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Detection and effect of ephedrine sulphate on the development rate of the forensic blow fly larvae *Chrysomya albiceps* (Diptera: Calliphoridae) colonize a dog carcass

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Abstract

Necrophagous insects may provide useful information to medico legal 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 fluids, normally taken during autopsies, are not available, due to decomposition of the carcass.
Using gas chromatography with flame ionization detector (GCFID), the present study demonstrated the detection of ephedrine from the forensically – important blow fly larvae *C. albiceps* which the most abundant fly attracted firstly and consume the flesh of carcass as their food substrate. In addition the effect of ephedrine on the development rate of these fly larvae was observed.
Ephedrine was detected in the homogenate of *C. albiceps* larvae that fed on ephedrine sulphate – dead dog carcass at 4.336 min. vs. at 4.339 min. in the standard samples as shown from chromatograms. Ephedrine was found to significantly prolong the pupal and total durations of *C. albiceps* colonized the treated carcass as compared to control.

Keywords: postmortem interval, dog carcass and necrophagous insects.

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 corpes. 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)**.

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 Nov.17, 2016 to Dec. 7, 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 injected 5 ml. of ephedrine sulphate 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

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 Agriclture, Dokki, Giza, Egypt.

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

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.

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

1. Larvae Ephedrine confirmation by a G.C. system (HP- 6890) combined with a flame ionization detector (FID) in National Research Center, Dokki, Giza, Egypt.

A. Preparation of sample by Ammonium sulphate method

10 gm. of frozen larvae is homogenized and mixed with 50 ml of water acidified with dilute hydrochloric acid. Solid ammonium sulphate is added to make a saturated solution. The mixture is warmed in a water-bath to 65° for 1 hour. Then leave the mixture over night at room temperature until the protein coagulates. When coagulation is complete, the mixture filtrated through glass funnel with what-man No. 5 paper. Then added ammonium hydroxide to reached pH (9-11), and then added solution to separation funnel with 50 ml. of chloroform to separation of ephedrine, and stand for evaporation of chloroform and then dried, then added 4µl of Ethanol HPLC and injected to GCFID **(Jackson, 1969)**.

B. Operating conditions of (G.C.F.I.D) for qualitative analysis

Column: a RTX-1 capillary column (30 m×0.32 mmID×0.25 µmdf, cross bond 100% dimethyl polysiloxane) 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 280 ºC.

Oven: The oven initial column temperature was 130 °C, which immediately increased to 205 °C at a rate of 15 °C/min and then to 240 °C at a rate of 10 °C min. then holed 2 min. The total run of one injection was 10.5 min.

Results

1. 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 Nov. 17, 2016 to Dec. 7, 2016. Temperatures were varied from 26 to 11 °C with an average of 19 °C, while the relative humidity was varied from 100 % to 7 % with an average of 55 % (Fig.2, 3).

