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TECHNICAL NOTE

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Nocturnal Oviposition Behavior of Necrophagous Dipterans in Kelantan, Malaysia*

ABSTRACT: The likelihood of dipteran maggots colonizing a corpse due to nocturnal oviposition can be used to challenge the postmortem interval (PMI) estimated assuming diurnal oviposition. Earlier experiments tested nocturnal oviposition behavior by exposing fresh baits once during a single night. In this pilot study, oviposition behavior was studied using beef baits, which, simulating the decay of the body seen in case situations, decomposed inside cages designed to open and close at scheduled intervals during consecutive night or twilight periods. Freshly hatched maggots from diurnally oviposited eggs emerged in control baits on the third day, while a limited number of maggots attributable to nocturnal or twilight oviposition were observed in experimental baits only on the fifth or sixth day, indicating a categorical delay. These results suggest that such delayed and limited nocturnal oviposition is not forensically significant since the larger maggots deriving from diurnal oviposition would be the ones considered when estimating PMI.

KEYWORDS: forensic science, forensic entomology, necrophagous dipterans, twilight and nocturnal oviposition, fly-proof cage, postmortem interval

The presence of insects and their life stages when colonizing carrion provide reliable parameters for scientific estimation of the time elapsed since death (1–7). Blowflies are generally diurnal and oviposit during the day (1,3–5), though many factors may influence oviposition behavior (3,5). Variables influencing the oviposition behavior of dipterans include differences in geographical area (3), species differences, seasonal changes, the asynanthropic or urban nature of the habitat and the stage of tissue decay (5). Even ensuring the uniformity of baits left out in fly cages fails to attract flies equally, suggesting the presence of individual idiosyncrasies (5). Although diurnal, blowflies often lay eggs in dark areas during daytime and it has sometimes appeared that turning off the light in a lab situation can induce egg laying (3). Findings of specific studies on colonization attributable to nocturnal oviposition are diverse; some support oviposition during the night (5,8,9), some find no evidence for such oviposition (1,2,10–14), and a few suggest the possibility of nocturnal oviposition under specific environmental conditions (3,4). Differences in the methodology of previous studies, such as including artificial lighting (11), ensuring total darkness (9), and allowing (8) or preventing (11) access to the bait by crawling insects may also influence oviposition behavior during the night time. As murders are not field experiments (1,5), the time of oviposition assumed in a crime situation becomes one of the pivotal points in a trial. The prevailing uncertainty relating to nocturnal oviposition prompts challenges to the estimated postmortem interval (PMI) in two ways. First, if PMI is estimated as usual, considering oviposition to be a daytime activity (1,2,10–14), it can be called into question by suggesting the possibility of nocturnal oviposition, as supported by the work of one group of researchers (5,8,9). Second, if PMI is estimated supposing oviposition to be nocturnal (5,8,9), it can be questioned by citing the lack of evidence for nocturnal oviposition reported by the other group of researchers (1,2,10–14). Either way, the estimated PMI can be argued to extend about 10–12 h earlier or later (5)—a duration sufficient to confuse an investigation, or convince the judge or jury of an alibi. As there has been no consensus on the nocturnal oviposition behavior of necrophagous flies (15), the debate remains inconclusive (16). Previous controlled experiments investigating dipteran colonization due to nocturnal oviposition used fresh baits such as rat carcasses (5,8,12), mutton (9), chicken (11), beef and pig (12), and illegally killed bear cubs (14) that were subjected to a single exposure during the night excluding the influence of decomposition. Be that as it may, in case situations, decomposition is the singular and unique factor that necessitates the use of insects to provide a "biological clock" (5) that more precisely measures the time since death. Furthermore, the time ranges for the nocturnal exposure of the bait in the previous studies (8–12) did not include the twilight period. Astronomical twilight occurs near dawn and dusk, and it is defined as the time when the center of the sun is 12–18° below the horizon. Before the beginning of the astronomical twilight in the morning and after the end of the astronomical twilight in the evening, the sun does not contribute to sky illumination (17). Such intermediate levels of illumination during the twilight may affect oviposition behavior differently. In Malaysia, forensically important entomological specimens recovered from human cadavers have been analyzed retrospectively (18–22), and a key was published that identifies prevalent larval species (23). This pilot study was designed to gather empirical evidence on dipteran colonization attributable to twilight and nocturnal oviposition in Kelantan, Malaysia. The same bait was kept in fly-proof cages and exposed during

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consecutive night and twilight periods, simulating the phenomenon of decomposition prevailing in maggot-infested dead bodies in case situations.

