore, 2011), the similarity in time for observing oviposition by *C. rufifacies* in the citronella-treated carcasses with that of the controls on day-2 of decomposition remains expected. Moreover, the fact that citronella applied onto the carcasses might have been washed off by rain and hence, regaining its attractiveness for oviposition by *C. rufifacies*, could not be ruled out. Because the oviposition by *C. rufifacies* by about the same time on the second day of decomposition was observed in all the control and citronella-treated carcasses, similar to other forensic entomological findings reported in Malaysia (Lee *et al.*, 2004, Mahat *et al.*, 2014), it can be assumed that the influence of citronella during that period may prove negligible. This observation may also due to the fact that *C. rufifacies* is a secondary colonizer of corpses and carcasses in Malaysia (Mahat *et al.*, 2009).

Since oviposition in the chlorpyrifos-treated carcasses was only evident during visits at 2 p.m. onwards on the second day of decomposition and because eclosion of eggs into larvae was not observed, taxonomic identification of species could not be ascertained. Moreover, neither oviposition nor evidence of eclosion was observed on any of the subsequent days of decomposition for chlorpyrifos-treated carcasses. Observations made on day-3 onwards revealed dissociation of the clumps of eggs (fell off onto the ground from the carcasses) (Figure 1), indicating their non-viability; eclosion of these eggs into larvae was not observed throughout the period of observation. Considering such condition, suitable identification as well as statistical comparisons for *C. rufifacies* in the

Table 4. Day and time of the first observation of the different stages of life cycle for *C. megacephala* in the control (C), citronella (T1) and chlorpyrifos treated (T2) carcasses

Replicate	Groups	The different stages of life cycle for *C. megacephala*							Total duration
		Eggs	First instar	Second instar	Third instar	Prepupae	Pupae	Teneral	
1	C	Day-1 (2 pm) [6 h]	Day-2 (8 am) [16 h]	Day-2 (4 pm) [8 h]	Day-3 (8 am) [18 h]	Day-4 (12 pm) [28 h]	Day-5 (10 am) [22 h]	Day-9 (10 am) [96 h]	188 hours [7.83 days]
	T1	Day-1 (6 pm) [10 h]*	Day-2 (10 am) [16 h]	Day-2 (6 pm) [8 h]	Day-3 (12 pm) [18 h]	Day-4 (12 pm) [24 h]	Day-5 (10 am) [22 h]	Day-9 (4 pm) [104 h]	192 hours [8.00 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						
2	C	Day-1 (12 pm) [4 h]	Day-2 (8 am) [20 h]	Day-2 (4 pm) [8 h]	Day-3 (8 am) [18 h]	Day-4 (10 am) [26 h]	Day-5 (10 am) [24 h]	Day-9 (10 am) [96 h]	192 hours [8.00 days]
	T1	Day-1 (6 pm) [10 h]*	Day-2 (12 pm) [18 h]	Day-2 (6 pm) [6 h]	Day-3 (4 pm) [22 h]	Day-4 (4 pm) [24 h]	Day-5 (4 pm) [28 h]	Day-9 (4 pm) [96 h]	194 hours
	T2	Day-2 (2 pm) [30 h] †	Larvae as well as completion of life cycle were not observed						
3	C	Day-1 (12 pm) [4 h]	Day-2 (8 am) [20 h]	Day-2 (4 pm) [8 h]	Day-3 (8 am) [18 h]	Day-4 (10 am) [26 h]	Day-5 (10 am) [24 h]	Day-9 (10 am) [96 h]	192 hours [8.00 days]
	T1	Day-1 (6 pm) [10 h]*	Day-2 (12 pm) [18 h]	Day-2 (6 pm) [6 h]	Day-3 (4 pm) [22 h]	Day-4 (4 pm) [24 h]	Day-5 (4 pm) [28 h]	Day-9 (4 pm) [96 h]	194 hours [8.03 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						
4	C	Day-1 (12 pm) [4 h]	Day-2 (8 am) [20 h]	Day-2 (4 pm) [8 h]	Day-3 (8 am) [18 h]	Day-4 (10 am) [26 h]	Day-5 (10 am) [24 h]	Day-9 (10 am) [96 h]	192 hours [8.00 days]
	T1	Day-1 (6 pm) [10 h]*	Day-2 (12 pm) [18 h]	Day-2 (6 pm) [6 h]	Day-3 (4 pm) [22 h]	Day-4 (4 pm) [24 h]	Day-5 (4 pm) [28 h]	Day-9 (4 pm) [96 h]	194 hours [8.03 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						

Values in brackets ( ) and square parentheses [ ] indicate the time and duration in hours for the first observation of a particular stage of development from the previous ones, respectively. Mann-Whitney U test of pooled replicate experiments revealed a significant delay in initial oviposition for *C. megacephala* in T1s (*) ($P < 0.05$) compared to Cs. While delayed in oviposition was observed in all the T2s, identification of the species could not be attempted since eclosion from eggs to larvae was not successful, rendering inability in conducting appropriate statistical comparison with the other groups (†). No significant differences ($P > 0.05$) in the durations for completing the life cycles for *C. megacephala* were found in Cs when compared with that of T1s. Significance level of 0.05 was used for determining the significant differences among groups.

