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Effect of Intoxication with Warfarin Rodenticide on Development and Survival of Forensically Important Fly Maggots in Egypt

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ABSTRACT

This study concerns with the effects of Warfarin rodenticide on development and survival of some fly maggots. Maggots were collected off warfarin intoxicated and non-toxicated rabbits. These were laboratory reared on tissues of control rabbits. Other group of control larvae was reared on pieces of beef mixed with 19 different concentrations of Warfarin. Intoxicated larvae of both groups had shorter developmental periods from first instar larvae to pupae indicating faster development of these larvae as compared to control ones. The overall duration periods (first instar larvae to Adults) for the fly species resulted from larvae fed rabbit tissues (specially *Lucilia sericata, Chrysomya albiceps* and *C. megacephala*) generally indicate faster development of intoxicated larvae than control ones ($P<0.01$). The different Warfarin concentrations had its effects on emerged adults: (1) 52.63% of larvae that were exposed to 20 to 200 ppm failed to complete its development to adult stage, (2) 31.58% of larvae yielded adults with sluggish movements and died within minutes, (3) 5.26% of larvae yielded adults with different forms of abnormalities and malformations and (4) 10.53% of larvae emerged normal adults. The study concluded that like other toxins and drugs, Warfarin has an effect in accelerating the development of dipteran larvae which is an important indication of cause of death and may be a useful tool for postmortem interval (PMI) estimation based on entomological evidences.

INTRODUCTION

In forensic entomology, Entomotoxicology is defined as the analysis of toxins in arthropods (mainly flies and beetles) that feed on a carrion. Using arthropods off corpse or at a crime scene, investigators can determine whether toxins were present in a body at the death time. In some circumstances, the flesh from the corpse can retain some types of drugs that had been consumed by the victim before he/she died and which may even have been the cause of death, these drugs may be recovered by analyzing the insects (Introna *et al.*, 1990). Moreover, necrophagous insects are valuable tools for postmortem interval (PMI) estimation

or for determining the cause of death (Souza *et al.*, 2011) for example, the potential use of blow flies larvae as indicators for detecting toxins and drugs in decomposing carcasses have been widely demonstrated (Nolte *et al.,* 1992). This technique is a major advance in forensic science so that the ongoing researches directed to study the effects of toxins on arthropod development has also allowed better estimations of PMI. Due to the increase in deaths related to drug abuse, it is necessity of considering the possible effects of drugs in tissues on growth rates of flies that feed on corpses when estimating the PMI to avoid errors in such estimates using entomological techniques (Goff *et al.,* 1991; Bourel *et al*., 1999; Souza *et al.*, 2011).

There are several studies on the effect of tocxins and drugs on the development and survival of several fly species. Carvalho *et al.* (2001) examined the effect of diazepam on the development of two necrophagous flies (*Chrysomya albiceps* and *C. putoria*) and reported that larvae feeding on rabbit tissues containing the drug developed more rapidly than larvae from the control colony for both fly species. Musshoff *et al.* (2001) examined the effects of consuming liver containing either a barbiturate (sodium methohexital) or a steroid (hydrocortisone) on the development of a flesh fly, *Sarcophaga tibialis* and showed that compared with controls, the length of the larval stage was increased, whilst pupariation was more rapid. In laboratory experiments, Arnaldos *et al.* (2005) showed that the length of time taken to complete individual larval stage in *Sarcophaga tibialis* was considerably longer, in contrast to those larvae which were not fed heroin. Heroin has also been shown to increase the growth rate (duration of pupal development) of other maggot species, e.g. *Boettcherisca peregrina*. El-Samad *et al.* (2011) studied the effect of Tramadol on the development of *Lucilia sericata* and indicated that the total developmental period increased in relation to increasing of the administrated dosage of Tramadol. de Carvalho *et al.* (2012) examined the effect of cocaine on the developmental rate of immatures and adults of *Chrysomya albiceps* and *C. putoria* and documented that the larvae of both species that fed on the cocaine-containing liver tissues developed rapidly than that fed on control tissues. Magni *et al.* (2014) indicated that methamphetamine (MA) produced a clear increase in the developmental time from eggs till reaching adult stage in *Calliphora vomitoria*. About 60% of larvae exposed to either dose of MA died during the pupation period and the lengths of larval and pupal stages were on average significantly larger than the controls. However, a study conducted by George *et al.* (2009) showed that pure morphine had no effect on certain blow fly species. Furthermore, a study by O'Brien and Turner (2004) on the effects of Paracetamol indicated only a slight acceleration in larval growth rate.

