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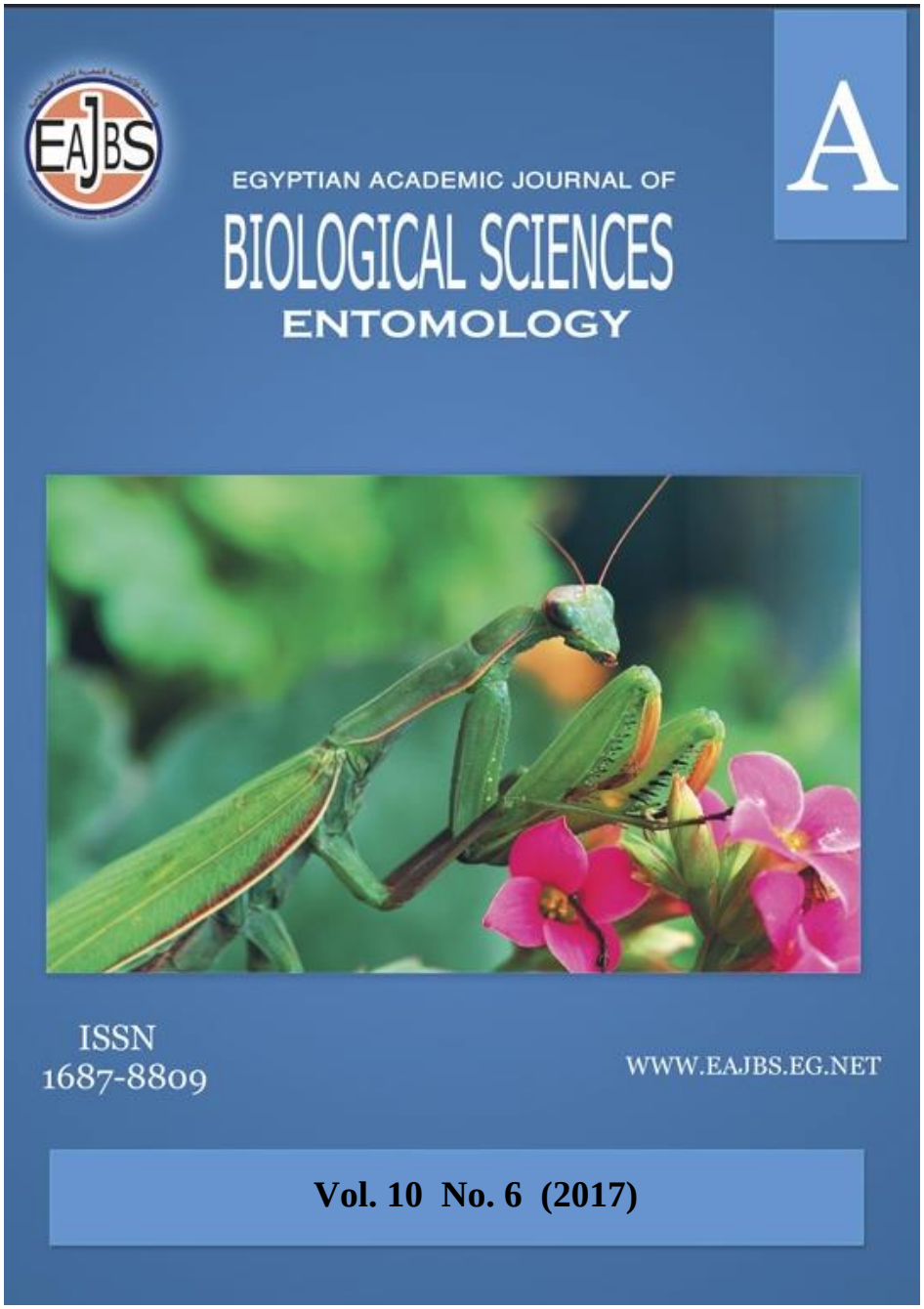

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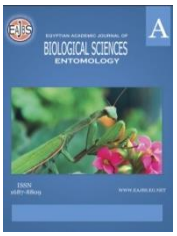

Entomotoxicological study on the forensic blow flies *Chrysomya albiceps* associated with dog carcass

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ABSTRACT

This study was carried out to identify malathion which may cause the death. Toxicological analysis was applied to the forensically – important blow fly larvae *C. albiceps* which is the most abundant fly attracted firstly and consume the flesh of carcass as their food substrate.

In addition to detection of malathion in tissues of blow fly larvae fed on treated – dog carcasses using gas chromatography with flam ionization detector (GCFID.), the effect of this chemical compound on insect succession, frequency of insects attracted to dog carcass and the development rate of *C. albiceps* larvae as the most important consumer of carcass tissues and can affect the estimate of postmortem interval (PMI) were investigated.

INTRODUCTION

Entomotoxicology is a new area of criminal investigation, where entomological evidence is analyzed to determine whether or not drugs or toxins were used prior to death.

Necrophagous insects may provide useful information about the time, place, and cause of death. In addition they can serve as reliable alternative specimens for toxicological analysis in cases where human tissue and fluid, normally taken during autopsies, are not available due to composition of the corpse. The true flies of families; calliphoridae (blow flies), sarcophigidae (flesh flies), and muscidae (houseflies) are highly motile, strong- flying insects and are typically the first to reach the dead body, often within minutes of death (Smith, 1986; Goff, 1993; Kabadaia, 2015).

Entomotoxicology includes the study of the effects of drugs, toxins and opiates on the development rate of carrion-feeding insects **(Goff and Lord 2001)**, and the use of these as alternative sample in the absence of other tissues. Insects lay eggs on or in human remains, as well as utilize the corpse for food or habitat. Insect development and successional patterns can be used as indication of PMI when time of death is unknown. Blow flies, especially *C. albiceps* play a fundamental role in the carcass decomposition (Kabadaia, 2015).

Studies have shown that antemortem use of various drugs and toxins affect maggot development rate, manifesting into an inaccurate PMI estimation based on insect development (Goff *et al*., 1989, 1991, 1993; Hedouin *et al*., 1999). Errors of up to 38 h. can occur in PMI estimates with heroin (Goff *et al*., 1991), up to 48 h. with methamphetamine (Goff *et al*., 1992), and up to 77 h. amitriptyline (Goff *et al*., 1993).

