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Impact of Diazepam on the Development of *Chrysomya albiceps* (Wiedemann 1819) (Diptera: Calliphoridae) in a Rabbit Carcass

Layla Ouda Al-Shareef[1]*, Ohoud Mohammed Alazwari[1], Sami Saeed Al-Ghamdi[2]

[1]Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia.
[2]Poison Controls and Forensic Chemistry Centre, Jeddah, Saudi Arabia.

***E-mail** ✉ loalshreef@uj.edu.sa

ABSTRACT

The larvae of necrophagous fly species serve as valuable forensic indicators for estimating the minimum postmortem interval (PMI). However, the presence of drugs within a corpse can affect larval growth, potentially leading to inaccuracies in PMI determination. This study investigated how diazepam affects the developmental progression of *Chrysomya albiceps* and assessed the suitability of this fly species for toxicological investigations. To achieve this aim, *C. albiceps* larvae were raised on rabbit carcasses containing a lethal dose of diazepam. The growth rate of the flies was monitored and samples of carcass tissues as well as different fly stages were analyzed for the presence of diazepam. The findings showed that the drug was detected in all tissue samples and at different life stages of *C. albiceps*, including larvae, pupae, and adults, demonstrating their reliability for qualitative toxicological assessment. The presence of diazepam led to an acceleration in larval development by 12 hours, while the pupal phase remained unchanged. Furthermore, a 12-hour discrepancy in the overall development period from the first larval instar to adulthood must be taken into account when diazepam is present in the deceased body.

Keywords: Entomotoxicology, Forensic entomology, Drugs, *Chrysomya albiceps*

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Introduction

In medico-legal investigations, entomological evidence is most commonly applied to estimate the minimum postmortem interval (PMI). Flies from the order Diptera, particularly those in the family Calliphoridae, are frequently utilized for this purpose due to their rapid arrival and colonization of remains. *Chrysomya albiceps* (Wiedemann, 1819) is among the first insects to lay eggs on human remains. Once the larvae hatch, they consume decomposing tissue. By determining the developmental stage of these flies, it becomes possible to approximate their colonization time, thereby aiding in PMI estimation [1-4]. Although the life cycle of many forensic fly species has been well documented, various environmental conditions can impact their growth rates. Factors such as temperature, humidity, body positioning, larval crowding, and the presence of toxic substances or drugs in the remains can influence insect development. Prior research has demonstrated that certain drugs can either accelerate or delay the growth rate of different fly species [5, 6]. Ignoring the presence of such substances in a corpse may result in inaccurate PMI estimations. Additionally, cases of poisoning often remain undetected until the remains reach an advanced state of decomposition, making traditional toxicological analysis of tissues and bodily fluids impractical. Recently, forensic entomologists have introduced insect evidence as a valuable tool for interpreting postmortem details [7-9]. Fly larvae that feed on a body absorb any toxic substances present, which can then be

analyzed to detect these compounds. This field, known as forensic entomotoxicology, examines how drugs and toxins affect the development of necrophagous insects and explores the use of these insects in detecting toxic substances within animal tissues [8-12]. The objective of this study was to evaluate how diazepam influences the development of *C. albiceps* when present in rabbit carcasses, as well as to determine the concentration of diazepam in rabbit tissues and different developmental stages of the fly, including larvae, pupae, and adults.

Materials and Methods

Experimental design

This research was conducted during the autumn season, spanning from November 23, 2017, to December 7, 2017, in Jeddah, a city situated along the western coast of Saudi Arabia (longitude 39.7° E, latitude 29.21° N) on the eastern shoreline of the Red Sea. Diazepam for the study was provided by the pharmacy of King Abdulaziz University Hospital, which operates under the Ministry of Health in Jeddah, Saudi Arabia. The experiment involved six domestic rabbits, each weighing between 3.75 and 3.96 kg, which were divided into two experimental groups, with three rabbits in each. The first group received an oral suspension of diazepam at a lethal dose of nine mg/kg body weight, as described in [13], while the second group served as a control and was administered distilled water. The rabbits were euthanized by mechanical cervical dislocation.