Methods

Materials

Beef liver purchased from the local supermarket was used as bait to test the performance of the fly-proof cage (Figs. 1 and 2) fabricated here. Bovine (*Bos* sps.) meat was used as bait for oviposition experiments, and it was purchased at a slaughter house at about 04:30 hours, where animals had been slaughtered 1 h earlier. During the first cycle of the experiment, four samples of beef, each weighing about 500 g were packed in separate clean plastic bags and transported to the experiment site within 30 min of purchase. Each sample was placed in a separate fly-proof cage. Similar samples of fresh beef were used during the subsequent cycle of the experiment. Fly-proof cages were fabricated using a wooden base of 30.5 × 30.5 cm with 18 cm high wooden frames on the four sides supporting a plastic mosquito net of commercial type (1.25 mm mesh size) along the sides and top. Wooden planks 2.5 cm high along the four sides of the base prevented physical contact between the decomposing beef and the net. The cage was mounted on a 1 m high wooden pole to prevent access by vertebrate scavengers. The bottom of the pole was embedded into a cylindrical tin container and was filled with cement concrete to ensure stability and transportability. The net in the cage was made of two parts, one part permanently secured to the wooden frame and covering two of the four sides, and the other covering the remaining two sides and the top and able to be opened or closed using Velcro (VTB®, Malaysia, 25 mm loop and hook) (Figs. 1 and 2). During observations, a digital camera (Canon PowerShot A70, Malaysia, 3.2 mega pixels) was used to photograph and videotape the specimens.

FIG. 1—*A closed fly-proof cage with the bait (Phase I).*

FIG. 2—*An open cage exposing the bait (Phase I). Strips of hook (gray arrow) and loop (black arrow) of Velcro fasten while closing the cage.*

Experimental Setup

The experiments were conducted in three phases (Table 1).

Phase I (December 12–24, 2005) was a study to verify the effectiveness of the netting in preventing fly access as well as contamination by flies dropping eggs or maggots through the mesh in the net while the cage was closed. Here, beef liver was used as the oviposition medium. A single fly-proof cage containing the beef liver was kept closed for the first 3 days and then opened from the fourth day onwards until the liver liquefied on the eighth day.

Phase II consisted of two cycles of experiments, the first cycle (January 9–18, 2006) and the second cycle (January 31–February 9, 2006). Each oviposition medium (500 g of beef) was placed inside designated fly-proof cages and exposed during twilight (dawn and dusk), night time, or daytime on consecutive days to test oviposition behavior. Despite decomposition, the amount of beef used here supported maggot growth and enabled observation for up to 10 days.

In Phase III (February 3–9, 2006), beef was laid open on the ground to determine oviposition and colonization timing for bait in conditions simulating typical body disposal in case situations. Observations lasted for 7 days, and samples of maggots were reared for species identification.

