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EFFECT OF DIAZEPAM ON THE DEVELOPMENT OF *WOHLFAHRTIA NUBA* (DIPTERA: SARCOPHAGIDAE) AND DETERMINATION OF DRUG LEVELS IN POSTMORTEM TISSUE, LARVAE AND PUP ARIA

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ABSTRACT

Larvae of *Wohlfahrtia nuba were* reared at constant temperature on liver tissue obtained from rabbits administered lethal and double lethal dosages of diazepam. Larvae from colonies fed on tissues from rabbits receiving lethal and double lethal dosages developed more rapidly between 24 and 72 hours after administration as compared to the control. The period required for completion of larval development was significantly shorter for the larvae from both colonies fed on liver tissue from rabbits receiving the drug than the control. However, no relationship was found between drug concentrations and developmental time of larvae. No significant differences were observed among the colonies in the duration of the pupal period. Toxicological analysis of the larvae, prepupae and empty puparial cases detected the drug in small quantities related to the dosages of the drug administered to the rabbits serving as food source.

INTRODUCTION

Over the past several years, human drug-related deaths have increased in different parts of the world. Information on the effects of drugs on larval development becomes essential, particularly in cases in the early stages of decomposition where Diptera are the predominant group present (Early & Goff, 1986). Moreover, insects can be used as alternate specimens for toxicological analysis when conventional postmortem samples are not available (Beyer *et ai,* 1980; Hedouin *et al,* 1999). In other cases where the death is rather old, the only abundant material are insect remains, and particularly puparial cases which stand up to climatic conditions and remain unchanged for a long time after adult emergence (Teskey and Turnbull, 1979).

Diazepam, a benzodiazepine drug, has sedative and tranquilizing effects. Diazepam is included in the long-acting compounds and it is widely prescribed as an antianxiety, antispasmodic and antiepileptic drug (Kudo *et al,* 1988; Knight, 1991). It is one of the tranquilizer drugs mostly associated with unexpected deaths caused by drug overdose (Bortolleto, 1993).

The aim of the present study was firstly to determine the effect of diazepam on the development and growth of larvae as well as to verify the time of pupation that can affect the estimate of postmortem interval and secondly to determine the concentrations of diazepam in feeding, post-feeding larvae and puparial cases of *Wohlfahrtia nuba* offered rabbit liver containing different concentrations of diazepam and finally to detect the correlations of diazepam

concentrations in the tissues used as food and the concentrations in the insects fed on this tissue.

MATERIAL AND METHODS

Four domestic rabbits (1.85-1.65 kg in weight) were used as experimental animals for the administration of diazepam (Valpam). The lethal and double lethal dosages for rabbits (9 mg/kg and 18 mg/kg, respectively) were recorded by Carvalho *et al.* (2001). Diazepam was diluted in 6 ml of saline solution and administered by ear vein injection. Control rabbits (1.65-1.55 kg in weight) received only 6 ml of normal saline solution. After three hours, the rabbits were killed mechanically and samples of blood, liver, muscle and urine were taken for chemical analysis of diazepam concentration.

Flies used in this study were from a colony of *W. nuba* established since 2000 from specimens collected from decomposing rabbit remains in the laboratory of Zoology Department, Sohag University. Beef liver was offered to this colony for a period of 30 minutes. To initiate the test colonies, 100 larvae were reared on50g of each test liver and were maintained under constant temperature of 24 ±1°C and 40-50 % R.H. according to (Khedre, 2003) At 12 h-intervals, 10 larvae were removed from each colony, gently washed in water, dried in paper towel and then weighed to establish the growth curves. Samples of ten 3rd instar (Full-grown larvae) as well as post-feeding larvae were removed from each colony and frozen for detecting the concentration of diazepam. Following completion of larval development, pupae were weighed and observed at 6 h-intervals to determine the duration of pupal period and percentage of adult emergence. After adult emergence, puparial cases were collected for toxicological analysis. Toxicological Analysis A-Extraction Method

The body fluid samples of rabbits injected with diazepam (lethal and double lethal dosages) comprising blood serum and urine were collected and kept in the refrigerator till toxicological analysis.