In spite of these studies and as far as we are aware, no available studies on the effect of Warfarin on development and survival of dipteran maggots. For this, the present study was planned and objected at examining the effect of Warfrin rodenticide on the developmental periods of fly larvae and its delayed effect on adult emergence. This is an important parameter in using the fly larvae as indicators for detecting toxins and drugs that may be the cause of human death.

MATERIALS AND METHODS

The Toxin:

The anticoagulant rodenticide, Warfarin (Coumadin, 4-hydroxy-3-(3-oxo-1-phenylbutyl) coumarin; 4-ydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one) (Figure 1) was used. Two experiments were carried out in which Warfarin was used: (1) The toxin was administrated to rabbits to collect fly larvae attracted to their carcasses along with larval collection on control rabbit carcasses that were asphyxiated via hanging

(Abd El-Gawad *et al*., In prep.) and (2) Different concentrations of Warfarin were applied to treat the fly larvae (mixed with beef as synthetic feeding media for larvae). For this experiments, two treatments were carried out: In the 1st treatment, to each of 5 gm beef 0.1, 0.2, 0.3, ….., 1mg Warfarin were added (concentration of 20 to 200 part per million, ppm) and in the 2nd treatment, 0.01, 0.02, 0.03, …., 0.09 mg Warfarin were added each to the 5 gm beef (concentration of 2 to 18 ppm).

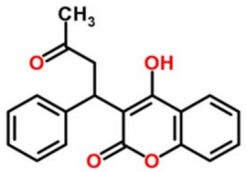

Fig. 1: Molecular structure of Warfarin

Fly Larvae:

Two groups of 1st instar larvae were collected for observation of their development and survival: (1) Larvae of the first group (intoxicated) were collected off rabbits intoxicated with Warfarin (Abd El-Gawad *et al.*, In prep). (2) Larvae of the second group were collected off control rabbits (control larvae).

The two larval groups were transported to the laboratory where: (1) Intoxicated and control larvae were reared separately in glass Jars (0.4 L.) supplied with a little damp tissue paper to prevent dehydration and pieces of control rabbit tissues for feeding of larvae. The top of the jar was covered by a piece of muslin netting fixed with a rubber band to prevent insects from escaping as long as the jar stays upright. Three replicates were prepared for both control and intoxicated larvae. Jars were kept in a room where mean temperature was 23.5°C (20-27°C). Observations were continued till adult emergence. Adults were identified to species level. (2) For other control larvae, a synthetic media was prepared by mixing of 5 gm beef and the desired concentration of Warfarin. Three replicates were prepared for each concentration included only one larva each. Check or control larvae were reared on beef alone. As all high Warfarin concentrations (20 to 200 pm) caused death in the pupal stage, a series of lower concentrations (2 to 18 ppm) was prepared and applied as above. Experiments were continued (for 2 months at 21-25°C room temperatures) till either pupal death or adult emergence.

Observations:

All larvae were observed daily for development to the next instars, pupation and adult emergence. The durations (in days) of the three larval instars and pupal stage were determined.

Statistical Analysis:

Means and Standard deviations of the developmental periods of different larval instars and pupal stage were determined for control and intoxicated larvae and compared by one-way Analysis of Variance (ANOVA). The SPSS software (Version 11 for windows, SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

The results of larvae that were fed rabbit tissues (Table 1) indicate that all developmental periods were significantly shorter for intoxicated larvae than for control ones ($P<0.05$ - $P<0.001$) except that from pupa to adult emergence (4.16 and 3.81 days for control and intoxicated larvae, respectively; $P>0.05$). The overall period for development of 1st instar larvae to adult emergence was 12.37 days for intoxicated larvae compared to 17.06 days for control ones ($P<0.001$). The mean duration periods (L_1-Adult) for the observed fly species (Table 2) generally indicate also shorter periods for intoxicated larvae ($P<0.01$) than for control ones (specially *Lucilia sericata, Chrysomya albiceps* and *C. megacephala*).