TABLE 1—*Details of the experiments and the corresponding bait exposures.*

Phase and Cycle of Experiment	Duration of Experiment	Purpose	Cage Details	Duration for Opening the Cage	Duration for Closing the Cage	Duration of Bait Exposure (approximate)
Phase I	December 17–24, 2005	To test the effectiveness of the cage netting in preventing fly access or colonization due to contamination	Single cage	Days 4–8	Days 1–3	Days 4–8
Phase II						
Cycle 1	January 9–18, 2006	To investigate colonization due to nocturnal oviposition	Dawn cage	06.02–06.14 hours	07.24–07.29 hours	1 h 15 min
			Day cage	07.24–07.29 hours	19.13–19.24 hours	12 h
			Dusk cage	19.13–19.24 hours	20.27–20.31 hours	1 h 10 min
			Night cage	20.27–20.31 hours	06.02–06.14 hours	9 h
Cycle 2	January 31 to February 9, 2006	Replicate to investigate colonization due to nocturnal oviposition	Dawn cage	06.02–06.14 hours	07.24–07.29 hours	1 h 15 min
			Day cage	07.24–07.29 hours	19.13–19.24 hours	12 h
			Dusk cage	19.13–19.24 hours	20.27–20.31 hours	1 h 10 min
			Night cage	20.27–20.31 hours	06.02–06.14 hours	9 h
Phase III	February 3–9, 2006	To assess the duration for colonization of bait at ground level in the study site	No cage—bait left openly on the ground	–	–	7 days

All the experiments were carried out in open fields on the premises of the Universiti Sains Malaysia, Health campus, Kelantan, Malaysia. This equatorial (latitude 6°10′ N, longitude 102°17′ E) tropical region is about 5 m above sea level with uniform temperature averaging 29 ± 3°C, a high relative humidity of about 75 ± 10% and annual rainfall of 1.3–2.0 m (24). The monthly average rainfall during December 2005 ranged from 600 to 800 mm while during January and February 2006 it was about 100–180 mm (25). Weather data were obtained from the government source—Malaysian Meteorological Department (25) situated about 4 km from the study site.

Cage Assignment and Duration of Bait Exposure

During the first cycle of the Phase II experiment, bait was allowed to decompose for 10 consecutive days in four fly-proof cages, each one opened and exposed at a scheduled time, either dawn, day (control), dusk, or night. The experiment was replicated in the second cycle using fresh bait to ascertain reproducibility of the results. The astronomical definitions of dawn, day, dusk, and night were used to set the opening and closing times for each cage (Table 1), with an allowance of ±15 min to permit researchers to move from one cage to another. The control cage remained open during the daytime (07.24–19.24 hours), facilitating normal diurnal oviposition, and was closed during the night and twilight periods. The night cage was kept open during the night time (20.27–06.14 hours) and remained closed during the twilight (both dawn and dusk) as well as the daytime. Of the remaining two cages, the dusk cage was kept open during the evening twilight (19.13–20.31 hours), and the dawn cage remained open during the morning twilight (06.02–07.29 hours). The cages were placed in areas devoid of artificial light, ensuring natural darkness during the night time. A distance of at least 50 m was maintained between each cage to prevent fly populations attracted by one cage from transferring to another (26). The cages were placed at least 20 m away from trees and bushes and 50 m away from the nearest building in current use.

Periodicity of Observation

Each cage was observed twice a day: once during the forenoon and once during the afternoon before dusk. At each time, ambient temperature (AT) and carcass surface temperature (CST) were recorded, in addition to current weather conditions (sunny, windy, cloudy, drizzling or, rainy). Data on daily rainfall were obtained from the Malaysian Meteorological Department (25). Entomological observations included the presence of adult flies on the bait when the cage remained open, or on the net when the cage remained closed, the presence of eggs on the bait and the number of maggots, specifically the initial observation of freshly hatched ones. In the control baits, the number of crawling maggots counted is likely to be lower than the number present, as maggots were only counted if they could be individually resolved. As it is possible that more maggots were present in the crevices of the bait or cage, control bait counts are likely to be a conservative estimate. In contrast, the number of maggots counted in the baits in all the other cages (dawn, dusk, or night) is likely to be close to 100% of the maggots present, as the maggot population itself was sparse. The number of maggots collected for rearing varied from cage to cage and was correspondingly low in the dawn, dusk, and night cages, as maggots were sparse and a few had to be left in the bait to ensure the continued decomposition central to this study. Collected maggots meant for preservation were maintained in vials containing 80% ethanol; those collected for rearing were maintained at room temperature (24°C) in cups half-filled with sand, with small cubes of beef liver as a rearing medium. Emerging adults were killed using a few drops of ethyl acetate soaked in a cotton ball (27), with the rearing cup used as a killing jar. Killed adults were dried in a desiccator, pinned onto a Styrofoam board with suitable tags for identification and display. Taxonomic identification was based on the standard description available in the literature (5,23).

