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Effects of various lethal doses of amitriptyline to the length of *Calliphoridae larvae*

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ABSTRACT

Background: Uses of insects to predict Post-mortem Interval (PMI) is important for non-natural causes of death such as drug abuses. Doses of drugs in a corpse are presumed to affect rates of growth and development of insects and relationships to predict the PMI.

Objective: This study aims to study effects of various doses of lethal amitriptyline to the length of *Calliphoridae* larvae stage as the first organism seen on a corpse as well as to assess other factors involved in insect growth such temperatures and humidity.

Methods: This study was an experimental research using 24 dead rats as larva's growth media. These rats were divided into four group: the control group, the first treatment (T1) group, the second treatment (T2) group, and the third treatment (T3) group. The control group was deceased by cervical dislocation representing natural cause of death, while the treatment groups were given various doses of amitriptyline orally. The doses given to T1, T2, and T3 groups were 75 mg, 100 mg and 125 mg, respectively. Next, the rat corpses were put into cages containing *Calliphoridae* larvae; daily observation was conducted every morning and afternoon until the larvae transformed into pupae. All obtained data were analysed by using a multivariate linear regression analysis, Spearman correlation and Kruskal-Wallis.

Results: This study showed that the more doses of amitriptyline, the longer larva cycle and the shorter length of larvae ($p<0,05$). The life cycle time in the control, T1, T2, and T3 groups were four, five, eight and nine days, respectively. Based on the first day of larva appearance in each group, the control group produced an average length of larvae longer than the T1, T2, and T3 groups, respectively 8.33 mm, 7.33 mm, 4.5 mm and 5.67 mm. However, differences of temperatures and humidity observed in the routine morning and afternoon did not have any differences.

Conclusion: Increasing more doses of amitriptyline extended the larva cycle that could cause the larva length to be shorter in the treatment group compared to the control group on the same day. Environmental factors in this study had smaller effects on the larva length growth of the Calliphoridae larvae.

Latar Belakang: *Penggunaan serangga dalam menentukan Postmortem interval (PMI) merupakan hal yang penting terutama bagi kematian tidak wajar seperti overdosis obat-obatan. Dosis obat dalam jasad diperkirakan berpengaruh terhadap pertumbuhan dan perkembangan serangga yang selanjutnya*

mempengaruhi penentuan PMI.
Tujuan: *Penelitian bertujuan untuk mengetahui pengaruh pemberian variasi dosis lethal amitriptilin terhadap panjang larva lalat famili Calliphoridae yang merupakan organisme yang pertama terlibat, serta menilai faktor lain yang terlibat dalam pertumbuhan serangga dalam hal ini suhu dan kelembapan ruangan.*
Metode: *Penelitian ini merupakan penelitian eksperimental menggunakan 24 bangkai tikus sebagai media tumbuh larva. Tikus tersebut dibagi menjadi empat kelompok yaitu kelompok kontrol, perlakuan pertama (T1), perlakuan kedua (T2), dan perlakuan ketiga (T3). Pada kelompok kontrol dibunuh dengan dislokasi servikal mewakilkan kematian secara alami, pada kelompok perlakuan diberi amitriptilin secara oral. Dosis pemberian untuk kelompok T1, T2 dan T3 masing-masing secara berurutan sebesar 75 mg, 100 mg dan 125 mg. Bangkai tikus dimasukkan ke dalam kandang berisi lalat famili Calliphoridae, selanjutnya diamati setiap pagi dan sore hingga larva menjadi pupa. Data dianalisis dengan analisis multivariat regresi linear, korelasi Spearman dan analisis Kruskal-Wallis.*
Hasil: *Didapatkan bahwa semakin besar dosis maka semakin lama siklus larva lalat dan semakin pendek larva lalat tersebut (p<0,05). Lama siklus hidup pada kelompok kontrol, T1, T2, dan T3 secara berurutan yaitu empat, lima, delapan dan sembilan hari. Sejak hari pertama munculnya larva, kelompok kontrol menghasilkan rata-rata larva yang lebih panjang dibandingkan kelompok T1, T2, dan T3 yaitu secara berurutan 8,33 mm, 7,33 mm, 4,5 mm dan 5,67 mm. Namun, perbedaan suhu dan kelembaban pengamatan pagi hari dengan pagi berikutnya tidak memiliki banyak perbedaan, begitu pula dengan pengamatan sore hari.*
Kesimpulan: *Hasil penelitian menunjukkan bahwa semakin besar dosis amitriptilin maka semakin panjang siklus larva untuk menjadi pupa sehingga semakin pendek panjang larva pada kelompok perlakuan dibandingkan dengan kelompok control pada hari yang sama. Serta, faktor lingkungan pada penelitian ini memiliki pengaruh yang lebih kecil terhadap pertumbuhan panjang larva lalat famili Calliphoridae.*