Table 1: Developmental periods of the different immature stages (fed rabbit tissues) of dipteran flies kept at 20-27°C room temperatures.

	Replicate	No	Mean development period (day)[a]					
			L_1-L_2	L_2-L_3	L_3-P	L_1-P	P-A	L_1-A
Control	1	62	4.06	7.06	4.87	12.13	3.92	17.04
	2	55	10.36	9.45	4.76	12.45	4.51	17.22
	3	112	9.69	5.18	4.04	13.62	4.04	16.91
	Mean±SD	229	8.04±3.46	7.23±2.14	4.56±0.45	12.73±0.78	4.16±0.31	17.06±0.16
Intoxicated	1	55	2.25	1.38	3.70	8.13	3.67	13.53
	2	20	2	3	3.75	9	3.75	11.75
	3	25	1.4	2.2	3.84	7.6	4	11.84
	Mean±SD	100	1.88±0.44	2.19±0.81	3.76±0.07	8.24±0.71	3.81±0.17	12.37±1.00
F (1,4)[b]			9.34*	14.53*	9.07*	54.25**	2.90	63.91***

[a]* L_1, L_2 and L^3: first, second and third instar larvae, respectively; P: pupa. A: Adult.
[b]*P<0.05, **P<0.01, ***P<0.001

Table 2: Means of developmental periods (L_1 to Adult, day) of dipteran flies as result of larvae collected off control and warfarin intoxicated rabbit carcasses that fed control rabbit tissues and kept at 20-27°C room temperature.

Species	Control		intoxicated		F (df)
	No	Period	No	Period	
Musca domestica	10	17.20±0.92			
Musca sorbens	5	17.80±0.45	1	13.00	
Calliphora vicina	1	14.00			
Lucilia sericata	75	16.24±1.51	34	14.35±0.65	8.59 (1,107)**
Lucilia cuprina	9	16			
Chrysomya albiceps	101	15.74±1.29	36	14.22±0.90	7.79 (1,135)**
Chrysomya megacephala	4	16.50±0.58	20	15.00±0.00	9.95 (1,22)**
Chrysomya rufifacies	17	16.06±0.24			
Sarcophaga argyrostoma	4	17.00±2			

**P<0.01

Results of larvae maintained on beef media revealed that: In its high concentration (20 to 200 ppm), Warfarin showed a range of 7 to 58 days as developmental periods from 1st larva to pupa that increased in relation to the decreasing concentration with the shortest period (7 days) at 200 and 180 ppm. This indicates faster development as compared with that of control larvae (17 days). In its low concentrations (2 to 18 ppm), Warfarin showed the same trend as with the high concentrations (Fig. 2) with the shortest period of 15 and 16 days at 18 and 16 ppm, respectively which also indicates faster development as compared with that of control larvae. Based on number of Warfarin concentrations, results indicated that some larvae (10/19: 52.63%) failed to complete its development to adult stage (Table 3, Fig. 3) and others completed development but yielded adults with sluggish movements and died within minutes (6/19: 31.58%). Still others (at 6 ppm, 1/19: 5.26%) yielded adults with different forms of abnormalities and malformation (Fig.'s 4-7). With the lowest Warfarin concentrations (2 and 4 ppm, 2/19: 10.53%), normal adults were emerged.

