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Research Article

Effect of microbial inoculant on the oviposition and completion of life cycle of *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) infesting rabbit carcasses

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ABSTRACT

This research was conducted to assess the influence of microbial inoculant (EM.1®) on the initial oviposition and developmental pattern of two prevalent necrophagous flies (*Chrysomya megacephala* and *Chrysomya rufifacies*) in Malaysia. Nine rabbit carcasses were equally divided into control and two treated groups. The control carcasses were sprayed with deionized water (about 100 mL), whereas the two other treated groups were individually sprayed with two concentrations of EM.1® at 1:500 and 1:100, respectively. Results revealed that the single application of EM.1® on both treated carcasses did not statistically impede oviposition by both necrophagous species, as well as their subsequent developmental patterns ($P > 0.05$) when compared with that of controls. Therefore, estimating the minimum post-mortem interval (mPMI) using empirical baseline data for the control animal model would remain appropriate for estimating mPMI in cases involving carcasses with a single application of EM.1®.

Keywords: Chrysomya megacephala, Chrysomya rufifacie, forensic science, Forensic entomology, Microbial inoculant, Post mortem interval, Oviposition and development

Introduction

Applying entomology in death investigations involves interpreting the insects' developmental data, particularly for providing an accurate estimation of minimum post-mortem interval (mPMI) [1]. In a forensic investigation, having an accurate estimation of mPMI would aid the investigators in setting the time of a homicide, validating statements from witnesses, and narrowing down the number of potential suspects [2]. Forensic entomological evidence becomes crucial when the time since death is more than 72 hours, rendering pathological changes (e.g., rigor mortis, algor mortis, and livor mortis) less reliable for estimating mPMI [3]. This can be done by examining the necrophagous insects infesting the corpse, observing their current life cycle stage, estimating the age of the immatures (e.g., larval instar), and subsequently estimate the mPMI [4]. In this context, the accuracy of mPMI estimate relies largely on accurately identifying the oldest necrophagous insect species, prior to utilizing the appropriate growth data. In Malaysia, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) has been identified

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as the first and dominant necrophagous species infesting corpses and carcasses, followed by *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) [5].

Many factors can affect initial oviposition, development pattern, and succession of species that, include the bio geo-climatic factors (e.g., rain) [6], presence of therapeutic drugs and toxic substances [1], as well as physical barriers such as covering or wrapping [7]. A review of the literature reveals that while insecticides such as malathion may delay initial oviposition and prolong the development of blowfly species [6], faster development of necrophagous insects has been associated with cocaine [8] and diazepam [9]. Besides, there are also chemicals (e.g., bleach and paraquat) that do not affect the development of necrophagous insects [10, 11]. The use of chemicals and corrosive substances can be observed in some planned and predetermined homicide cases. In general, planned murder can be characterized as instrumental murders in which the murders are planned and committed for certain benefits such as financial gain, power, and wealth status [12]. Such murderers may tend to undertake precautionary behaviors that are consciously intended to confuse, hamper, or defeat investigative or forensic efforts to conceal their identity, connection to the crime, or the crime itself [13]. The precautionary behaviors include various crime scene concealment and other deliberate alteration methods (e.g., pouring chemicals over the victim's body) to mislead the authorities or redirect an investigation [13, 14]. In this context, it is pertinent to indicate here that any factors that alter the oviposition and development pattern of necrophagous insects would subsequently result in the erroneous estimation of PMI. Hence, investigating the different chemicals and/or formulations that are commonly available in the market for their potential influences on the oviposition and duration for completing the life cycle among necrophagous insects merits forensic consideration.