Results

The AT, CST, and rainfall recorded during the three phases of the experiments were compared (Table 2) using an ANOVA test. No significant difference in temperature occurred during the study period. Rainfall was significantly higher in Phase I than Phase II. In Phase I, rainfall was high on the first 3 days (45, 26, and 35.3 mm, respectively)—intermittent during the first 2 days with continuous drizzling on the third day. During the second cycle of Phase II, rainfall was high on the first and second days, as well as the seventh, tenth, and eighth days (10, 10, 8, 7, and 4 mm, respectively). However, on the first day, the daytime remained "sunny" while on the second day, it was "sunny" and "windy," with rainfall restricted to the night time in both cases.

In Phase I, maggots were not observed in the bait during the first 3 days, when the fly-proof cage was maintained closed. The cage

TABLE 2—*Ambient temperature (AT), carcass surface temperature (CST), and rainfall during the three phases of experiments.*

Variable	Mean (SD)	*F* statistics	*p*-Value
AT			
Phase I: Pilot study	26.31 (1.62)	1.174	0.323
Phase II: Cycle 1	27.79 (2.30)		
Phase II: Cycle 2	26.94 (2.77)		
Phase III: Open study	27.21 (2.75)		
CST			
Phase I: Pilot study	26.68 (1.46)	2.551	0.060
Phase II: Cycle 1	29.28 (3.44)		
Phase II: Cycle 2	27.55 (3.14)		
Phase III: Open study	28.78 (4.14)		
Rainfall			
Phase I: Pilot study	29.34 (27.07)	31.30	0.000
Phase II: Cycle 1	0.40 (0.81)		
Phase II: Cycle 2	4.22 (3.97)		
Phase III: Open study	2.14 (3.53)		

was opened on the third day at 17.00 hours, and eggs and freshly hatched maggots were seen on the fourth day (time of observation, 15.30 hours).

The number of maggots observed and counted in the baits by day and cage during the two cycles of Phase II experiments is provided in Table 3. In the first cycle of Phase II, five freshly hatched maggots were first sighted in the night cage on the sixth day (time of observation, 09.41 hours), while maggots were not observed in the dawn and dusk cages at any point during the 10-day experiment. In the first cycle control cage, eggs were seen on the second day (time of observation, 15.03 hours), and freshly hatched maggots were seen on the third day (time of observation, 11.17 hours). By the sixth day, when five freshly hatched maggots were sighted in the night cage, the number of maggots in the control cage was 21, a significant difference ($\chi^2 = 9.846$, $p = 0.002$). Furthermore, by the sixth day, the largest of the control cage maggots had reached the prepupal stage, wandering away from the bait, while on that day, the night cage maggots had only reached the first instar stage.

In the second cycle of Phase II, freshly hatched maggots were first sighted on the fifth day in the night, dawn, and dusk cages: seven maggots in the night cage (time of observation, 11.38 hours), five maggots in the dusk cage (time of observation, 11.48 hours), and six maggots in the dawn cage (time of observation, 11.55 hours). In the control cage, eggs were seen on the third day (time of observation, 11.17 hours), and freshly hatched maggots were observed a few hours later (time of observation, 18.06 hours). In this cycle, when five to seven freshly hatched maggots were first observed in the dawn, dusk, and night cages, the number of maggots in the control cage was 22, also a significant difference ($\chi^2 = 7.759$, $p = 0.005$). The largest maggots among those in the control cage were in the third instar stage.

Cross-tabulation of both cycles of Phase II experiments, comparing the number of maggots present in each cage type on the fifth and sixth day, reveals that there is no significant difference in the pattern of infestation between the two cycles of experiments (2×2 $\chi^2 = 19.85$, $p = 0.000$).

In Phase III, where bait was laid open on the ground, eggs were seen about 30 min after the bait was exposed and freshly hatched maggots were observed on the second day.