To detect the concentration of diazepam in these samples the method of Wilson *et al.* (1993) was used. Solid tissue such as liver and muscles (0.5 gm each) were frozen on dry ice and stored at -20 °C. They were then immersed in boiling acetic acid (0.5 M) and heated to 56 °C for 10 min in a water bath to inactive proteolytic enzymes. The tissues were chilled on ice and homogenized with a glass potter. The homogenates were centrifuged (4800 X g, 4 °C, 30 min) and the supernatants were prepurified on a Sep-Pak Cig cartridge (BIO-RAD, USA) equilibrated with 50% acetonitrile. The cartridge was rinsed with 0.12% trifluoroacetic acid (TFA) and the peptide fraction was eluted with 50% acetonitrile. Samples of the dried extracts were stored at room temperature until analysis.

Larvae and puparial cases (10 each) which had fed on drug-laden liver were accurately washed by distilled water and the samples were homogenized. The homogenate was made up to 20 ml with distilled water and homogenized again and vibrated for 10 minutes by senicator (HP-Japan). Quantities of 4 ml of the homogenate were used for extraction of diazepam from solid tissue according to Wilson *et al.* (1993).

B- Quantitative Diazepam / Benzodiazepine

An analytical method was able to detect various benzodiazepine compounds in a single run. Enzymatically hydrolyzed urine or blood serum underwent a liquid-liquid extraction procedure with chloroform/isopropanol (9:1) at pH 8-9 followed by a solid-liquid clean up (Bond Elute TCA ® C 18) to obtain appropriate extracts for GC/MS (TDX, Viva) analysis. Mobile phase composition was optimized by means of the linear solvation energy relationship methodology based on Reichardt's normalized solvatochromatic parameter (ETN) (Wilson *et al,* 1993).

RESULTS

Results presented in Table 1 and Figure. 1 show that the rate of development of *W. nuba* larvae, based on larval weight measurements, was not significantly different among samples of larvae reared on liver tissues of rabbit receiving lethal dosage (R_1), twice lethal dosage (R_2) of diazepam or the control (R_o) at the early larval instar. At about 24h following the molt to the second instar, significant differences ($P < 0.05$) in the mean larval weights were observed in larvae reared on liver tissues of rabbit receiving (R_1) and (R_2) of diazepam compared to the control (R_o). The acceleration of larval development continued for the following 72 h after larviposition. However, at hours 84 and later no significant differences ($P >0.05$) were observed among larvae from different colonies. On the other hand, drug concentrations had no effect on larval development as the larvae reared on liver tissue receiving R_1 and R_2 developed at similar rates through the whole larval stage.

Maximum total weights were recorded at hour 84 after drug administration for larvae reared on R_1 and R_2 and at hour 96 for larvae reared on R_o. This corresponded to the end of larval growth and the beginning of the prepupal stage, marked by cessation of feeding, a decrease in body weight and migration away from the food source. At hour 96 prepupae were first observed for larvae reared on R_1 and R_2 and at hour 108 for larvae reared on R_o. As the larvae entered the pupal stage, a rapid decrease in weight was observed. Statistical analysis showed that there was no significant difference ($P>0.05$) in the mean weights of pupae among the different colonies. Pupation was first observed in R_1 and R_2 colonies at hour 144, followed by the R_o colony at hour 162 (Table 2). The initial time required for pupation was significantly different ($P< 0.05$). However, the pupation time was not affected by the drug concentration as larvae reared on liver tissues receiving the lethal or double

lethal dosage of diazepam started and finished their pupation time almost at the same time. No significant differences (P>0.05) were observed in the total time required for ending pupation between R_o and both R_1 and R_2 colonies. Associated with early pupation, adult emergence was earlier in pupae from the colonies reared on tissue from rabbits receiving dosage of diazepam than in pupae from the control. However, there were no significant differences (P>0.05) in the duration of the pupal stage among the different colonies (Table 2). Larval mortality was higher for the R_2 colony (27.5%) followed by the R_1 colony with 18.1%. The control colony had the lowest larval mortality (9.1%), and the pupal mortality (8.05%). Meanwhile, the R2 colony had the highest mortality percentage for both larvae and pupae (Table 2). Results obtained showed no significant differences (P>0.05) between RQ and R_1 in both larval and pupal mortality. However, significant differences were observed between R_o and R_2.