This study aimed to detect the causative toxic substance which causes the death in tissues of *C. albiceps* larvae which firstly attracted to the carcass. In addition, the effect of the toxic substance (Malathion) on insect succession and frequency on dog carcass and on development rate of *C. albiceps* were investigated.

MATERIALS AND METHODS

Study site:

The study site was located in University of Al-Azhar, Nasr city, Cairo, Egypt. The experiments were carried out during the period from Sep. 20, 2016 to Oct. 3, 2016. Each experiment was continued until the entire carcass was consumed. Sites for carcass placement were chosen in a botanical garden of the animal house at the Department of Zoology and Entomology, Faculty of Science, Al-Azhar University.

Experimental design:

For the experiment four dogs (*Canis lupus familiaris*), weighing approximately 5 kg were used. All dogs reared on the roof of the central lab building, Faculty of Science, Al-Azhar University, dogs were taken alive to the study site; two dogs were killed with antemortem 15 ml. of Malathion (insecticide) soluble as over dose for each dog, and the other two were killed with blow on the head. Care was taken to prevent external bleeding that might alter the attractiveness of the carcasses to flies or provide alternate sites for oviposition or larviposition.

After death, carcasses were placed in a botanical garden of the animal house, and immediately placed into mesh cages to prevent scavenging by large vertebrates and left exposed to natural conditions. The dog carcasses were separated by approximately 10 m.

Collection, sampling and identification:

Adult insects were collected on a daily basis until apparent insect activity had ceased. Insect collection was carried out twice daily, one in the morning from 8 to 9 am and the other collection was in the afternoon before sunset, from 4 to 5 pm. The numbers of adult insect collected were counted and representative samples were preserved in 70% ethanol and taken to the laboratory for identification. Adult Diptera and Hymenoptera were collected using a hand net, while adult Coleoptera and larvae were collected using hand picking forceps and vial glasses.

Identification and taxonomic determinations were made by using current keys *(*Greenberg, 1971; Mosallam, 1980; Shaumar et al., 1989; Whitworth et al., 2006 and Carvalho& Mello-Patiu, 2008), and by specialists in Cairo University and insect collection of Ministry of Agriculture, Dokki, Giza, Egypt. All insects were identified to the minimum of the family level. All efforts were made to identify Diptera and Coleoptera to the species level as they were considered of forensic importance.

Carcass decomposition:

Carcasses were examined twice daily; in morning and afternoon in order to determine the duration of each decompositional stage. Images of carcasses throughout decomposition study were captured using a digital camera (Fig. 1).

Climatic conditions :

The ambient conditions of temperature and relative humidity outdoor habitat (of Nasr city) were obtained monthly from the meteorological station of Kobri El-Kobba in Cairo, Egypt.

Insect succession tables:

Insect succession tables were developed by combining data from sweeping nets and hand collections. The different insect species that collected from each carcass were distributed according to the decomposition stages of carcasses i.e. according to postmortem interval (PMI) giving their numbers.

Statistical analyses:

All data obtained for the mean values of durations for the larvae and pupae and T test were statistically analyzed by the method of one way ANOVA using (graph pad instate). Graphs and tables were prepared using Microsoft Excel 2010.

Sample Preservation:

Entomological samples (Larvae of *C. albiceps*) were analyzed in similar standards to human tissue samples. Once the specimens have been removed from the body carcasses they were washed with deionized water and the specimens are then frozen for storage at a temperature of -20°C until they were needed for analysis (Gagliano-Candela and Aventaggiato, 2001).

Analysis of Samples:

Larvae Malathion confirmation by a G.C. system (HP- 6890) combined with a flame ionization detector (FID) in national research center, Dokki, Giza, Egypt.

1- Preparation of sample:

5 g of frozen larvae is homogenized and immersed over night with petroleum ether: diethyl ether (40:10) in closed conical with aluminium paper and then filtrated and stand for evaporation of solvents and then dried, then added 4µl of Ethanol HPLC and injected to GFID (Jackson, 1969).

2-Operating conditions of Gas chromatography flam ionization detector (GCFID) for qualitative analysis:

Column: weakly polar HP-5 column (5% phenyl-methylsiloxane; 30 m ×0.25 mm; film thickness 0.25 µm) was used for separation.

Carrier gas: Nitrogen fumes were used as carrier gas at constant flow of 1.0 mL/min. Injection was set as the split/split less mode with split time of 0.5 min.

Temperatures Injector: The injector and detector temperature were 250 °C.

Oven: The oven initial column temperature was 80 °C, which immediately increased to 290 °C at a rate of 10 °C/min, holed 5 min. and detector at 250 °C. The total run of one injection was 26 min.

Fig. (1): Larvae of *C. albiceps* on dead dog carcass.