INTRODUCTION

Post-mortem Interval (PMI) is average interval time between onset of death and founding of corpse.[1] Death according to causes of death was divided into natural death, unnatural death (murder, suicide, and accident), and unexplainable.[2] Drug overdoses are one of the most commonly used as suicide methods in most countries in Europe.[3] Based on data in UK in 2015, tricyclic antidepressants are one of the most commonly used drugs.[4] There are times of drugs related to deaths that are discovered after a period of time and the corpse could be highly decomposed; this often occurs when a person died in isolated places or suicides.[5,6] That condition causes organic source predictors of PMI such as blood, urine, or internal organ are not readily available, so insects may be an alternative specimen to do a toxicological analysis and to determine the PMI.[6,7] *Sarchophagidae* (flesh flies) and *Calliphoridae* (blow flies) families are the most important families because those are the first insects found in a corpse.[8] The *Calliphoridae* can find the source of odours with excellent special accuracy and lay eggs in the body within minutes to hours since death.[9]

The presence of various metabolic drugs and toxic inside the insects may change the development time that can be predicted before. If chemical compounds in a corpse tissue is consumed by larvae, they may be able to change the development of these insects into several hours or day.[10] This can make estimation of PMI wrong if effects of drugs are not considered.[11] Furthermore, another factor can accelerate and/or decelerate the development of insects such as environmental factors (i.e temperature and humidity), food quality and quantity.[8,12] High temperatures and low humidity can increase the development of larvae and pupae if there are sufficient food supplies, as well as the opposite.[13] A prior study by Goff in 1993 had studied effects of imparting amitriptyline to the length of *Parasarcophaga ruficornis* larvae (the flies from the *Sarchopagidae* family). Its result showed that there were extended time of pupae and post feeding stage. Amitriptyline also causes greater length and weight than larvae in the control group.[8] Another study in Indonesia using musca sp from Muscidae family found that amitriptyline could shorten the growth of *musca sp* larvae.[14] Studies to investigate effects of amitriptyline to the growth of the *Calliphoridae*

family as the most important family in forensic investigation need further research. Therefore, the authors aimed to investigate effects of lethal amitriptyline in various doses to the length of *Calliphoridae* larvae and to asses other factors involved in larvae growth such as temperatures and humidity.

METHODS

This study is a true experimental research in an outdoor setting, at the Biology Garden of Universitas Mataram in August 2019. Ethical clearance was signed by Ethics Committee, Faculty of Medicine, Universitas Mataram, (number 179/UN18.F7/ETIK/2019, dated 07/25/2019). The growth media used were 24 dead male rats of Rattus norvegicus strain wistar aged 8-12 weeks old with a weight of 150-200 grams. The male rats were chosen over female rats due to hormonal fluctuations that can cause variable nature of female data.15 The rats were randomly divided into four groups: the control group, the first treatment group (T1), the second treatment group (T2), and the third treatment group (T3). Their sample sizes were calculated based on Federer's formula described as (T - 1) (r - 1)> 15 where T was the number of research groups and r was the number of samples for each group, so the number of samples for each group was six rats with 24 rats of all experimental animals used.[16] However, one experimental animal to each group was added to prevent loss of experimental units, so that the total number of rats used was 28 rats. There were 28 experimental animals, but when this study was conducted there were dead rats due to aspiration during the treatment, so four rats were excluded from this study. 24 experimental animals with six experimental animals in each group were analysed (Figure 1).

The control group deceased by cervical dislocation represented natural causes of death while the treatment group was given various doses of amitriptyline orally.[17] The doses given to T1, T2 and T3 groups were 75 mg, 100 mg and 125 mg, respectively; they were counted to be equal to 4200 mg, 5600 mg, and 7000 mg in human, respectively.[18] Each rat was given amitriptyline once for each dose according to the dose determined in each group. The rats were then left for thirty minutes with an estimation that all the drug had been distributed in the rat body, and the rats were died by overdoses of the drug.