Toxicological analysis revealed that all blood, urine and tissue samples from rabbits receiving dosages of diazepam were positive for the drug. For each rabbit, diazepam tissue concentrations were consistent with the dosage of diazepam administered (i.e. increased with dose) (Table 3). Also, diazepam could be detected in both 3rd instar and prepupal stage. However, the drug was found in the 3rd instar in a higher concentration than in prepupal stage. Moreover, a small quantity of diazepam was detected in the cuticle of the puparium. On the other hand, the concentrations of diazepam in the developmental stages were lower than those in the liver tissues (Table 3).

DISCUSSION

Results obtained during this study revealed that the presence ofdiazepam in rabbit liver tissues shortens the duration of larval stage of *Wohlfahrtia nuba* feeding on them. This suggests that the drug accelerates the rate of larval development. This acceleration occurred between hours 24 and 72. In this period the larvae reared on the liver treated with diazepam weighed significantly more than the control. It can be concluded that the drug is probably incorporated in and affected, the larval development after 24 h of exposure to the drug. In this respect, Carvalho *et al.* (2001), dealing with the effects of diazepam on the growth of *Chrysomya albiceps* and *C. putoria*, reported that between 18 and 54h, estimates of larval age based on total weight can be significantly mistaken, if presence of diazepam in tissues is not considered. Also, O'brien and Turner (2004) reported that paracetamol accelerated larval development of blowfly *Calliphora vicinia*, particularly during days 2-4 of development in comparison to the control group.

The present study revealed that the time required for starting pupation was shortened in larvae reared on liver from rabbits receiving different dosages of diazepam. Similar results were observed by Goff *et al.* (1989, 1991) who indicated that pupation occurred earlier in larvae of the flesh fly *Boettcherisca peregrina* feeding on tissues from rabbits receiving a dosage of cocaine or

heroin than in the control. Moreover, Goffer *et al.* (1992) reported that changes in the development rates for *Parasarcophaga ruficorins* larvae reared on liver tissues from rabbits administered different dosages of methamphetamine were sufficient to alter the postmortem interval estimates based on larval development by up to 18 h. On the contrary, the study carried out by Goff *et al.* (1993) on the effect of amitriptyline (a tricyclic antidepressant) on the *P. ruficornis* showed no significant differences among colonies in the rate of development to maximum size. Once maximum size had been attained, a prolonged post-feeding period was recorded and thus duration of the larval stage was significantly longer. Also, Bourel *et al.* (1999) reported that the presence of morphine in rabbit tissues appears to retard the normal growth rate for *Lucilia sericata* during larval stages.

The present study showed that there was no relationship between the concentration of diazepam in tissues and the duration of larval stage. Similarly, Musvasva *et al.* (2001) reported no linear dose-dependent relationship was found between dosages of drugs (hydrocortisone, sodium methohexital) and development time of larvae, *Sarcophaga tibialis*, when they were reared on liver treated with these drugs. However, Goff *et al.* (1989, 1992) demonstrated that the larval development was accelerated and pupation occurred earlier on *B. peregrina* and *P. ruficornis* feeding on tissues with higher concentrations of cocaine and methamphetamine. Moreover, lower concentrations of methylenedioxymethamphetamine (MDMA) delayed larval development of *P. ruficornis (Goffet al.,* 1997). On the other hand, larvae of I. *sericata* fed on the carcass receiving the greatest dosages of morphine were the slowest to develop (Bourel *et al.,* 1999).

The present data indicated that there were no differences in the weights of pupae related to the presence of diazepam in the tissues. These results were in agreement with those obtained by Bourel *et al.* (1999) during their studies on the effect of morphine on development of *L. sericata.* In contrast, Goff *et al.* (1993) showed that colonies of *P. ruficornis* larvae reared on tissues receiving the median lethal and twice median lethal dosages of amitriptyline produced pupae which were significantly greater both in terms of length and weight. This appears to indicate different responses to the drug based on systematic differences among the Diptera.

Results of the present study indicated that the duration of pupal stage was not affected by the presence of diazepam in tissues. Similarly, Goff *et al.* (1989, 1997) reported that there were no differences noted in the duration of pupal stage for colonies of *B. peregrina* and *P. ruficornis* fed on tissues containing cocaine and methylenedioxymethamphetamine, respectively. On the other hand, studies dealing with heroin (Goff *et al.,* 1991) and amitriptyline (Goff *et al.,* 1993) revealed that the duration of pupal stage was longer for colonies fed on tissues containing these drugs.