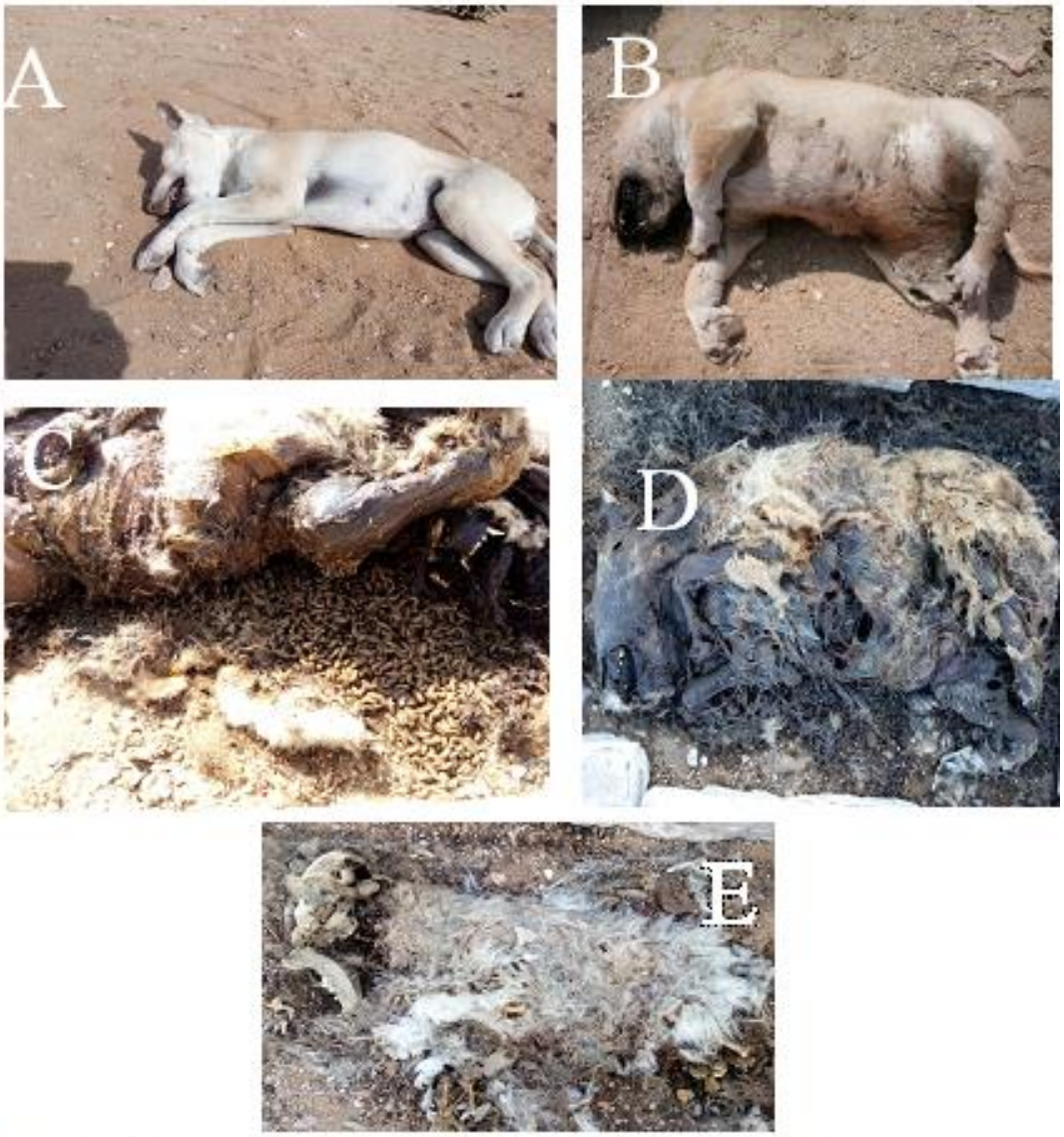

Fig.(2): Decmpositional stages of dog carcass(A) frsh stage, (B) bloated stage, (C) decay stage, (D) advanced decay and (E) dry stage.

RESULTS

Climatic conditions (temperature and humidity).

Ambient temperatures and relative humidity around dog carcasses placed outdoor were obtained from Meteorological Authority, Ministry of Civil Aviation, Egypt, during the study period from Sep. 20, 2016 to Oct. 3, 2016. Temperatures were varied from 40 to 21 °C with an average of 28 °C, while the relative humidity was varied from 94 to 9 % with an average of 56 % (Fig.3, 4).

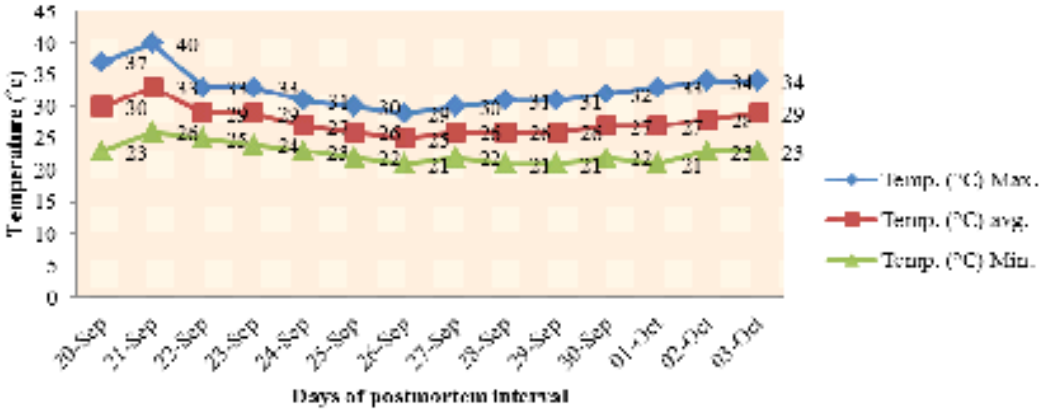

Fig. (3): Ambient temperatures during the study period from Sep. 20, 2016 to Oct. 3, 2016

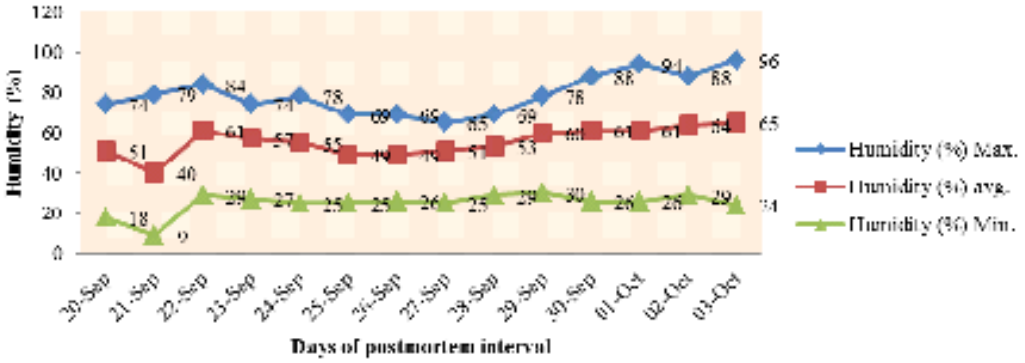

Fig. (4): Relative humidity during the study period from Sep. 20, 2016 to Oct. 3, 2016.