The use of microbial inoculants that contain effective microorganisms (EM) to reduce offensive odor, especially from waste and manure at livestock farms, has been suggested [15]. The selected species of microorganisms included in microbial inoculants are lactic acid (*Lactobacillus casei*) and photosynthetic bacteria (*Rhodopseudomonas palustris*), as well as yeast [16]. The individual functions of the effective microorganisms include inhibiting the propagation of harmful microbes and accelerating decomposition (i.e., lactic acid bacteria), producing amino acids that feed other microbes (i.e., yeast) as well as playing an important role in the nitrogen and carbon cycle (i.e., photosynthetic bacteria) [17]. Because scientific information has already become the public domain, and since criminals nowadays are getting better informed, it is possible that these cunning criminals may resort to the use of commercial formulations like microbial inoculants for delaying necrophagous insect activity. The application of microbial inoculants in the surrounding area can lead to alteration in the successional patterns of necrophagous flies infesting a corpse. This issue may appear pertinent to forensic entomologists since alteration in insect activity (especially the initial oviposition) may lead to inaccurate estimation of mPMI, and subsequently, result in the miscarriage of justice. The fact that such microbial inoculants are readily accessible in the market and since the review of the literature does not reveal any specific study focusing on its influence on oviposition and development pattern of *C. megacephala* and *C. rufifacies*, this specific research that was designed to elucidate such an aspect appears forensically relevant.

Material and Methods

Experimental design

Nine freshly slaughtered male domestic rabbits (*Oryctolagous cuniculus* (L.)) were purchased as carcasses (weighing 1.5-2.0 kg) from a local meat seller, and they were equally divided into three different groups designated as control (C), treated 1 (T1) and treated 2 (T2). For one replicate experiment, C was sprayed with deionized water (about 100 mL) while T1 and T2 were sprayed with 1:500 and 1:100 (ratios to deionized water) of the same microbial inoculant (EM.1®), respectively. The chosen two concentrations of microbial inoculant were that of suggested by the manufacturer and distributor for cleaning yards of livestock rearing facilities [18].

Upon completion, each carcass was left to decompose in a shaded habitat that was fully protected from rain. To minimize interruptions from the adjacent colonies, each carcass was separated by at least 20 meters apart. The carcass was covered with a slotted plastic basket with 3–4 bricks placed on top of the basket to protect it from being scavenged, yet enabling free ingress and egress of

adult flies. Replicating such a procedure, three replicate experiments performed during 4–14 September 2017, 25 September–5 October 2017 and 16–26 October 2017 were included in this present research.

Decomposition site and entomological observation.

The decomposition site was a cemented area under a reinforced concrete roof (i.e., fully protected from rain) with all sides remaining open. The area located within the lower ground floor of a six-story building within Universiti Teknologi Malaysia Johor Bahru Campus (103.635°E, 1.563°N, ~34 m above sea level). Such a habitat was chosen to exclude rain from being a factor that would wash away or further dilute the concentrations of the sprayed microbial inoculant.

Following the method prescribed by previous researchers [6, 10] with a minor modification, entomological observations along with data on daily ambient temperature and outside daily total rainfall were recorded using a data logger and a rain gauge until the first emergence of tenerals for *C. megacephala* and *C. rufifacies*. Entomological observation was made at every 2-hour interval during daytime (between 8.00 a.m. to 6.00 p.m.) until the observation of the second instar larvae of *C. rufifacies*. Then, the observation was made at every 4-hour interval until *C. rufifacies* started to pupate. Considering the limitation indicated by Mahat et al. [6], two periods of observations (10 a.m. and 4 p.m.) were performed during the pupation period until the emergence of tenerals, as opposed to once daily around noon reported by the authors [6]. In every visit, entomological observations such as presence of eggs, the different instars of larvae, prepupae, pupae, empty pupal cases and tenerals were documented [10].

Sample collection and preservation

Whenever applicable representative specimens of larvae demonstrating differences in appearances and sizes were collected randomly from each carcass. Apart from the larvae, pre-pupae, pupae and tenerals were also collected. To ascertain the instars of the larvae, the number of spiracular slits in their posterior spiracles was observed [3]. The completion of a particular development stage was decided if at least 10% of the specimens sampled had reached that stage [19]. All larvae sampled from each carcass were killed using hot water (~80°C) and then transferred into specimen containers containing 80% ethanol for preservation until further taxonomic identification. Following the method described by previous researchers [6], samples of the wandering third instar larvae, prepupae as well as pupae were collected and reared in rearing cups until the emergence of tenerals for taxonomic identification. The remaining pupae were observed *in situ* each day until the emergence of tenerals. The rearing cups containing the larvae/pupae were placed at the same decomposition site as the carcasses to ensure similarity in ambient conditions during their development. The tenerals of *C. megacephala* and *C. rufifacies* were identified using the taxonomic keys provided by Kurashi et al. [20] and Nazni et al. [21], while their larvae were identified using the keys provided by Omar [22].