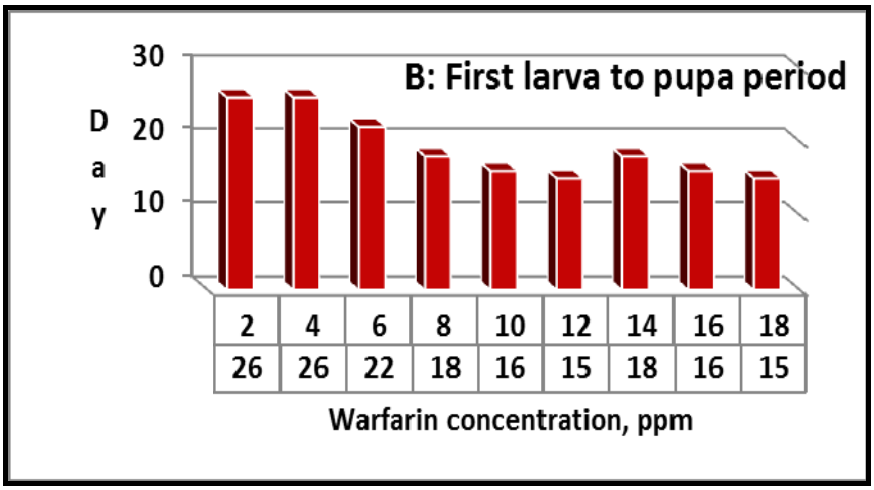

Fig. 2: Developmental periods of larvae intoxicated with different concentrations of Warfarin.

Table 3: Conditions of emerged adults from intoxicated larvae treated with different concentrations (ppm) of warfarin

ppm	Adult	No	%
20-200	Failed to emerge	10	52.63
08-18	Emerged with sluggish movements and died within minutes	6	31.58
06	Emerged but malformed	1	5.26
02 & 04	Emerged normally	2	10.53

Fig. 3: Pupal stage that failed to reach adult stage

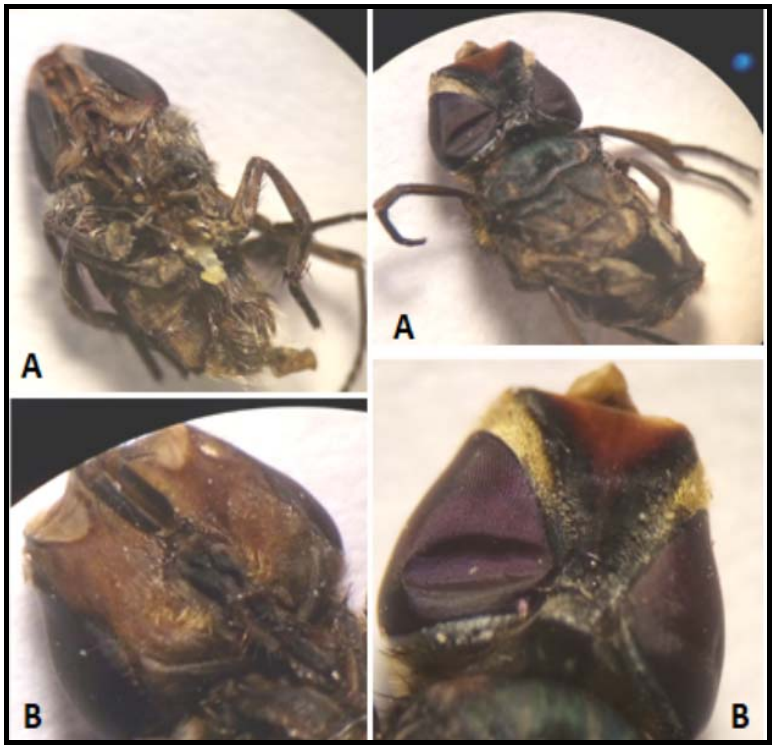

Fig. 4: Malformed Muscidae: Adults (A) and Compound eyes (B)