As a general rule, if the larvae feed on a tissue containing some kind of drug or substance, there are two processes that may happen: a bioaccumulation or an excretion of the drug (Carvalho *et al*, 2001). Also it is clear that the measured drug levels must represent absorption into the larval tissues. The present study indicated that there was a bioaccumulation in the larval tissues since the presence of the drug had an effect on. larval growth and larval mortality. Although toxicological analysis detected tie drug through the larval stage of *W. nuba,* the concentrations of the drug in the larval tissues were lower than in the tissues used as food source. Similar results were obtained earlier for *Lucilia sericata* (Hedouin *et al.,* 1999) for *Phormia taeraenovae* and *Calliphora vicina* (Hedouin *et al.,* 2001); for *C. aflticepes* and *C. putorim* (Carvalho *et al.,* 2001). The obtained results in the present work differ from those obtained by Introna *et al.* (1990) in which the concentrations of the drug (morphine) in *C. vicina* larvae were quite similar to those in the human tissues used.

The present results indicated that *W. nuba* larvae metabolize and eliminate the drug with varying level, of efficiency since larval drug concentrations vary considerably throughout larval development with a clear decrease in the drug concentration measured in post-feeding larvae. This result suggested that actively feeding larvae on corpse should be sampled for toxicological analysis because they represent the best source of drug residues as reported by Introna *et al.* (2001).

The present study revealed that the drug was found in very low concentrations in the pupal cases. The result was similar to those reported by Hedouin *et al.* (2001) in which a small amount of morphine was measurable in puparial stage. This result just confirmed that larvae excrete the drug during the post-feeding stage. This elimination before the process of metamorphosis was also observed by Sadler *et al.* (1997).

CONCLUSIONS

The present study and earlier studies demonstrated that there are differences in the rates of development of necrophagous flies when they feed on tissues containing drugs. Different species appear to have different responses to drugs and rates of development can be either increased,, retarded or unchanged. The present study demonstrated that differences observed in the rates of larval development were sufficient to alter postmortem interval estimates based on larval development by 18 h. Also based on the results obtained from this study, between hours 24 and 72, estimations of larval age based on total weight can be significantly in error if the presence of diazepam in the tissues is not considered. This study demonstrated the necessity of considering the possible effects of drugs in tissues on insect growth rates when estimating the postmortem interval using entomological techniques.

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Table 1. Mean total body weights (mg) of *W. nuba* larvae and newly formed pupae reared at temperature of 24 °C±1 on liver tissue of rabbit administered different dosages of diazepam via ear vein injection (± SE).

Time of sampling (h) after diazepam administration	Mean total body weights		
	R_0 (Control)	R_1	R_2
12	0.625±0.08	0.65 ±0.08	0.70 ±0.05
24	1.66 ±0.11	2.18 ±0.08	2.42 ±0.14
36	4.78 ±0.73	7.38 ±0.52	7.33 ±0.68
48	13.89±1.93	26.12±1.60	26.13 ±2.14
60	30.87±4.9	54.42±3.20	51.79 ±3.70
72	69.49±5.56	90.32±3.07	87.76 ±3.70
84	87.44±5.12	97.59±3.75	100.09±4.86
96	97.53±4.84	92.16±3.03	85.85 ±3.56
108	83.27±4.12	80.22±3.97	77.90 ±3.83
120	78.43±4.56	78.30±4.87	76.59 ±5.82
132	68.72±2.32	67.06±2.43	68.02 ±2.61
144	67.58±3.43	57.71±1.19	54.58 ±1.56
156	61.08±2.03	---------	--------
162	53.79±2.73	--------	--------

Table 2. Duration of larval stage, larval mortality, duration of pupal stage and pupal mortality for colonies of *W. nuba larvae* reared on rabbit liver tissue containing varying amounts of diazepam at temp. of 24 °C (± SE).