Statistical analysis

Data gathered in this study was analyzed using the IBM SPSS version 20.0 software. The data analyzed included the mean daily ambient temperature, outside daily total rainfall and onset of initial oviposition as well as duration taken for completion of life cycles for the two species in all groups of rabbit carcasses. The normality of the data was determined using Shapiro-Wilk test, prior to conducting the hypothesis testing. Normally distributed data were analyzed using ANOVA with Tukey-Kramer post-hoc test, while the data that violated the assumption of normality were analyzed using Kruskal-Walls H test with pairwise comparison using Mann-Whitney U test. To determine the significance of the findings, the level of significance (α) was set at 0.05.

Results and Discussion

Temperature and rainfall at the decomposition site

Necrophagous insects (e.g., *C. megacephala* and *C. rufifacies*) are unable to regulate their body temperature, and therefore, they rely largely on heat from the ambient to support their physiological and biochemical processes [3]. Specifically, extrinsic abiotic factors such as ambient temperature and rainfall play pivotal roles at affecting their developmental patterns, a significant aspect for consideration when estimating mPMI [23]. Hence, the data on daily ambient temperature at the chosen rain-protected decomposition site (but exposed at all sides to the fluctuation of temperature)

Table 1. Daily ambient temperature during the three-replicate experiments

Day	Daily ambient temperature (°C)					
	Replicate experiment 1 (4^{th} - 14^{th} Sept 2017)		Replicate experiment 2 (25^{th} Sept – 5^{th} Oct 2017)		Replicate experiment 3 (16^{th} – 26^{th} Oct 2017)	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
1.	27.98 ± 0.82	26.8-29.4	26.81 ± 1.44	25.1-29.7	28.18 ± 1.69	26.0-31.1
2.	27.64 ± 1.23	26.2-29.9	25.80 ± 1.71	24.1-29.7	28.31 ± 2.23	25.5-31.9
3.	27.42 ± 1.14	26.1-29.7	26.98 ± 1.25	24.9-29.0	28.89 ± 2.08	26.1-32.2
4.	26.93 ± 1.45	25.3-29.7	26.86 ± 0.96	25.5-28.7	28.37 ± 1.94	25.8-31.2
5.	26.67 ± 1.03	25.7-29.3	26.10 ± 0.88	25.0-28.0	28.85 ± 1.70	26.6-31.5
6.	26.67 ± 0.90	25.8-28.8	27.08 ± 1.38	24.9-29.2	28.71 ± 1.79	26.3-31.9
7.	26.89 ± 0.94	25.5-28.6	26.44 ± 1.12	24.7-28.8	28.49 ± 1.63	26.4-30.9
8.	26.35 ± 0.77	24.9-27.9	26.73 ± 1.49	25.0-29.7	27.64 ± 1.52	25.7-30.3
9.	27.32 ± 1.53	25.5-30.0	26.28 ± 1.09	25.0-28.8	26.92 ± 1.45	25.0-29.7
10.	27.86 ± 1.28	25.9-30.1	25.15 ± 0.91	23.9-26.6	27.39 ± 1.52	25.8-30.7
11.	27.78 ± 0.84	26.3-29.5	27.01 ± 1.36	24.9-28.6	27.33 ± 1.51	25.2-30.2

Notes: The ambient temperature was recorded *in situ* at every 2-hours interval throughout the experiments using a data logger (TEN1720, KK Instruments Malaysia).

Table 2. Overall ambient temperature and rainfall for the three replicate experiments.