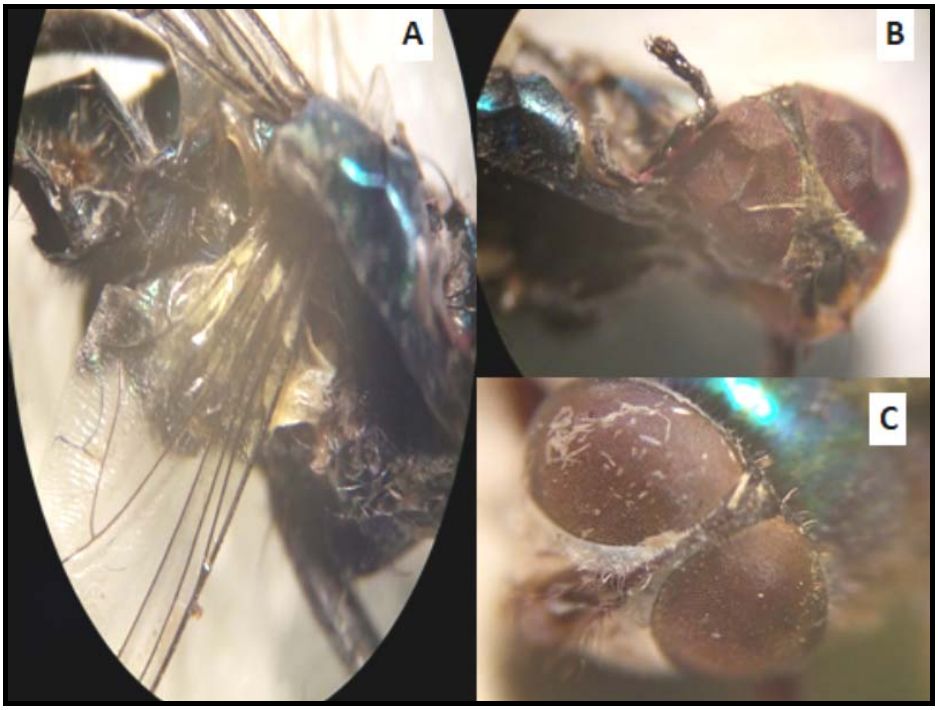

Fig. 5: Calliphoridae: Abnormal abdomen (A), Abnormal compound eyes (B) and Normal compound eyes (C)

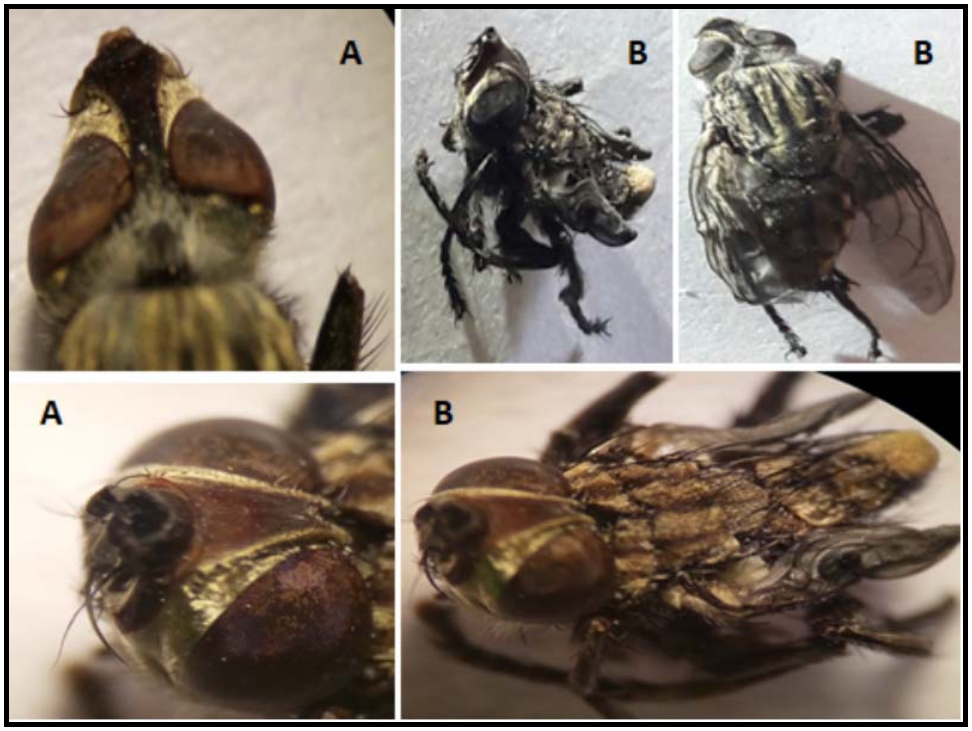

Fig. 6: Malformed Sarcophagidae: Compound eyes (A) and Adults (B)