Detection and effects of malathion on insect succession, frequency and development of insects associated with carcass:

Unintentional death cases usually occurred from over dose usage of certain toxic agents. Toxicological analysis was applied to the forensically- important blow flies (*C. albiceps*) in order to identify malathion present on intoxicated tissues, and effects caused by such substance on insect succession, frequency and development on the carcass.

A-Detection:

Chromatograms of Malathion analysis are shown in (Fig. 5&6). Malathion was eluted at 6. 435min. and 6.٥٦٨ min. in control and treated samples, respectively.

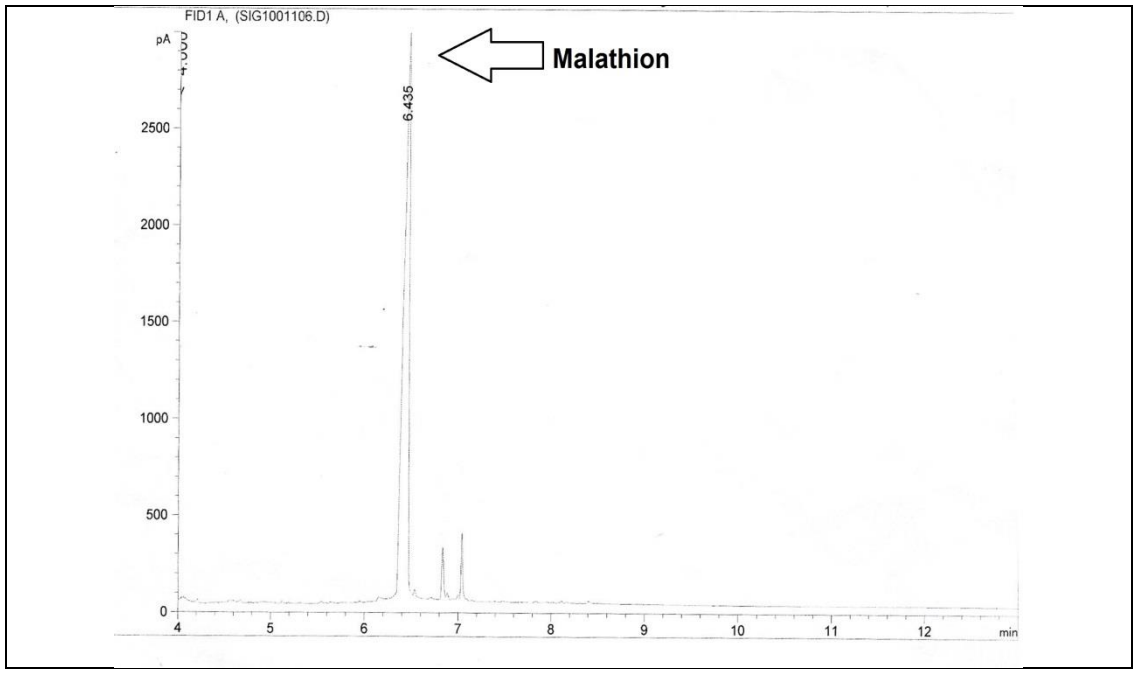

Fig. 5: Chromatogram obtained from analysis of standard Malathion.

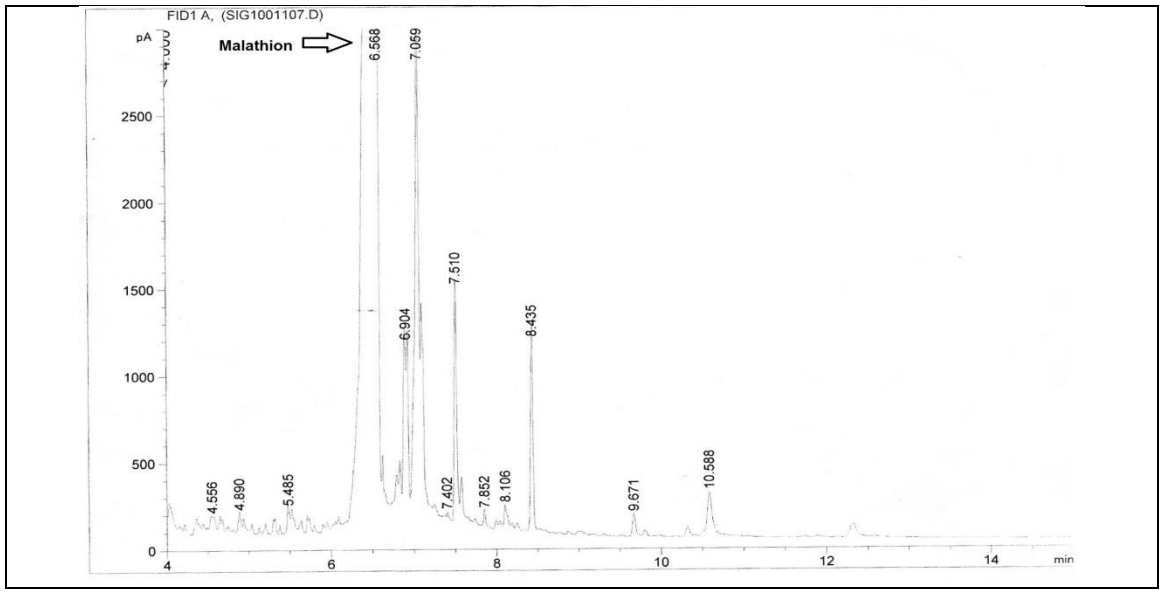

Fig.6: Chromatogram obtained from analysis of third instar larvae of *C. albiceps* fed on dog carcass died with Malathion

B- Effect of malathion on insect succession associated with dog carcass

Succession table of forensically significant insects on dog carcass died by Malathion as compared to succession table for dog carcasses died normally (control) are given in tables (1, 2).

As shown from the results, the number of adult fly was 184 and 142 for treated and control dog carcasses, respectively (Tables 1, 2).