	Replicate experiment 1 (R1)	**Replicate experiment 2 (R2)**	**Replicate experiment 3 (R3)**	**R1 Vs. R2**	**R1 Vs. R3**	**R2 Vs. R3**
Ambient temperature (°C)	27.23 ± 0.54 (24.9-30.1)	26.48 ± 0.58 (23.9-29.7)	28.10 ± 0.65 (25.0-32.2)	$P < 0.05$	$P < 0.05$	$P < 0.05$
Rainfall (mm)	11.00 (0.0-102.0)	3.30 (0.0-25.0)	0.00 (0.0-1.2)	$P < 0.05$	$P < 0.05$	$P < 0.05$

were recorded during the 11 days observation period for each replicate experiment (Table 1). Taking into account the importance of rainfall at influencing blowflies' activity [3], data on the daily total rainfall (Table 2 and Figure 1) outside of the decomposition site were recorded too. The data on daily mean ambient temperature recorded during the three replicate experiments along with their statistical inference are provided in Table 1 and Table 2, respectively. While Figure 1 depicts the daily total rainfall, its median value as well as the appropriate statistical inference are presented in Table 2.

The means and ranges of ambient temperature for replicate experiments 1, 2 and 3 were 27.23 ± 0.54°C (24.9–30.1), 26.48 ± 0.58°C (23.9–29.7) and 28.10 ± 0.65°C (25.0–32.2), respectively (Table 2). The daily total rainfall recorded during replicate experiments 1, 2 and 3 ranged between 0.0–102.0 mm (median: 11.0 mm), 0.0–25.0 mm (median: 3.30 mm), and 0.0–1.2 mm (median: 0.0 mm), correspondingly (Table 2). It is pertinent to indicate here that although the one-way ANOVA paired with Tukey-Kramer post-hoc test revealed statistically significant differences ($P < 0.05$) in the ambient temperature among the three replicate experiments, the mean values actually differed marginally by 0.75–1.62°C only. Such a small variation, despite being statistically significant, may probably small enough to cause any observed variations in the growth pattern of *C. megacephala* and *C. rufifacies* investigated in this present research. It is interesting to note that [24] reported his laboratory controlled experimental data in Malaysia that *C. megacephala* and *C. rufifacies* consistently completed their life cycles at 7.15 and 9 days respectively, despite the 2°C variations in the rearing temperature (30 ± 2°C). Moreover, findings reported by a study in Malaysia [25] revealed that *C. megacephala* completed its life cycle in 9.92 days when reared at constant temperature of 27°C when compared with that of 9.13 days at 30°C, a marginal difference of 0.79 day even with a constant 3°C variation in the rearing temperature. In addition, a study conducted in Western Australia [26] revealed no significant difference in the rate of larval development for *Calliphora dubia* reared at the mean constant temperature of

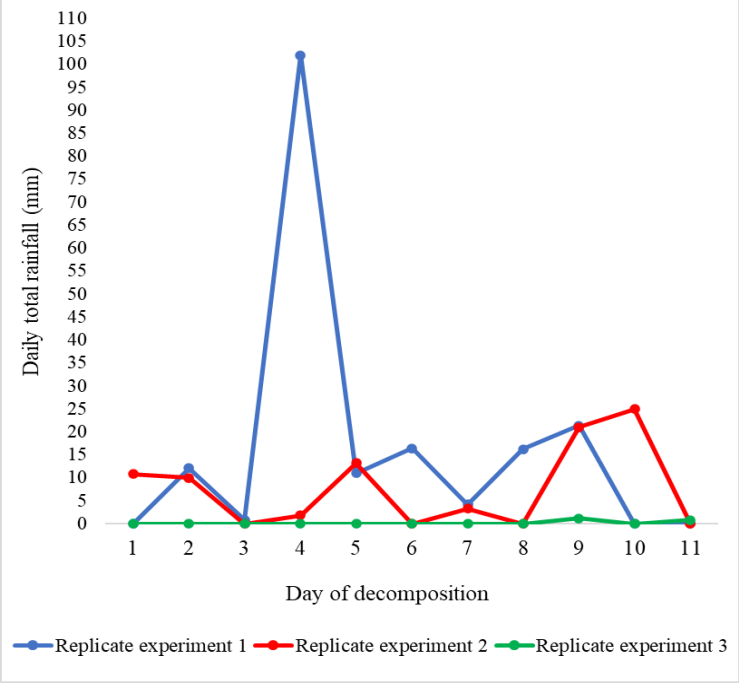

Figure 1. Data on daily total rainfall (mm) recorded outside of the decomposition site during the three replicate experiments.