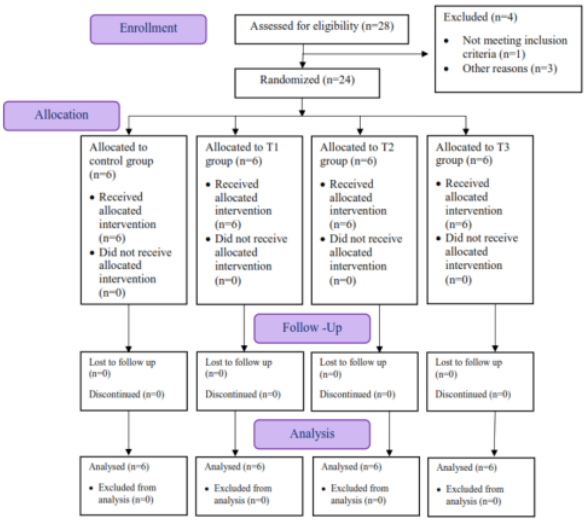

Figure 1. The arrangement flowchart of the subjects

To fasten the flies laying eggs on carcass, post mortem incision was made sagittally in the truncus midline from neck to anus until the visceral organs were visible. The rat corpses then were put into cages containing fifteen flies from *Calliphoridae* larvae. Daily observation was conducted every morning (06.00- 07.00 local time) and afternoon (16.00 -17.00 local time) until final stage of fly cycles of pupae transformed.[19] Every morning and evening, observation of the length of larvae and temperatures and humidity of the room was conducted. One largest larva was selected by manual comparison from each sample, so 24 larvae were assessed every day. Temperatures and humidity were measured twice daily by using beurer HM16 thermohygrometer.

Multivariate linear regression was used in this study to see the variables having the strongest influence among variations of amitriptyline doses, temperatures, and room humidity on the length of *Calliphoridae* larvae. Correlations between dependent variables and independent variables were analysed by using a Spearman correlation. To determine a significant difference in the larva length of each group, an analysis was conducted by Kruskal-Wallis analysis.

RESULTS

Larva Length

The average length of Calliphoridae larvae in the control and treatment groups was described below (table 1). Table 1 demonstrated that larva growth in the control media could reach an average maximum length of 16.33 mm on the fourth day in the morning, and then it had become a pupa on the fifth day in the morning. T1 group could reach an average maximum length

Table 1. The average length of larvae in each group

	Length (mm) ± 1SD			
Days	**Control**	**Amitriptyline 75 mg (T1)**	**Amitriptyline 100 mg (T2)**	**Amitriptyline 125 mg (T3)**
1m	NS	NS	NS	NS
1a	NS	NS	NS	NS
2m	7,17 ± 1,17	6,83 ± 0,90	4,00 ± 0,82	NS
2a	8,33 ± 3,39	7,33 ± 0,75	4,50 ± 0,96	NS
3m	13,67 ± 4,27	14,50 ± 1,38	5,83 ± 0,90	NS
3a	14,83 ± 2,48	15,67 ± 0,47	6,33 ± 0,47	NS
4m	16,33 ± 0,52	16,17 ± 0,69	10,33 ± 4,68	NS
4a	15,67 ± 1,03	16,67 ± 0,47	11,00 ± 3,42	5,67 ± 0,82
5m	NF	17,33 ± 0,74	13,33 ± 2,56	8,00 ± 0,63
5a		15,83 ± 0,68	14,00 ± 2,65	9,50 ± 0,55
6m		NF	11,67 ± 1,25	13,17 ± 1,60
6a			12,00 ± 1,15	15,17 ± 1,17
7m			10,83 ± 1,07	17,33 ± 0,82
7a			11,17 ± 0,69	17,33 ± 0,82
8m			NF	17,00 ± 0,89
8a				17,17 ± 0,98
9m				16,17 ± 0,75
9a				17,00 ± 0,89
10m				NF

m= morning ; a= afternoon; NS= larvae are not seen in daily observation; NF= next stage forms of pupae

of 17.33 mm on the fifth day in the morning, and then it became pupae on the sixth day in the morning. T2 group can reach an average maximum length of 14.00 mm on the sixth day in the morning, and then it became pupae on the eight day in the morning. Meanwhile, T3 group could reach an average maximum length of 17.33 mm on the seventh day in the morning, and then it became pupae on the tenth day in the morning. This indicated that the life cycle of the *Calliphoridae* family from eggs to pupae extended in the treatment group compared to the control group. In addition, it could be observed that the larva infestation time to the corpse became shortened in the T3 group, which took three and a half days until the larvae shown in corpse compared to the control, T1, and T2 group which took one day.