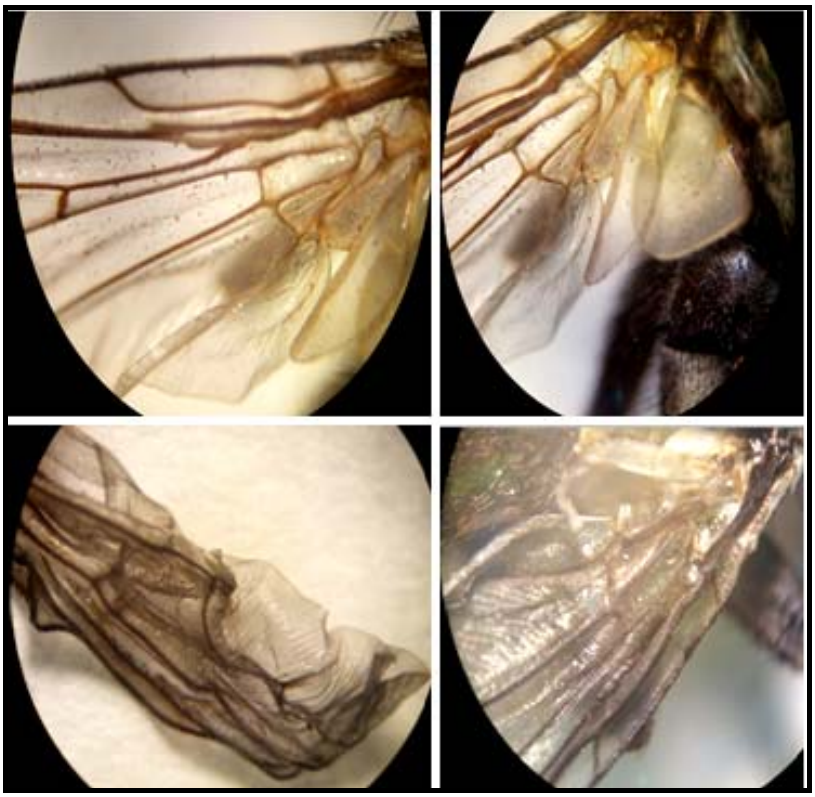

Fig. 7: Wing of Sarcophagidae: normal (Above) and abnormal (below).

DISCUSSION

Research of Entomotoxicology trends have been directed towards elucidating the effects of drugs of use and abuse on development of fly larvae (mainly blow flies) that can be large enough to significantly bias PMI estimates. Necrophagous insects are valuable tools for PMI estimation or for determining the cause of death. Due to the increase in deaths related to drug abuse, it is crucial to know how these substances affect the development of flies that feed on corpses, to avoid errors in the PMI estimates (Souza *et al*., 2011). It was reported (Bourel *et al.,* 1999) that an underestimation of the PMI of 24 h is possible if the presence of morphine in tissues is not considered. This demonstrates the necessity of considering the possible effects of drugs in tissues on insect growth rates when estimating the PMI using entomological techniques.

The present study revealed that Warfarin accelerated the larval development and lead to a malformed adults. As we are aware, no previous studies concerning the effect of Warfarin on development of fly maggots. However, several studies (Goff *et al.*, 1991; Bourel *et al.,* 1999; Carvalho *et al.,* 2001 O'Brien and Turner, 2004; Arnaldos *et al.*, 2005; George *et al.*, 2009; El-Samad *et al.*, 2011; de Carvalho *et al.*, 2012; Magni *et al.,* 2014) examined the effect of other toxins (Methamphetamine, cocaine, Tramadol, diazepam, heroin, morphine and Paracetamol) on the development of several fly larval species (*Sarcophaga tibialis*, *Boettcherisca peregrine, Lucilia cuprina*, *L. sericata, Chrysomya rufifacies, C. megacephala,. C. albiceps, C. putoria* and *Calliphora vomitoria*) and reported the same observations of accelerating the development of examined larval species.