Table 5. Day and time of the first observation of the different stages of life cycle for *C. rufifacies* in the control (C), citronella (T1) and chlorpyrifos treated (T2) carcasses

Replicate	Groups	The different stages of life cycle for *C. megacephala*							Total duration
		Eggs	First instar	Second instar	Third instar	Prepupae	Pupae	Teneral	
1	C	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (4 pm) [104 h]	204 hours [8.50 days]
	T1	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (4 pm) [104 h]	204 hours [8.50 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						
2	C	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (10 am) [98 h]	198 hours [8.25 days]
	T1	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (4 pm) [104 h]	204 hours [8.50 days]
	T2	Day-2 (2 pm) [30 h] †	Larvae as well as completion of life cycle were not observed						
3	C	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (10 am) [98 h]	198 hours [8.25 days]
	T1	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (10 am) [98 h]	198 hours [8.25 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						
4	C	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (10 am) [98 h]	198 hours [8.25 days]
	T1	Day-2 (8am) [24 h]	Day-2 (6 pm) [10 h]	Day-3 (12 pm) [18 h]	Day-4 (8 am) [20 h]	Day-5 (4 pm) [32 h]	Day-6 (8 am) [20 h]	Day-10 (10 am) [98 h]	198 hours [8.25 days]
	T2	Day-2 (6 pm) [34 h] †	Larvae as well as completion of life cycle were not observed						

Values in brackets ( ) and square parentheses [ ] indicate the time and duration in hours for the first observation of a particular stage of development from the previous ones, respectively. Mann-Whitney U test of pooled replicate experiments revealed no significant delay in initial oviposition for *C. rufifacies* in the T1s ($p > 0.05$) when compared with that of Cs. While delayed in oviposition (by 6-10 hours) was observed in all the T2s, identification of the species could not be attempted since eclosion from eggs to larvae was not successful, rendering inability for conducting suitable statistical analysis (†). No significant difference ($p > 0.05$) in the durations for completing the life cycles for *C. rufifacies* was found in T1s when compared with that of Cs. Significance level of 0.05 was used for determining the significant differences among groups.

Figure 1. Dissociation of the clumps of eggs that fell off onto the grass (circle) observed in a chlorpyrifos treated carcass on day-3 (~8 am).

chlorpyrifos-treated carcasses with that of controls and citronella-treated carcasses could not be attempted in this present research. Review of the literature revealed about delayed egg-laying, decreased egg production and eggs failed to hatch in *Musca domestica* when exposed to chlorpyrifos (Dad *et al.*, 2011). In addition, a study focusing on *Drosophila melanogaster* reported that reduction in fecundity and reproductive performance were observed following the exposure of such fly species towards organophosphorus insecticides such as chlorpyrifos (Gupta *et al.*, 2007).

Influence of citronella and chlorpyrifos on completion of life cycles of *C. megacephala* and *C. rufifacies*

In control carcasses, the first instar larvae of *C. megacephala* were observed at about 8 a.m. during the second day of decomposition followed by the second instar larvae later that day (day-2, ~4 p.m.) and the third instar larvae on the following day (day-3, ~8 a.m., Table 4). The prepupal and pupal stages of *C. megacephala* were observed on day-4 (~10 a.m. –12 p.m.) and day-5 (~10 a.m.), respectively with the completion of its life cycle observed in the 9th day (~10 a.m.) of decomposition; bringing the total duration for completing its life cycle (from eggs to tenerals) ranged between 188–192 hours (7.83–8.00 days) (Table 4). As for the citronella-treated carcasses, the first, second, and third instar larvae, prepupae, pupae, and tenerals of *C. megacephala* were observed on day-2 (~10 a.m. – 12 p.m.), day-2 (~6 p.m.), day-3 (~12 – 4 p.m.), day-4 (~12 – 4 p.m.), day-5 (~10 a.m. – 4 p.m.) and day-9 (~4 p.m.), respectively. The total duration for completing the life cycle for *C. megacephala* in citronella-treated carcasses ranged between 192–194 hours (8.00–8.03 days, Table 4). Statistical analysis using the Mann – Whitney U test revealed no significant differences in the total durations for completing the life cycle as well as the individual stages of development for *C. megacephala* in citronella-treated carcasses when compared with that of controls (Table 4, $P > 0.05$).