In both dog carcasses (Malathion – dead dog and control dog), the insect succession firstly attracted to both carcasses were dipterous flies which were represented by the following insects; *C. albiceps*, (Calliphoridae), *Musca domestica* (Muscidae), *Sarcophaga carnaria* and *Wolhfartia magnifica* (Sarcophagidae), and *Piophila casei* (Piophilidae). The dipterous insects were followed by the coleopteran

insects: *Dermestes maculatus* (Dermestidae), *Hister* sp. (Histeridae) and *Necrobia rufipes* (Celeridae). Hymenoptran insects were the last insects attracted to both dog carcasses. They were represented by *Vespa orientalis* and *Dolichovespula* sp. (Fam. Vespidae) and *Monomorium pharoensis* and *Cataglyphis bicolor* (Fam. Formicidae).

However, statistical analysis showed that the total number of individuals of insect species attracted to both dog carcasses (Tables 1, 2) was insignificant i.e. this mean that Malathion showed no effect on the number of insect individuals which attracted to the dog carcass.

Table (1): Insect succession on control dog carcass during the period from Sep. 20, 2016 to Oct. 3, 2016

Order	Family	Species	No. of individuals on Decompositional stages / Days postmortem					Total
			Fresh	Bloated	Active decay	Advanced decay	Dry	
			0-0.125	1	2-3	4-6	7-13	
Diptera	Calliphoridae	*Chrysomya albiceps*	0	29	4	0	1.9	142
	Muscidae	*Musca domestica*	0	213	32	0	0	235
	Sarcophagida	*Sarcophaga carnaria*	0	0	0	1	1	2
		Wohlfartia magnifica	6	13	0	0	0	19
	Piophilidae	*Piophila casei*	7	36	5	0	0	48
Coleoptera	Dermestidae	*Dermestes maculatus*	0	1	5	7	15	28
	Histeridae	*Hister sp.*	0	2	9	7	4	22
	Celeridae	*Necrobia rufipes*	0	0	9	10	8	27
Hymenopte	Vespidae	*Vespa orientalis*	0	0	3	1	3	7
	Formicidae	*Monomorium pharoensis*	0	0	**0**	10	82	92
		Cataglyphis bicolor	3	2	4	3	18	30
Total								652

Table (2): Insect succession on Malathion - treated dog carcass during the period from Sep. 20, 2016 to Oct. 3, 2016

Order	Family	Species	No. of individuals on Decompositional stages / Days postmortem					Total
			Fresh	Bloated	Active decay	Advanced decay	Dry	
			0-0.25	1-2	3-7	8-10	11-13	
Diptera	Calliphoridae	*Chrysomya albiceps*	0	100	26	1	57	184
	Muscidae	*Musca domestica*	0	190	31	0	1	222
	Sarcophagida	*Sarcophaga carnaria*	0	0	1	0	0	1
		Wohlfartia magnifica	0	4	7	0	1	12
	Piophilidae	*Piophila casei*	0	13	8	0	0	21
Coleoptera	Dermestidae	*Dermestes maculatus*	0	11	15	11	15	52
	Histeridae	*Hister sp.*	0	5	39	3	7	54
	Celeridae	*Necrobia rufipes*	0	1	0	3	3	7
Hymenoptera	Vespidae	*Vespa orientalis*	0	4	1	0	5	10
		Dolichovespula sp.	0	0	2	0	0	2
	Formicidae	*Monomorium pharoensis*	0	200	0	0	0	200
		Cataglyphis bicolor	1	6	7	2	1	17
Total								782

C- Effect of malathion on frequency of insect species on dog carcass

Data given in tables (5, 6) and illustrated in Figs. (7,8,9) indicate the number of occurrence (frequency) or abundance of different insect species (adults) that collected from carcasses – died from malathion and dog carcass died normally (control) during the period from Sep. 20, 2016 to Oct. 3, 2016.

According to the number of occurrence (frequency) the different adult species collected from dog carcass died with malathion were arranged as follows: *Musca domestica* (222), *Monomorium pharoensis* (200), *C. albiceps* (184), *Hister* sp. (54), *Dermestes maculatus* (52), *Piophila casei* (21), *Cataglyphis bicolor* (17), *Wolhfartia magnifica* (12),*Vespa orientalis* (10),*Necrobia rufipes* (7), *Dolichovespula* (2) and *Sarcophaga carnaria* (1).

On the other hand the frequency of different adult species collected from control dog carcass were arranged as follows: *Musca domestica* (235), *C. albiceps* (142), *Monomorium pharoensis* (92), *Piophila casei* (48), *Cataglyphis bicolor* (30), *Dermestes maculatus* (28), *Necrobia rufipes* (27), *Hister* sp. (22), *Wolhfartia magnifica* (19), *Vespa orientalis* (7), *Sarcophaga carnaria* (2).

From the aforementioned results it is appeared that malathion had no effect on the frequency of insects firstly attracted to the carcass, where *C. albiceps* was found to be predominated on both malathion- dead dog and normal (control) dog carcasses.

It was interesting to note that all species of order Coleoptera, and 25% of total adult *C. albiceps* collected from malathion dead dog carcass were dead around the treated carcass.

D- Effect of malathion on development of *C. albiceps* larvae:

Data given in table (3) and illustrated in fig. (10) represent the effect of dog – carcass flesh treated with malathion on the development of *C. albiceps*.

Statistical analysis revealed that incubation period of *C. albiceps* was insignificant on both malathion - treated dog and normal (control) dog carcasses, while larvae which fed on malathion- treated dog carcass showed highly significant longer duration (5. 50 ± 0.25 days) compared to (4. 50 ± 0.25 days; p= 0.0080) for control, pupae that produced from larvae fed on malathion - dead dog carcass showed insignificant shorter duration (3. 50 ± 0.25 days) compared to (4 ± 0.25 days; p=0.0705) for control. The total duration of *C. albiceps* which fed on malathion - dead dog was significantly longer (10± 0.25 days) than control (9. 50 ± 0.25 days; p=0.0705).

From the aforementioned results it is appeared that malathion affected duration of the developmental stages of *C. albicep* which could be used as a model for determination the minimum postmortem interval (PMI) taking into consideration the increase of larval duration.