24°C when compared with that of fluctuating temperature between 19°C and 30°C. The authors attributed the similarity in larval growth of the said species with the fact that "fluctuating regime lie within the threshold and optimum temperature for the larval development of *C. dubia*" [26]. Therefore, considering all these facts, the proposition that such a small variation in the ambient temperature as 0.75–1.62°C may not likely to cause considerable/ significant differences in the growth patterns of *C. megacephala* and *C. rufifacies*, appears supported.

In addition, Kruskal-Wallis H with pairwise comparison using Mann-Whitney U test revealed a statistically significant difference ($P < 0.05$) in daily total rainfall among three replicate experiments. This may be due to the scarcity in the daily total rainfall observed during replicate experiment 3, as well as the heavy rainfall recorded on one particular day (day-4) during replicate experiment 1 (Figure 1 and Table 2). The fact that the decomposition site was fully protected from rain and because initial oviposition by *C. megacephala* and *C. rufifacies* invariably occurred on day-1 and day-2, respectively, such influence of rain on the flying activity for both necrophagous species infesting carcasses in such habitat may prove as irrelevant.

Influences of microbial inoculant on initial oviposition and completion of life cycles of C. megacephala and C. rufifacies

The durations taken for initial oviposition as well as the onset of other stages of life cycle for *C. megacephala* and *C. rufifacies* among the three groups of carcasses, as well as the suitable statistical comparisons are presented in Tables 3, 4 and 5, respectively. It was observed that C. megacephala being the first necrophagous fly species that oviposited in all the control, T1 and T2 carcasses during the three replicates, followed by C. rufifacies. While the one-way ANOVA with Tukey-Kramer post hoc test was used for comparing the ambient temperature data among the three replicate experiments, Kruskal-Wallis H with pairwise comparison using Mann-Whitney U test was used for comparing the rainfall data. The ambient temperature data are presented as mean ± standard deviation (range). On the other hand, the rainfall data are presented as median (range). Significant differences ($P < 0.05$) in the rainfall data among the three replicates experiment were observed.

For both the control and T1 carcasses, the oviposition by *C. megacephala* occurred consistently within 8 hours on the first day of decomposition; the same was within 6-10 hours for T2 carcasses (Table 3). Despite such a small variation for ovi-

position of *C. megacephala* observed in T2 (median: 10 hours) than that of control (median: 8 hours) and T1 (median: 8 hours) carcasses, Kruskall-Wallis H test did not reveal any statistical significance ($P > 0.05$) among the groups (Table 5). As for *C. rufifacies*, oviposition for all the groups of carcasses was invariably observed between 24–26 hours of decomposition (Tables 5, Kruskall-Wallis H test: $P > 0.05$). The durations of oviposition for *C. megacephala* and *C. rufifacies* observed here appear consistent with that reported for the same species in Malaysia [5, 27].

Studies reported that odors emanating from the decaying tissues attracted gravid blow flies to oviposit [28], due to the production of volatile ammonia-rich compounds (i.e., strong attraction signals) associated with tissue breakdown by proteolytic bacteria [29]. Although EM may reduce malodor by suppressing the growth and activity of putrefactive microorganisms [30] its effectiveness depends largely on the consistent application of the product for building the suitable environment for the EM to self-propagate [18]. In this present research, the dilutions of microbial inoculant utilized for the T1 (1:500) and T2 (1:100) carcasses

Table 3. Day and time for the first observation of the different stages of life cycle for *C. megacephala* in control, treated 1 and treated 2 carcasses for all three replicate experiments.