All observational data were not normally distributed (Shapiro-Wilk, p<0,05), so that the correlation test used was the Spearman correlation test (Table 2). The Spearman correlation test was conducted to determine the correlation between amitriptyline dose variations and the length of larvae in the morning and evening observation. Significant differences in the length of larvae from each group were identified through the non-parametric Kruskal-Wallis test followed by a Post Hoc analysis for Kruskal Wallis that was Mann- Whitney (Table 3).

Table 2. Spearman correlation analysis in the morning and evening observations

Spearman Correlation Analysis for Morning Observation	**Observation days-**					
	Two	**Three**	**Four**	**Five**	**Six**	**Seven**
Correlation (r)	-0,901	-0,878	-0,835	-0,938	0,447	0,885
P	0,000	0,000	0,000	0,000	0,145	0,000
N	24	24	24	18	12	12
Spearman Correlation Analysis for Afternoon Observation	**Observation days-**					
	Two	**Three**	**Four**	**Five**	**Six**	**Seven**
Correlation (r)	-0,873	-0,879	-0,755	-0,755	0,818	0,885
P	0,000	0,000	0,000	0,000	0,001	0,000
n	24	24	24	18	12	12

The morning observation showed a significant correlation between the larva length and amitriptyline doses from the second day to the seventh day observation except on the sixth day (Spearman, p <0.05). The second to fifth day observation showed a strong negative correlation, while the seventh day showed a positive correlation value. The spearman correlation analysis in the afternoon observation showed a significant correlation (p <0.05) on the second to the seventh day observation. The second to the fifth day observation showed strong negative correlations, while the seventh and the sixth days showed a strong positive correlation value. The negative correlation showed that the relationship was not unidirectional, meaning that the greater amitriptyline doses, the smaller larva length, while the positive correlation is the opposite.

There were significant differences in the larva length between the control group and the treatment group for the second day observation until the seventh day observation (except the sixth day) in all groups (Kruskal-Wallis, p<0,05). There were no significant differences between the control group and the T1 group on the second to seventh day observation (Mann Whitney, p>0,05). Significant differences were seen between the control group and the T2 group in the morning observation for the second and the third days, and in the afternoon observation for the second, third and fourth days (Mann Whitney, p <0.05). Significant differences between the control group and the T3 group were only found on the fourth day in the afternoon (Mann Whitney, p <0.05). Significant differences

between the T1 group and the T2 group showed significant values in the morning observation for the second day, third, fourth and fifth days, and in the afternoon observation for the second, third and fourth days (Mann Whitney, $p < 0.05$). Significant differences between the T2 group and the T3 group were found in the morning observations for the fifth and seventh days, and in the afternoon observation for the fourth, fifth, sixth, and seventh days (Mann Whitney, $p < 0.05$).

Table 3. Results of Kruskal-Wallis test followed by Mann-Whitney test

Days	Kruskal-Wallis test result for all groups	Significance of differences in length between groups using the Mann-Whitney test					
		C-T1	C-T2	C-T3	T1-T2	T1-T3	T2-T3
2m	P= 0,000	0,613	0,004	-	0,003	-	-
2a	P= 0,000	1,00	0,006	-	0,004	-	-
3m	P= 0,000	0,870	0,05	-	0,004	-	-
3a	P= 0,000	0,727	0,003	-	0,003	-	-
4m	P= 0,000	0,589	0,014	-	0,007	-	-
4a	P= 0,000	0,080	0,016	0,003	0,003	0,003	0,004
5m	P= 0,001	-	-	-	0,012	0,004	0,005
5a	P= 0,006	-	-	-	0,253	0,003	0,036
6m	P= 0,138	-	-	-	-	-	-
6a	P= 0,007	-	-	-	-	-	0,007
7m	P= 0,003	-	-	-	-	-	0,003
7a	P= 0,003	-	-	-	-	-	0,003

m= morning; a= afternoon; C = control group; T1 = Treatment 1 Group; T2 = Treatment 2 Group; T3 = Treatment 3 Group

Based on the larva length data (Table 1), differences of larva length in each treatment group were found by comparing data based on the first day of larva appearance in each group. Data on table 1 demonstrated that the control group produced an average length of larvae that were longest than the other groups since the first day of larva appearance compared to the T2 and T3 groups. Comparing the treatment groups, larva length of the T1 group appeared to be the longest until the third day than the T2 and T3 groups. Since larva first appearance, the T2 group became the group with the lowest length when compared to the control, T1 and T3 groups. Since the first day of larva appearance, the T3 group continued to grow until it reached its maximum length on the fourth day, and subsequently it showed a slight decrease before finally becoming pupae on the sixth day since the first larva was found.