As for *C. rufifacies* in the control carcasses, the first, second and third instar larvae, prepupae, pupae and tenerals were observed on day-2 (~6 p.m.), day-3 (~12 p.m.), day-4 (~8 a.m.), day-5 (~4 p.m.), day-6 (~8 a.m.) and day-10 (~10 a.m. – 4 p.m.) of decomposition, respectively with the total duration for completing its life cycle (from eggs to tenerals) ranged between 198–204 hours (8.25–8.50 days, Table 5). As for the citronella-treated carcasses, the first, second, and third instar larvae, prepupae, pupae, and tenerals of *C. rufifacies* were observed on day-2 (~6 p.m.), day3 (~12 p.m.), day-4 (~8 a.m.), day-5 (~4 p.m.), day-6 (~8 a.m.) and day-10 (~10 a.m. – 4 p.m.), respectively. The total duration for completing the life cycle for *C. rufifacies* in citronella-treated carcasses ranged between 198–204 hours i.e. 8.25–8.50 days (Table 5). No significant differences in the total durations for completing the life cycle as well as individual stages of development for *C. rufifacies* in citronella-treated carcasses were observed when compared with that of the controls (Table 5, Mann-Whitney U test, $P > 0.05$).

Considering that the Mann-Whitney U test did not reveal significant differences in the total durations for completing the life cycles for *C. megacephala* and *C. rufifacies* as well as the individual stages of development in the citronella-treated carcasses than that of controls, the development of these species was evidently independent of the influence of citronella. In addition to the fact that rain may have washed off substantial amount of citronella residues on the carcasses, rapid evaporation of compounds such as citronellol, citronellal and geraniol (Koba *et al.*, 2009; Solomon *et al.*, 2012) may further limit the bioactivity of citronella preparation in delaying the completion of life cycles for *C. megacephala* and *C. rufifacies*. Moreover, the total durations for completing the life cycles for *C. megacephala* and *C. rufifacies* observed in this present research were comparable to the durations reported by previous researchers in Malaysia (Omar *et al.*, 1994; Mahat *et al.*, 2009; 2014; 2016). Since rain can be an impeding factor to elucidate the real influence of citronella preparation on *C. megacephala* and *C. rufifacies*, replicating the same experimental design for shaded habitat that is completely protected from rain may prove useful. However, considering that bodies are also found in sunlit locations, the findings of this present study may still be useful for forensic entomologists to interpret the estimated minimum PMI. In addition, further studies to investigate the possible resistance of these two blowflies towards citronella merit forensic consideration.

The developmental stages beyond the egg stage were not observed in chlorpyrifos-treated carcasses, rendering difficulties in identifying them as *C. megacephala* and/or *C. rufifacies* (Tables 4 and 5). It has been reported that, depending on the soil type, climate and other conditions, chlorpyrifos can be moderately persistent in the environment and its half-life can last for more than 120 days (Gilani *et al.*, 2010). Being an acetyl cholinesterase inhibitor, chlorpyrifos can interrupt biochemical processes in insects since they do not have specific pathways for detoxifying this poison (Hui *et al.*, 2010). This factor can potentially explain the lack of larval stages as well as completion of life cycle for these necrophagous flies in all the chlorpyrifos-treated carcasses. It was also possible that the dose of chlorpyrifos sprayed onto the carcasses (i.e. 0.42 g/L) in this present research may be too toxic for permitting growths of necrophagous flies. Because the lethal dose (LD50) for chlorpyrifos in *Chrysomya* species remains unreported, further studies for exploring this aspect may prove useful.

CONCLUSION

When compared with control carcasses, (a) the initial oviposition of *C. megacephala* was significantly delayed by 4-6 hours in all the citronella-treated carcasses ($P < 0.05$) although (b) significant prolongation in the duration for completing its life cycle was not observed. As for *C. rufifacies*, (c) neither delay in initial oviposition nor (d) prolongation in the duration of life cycle was

observed in the citronella-treated carcasses. While (e) delay in initial oviposition was observed in all the chlorpyrifos-treated carcasses, the species that oviposited could not be ascertained since the eclosion of eggs into larvae, as well as completion of life cycle were not evident. Since forensic entomological baseline data for Johor as well as the individual influences of citronella and chlorpyrifos on oviposition and completion of the life cycle for *C. megacephala* and *C. rufifacies* remain lacking, the results reported here may prove useful for estimating PMI *via* entomological assessment within this region, especially whenever presence of these two chemical agents is suspected.

LIMITATION

Although morphological differences in aspect ratios, presence of fur, as well as weight would limit suitable discussions of the findings for human forensic (Mahat *et al.*, 2016), rabbit carcasses were used due to easy handling for administering citronella and chlorpyrifos, as well as having sufficient body size to support insect infestation (Bharti & Singh, 2003). Therefore, replicating the same experiment using pigs that are similar to human bodies would be necessary for providing better insights into rates of decomposition and patterns of fly colonization. The fact that only one level of concentration was used for both citronella and Chlorpyrifos, and because biogeoclimatic variations like rain and habitat may also affect oviposition and completion of life cycle, further studies replicating the experimental model addressing such factors prove necessary.

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