Replicate	Group	Day and time for the first observation of							
		Eggs	1st instar	2nd instar	3rd instar	Prepupae	Pupae	Tenerals	Total duration
1	C	Day-1/ (4pm) [8h]	Day-2/ (8am) [16h]	Day-2/ (4pm) [8h]	Day-3/ (2pm) [22h]	Day-4/ (4pm) [26h]	Day-5/ (4pm) [24h]	Day-9/ (4pm) [96h]	192 hours/ 8.00 days
	T1	Day-1/ (4pm) [8h]	Day-2/ (8am) [16h]	Day-2/ (2pm) [6h]	Day-3/ (12pm) [22h]	Day-4/ (4pm) [28h]	Day-5/ (4pm) [24h]	Day-9/ (4pm) [96h]	192 hours/ 8.00 days
	T2	Day-1/ (6pm) [10h]	Day-2/ (10am) [16h]	Day-2/ (4pm) [6h]	Day-3/ (2pm) [22h]	Day-4/ (4pm) [26h]	Day-5/ (6pm) [26h]	Day-9/ (4pm) [94h]	190 hours/ 7.92 days
2	C	Day-1/ (4pm) [8h]	Day-2/ (10am) [18h]	Day-2/ (6pm) [8h]	Day-3/ (4pm) [22h]	Day-4/ (4pm) [24h]	Day-5/ (4pm) [24h]	Day-9/ (4pm) [96h]	192 hours/ 8.00 days
	T1	Day-1/ (4pm) [8h]	Day-2/ (10am) [18h]	Day-2/ (4pm) [6h]	Day-3/ (4pm) [24h]	Day-4/ (4pm) [24h]	Day-5/ (4pm) [24h]	Day-9/ (4pm) [96h]	192 hours/ 8.00 days
	T2	Day-1/ (6pm) [10h]	Day-2/ (10am) [16h]	Day-2/ (4pm) [6h]	Day-3/ (2pm) [22h]	Day-4/ (2pm) [24h]	Day-5/ (2pm) [24h]	Day-9/ (4pm) [98h]	190 hours/ 7.92 days
3	C	Day-1/ (4pm) [8h]	Day-2/ (10am) [18h]	Day-2/ (6pm) [8h]	Day-3/ (6pm) [24h]	Day-4/ (4pm) [22h]	Day-5/ (4pm) [24h]	Day-9/ (4pm) [96h]	192 hours/ 8.00 days
	T1	Day-1/ (4pm) [8h]	Day-2/ (8am) [16h]	Day-2/ (4pm) [8h]	Day-3/ (4pm) [24h]	Day-4/ (4pm) [24h]	Day-5/ (4pm) [24h]	Day-9/ (10am) [90h]	186 hours/ 7.75 days
	T2	Day-1/ (2pm) [6h]	Day-2/ (8am) [18h]	Day-2/ (2pm) [6h]	Day-3/ (2pm) [24h]	Day-4/ (12pm) [22h]	Day-5/ (12pm) [24h]	Day-9/ (4pm) [100h]	194 hours/ 8.08 days

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. Kruskal-Wallis H test does not reveal any statistical significance ($P > 0.05$) in the initial oviposition and duration for completing life cycle for *C. megacephala* among the three groups of carcasses.

were of that suggested for cleaning animal enclosures [18]. Since the abundance of putrefactive bacteria on the vigorously decomposing rabbit carcasses may be larger than that of animal enclosures, the single application of low concentrations of microbial inoculant performed here may not be adequate to alter the dynamics of necrobiomes for delaying the infestation by *C. megacephala* and *C. rufifacies*. Considering that murderers may avoid multiple entries to the crime scenes to minimize the possible transfer of trace evidence, the proposition of multiple applications of microbial inoculant to confuse forensic entomologist may appear unlikely. Therefore, the findings reported here indicating similarity in oviposition by *C. megacephala* and *C. rufifacies* in all the control, T1 and T2 carcasses, despite the presence of two different concentrations of microbial inoculant, prove as forensically relevant.