Significant correlation between larva length and amitriptyline from the first day of larva appearance to the sixth day were found on the first, second, third and sixth day for morning observation (Spearman, $p<0.05$). Negative correlation was found on the first day of larva appearance until the third day (-0.901, -0.594, and -0.608, respectively), while on the sixth day it showed a strong positive correlation value (0.885). Meanwhile, in the afternoon observation the significance correlation was found on the first day of larva appearance until the sixth day, except on the third day (Spearman, $p<0.05$). Strong negative correlation was found on the first day of larva appearance until the second day (-0.600 and -0.653, respectively); in contrary to the fourth to sixth day, it showed a strong positive correlation value (0.627, 0.833, and 0.881, respectively).

There were significant differences in

larva length between the control group and the treatment group on the first day of larva appearance to the sixth day (Kruskal Wallis, p <0.05), except on the fourth and fifth days. Significant difference between the control and T1 group was only seen on the third day in the afternoon (Mann Whitney, p< 0.05). Significant differences between the control and the T2 groups were found on the first day of larva appearance until the third day (Mann Whitney, p< 0.05). Significant differences between the control and T3 group, between the T1 and T2 group, between T1 and T3 group were found on the first day of larva appearance until the third day in the morning (Mann Whitney, p< 0.05). Significant differences between the T2 and T3 groups were found on the first day of larva appearance until the third day (Mann Whitney, p< 0.05), except on the third day in the morning.

Room Temperature and Humidity

This study also measured the temperatures and humidity of the room where the control and treatment groups were placed (Table 4).

Table 4. Room temperatures and humidity data

Days	Temperature (°C)	Humidity
1 m	22,8	82%
1 a	29	66%
2 m	24	80%
2 a	29,1	62%
3 m	23,5	81%
3 a	27,8	65%
4 m	22,3	85%
4 a	27,2	65%
5 m	23,9	85%
5 a	28,9	68%
6 m	22,8	82%
6 a	28,8	66%
7 m	23,1	82%
7 a	28,7	66%
8 m	24,8	81%
8 a	28	71%
9 m	25,2	79%
9 a	28,2	69%
10 m	23	82%

m= morning ; a= afternoon

Table 4 showed that the humidity in the morning was higher than in the afternoon, while the temperatures was smaller in the morning observation than in the afternoon. The average temperatures in the morning observation was 23.6°C compared to the afternoon observation which was 28.4°C. The average humidity in the morning observation was 81.8% compared to the humidity in the afternoon which was 66.4%. However, there were no differences of temperature and humidity in the morning observation with the following morning and in the afternoon observation. The average difference of temperatures observed in the morning and afternoon with the following morning and afternoon observation was 1.1°C

and 0.42°C, respectively. Meanwhile, the average difference of humidity observed in the morning and afternoon with the following morning and afternoon observation was 1.78% and 2.37%, respectively. The highest temperature in the morning observation was found on the ninth day observation with 25.2°C and the lowest was on the fourth day observation with 22.3 °C. The highest humidity in morning observation was found on the fourth and fifth days observation which was 85%, and the lowest was on the ninth day observation which was 79%.

Multivariate linear regression was conducted to see variables that have the strongest influence among variations of the amitriptyline doses, temperature and room humidity on the length of *Calliphoridae* larvae. First, a bivariate analysis was performed in the morning and evening data, and all variables (dose, temperatures in the morning, and humidity in the morning) showed that they met the criteria for the multivariate analysis ($p < 0.05$). In the multivariate analysis in morning and evening data, the variables that had the strongest influence on larvae length was the dose variable (morning correlation coefficient: -0.621; and afternoon: -0.575) which had a correlation coefficient value exceeding the temperature variable (morning correlation coefficient: -0.352 and evening: -0.518). Meanwhile, the least significant variable was the humidity variable.