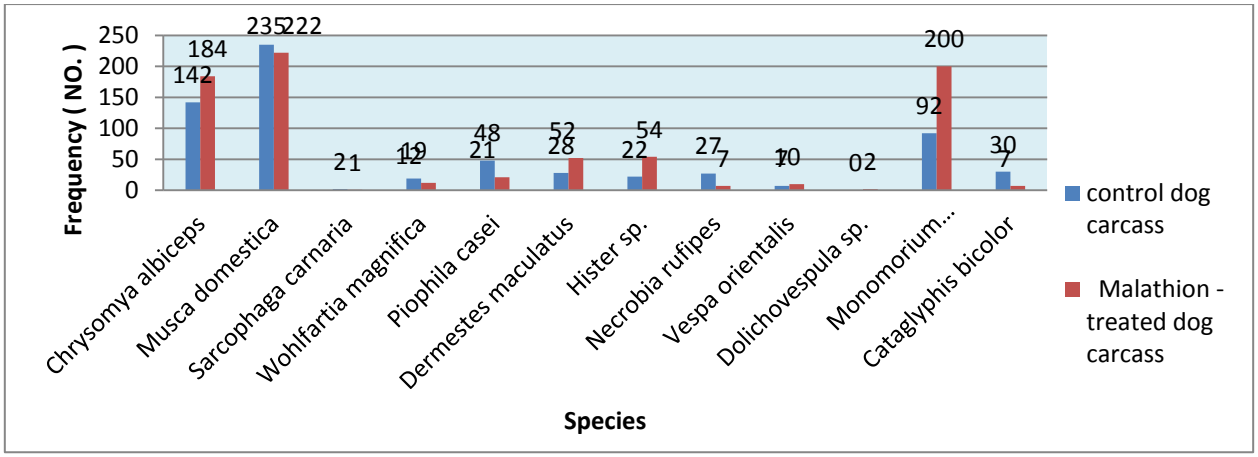

Fig. (7): Frequency of forensic insect species on control dog carcass and Malathion - treated dog carcass during the period from Sep. 20, 2016 to Oct. 3, 2016.

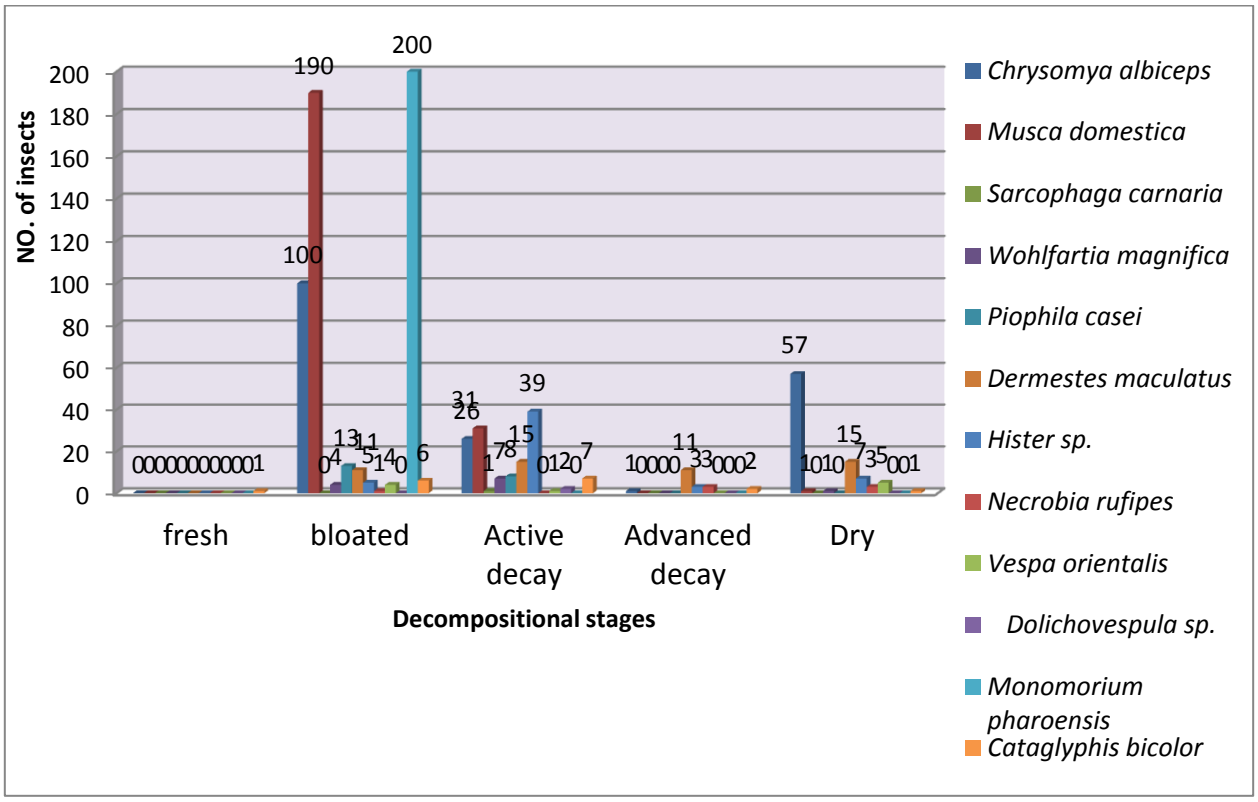

Fig. (8): Frequency of forensic insects on Malathion - treated dog carcass by species through decompositional stages during the period from Sep. 20, 2016 to Oct. 3, 2016.