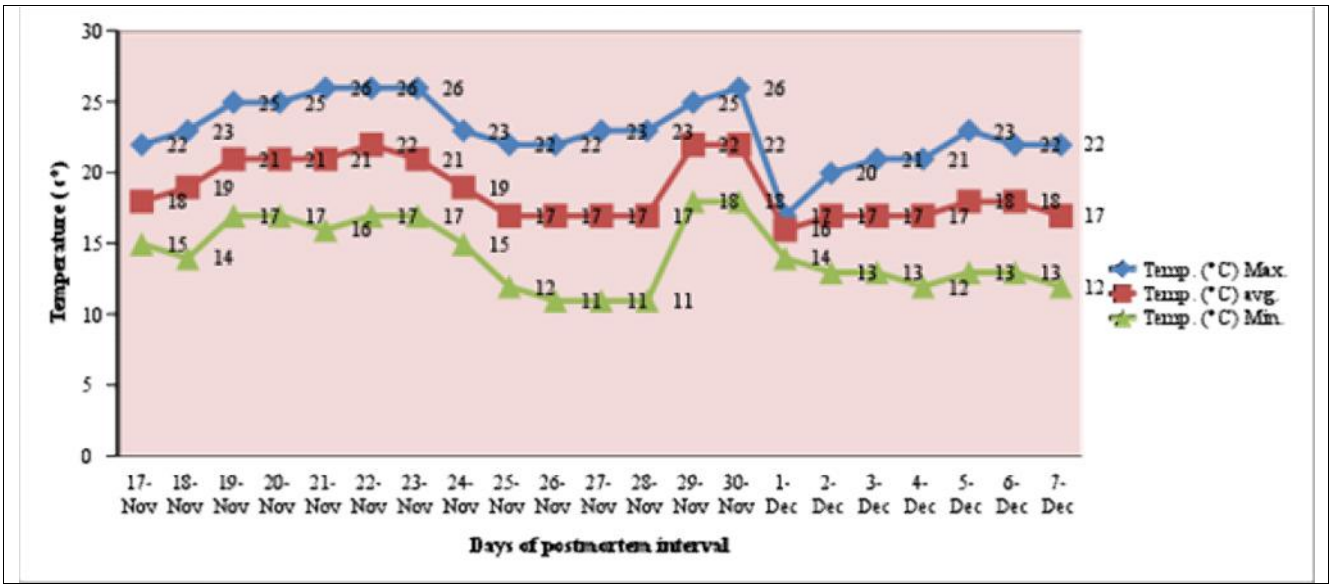

Fig. (2): Ambient temperatures during the study period from Nov. 17, 2016 to Dec. 7, 2016

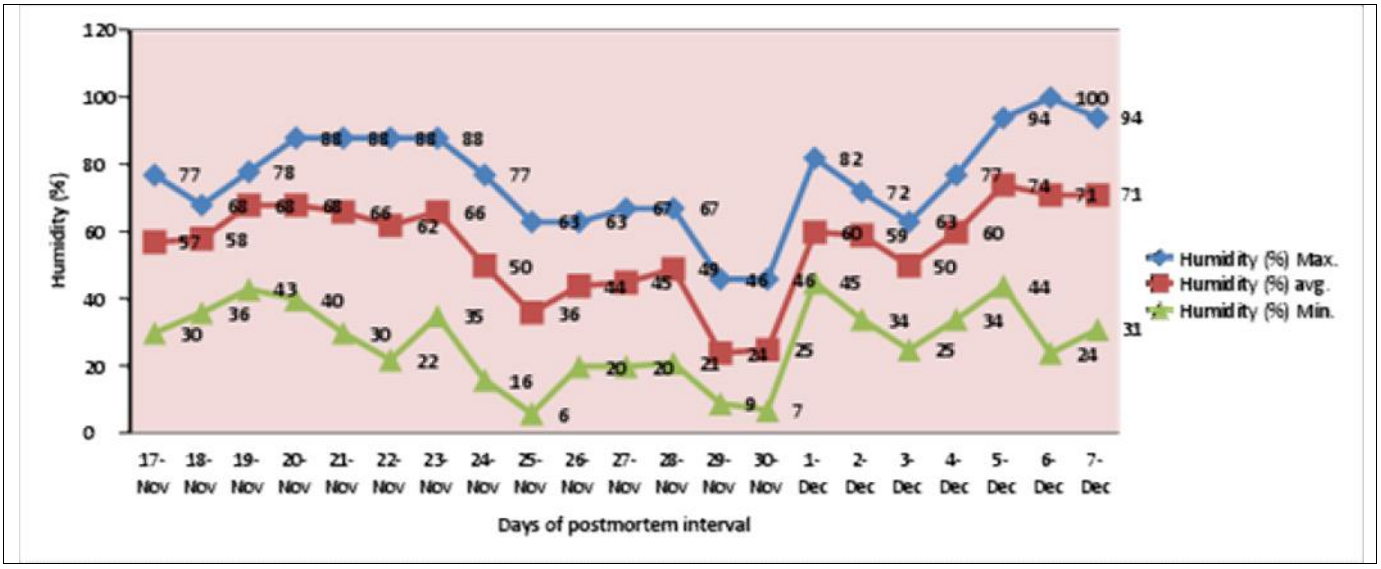

Fig. (3): Relative humidity during the study period from Nov. 17, 2016 to Dec. 7, 2016