In both the control and treated groups, the carcasses were dissected to collect three tissue samples, each weighing 5.003 kg, from the heart, lungs, liver, kidneys, and skeletal muscle. The carcasses were then moved outdoors and placed inside specialized metal cages with soil flooring. These cages, made with a 2 cm^2 mesh, allowed insect access while preventing scavengers from disturbing the remains. The caged carcasses were arranged in two separate rows, with a 10-meter distance between them. Daily environmental data, including temperature and relative humidity for the study location, were sourced from the general authority for meteorology and environmental protection in Jeddah.

Determination of the developmental period and accumulated degree hours (ADH) for the immature stages of C. albiceps

Carcasses at the study site were observed until *C. albiceps* flies arrived and deposited eggs. Subsequently, examinations were conducted every 12 hours to track the immature stages of *C. albiceps*. The duration of each developmental phase—including the pre-incubation period, incubation period, first, second, and third larval instars, pupal stage, and the total time from the first larval instar to adult emergence—was documented.

The lower developmental threshold temperature (Dz) was obtained from previous research. For the incubation period, a Dz of 9.72 °C was used [14], and the same value was applied to the pre-incubation phase due to the absence of specific data in prior studies. The first larval instar had a Dz of 11.14 °C [15], while the second larval instar was recorded at 13.00 °C [15]. Since no Dz value was available for the third larval instar, the Dz of the overall larval stage was used, which averaged 14.48 °C based on values of 15.04 °C [16] and 13.92 °C [15]. For the pupal stage, Dz values reported were 17.39 °C [16], 11.65 °C [14], and 13.65 °C [15], with an average of 14.317 °C. The Dz for the entire period from the first larval instar to adult emergence was recorded as 15.38 °C [16].

Given that the experiment took place under fluctuating temperatures, the accumulated degree hours (ADH) for each developmental stage were determined using the equation:

$$DH = [(\text{maximum temperature} + \text{minimum temperature}) / 2] - Dz \quad (1)$$

ADH = DH × D where Dz represents the lower developmental threshold temperature (°C), and D denotes the duration of development (hours) [17].

Toxicity assessment in insects and rabbit carcass tissues

For both the treated and control rabbit carcasses, three replicate samples, each weighing 3 grams, were collected from insect specimens (third larval instar, pupae, and adult *C. albiceps*) and various carcass tissues (liver, heart, kidney, lung, and skeletal muscle). These samples were rinsed twice using distilled water and a 0.9% NaCl physiological solution, then blotted dry with filter paper. Afterward, they were placed in plastic containers and stored in a deep freezer at -20 °C until further analysis.

LC-MS-MS method

A Thermo LCQ Fleet system combined with an MS/MS Ion Trap was utilized. The LC was equipped with a Gemini C18 column (5 µm particle size, 150 mm × 2.0 mm) and safeguarded by a guard column containing packing material (4 mm × 2 mm), with the column temperature set at 25 °C. A single gradient composition and column were employed to optimize the LC process. Mass spectrometry was conducted in positive ion mode for all drugs, with a probe voltage of 4.5 kV. Full-scan MS/MS was applied for drug screening.

Drug tuning and optimization of drug separation

Tuning was performed separately for the drug and its internal standard to optimize the sheath gas flow rate, auxiliary gas flow rate, and collision energy. During the tuning process, working solutions of 10 mg/L were utilized for each drug. Compound separation was carried out within the LC system. Optimization of the mobile phase type and flow rate was achieved by preparing drug solutions at a concentration of 1 mg/L, including the internal standard.

Sample preparation

A total of fifteen milliliters of deionized water was added to 1g of the sample in a stomacher bag, and the mixture was homogenized at high speed for 2 minutes. A 2 ml portion of the homogenized sample was transferred to a centrifuge tube, to which one milliliter of phosphate buffer and fifty microliters of internal standard (I.S.) were added. The tube was vortexed and then centrifuged for 5 minutes at 4500 revolutions per minute.