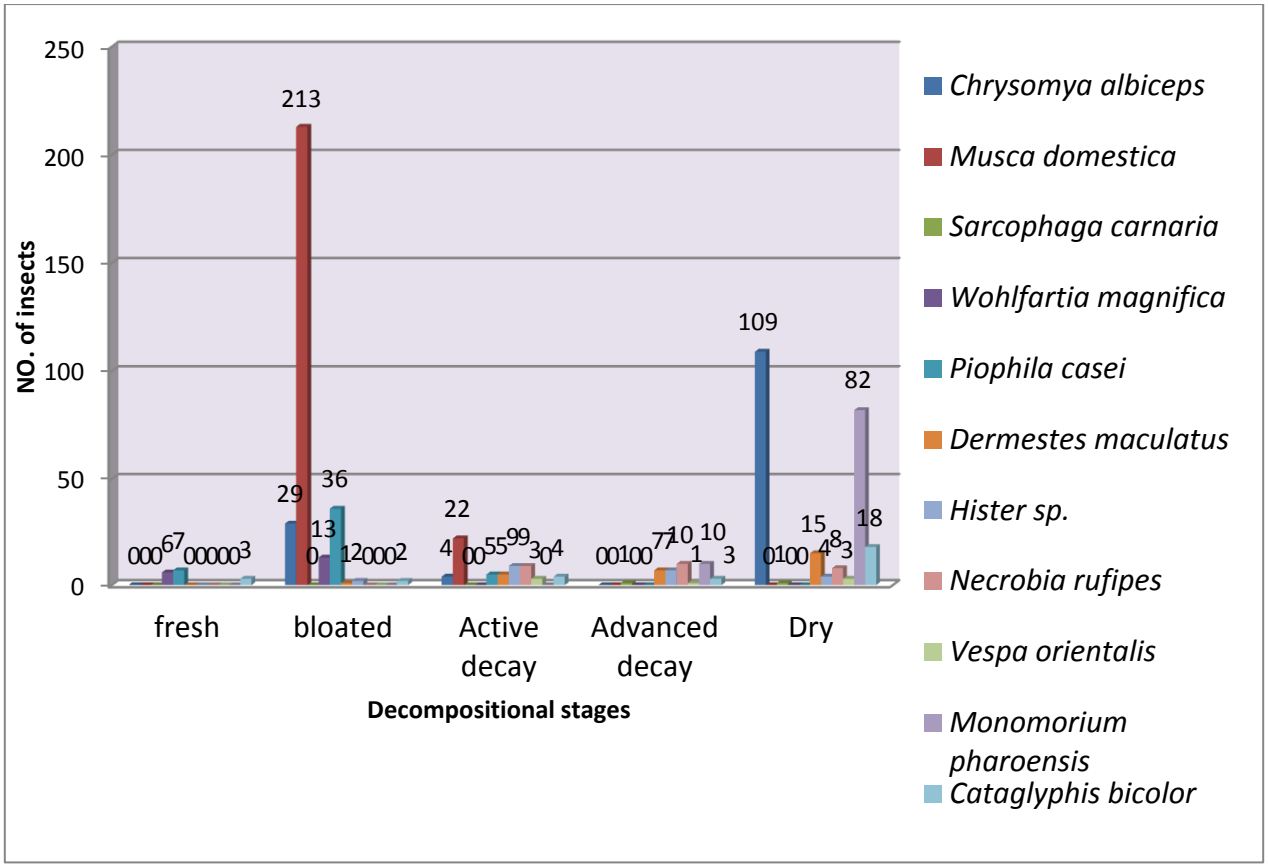

Fig.(9): Frequency of forensic insects on control dog carcass by species through decompositional stages from during the period Sep. 20, 2016 to Oct. 3, 2016.

Table (3): Incubation period and larval and pupal duration of *C. albiceps* reared on control dog carcass and Malathion- treated dog carcass from during the period Sep. 20, 2016 to Oct. 3, 2016

Developmental stages	Duration in days ± S.D		Mean	
	Control-dog carcass	Malathion – treated dog carcass	Temp. (c°)	R.H (%)
Incubation period	1 ± 0.25	1 ± 0.25^{ns}	28	56
Larval duration	4.5 ± 0.25	5.5 ± 0.25**		
Pupal duration	4 ± 0.25	3.5 ± 0.25^{ns}		
Total duration	9.5 ± 0.25	10 ± 0.25^{ns}		

S.D= Standard Deviation.
ns = not significant.
** = highly significant. (P < 0.01)

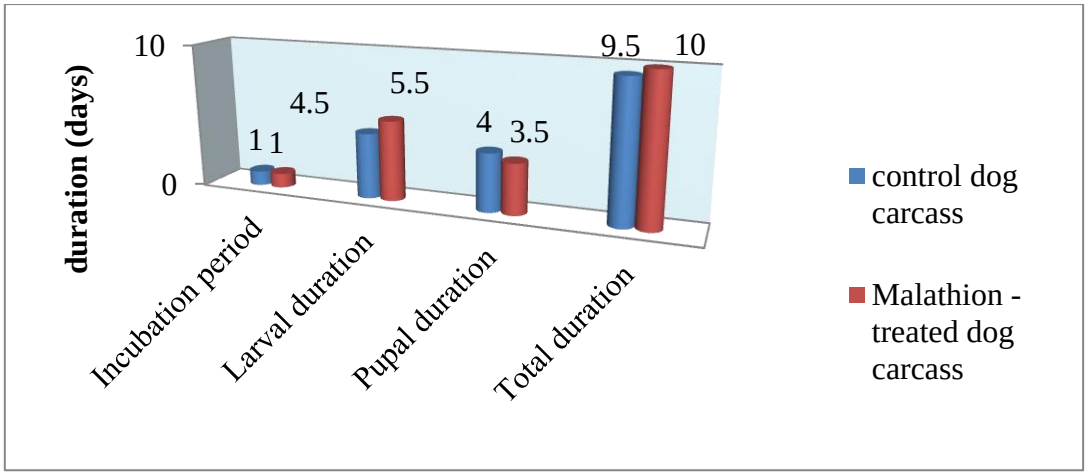

Fig. (10): Incubation period and larval and pupal duration of *C. albiceps* reared on control dog carcass and Malathion - treated dog carcass during the period from Sep. 20, 2016 to Oct. 3, 2016.

DISCUSSION

The most common application of entomological evidence in forensic medicine is the estimation of the time of death. There are additional applications which include determination of the place of death or detection of an antemortem trauma (Goff, 1993). Insects may serve as important alternative species for toxicological analysis in case where human sample are not available for this purpose (Goff and Lord, 1994). Common species are the true flies (Diptera) of families; calliphoridae (blow flies), sarcophigidae (flesh flies), and muscidae (house flies).They are highly motile, strong- flying insects and are the first to reach the dead body, often within minutes of death (Smith, 1986).