2. Detection and effects of ephedrine on development of insects associated with carcass.

Unintentional death cases usually occurred from over dose usage of certain amphetamines. Toxicological analysis was applied to the forensically- important blow flies (*C. albiceps*) in order to identify ephedrine present on intoxicated tissues, and effects caused by such substance on the development rate of *C. albiceps* reared on ephedrine - treated dog carcass.

A. Detection

As shown from Chromatograms of ephedrine analysis in homogenates of 3rd instar larvae of *C. albiceps* from dog carcass died from ephedrine sulphate and standard sample of ephedrine sulphate (Fig. 4&5). Ephedrine sulphate was eluted 4.339 at min. and 4.366 min., respectively.

B. Effect of ephedrine on development of *Chrysomya albiceps* larvae

Data given in table (1) and illustrated in fig. (6) represent the effect of dog – carcass flesh treated with ephedrine sulphate on the development of *C. albiceps*.

Statistical analysis revealed that incubation period of *C. albiceps* was not significant on both ephedrine sulphate dead dog and normal (control) dog carcasses.

Also larval duration was not significant for both larvae. Pupae that produced from larvae fed on ephedrine sulphate - dead dog carcass showed highly significant longer duration (6. 50 ± 0.25 days) compared to (5. 50 ± 0.25 days; p=0.0080) for control. The total development of *C. albiceps* which fed on ephedrine sulphate - dead dog carcass was highly significantly longer (17 ± 0.25 days) as compared to (16 ± 0.25 days; p=0.0705) for control.

From the aforementioned results it is appeared that ephedrine sulphate affected only the pupal duration of *C. albicep* which could be used as a model to determine the minimum PMI.