Rearing the maggots collected from each cage as well as the bait in Phase III produced adults belonging to the two families, Calliphoridae (*Chrysomya megacephala* [Fabricius]) and Sarcophagidae (*Sarcophaga sps.*).

Discussion

Both groups of earlier researchers, those who reported positive evidence supporting dipteran colonization due to nocturnal oviposition (8,9) as well as those who found a lack of evidence for such oviposition (10–14), relied on solitary exposure of fresh baits for defined periods in a single night. As the application of forensic entomology in case situations is necessitated when delayed discovery of a body creates a challenge in determining the time of death, it is pertinent to test dipteran nocturnal oviposition behavior using a decomposing bait, simulating the decomposition that occurs in case situations.

Here, the use of 500 g of beef, a bulkier oviposition medium than rats (8,12) or mutton pieces (9), ensures better attraction of the flies to the bait and offers prolonged support for maggots during decomposition. Beef was purchased from the slaughterhouse at a time without fly activity (04.30 hours), preventing accidental oviposition. While previous researchers have exposed bait for varying periods—3 h (8), 5 h (9), or 3, 7, and 8 h (12)—in a single night, here, natural conditions were simulated by exposing each bait for fly attraction during a designated interval, either the whole of the astronomical night (about 9 h) or dawn or dusk (each about 1 h 15 min), and the exposure was continued on all the consecutive days until the completion of the experiment. Replacement with fresh bait enabled the replication of a similar continuum of decay in a second cycle of the experiment. Applying astronomical classifications of time defined the periods of night, dawn, and dusk precisely and in terms easily understood by the locals. Incidentally, the understanding of dawn, dusk, and night time by the Malay population as influenced by the Islamic prayer times prescribed by the authorities (28) aligns with the time schedule described by the astronomical classification.

The possibility of daytime colonization contaminating the twilight and night time cages is discounted by the absence of colonization of the bait in Phase I during the first 3 days when the cage remained closed. Furthermore, colonization failed to occur in the baits in the dawn and dusk cages in the first cycle of Phase II

TABLE 3—*Number of maggots counted in the cages by day during the cycles of experiments in Phase II.*

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Cycle 1										
Control cage	0	0	10	15	23	21	18	9	4	1
Dusk cage	0	0	0	0	0	0	0	0	0	0
Night cage	0	0	0	0	0	5	6	0	3	1
Dawn cage	0	0	0	0	0	0	0	0	0	0
Cycle 2										
Control cage	0	0	8	13	22	10	17	30	13	0
Dusk cage	0	0	0	0	5	0	0	9	35	1
Night cage	0	0	0	0	7	4	0	12	7	3
Dawn cage	0	0	0	0	6	1	0	13	0	12

through the tenth day, and, if the nets were not a sufficient barrier to nocturnal or diurnal oviposition, colonization would have been observed. Thus, contamination from eggs or larvae dropped through the mesh openings in the net is discounted; the cages are verified as fly-proof.

Rainfall was significantly higher during Phase I, and it may appear that high rainfall could be a factor preventing colonization during the first 3 days of Phase I. However, rain was intermittent during the daytime on the first 2 days with drizzling being continuous on the third day. Similarly, higher rainfall of 10.0 mm on the first and second day of the second cycle in Phase II was restricted to the night time with the daytime being "sunny." Although eggs were observed 1 day later in the control cage of the second cycle than the first cycle, the first set of maggots was observed on the same day in both cycles, indicating that the rain had not adversely affected the colonization pattern.

Studies have revealed that diurnal oviposition can occur within "minutes," "2–3 h," "12 h after death," or that it "may not occur at all" (5). The experiment in Phase III assessed the time required for oviposition when bait was laid open in the study site. Elevating the cages appeared to have delayed oviposition in the control baits of both Phase II cycles by at least 1 day, as oviposition was observed within 30 min when the bait was laid open on the ground (Phase III). It seems possible that nocturnal oviposition at ground level would also be advanced by a day, compared with the time taken for nocturnal oviposition in an elevated cage, but it is reasonable to assume that nocturnal versus diurnal differences in the growth and number of maggots would remain similar, regardless of cage elevation. However, experiments designed to study oviposition behavior on decomposing baits at ground level are necessary to confirm the above supposition.