Colony	Duration of larval stage (h)	Larval mortality	Duration of pupal stage (h)	Pupal mortality
R_0 (Control)	191.18±23.7 (a) (162-246)	9.1%	364.8±2.8 (a) (324-408)	8.05%
R_1	177.3±17.6 (b) (144-234)	18.01%	359.3±3.2 (a) (312-408)	15.8%
R_2	173.2±18.5 (b) (144-234)	27.2%	360±3.0 (a) (312-408)	25.5%

[a] Means in a column followed by the same letters are not significantly different (P>0.05).

Table 3. Concentrations of diazepam in samples of rabbit tissues administered different dosages of the diazepam and from larvae and puparial cases of *W. nuba* fed on liver tissues (ng/mg).

Samples	Concentration of benzodiazepine (ng/mg)		
	R_o (Control)	R_1	R_2
Cardiac blood	ND	3780	4010
Liver	ND	10810	17100
Muscle	ND	8170	13620
Urine	ND	4200	4600
Larvae	ND	630	760
Prepupae	ND	467	509
Pupal cases	ND	327	399

ND = None detected.

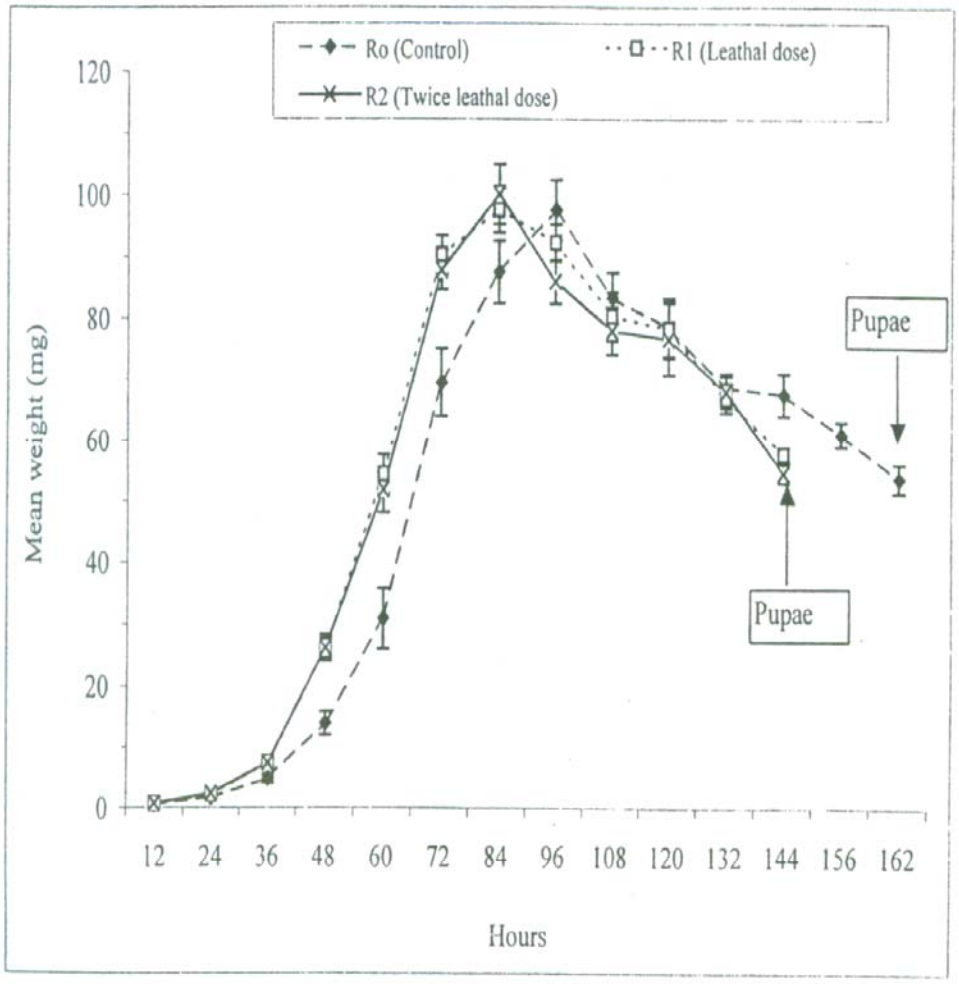

Fig. 1. Changes in mean weights of larvae and pupae of *Wohlfahrtia nuba* reared on liver tissues of rabbit administered different amounts of diazepam via ear vein injection.