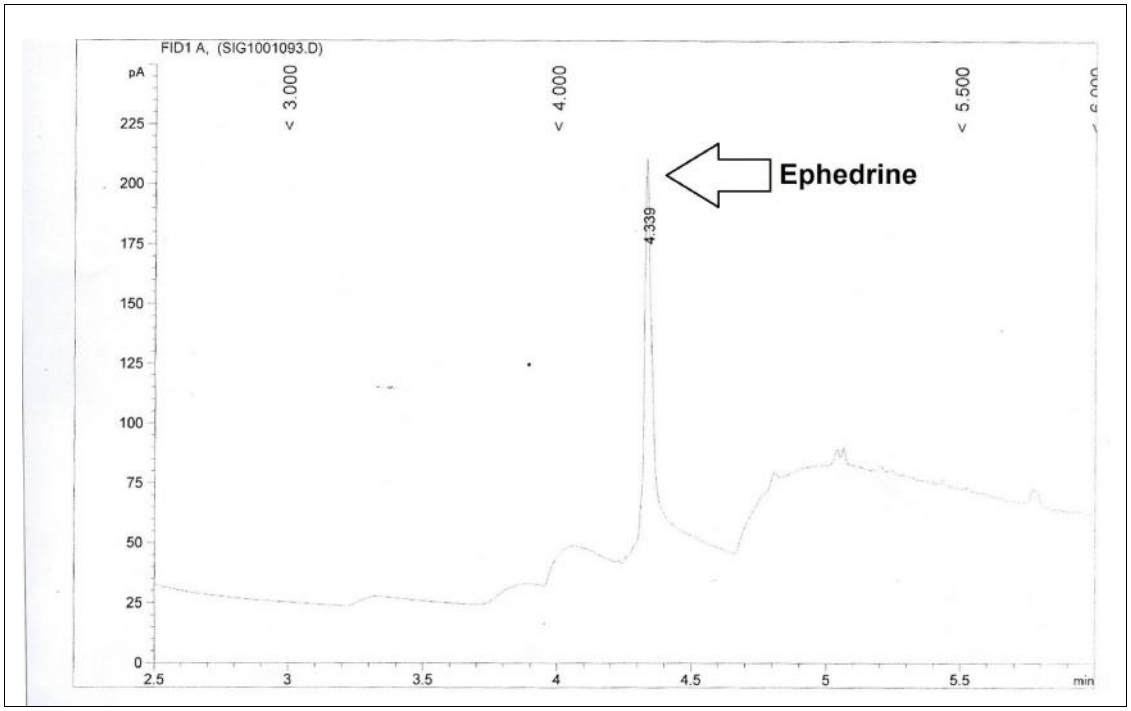

Fig. 4: Chromatogram obtained from analysis of standard ephedrine sulphate.

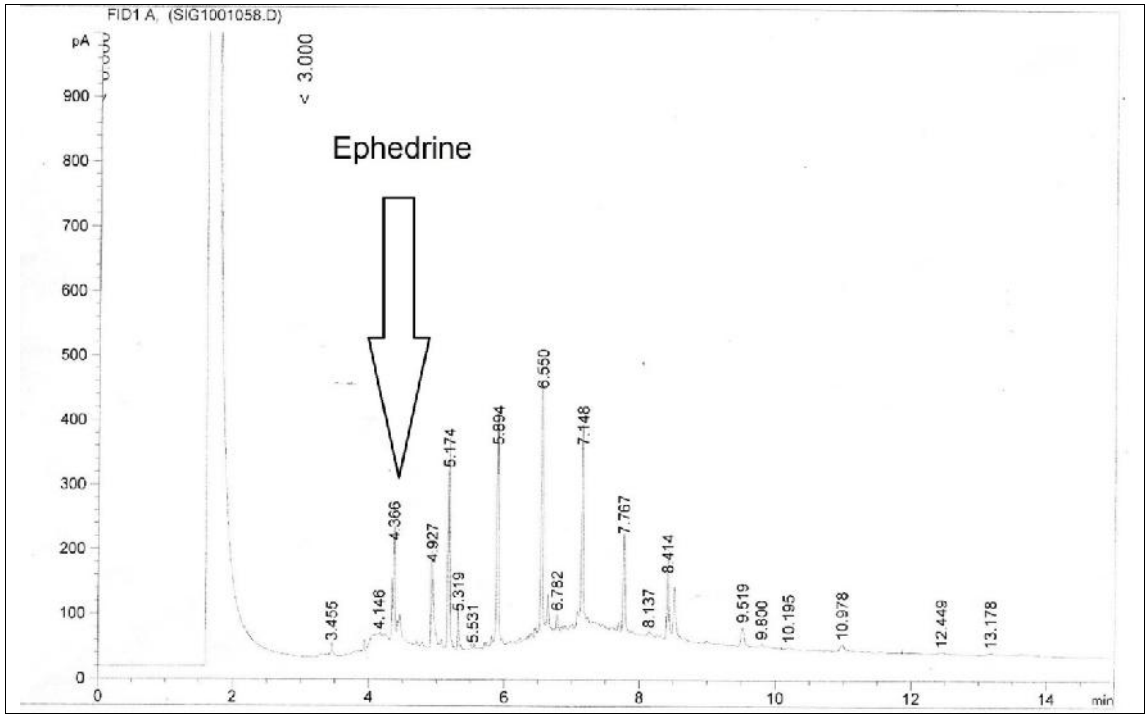

Fig. 5: Chromatogram obtained from analysis of third instar larvae of *Chrysomyia albiceps* fed on dog carcass died with ephedrine sulphate

Table (1): Incubation period and larval and pupal duration of *C. albiceps* reared on control dog carcass and Ephedrine sulphate - treated dog carcass during the period from Nov. 17, 2016 to Dec. 7, 2016