Maggot counts for the dawn and dusk cages (Table 3) represent the maximum number of maggots that could be observed during that visit. Counts include the maggots collected for preservation or rearing but exclude any dead maggots. Maggot mortality was observed in both Phase II cycles and was higher in the control cages, starting from the sixth day in the first cycle and from the seventh day in the second cycle. Dead maggots and their tissue residue were found along the periphery of the wooden platform of the cage, indicating that the migrating maggots had been obstructed by the cage and its net. The fluctuation in the number of maggots observed after the sixth day (Table 3) is attributable to both mortality and removal for preservation and rearing. To control for these circumstances, only the number of freshly hatched maggots first observed and counted on the third and sixth days of the first cycle and the third and fifth days of the second cycle (Table 3) are considered in testing the significance of difference in the pattern of infestation by cross-tabulation.

Separate bait exposure during the dawn and dusk periods allowed a study of the flies' tendency to oviposit during the twilight periods. The presence of significantly fewer maggots in the dawn and dusk cages reinforces earlier observations that fly activity declines in late afternoon, probably in response to diminishing light (3,5), that the flies settle in for nocturnal rest by evening (5), and that they are not early risers (1,5). The novel finding of this study is that colonization due to nocturnal oviposition is limited and categorically delayed relative to a control when decomposing baits are exposed during consecutive nights. In both cycles of experiments, by the time that freshly hatched maggots could be sighted in the dawn, dusk, and night cages, maggots in control baits (where diurnal oviposition was permitted) had reached the third instar. This provides empirical evidence that colonization attributable to nocturnal oviposition is not a forensically significant phenomenon in decomposing bait that simulates the corpse of a case situation. As seasonal variations influence dipteran oviposition behavior (5), nocturnal oviposition should be studied over the course of a year to determine any annual variations, allowing inferences to be drawn to case situations across a variety of seasons and weather conditions.

Limitations

Although the oviposition medium used here was bulkier than those used by earlier researchers (8,9), the attraction of flies to 500 g of beef cannot be equated to their attraction to a human corpse, irrespective of diurnal or nocturnal behavior, as it is known that the potential of a carcass to attract oviposition varies from animal to animal and may vary with time even in the same animal (3,5). Previous research also demonstrates that fly arrival time on small mammals may not be valid for human corpses (5). However, this limitation is a common one in oviposition studies using media other than adult pigs.

Although sarcophagid adults emerged while rearing maggots collected from the cages, their proportion in relation to the calliphorid adults could not be precisely determined as the collection of samples was nonrandom, being restrained by the smaller number of maggots available in the dawn, dusk, and night cages, especially as some had to be left in the bait to ensure the continuum of decomposition. Further research designed to explore the relative proportions of species among nocturnally oviposited maggots would permit inferences about the tendency of the various species to oviposit nocturnally.

Conclusion

Previous workers researching nocturnal oviposition by necrophagous flies have used fresh bait for solitary exposure during a single night. Here, use of a decomposing oviposition medium simulates a corpse that is discovered after some delay. This study reveals that colonization due to oviposition during the dawn, dusk, and night is limited and is categorically delayed by about 2–3 days when compared with the colonization of the control baits exposed during the daytime. By the time that freshly hatched maggots are observed in the dawn, dusk, or night cages, maggots in day-exposed cages had reached the second instar stage. Due to their larger size, it is these diurnally oviposited maggots that would be considered when estimating PMI, according to customary forensic practice. The novel approach of this study, where the same baits were exposed on consecutive days and nights, allowed nocturnal oviposition behavior to be studied during the course of decomposition as it would occur in case situations. These findings provide empirical evidence that, in the climatological conditions prevailing in Kelantan, Malaysia, limited colonization due to nocturnal oviposition occurs in a decomposing bait only after the fourth or fifth day, while maggots due to diurnal oviposition colonize the bait on the second or third day. Such delayed and limited dipteran nocturnal oviposition is not a forensically significant phenomenon when estimating PMI.

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