Results of larvae maintained on beef media mixed with different Warfarin concentrations revealed that the highest concentration (200 and 180 ppm and 18 and 16 ppm) resulted in shortest developmental periods estimated for L_1 to pupa which indicated faster development as compared with that of control larvae. Such faster development at the higher concentrations in comparison to the other tested concentrations may be a defense behavior of larvae to avoid the lethal effect of such high concentrations, i.e. faster development to the quiescent puapal stage. It was reported that the total developmental period of *Lucilia sericata* increased in relation to the increasing the administrated dosage of Tramadol (El-Samad *et al.,* 2011). In the present study however, it was found that the developmental periods (L_1 to pupa) increased as Warfarin concentrations decreased (from 200 to 20 ppm or from 18 to 3 ppm).

In the present study with warfarin treatment, 52.63% of larvae (out of the 19 tested concentrations) that were exposed to 20 to 200 ppm (100% of such concentrations) failed to complete its development to adult stage which is in agreement with the observations of Magni *et al* (2014) that about 60% of *Calliphora vomitoria* larvae that were reared on liver substrates with measured amounts of Methamphetamine died during the pupation period. Other larvae (31.58%) yielded adults with sluggish movements and died within minutes and still others (5.26%) that were exposed to 6 ppm, yielded adults with different forms of malformation. The lowest Warfarin concentrations (02 and 04 ppm) had no effect as 10.53% of larvae emerged normal adults.

Sukontason *et al.* (2008), observed dark spots in the integument of the third instar larvae of *Lucilia cuprina*, *Chrysomya rufifacies* and *Chrysomya megacephala* which was thought to be due to the intoxicated substances found in the cadaver. In the present study, no morphological anomalies appeared at any larval instars but only some dead pupae and some adult flies of families Muscidae, Calliphoridae and Sarcophagidae which emerged with malformed appearance, so that Warfarin is clearly sequestered in the larval body and

retained in the next life stage and lately affected adults.

CONCLUSION

Like other toxins and drugs, Warfarin has an effect in accelerating the development of dipteran larvae which is an important indication of cause of death and may be a useful tool for PMI estimation based on entomological evidences.

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ARABIC SUMMERY

تأثير التسمم بمبيد القوارض وارفارين على نمو واعاشة يرقات ذات الجناحين وذات ألأهمية الشرعيه في مصر

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هذه الدراسة تتعلق بتأثير مبيد القوارض وارفارين على نمو واعاشة بعض يرقات الذباب الممجمعه من على ألأرانب المسممة وغير المسممة بالوارفارين والتى تم تربيتها على أنسجة الأرانب غير المسممة. مجموعة أخرى من اليرقات الممجمعه من على الأرانب غير المسممة تم تربيتها على قطع من لحم البقر مختلطة مع تركيزات مختلفة من الوارفارين. اظهرت اليرقات المسممة من المجموعتين فترات نمو أقصر (من يرقات الطور الأول إلى الخادرات) مما يشير إلى تطور أسرع لهذه اليرقات بالمقارنة مع تلك غير المسممة. أما الفترات الزمنية الكلية (من يرقات الطور الأول إلى الطور اليافع) لأنواع الذباب الناتجه من اليرقات المغذاه على أنسجة الأرانب (خاصة لوسيليا سيريكاتا و كريسوميا ألبيسيبس و ك. ميغاسيفالا) تشير بشكل عام إلى تطور أسرع لليرقات المسممة عن تلك غير المسممة ($P<0.01$) وقد تركت تركيزات الوارفارين المختلفة آثارها على الطورااليافع: (١) ٥٢.٦٣٪ من اليرقات التي تعرضت ل ٢٠ إلى ٢٠٠ جزء في المليون فشلت في استكمال تطورها إلى مرحلة اليافع، (٢) ٣١.٥٨٪ من اليرقات أنتجت حشرات بطيئة الحركه والتى ماتت في غضون دقائق، (٣) ٥.٢٦٪ من اليرقات أسفرت عن الطور اليافع مع أشكال مختلفة من التشوه و (٤) ١٠.٥٣٪ من اليرقات أنتجت حشرات عاديه. وخلصت الدراسة إلى أن الوارفارين، شأنه في ذلك شأن السموم والأدوية الأخرى، له تأثير في تسريع تطور اليرقات وهو مؤشر هام لسبب الوفاة، وقد يكون أداة مفيدة لتقدير االفتره الزمنيه بعد الوفاة استنادا إلى الأدلة الحشرية.