Results revealed that the earliest batch of 1st instar larvae for *C. megacephala* was observed during the first two visits on the second day of decomposition (8–10 am) in all the control, T1 and T2 carcasses, about 16–18 hours following the observations of eggs. Observation of the first batch of 2nd instar larvae for *C. megacephala* in all the groups of carcasses was recorded around 2–6 pm later that day. While the first batch of 3rd instar larvae for *C. megacephala* in all the groups of carcasses was invariably observed on day-3 of decomposition (by about 12–6 pm, i.e. 22–24 hours from the observation of 2nd instar larvae), the same for prepupae was observed on day-4 (12–4 pm). The pupation period for *C. megacephala* in

Table 4. Day and time for the first observation of the different stages of life cycle for *C. rufifacies* in control, treated 1 and treated 2 carcasses for all three replicate experiments.

Replicate	Group	Day and time for the first observation of							Total duration
		Eggs	1st instar	2nd instar	3rd instar	Prepupae	Pupae	Tenerals	
1	C	Day-2/ (10am) [26h]	Day-2/ (6pm) [8h]	Day-3/ (12pm) [18h]	Day-4/ (10am) [22h]	Day-5/ (6pm) [32h]	Day-6/ (6pm) [24h]	Day-10/ (4pm) [94h]	198 hours/8.25 days
	T1	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (12pm) [18h]	Day-4/ (8am) [20h]	Day-5/ (4pm) [32h]	Day-6/ (4pm) [24h]	Day-10/ (4pm) [96h]	200 hours/ 8.33 days
	T2	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (2pm) [20h]	Day-4/ (8am) [18h]	Day-5/ (4pm) [32h]	Day-6/ (4pm) [24h]	Day-10/ (4pm) [96h]	200 hours/ 8.33 days
2	C	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (4pm) [22h]	Day-4/ (8am) [16h]	Day-5/ (12pm) [28h]	Day-6/ (4pm) [28h]	Day-10/ (4pm) [96h]	200 hours/ 8.33 days
	T1	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (4pm) [22h]	Day-4/ (8am) [16h]	Day-5/ (12pm) [28h]	Day-6/ (12pm) [24h]	Day-10/ (10am) [94h]	194 hours/ 8.08 days
	T2	Day-2/ (10am) [26h]	Day-2/ (6pm) [8h]	Day-3/ (4pm) [22h]	Day-4/ (8am) [16h]	Day-5/ (12pm) [28h]	Day-6/ (4pm) [28h]	Day-10/ (4pm) [96h]	198 hours/ 8.25 days
3	C	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (12pm) [18h]	Day-4/ (8am) [20h]	Day-5/ (12pm) [28h]	Day-6/ (4pm) [28h]	Day-10/ (4pm) [96h]	200 hours/ 8.33 days
	T1	Day-2/ (8am) [24h]	Day-2/ (6pm) [10h]	Day-3/ (12pm) [18h]	Day-4/ (8am) [20h]	Day-5/ (4pm) [32h]	Day-6/ (4pm) [24h]	Day-10/ (4pm) [96h]	200 hours/ 8.33 days
	T2	Day-2/ (10am) [26h]	Day-2/ (6pm) [8h]	Day-3/ (4pm) [22h]	Day-4/ (12pm) [20h]	Day-5/ (4pm) [28h]	Day-6/ (4pm) [24h]	Day-10/ (4pm) [96h]	198 hours/ 8.25 days

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. Kruskal-Wallis H test does not reveal any statistical significance ($P > 0.05$) in the initial oviposition and duration for completing life cycle for *C. rufifacies* among the three groups of carcasses.

Table 5. Comparison of the durations for initial oviposition and the onset of other stages of life cycle for *C. megacephala* and *C. rufifacies* among the three groups of carcasses.

Groups / Stages	*C. megacephala*			*C. rufifacies*		
	C	**T1**	**T2**	**C**	**T1**	**T2**
Eggs	8 (8-8)	8 (8-8)	10 (6-10)	24 (24-26)	24 (24-24)	26 (24-26)
1st instar	18 (16-18)	16 (16-18)	16 (16-18)	10 (8-10)	10 (10-10)	8 (8-10)
2nd instar	8 (8-8)	6 (6-8)	6 (6-6)	18 (18-22)	18 (18-22)	22 (20-22)
3rd instar	22 (22-24)	24 (22-24)	22 (22-24)	20 (16-22)	20 (16-20)	18 (16-20)
Prepupae	24 (22-26)	24 (24-28)	24 (22-26)	28 (28-32)	32 (28-32)	28 (28-32)
Pupae	24 (24-24)	24 (24-24)	24 (24-26)	28 (24-28)	24 (24-24)	24 (24-28)
Tenerals	96 (96-96)	96 (90-96)	98 (94-100)	96 (94-96)	96 (94-96)	96 (96-96)
Completion	192 (192-192)	192 (186-192)	190 (190-194)	200 (198-200)	200 (194-200)	198 (198-200)