SPE extraction

Clean screen columns were placed in the SPE vacuum manifold. For activation, each column was treated with three milliliters of methanol, followed by three milliliters of deionized water, and then one milliliter of phosphate buffer (pH 6.0), all at a flow rate of 2 ml/min. The sample was then loaded onto the columns at a flow rate of 1 ml/min. Following this, deionized water was added to the column, followed by two milliliters of 0.1 M acetic acid. The columns were dried under a high vacuum for five minutes. In the first elution step, labeled collecting tubes were placed into the manifold, and 2 ml of a 50% ethyl acetate and 50% hexane mixture was added under low or no vacuum pressure. In the second elution step, the columns were dried under vacuum for 2 minutes before 2 ml of DCM/IPA/NH4OH (78/20/2) was added at a flow rate of 1 ml/min. The elution was dried using nitrogen or a Genevac vacuum system, then reconstituted by adding two hundred microliters of the initial mobile phase and vortexed for thirty seconds. The reconstituted sample was transferred to an HPLC vial, and twenty microliters of the sample were injected into the LC-MS/MS. The concentration of Diazepam in the samples was determined by referring to the calibration curve.

Statistical analysis

The duration of each stage, including pre-incubation, incubation, and the three larval instars, as well as the pupal phase and the period from the first larval instar to adult eclosion, was calculated by determining the mean value based on the method of Arkin and Colton [18]. For the analysis of diazepam levels in the tissues of rabbit carcasses and different developmental stages of *C. albiceps*, factorial experiments were employed using a randomized complete block design with 3 replicates for each factor. The diazepam content was assessed across eight levels, corresponding to the various tissues of the rabbit carcass and different stages of *C. albiceps*. Statistical analysis included the use of the F test, with the results presented in an ANOVA table, followed by Duncan's test for mean comparisons, following Snedecor [19].

Results and Discussion

In this study, *C. albiceps* larvae were reared on both diazepam-treated and control rabbit carcasses. The experiment took place under varying temperature conditions, with minimum temperatures ranging from 21.8 °C to 25.8 °C and maximum temperatures fluctuating between 27.37 °C and 31.8 °C. The relative humidity levels ranged from 68-73.5%.

Impact of diazepam on the developmental rates of C. albiceps immatures

The data presented in **Table 1** reveals that the pre-incubation period for *C. albiceps* on diazepam-treated rabbit carcasses (20 hours) was shorter than that on control carcasses (30 hours), while the incubation period was longer in the treated group (18 hours) compared to the control group (12 hours). The duration of the first larval instar and the late third larval instar in the treated group (12 hours and 24 hours, respectively) was half that of the control group (24 hours and 48 hours, respectively). Conversely, the 2nd larval instar and early third larval instar in the treated group each took twice as long (24 hours) compared to the control group. The overall larval stage duration in the treated group (84 hours) was shorter than in the control group (96 hours). Both groups had similar pupal stage durations (24 hours). From the first larval instar to adult eclosion, the treated group (108 hours) took less time compared to the control group (120 hours) **(Figure 1)**.

According to the data in **Table 1**, the accumulated degree-hours (ADHs) for the pre-incubation period in control rabbits (482.4 degree-hours) was higher than in the treated rabbits (319.6 degree-hours), while for the incubation period, the accumulated degree-hours in the control group (220.56 degree-hours) were lower than in the treated group (306.72 degree-hours). The accumulated degree-hours required for the first larval instar and late third larval instar in the diazepam-treated group (187.32 degree-hours and 298.08 degree-hours, respectively) were approximately half of those in the control group (374.64 degree-hours and 534.48 degree-hours, respectively). However, the second larval instar and early 3rd larval instar in the treated group required about twice the ADHs compared to the control group (337.2 degree-hours and 295.68 degree-hours in the treated group vs. 172.8 degree-hours and 147.84 degree-hours in the control group). The accumulated degree hours for the pupal stage were 320.88 degree-hours for the control group and 299.28 degree-hours for the treated group. Lastly, the first larval instar in the control group required more heat to reach adulthood (1412.4 degree-hours) compared to the treated group (1238.76 degree-hours).