DISCUSSIONS

These data showing unnormally distributed indicated that there were various growth rates of intra-species of the data. These strongly indicated that there were habitat's competition in taking nutrition and living needs between the larvae, called population density. This population density could cause a slow growth rate, a smaller body size, and an increasing mortality rate. Also, various nutrition composition was provided by the dead rats in a specific organ/ tissue, for example: brain tissue was less nutritious than visceral organs.[8]

The relationship between dose variations of amitriptyline and the larva length showed a significant association ($p < 0.05$ value), in the morning and evening observation for the second to seventh day, except on the sixth day in the afternoon (Spearman, $p<0.05$). Correlation values on the second to fifth day, both in the morning and evening observation, showed a strong negative correlation. Negative correlation indicated that the greater the dose of amitriptyline, the smaller the larva length. Meanwhile, on the seventh day in the morning and the sixth to the seventh day of the afternoon, a positive correlation value was obtained. The positive correlation value indicated that the greater dose of amitriptyline, the greater larva length.

This result can be linked to data in table 1 which showed extension of the life cycle of *Calliphoridae* larvae in the treatment group compared to the control group; therefore, it was assumed to cause shortening of larva length. These data are also similar to a prior study by Rahman et al. showed that amitriptyline could decrease the growth of musca sp larvae.[14] This could be caused by the amitriptyline in these experiments which have effects of inhibiting serotonin; serotonin in *Calliphoridae* family had control over the salivary glands in the flies by increased fluid secretion from salivary glands and various physiological and behavioural aspects related to the process of feeding.[20–22] Intense of feeding process in soft tissue was generally characterized by fast and efficient food assimilation which further contributed to acceleration of growth rates.[8]

The effects of the drugs could also be seen from the times of insects to invest the corpses. The T3 group had the longest time to invest the corpses, approximately for 89 hours. This could be assumed that it was caused by the scent of corpses containing amitriptyline different from the scent of corpses in the control group. Insects use chemical signals to determine the body and then decide whether it was a suitable as a food source, a place to lay eggs, and larva development. Searching for the food in adult flies was directly related to ability of olfaction.[8]

Based on the results of linear regression multivariate analysis on the second to fourth

day observation in the morning and evening observation showed that the most independent variable that influenced the larva length were the dose variations of amitriptyline. This showed that environmental factors in this study, especially the ambient temperature, had a smaller effect than the variation of the amitriptyline dose on the larva length of the Calliphoridae family. These results due to the study site used were the same place, time of observation was conducted in a short period in which there was no extreme weather changes during the study, and larvae length measurements were done almost at the same hour. Also, there was not difference in the temperatures or humidity data. The average temperature recorded in the morning and afternoon observation was 23.6°C and 28.4°C, indicating that the temperatures at the time of observation did not exceed the upper limit of temperatures that could cause death in larvae with 35°C. Changes in environmental condition, especially temperature, did not necessarily cause death in insects; insects also had a way of adaptation to overcome or avoid extreme heat or cold.[8]

The result of this study is useful to determine the time of deceased in forensic investigation, especially death due to amitriptyline overdose. In an investigation using insects, especially *Calliphoridae* fly larvae were as a PMI determinator. The family *Calliphoridae* is preferred to be used over the *Sarcophagidae* family because it is the first insect found in the corpse due to ability to find sources of odours with excellent special accuracy and lay eggs in the body within minutes to hours since death. The data can be used to determine PMI shifts due to amitriptyline. The data can be used to determine the shift in PMI due to amitriptyline by calculating the length and life cycle of *Calliphoridae* larvae in corpses that have overdosed amitriptyline, compared to the length of larvae and life cycles in corpses without drugs. Limitation of this study is in order to ease the investment of flies into rat carcasses, a ventral midline incision was carried out in all groups, this did not occur in actual suicides cases. Also, in this study, sampling was performed by killing the larvae that can cause reduced larvae in later measurements.

CONCLUSION

Increasing doses of amitriptyline extended development of the life cycle from larvae into pupae; this caused the larvae length to be smaller in the treatment group compared to the control group on the same day. The growth of fly larvae length was also influenced by environmental factors including temperatures and humidity. However, the environmental factor in this study had a smaller effect on the larva length growth of the *Calliphoridae* larvae.

CONFLICT OF INTEREST

Authors declare there was no conflict of interest.

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