Data for the duration (hours) of the first observation of a particular stage of development are presented as median (range). Kruskal-Wallis H test does not reveal any statistical significance ($P > 0.05$) in the initial oviposition and duration for completing the other stages of life cycle for *C. megacephala* and *C. rufifacies* among the three groups of carcasses

all the carcasses started on day-5 of decomposition (12–6 pm), with the first emergence of tenerals observed on the 9th day (10 am–4 pm), bringing the total durations for completing the life cycles ranging between 7.75–8.08 days (186–194 hours) (Table 3). As for *C. rufifacies*, the earliest 1st, 2nd and 3rd instar larvae, as well as prepupae, pupae and tenerals for the control carcasses, were observed on day-2 (6 pm), day-3 (12–4 pm), day-4 (8–10 am), day-5 (12–6 pm), day-6 (4–6 pm) and day-10 (4 pm), respectively. The same stages of develop ment for *C. rufifacies* for the T1 and T2 carcasses were observably occurring at similar time with that of the control carcasses; the differences in du rations of 2–4 hours were statistically insignificant (Tables 4 and 5, Kruskall-Wallis H test: $P > 0.05$). The total durations for completing the life cycle for *C. rufifacies* in control, T1 and T2 carcasses ranged between 8.25–8.33 days (198–200 hours), 8.08–8.33 days (194–200 hours) and 8.25–8.33 days (198–200 hours), respectively (Table 4).

While small variations in the durations taken for completing the individual stages of the life cycle for *C. megacephala* and *C. rufifacies* among the control, T1 and T2 carcasses were observed, such variations remained statistically insignificant (Table 5, Kruskall-Wallis H test: $P > 0.05$). Notwithstanding, the durations observed here were consistent with the prevailing life cycle data for *C. megacephala* and *C. rufifacies* reported in Malaysia [5, 6, 10, 24, 31]. Therefore, it appears that the single application of diluted microbial inoculant (EM.1) at 1:500 (T1) and 1:100 (T2) onto rabbit carcasses may not significantly alter the duration for completing the life cycle for *C. megacephala* and *C. rufifacies*. One possible explanation for this phenomenon can be attributable to the acute and limited amount of EM that may not be sufficient to alter the abundance and dynamic structure of necrobiomes on the decomposing carcasses. In this context, previous studies [32, 33, 34, 35] on the influence of bacterial isolates on larval development reported that different species of fly larvae reacted differently towards the different types of bacteria. The fact that previous studies pertaining to the contributions of microbes to larval nutrition and development were limited to *Drosophila* sp. and other non-necrophagous insects alone [29], suitable comparisons with that of necrophagous dipterans like *C. megacephala* and *C. rufifacies* evaluated here could not be made. Therefore, *in vitro* examination of the possible interactions between the individual species of bacteria with oviposition behavior and the life cycle of *C. megacephala* and *C. rufifacies* should form an interesting future study.

Conclusion

The *C. megacephala* was the first necrophagous fly to oviposit in all carcasses, followed by *C. rufifacies*. However, a single application of microbial inoculant on T1 (1:500) and T2 (1:100) carcasses did not statistically impede oviposition by either species or their subsequent developmental patterns ($P > 0.05$). The duration of initial oviposition and life cycle completion for these two species were concurrent with that reported by previous studies in Malaysia. The fact that this is the first research that evaluated oviposition behavior and completion of life cycle for *C. megacephala* and *C. rufifacies* in the presence of microbial inoculant, the findings reported here provided empirical evidence for estimating mPMI in Malaysia when a single direct application of microbial inoculant was suspected.

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