Detection of diazepam in carcass tisseus and C. albcipes stages

Diazepam was detected in all carcass tissues and developmental stages of *C. albiceps* analyzed, as indicated in **Table 2**. Among the carcass tissues, the liver contained the highest diazepam concentration (472), followed by pupae (211.5), kidney (182.6), lung (180.3), and heart (162.3). The lowest levels of diazepam were found in adult flies (38.833), third larval instar (35), and skeletal muscle (32.8), with significant differences observed.

Table 1. Development duration and thermal requirements (ADH) for different stages of *C. albiceps* feeding on control and treated rabbit carcasses with diazepam at fluctuating temperatures

The stages	Base Temp. (Dz)	Control rabbit carcasses					Treated rabbit carcasses				
		Temp (°C)		R.H. (max %)	Duration (hour)	ADH (°C)	Temp. (°C)		R.H. (max %)	Duration (hour)	ADH (°C)
		Min	Max				Min	Max			
Pre-incubation period	9.72	21.9	29.7	68	30	482.4	21.8	29.6	70	20	319.6
Incubation period	9.72	25.8	30.4	69.7	12	220.56	23.5	30.02	69.7	18	306.72
1st larval instar	11.14	23.5	30	69.8	24	374.64	23.5	30	69.8	12	187.32
2nd larval instar	13.00	24.6	30.2	69.5	12	172.8	24	30.1	69.5	24	337.2
Early 3rd larval instar	14.48	23.6	30	72	12	147.84	23.6	30	73.5	24	295.68
Late 3rd larval instar	14.48	23.5	27.73	70	48	534.48	23.5	30.3	71.3	24	298.08
Larval stage	14.48	23.8	29.5	70.3	96	1168.32	23.65	30.1	71.0	84	1041.18
Pupal stage	14.23	23.4	31.8	71	24	320.88	23.4	30	70	24	299.28
1st larval instar to adult eclosion	15.38	24.2	30.1	70	120	1412.4	23.6	30.1	70.8	108	1238.76

N = 30 insects for each development stage

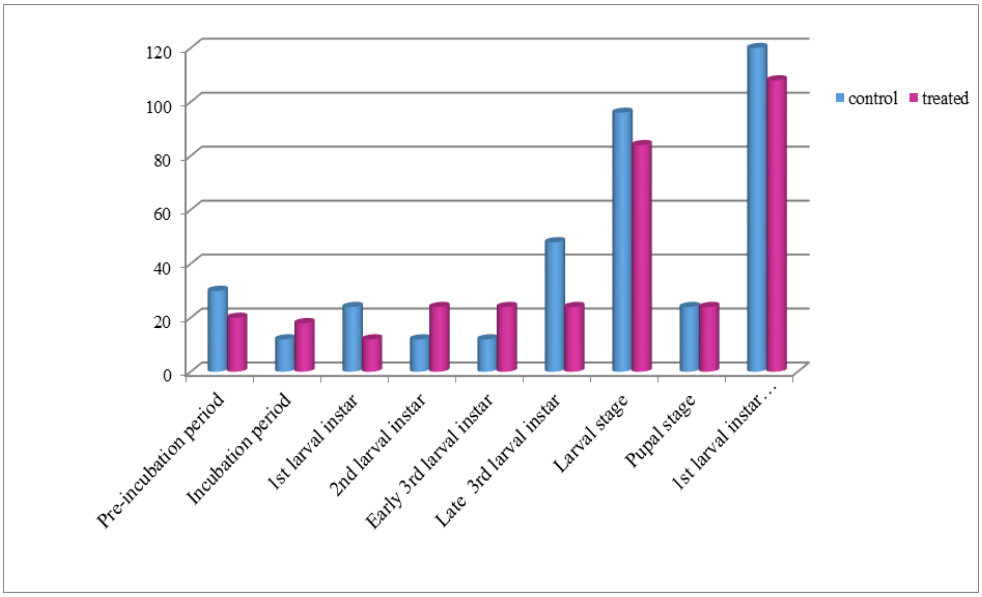

Figure 1. Development duration for different stages of *C. albiceps* feeding on control and treated rabbit carcasses with diazepam at fluctuating temperatures.