The time of death can be determined by estimating the minimum post-mortem interval (PMI) using age of the oldest blow flies larvae obtained from dead remains. The content of the larvae provides clues to the possible cause of death using toxicological analysis (Yi et al., 2013).

Toxicological analysis was applied to the forensically – important blow flies in order to identify drugs, toxins and opiates present on intoxicated tissues, and the effects caused by such substances on development. *Chrysomya* sp. were among the most blow flies reported to colonize dead remains (Omar *et al*., 2002), and they are available in large number of decomposition sites (Gosselin *et al.*, 2011).

The study reports the results of drugs, toxins, and opiates analyses in sample of *C. albiceps* 3rd instar larvae collected from dead dog bodies in an attempt to know the cause of death.

Several publications have described the detection of toxic, drugs and opiates through analyses of arthropods (Goff, 1994 and Tracqui *et al.*, 2004)

The results of the present study may be discussed as follows:

1- Detection of Malathion in the larvae of blow fly, *C. albiceps*.

Using gas chromatography flam ionization detector (GCFID), the organic substance used in the present study had been detected in *C. albiceps* larvae collected from treated – dog carcasses. This organic compound was malathion (insecticide).

The detections of malathion in tissues of *C. albiceps* larvae correspond to those demonstrated in other studies where organic compounds were identified (Goff, *et al.*, 1997; Campobasso, *et al.*, 2004; Tracqui *et al.*, 2004 and Mahmood *et al*., 2015). The blow fly samples used for chemical analysis in detecting tested organic compounds grow on the dog carcasses and consume the flesh of the carcasses as their food substrate. When larvae fed on a tissue that was intoxicated with some kind of drugs or poison, there were two processes are bioaccumulation or excretion of the drugs as well as its metabolites (Carvalh *et al.*, 2001). In this study, bioaccumulation was occurred, this was revealed from detection of this substance in the larval homogenates these results are in consistence with those obtained by **Mahmood *et al.*, (2015)** as they detected paraquat dichloride in tissues of Ch. *rufifacies* larvae feed on intoxicated rabbit carcass.

2- Effect of malathion intoxicated dog bodies in succession of insect species attracted to the carcass.

The present study indicated that malathion which used in killing of dogs as over doses did not affect the predictable sequence of insect species that arrive on carcass, where insect colonize the carcasses are separated into four ecological categories as noted by Catts and Goff (1992). The first category which contain the greatest number of individuals and of high significance in determining time since death; necrophagous species that feed directly on the carcass, the second category was predators and parasites of necrophagous species, the third category consist of omnivorous species (Wasps, ants and some beetles) that feed on both carcass and associated insects and the fourth category is composed of incidental species having no relationship to the carcass.

As shown from the present study, the succession table of forensically significant insects on both treated-dog carcass and control carcass, blow fly, *C. albiceps* was the most abundant fly attracted firstly to both dog carcasses. Generally, the insect succession firstly attracted to both dog carcasses were dipterous flies which were represented by the following families, Calliphoridae, Muscidae, and Piophilidae. The depterous insects were followed by Coleopteran families; Dermestidae, Histeridae, and Celeridae. Hymenopteran insects were the last insects attracted to both carcasses. They were represented by Fam. Vespidae and Fam. Formicidae. This succession as shown was similar on both dog carcasses (treated and control). The same insect succession was obtained by Kabadaia (2015) on dog carcasses at the same site of the present study. Also, the total numbers of insect species which attracted to dog carcasses died with over doses of drugs, toxins, and opiates and, to control dog carcasses were insignificant which means that numbers of insect individuals did not affect by chemical used in this study. It is suggested that insects arrive on a carcass in a predictable sequence are depending on the stage of decomposition.

3- Effect of malathion intoxicated dog bodies on frequency (abundance) of insect species attracted to the carcass.

Adults of the blow fly *C. albiceps* were attracted to both treated – dog and control – dog carcasses. These results are similar to those obtained by Carvalho and Linhares (2001) working on normal pig carcass and Kabadaia (2015) working on normal dog and rabbit carcasses in the same site of the present study. The high

frequency of *C. albiceps* may reflect the high dispersal ability and arrival at carcasses shortly following death (Carvalho and linhares, 2001).

In according with the previous mentioned results, the present study showed that malathion that used in killing dogs had no effect on the frequency of insects firstly attracted to both treated – and control – dog carcasses, where *C. albiceps* was found to be predominated. The occurrence (frequency) of the different adult species collected from both dog carcasses (treated and control) were arranged as follows: *C. albiceps*, *Musca domestica*, *Piphila casei*, *Dermetes maculatus*, *Cataglyphis bicolor*, *Hister* sp., *Necrobia rufipes*, *Wolhfartia magnifica*, *Sarcophaga carnaria*, *calliphora* sp., *Vespa orientalis* and *Dolichovespula*.

4- Effect of malathion intoxicated dog bodies on the development of immature stages of *Ch. albiceps*.

Previous studies have demonstrated the presence of drugs, toxins, and opiates can alter developmental rates of carrion – insects feeding on decomposed tissue from cadavers (Catts and Goff, 1992; Goff *et al*., 1992; Boural *et al.*, 1999; Goff and Lord, 2001; Byrd and Castner, 2010; O' Brin and Turner, 2004; Tabor *et al.*, 2004; Tabor *et al.*, 2005; EL- samad *et al.*, 2011and De carvalho *et al.*, 2012). Because *C. albiceps* is 1st insect arrive at dog carcass and its larvae grow on the carcass and consumes the flesh of the carcass as their food substrate, the present study had investigate the life table of this fly on malathion, - treated dog and control – dog carcasses. The results indicated variable effects of malathion on the duration of immature stages of the blow fly. Malathion affected the pupal duration, where it significantly prolonged the pupal duration as compared to control pupae. These results are in harmony with many researches for example, Carvalho *et al*. (2001), Tabor *et al*. (2005), Kharbouche *et al*. (2008) and De carvalho *et al*. (2012). However, the present results indicated that the estimate of PMI can be significantly affected by the presence of toxins in *C. albiceps* larval feed.

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