Developmental stages	Duration in days ± S.D		Mean	
	control dog carcass	Ephedrine - treated dog carcass	Temp. (°C)	R.H (%)
Incubation period	3 ± 0.25	3 ± 0.25^{ns}	19	55
Larval duration	7.5 ± 0.25	7.5 ± 0.25^{ns}		
Pupal duration	5.5 ± 0.25	6.5 ± 0.25**		
Total duration	16 ± 0.25	17 ± 0.25**		

S.D= Standard Deviation.
ns = not significant.
* = significant. (P < 0.01)
** = highly significant. (P < 0.01)
*** = very highly significant. (P < 0.001)

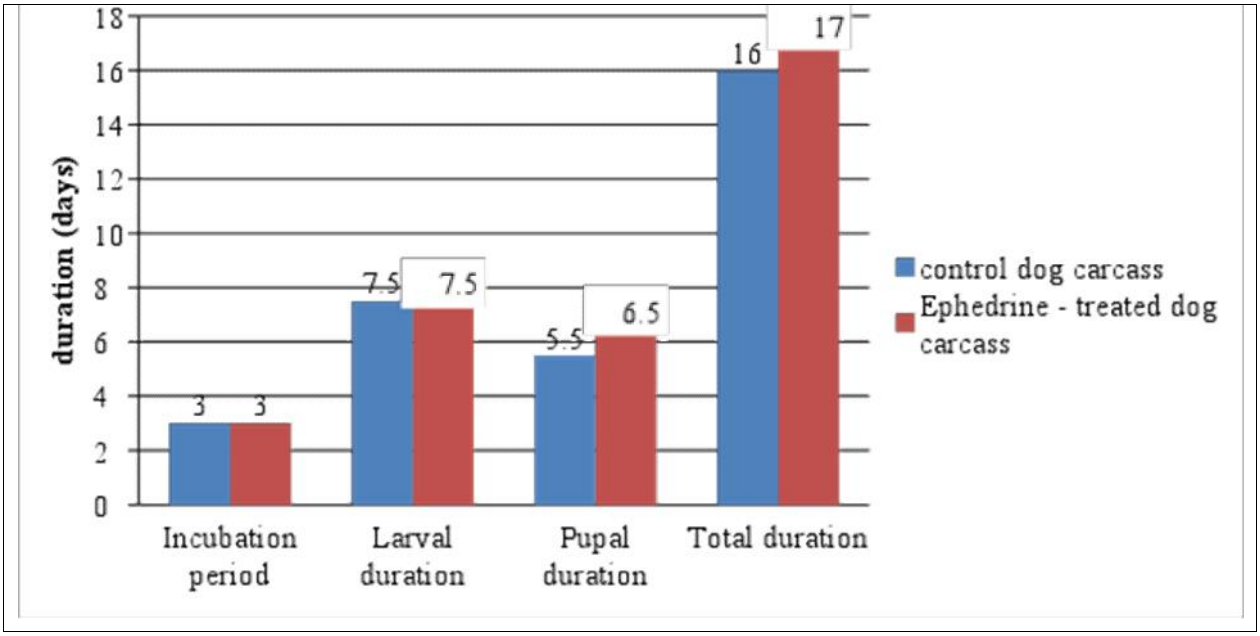

Fig. (6): Incubation period and larval and pupal duration of *C. albiceps* reared on control dog carcass and Ephedrine sulphate - treated dog carcass during the period from Nov. 17, 2016 to Dec. 7, 2016.

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 ephedrine analyses in sample of *Ch. 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 Ephedrine in the larvae of blow fly, *C. albiceps*.

Using gas chromatography flam ionization detector (G C F I D), the organic substance used in the present study had been detected in *C. albiceps* larvae collected from treated – dog carcasses. This organic compound was ephedrine (amphetamine). The detections of ephedrine 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 ephedrine intoxicated dog bodies on the development of immature stages of *C. 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 ephedrine, - treated dog and control – dog carcasses. The results indicated variable effects of ephedrine on the duration of immature stages of the blow fly. Ephedrine 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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