Table 2. Diazepam quantitation (ug/mg) in samples of rabbit tissues and deferent stages of *C. albiceps*

Type of tissue	Mean of diazepam content
Heart	162.3 B
Lung	180.3 B
Liver	472 A
Kidney	182.6 B
Skeletal muscle	32.8 C
3rd larval instar	35 C
pupae	211.5 B
Adult flies	38.833 C

Different letters indicate that there are significant differences between the averages.

In this study, *C. albiceps* females laid their eggs on control carcasses 30 hours after death, while in the treated groups, egg deposition occurred 20 hours post-mortem. This finding aligns with the work of Ekrakene and Odo [20], who demonstrated that when rabbits were treated with cypermethrin and tramadol, *C. albiceps* eggs appeared at 24 hours post-death in the control group and at 6.25 hours in the treated group. In contrast, the present study found the incubation periods to be 12 hours in the control group and 18 hours in the treated group. Fouda *et al*. [21] observed that the incubation period of *C. albiceps* did not show significant differences between dog carcasses treated with ephedrine sulfate and normal carcasses.

This research also highlighted that diazepam accelerated larval development during the early and late stages, but slowed growth during the intermediate phases. Overall, the larval stage duration was reduced from 96 hours in the control group to 84 hours in the treated group. These findings are consistent with earlier studies, such as that of Carvalho *et al*. [11], who found that *C. albiceps* larvae reared on rabbit tissues containing diazepam developed more quickly compared to those from control groups. Fathy *et al*. [22] recorded faster larval development in *C. albiceps* after they consumed diazepam-treated rabbit carcasses, with the treated group taking 72 hours and the control group 96 hours. Similarly, de Carvalho *et al*. [23] observed that *C. albiceps* larvae fed on cocaine-laced livers developed faster than those fed on livers from untreated animals.

The present study showed that diazepam didn't influence the duration of the pupal stage. This finding is in line with the observations of Goff *et al*. [24, 25], who reported no differences in the length of the pupal stage for *B. peregrina* and *P. ruficornis* colonies fed on tissues containing cocaine and methylenedioxymethamphetamine, respectively. However, this result contrasts with that of Carvalho *et al*. [11], who found that feeding *C. albiceps*

on diazepam-treated rabbit carcasses inhibited growth during the pupal stage when compared to the control group. Additionally, Ekrakene and Odo [20] noted that both tramadol and cypermethrin in rabbit carcasses extended the pupal stage relative to the control carcasses.

The observed variation in diazepam's effect on different stages of fly development aligns with findings by Kharbouche *et al*. [26], who noted that larvae of *Lucilia sericata* fed on liver samples treated with codeine developed faster than control larvae, though codeine had no impact on the pupal development. Similarly, Khedre [27] understood that when larvae of Wohlfahrtia nuba were reared on liver tissues from rabbits administered a lethal dose of diazepam, the presence of diazepam shortened the larval stage duration, but didn't affect the pupal stage. Furthermore, Fouda *et al*. [21], in their study on dog carcasses treated with ephedrine sulfate, found that while the larval stage was not significantly affected, the pupal stage duration in the treated group was notably longer than that in the control group.

This study demonstrated that the presence of diazepam in *C. albiceps* resulted in a 12-hour acceleration in the life cycle compared to the control group. Previous studies involving insects and various drugs have suggested that certain substances act as feeding stimulants for specific fly species. For instance, Carvalho *et al*. [11] observed that *C. albiceps* larvae fed on tissues containing diazepam experienced a faster adult emergence compared to the control group. Similarly, O'Brien and Turner [28] reported that *Calliphora vicina* larvae fed on pig liver containing paracetamol showed a 12-hour variation in life cycle duration [28]. Additionally, Fathy *et al*. [22] understood that when *C. albiceps* larvae fed on rabbit carcasses treated with codeine phosphate, the life cycle was accelerated, resulting in a 24-hour variation in PMI estimation based on larval development and 48 hours for pupal development. Abd El-Gawad *et al*. [29] observed that *C. albiceps* larvae on warfarin-treated rabbit carcasses developed more quickly than those on control carcasses. In contrast, other studies have reported opposite findings. Rashid *et al*. [30] found that larvae of *Chrysomya megacephala* in the control group developed faster (7 days) compared to those treated with malathion (10 days). Rezende *et al*. [31] understood that *C. albiceps* larvae reared on an artificial diet containing phenobarbital and methylphenidate hydrochloride exhibited a longer developmental time compared to the control group. Furthermore, Ekrakene and Odo [20] noted that cypermethrin and tramadol in rabbit carcasses extended the developmental period from egg to adult in *C. albiceps* compared to the control. Similarly, Fouda *et al*. [21] understood that *C. albiceps* fed on ephedrine-sulfate-treated dog carcasses experienced significantly longer development (17 ± 0.25 days) compared to the control (16 ± 0.25 days).

In the current study, the thermal requirements at fluctuating temperatures (ranging from a minimum of 23.4 °C to 24.6 °C, and a maximum of 27.73 °C to 31.8 °C) for each larval stage, pupal stage, and the duration from the first larval instar to adult emergence were found to be 1168.32 degree-hours (DH), 320.88 DH, and 1412.4 DH, respectively. In contrast, Al-Shareef and Al-Qurashi [32] reported the heat requirements at a constant temperature of 29.5 °C for the same stages, with 75.01 degree-days (DD) for the larval stage, 62.73 DD for the pupal stage, and 129.138 DD for adult eclosion [32]. Additionally, Shiravi *et al*. [33] recorded data from Tehran at 28 °C, with values of 30-45 DD for the larval stage, 62-93 DD for the pupal stage, and 130-195 DD for adult emergence. When compared to Shiravi *et al*. [33], the thermal requirements observed in this study differ, reflecting variations in developmental conditions across different geographic locations.

Detection of diazepam in carcass tisseus and C. albcipes stages

In the present study, toxicological testing showed that all tissue samples from treated rabbit carcasses, as well as various developmental stages of *C. albiceps* (third instar larvae, pupae, and adult flies), tested positive for diazepam. However, the diazepam concentrations found in the different stages of *C. albiceps* were lower compared to those in the rabbit tissues. This finding aligns with the results of previous studies, for example, Khedre [27], who demonstrated that diazepam concentrations in liver tissues were higher than those found in the developmental stages, including third instar larvae, prepupae, and empty puparia of *Wohlfahrtia nuba*. Similarly, Kharbouche *et al*. [26] reported that codeine concentrations in treated pig liver were notably higher than in the larvae. Mahat *et al*. [34] also observed that the concentration of malathion in *C. megacephala* larvae that fed on rabbit carcasses treated with the drug was lower than in the rabbit tissues.

The findings of this study revealed that the liver in rabbit tissues had the highest diazepam concentration, followed by the heart, lung, kidney, and skeletal muscles. For the insect stages, the drug was most concentrated in the pupal stage, surpassing both the third instar larvae and adult flies. These results are consistent with Rashid *et al*. [30] who demonstrated that after *Chrysomya megacephala* larvae fed on a rat carcass treated with malathion, the liver contained the highest concentration of the chemical, followed by the heart and lung. Similarly, the drug

concentration in pupae was higher than in larvae and adult flies. Likewise, Carvalho *et al*. [23] found that after rabbits were administered a lethal dose of diazepam, the highest concentrations of the drug were in the heart and liver, while in the insects, the pupal stage had higher concentrations than the larvae and adult flies. The elevated diazepam levels in liver tissues can be attributed to the liver's role in metabolizing drugs and toxins [35]. The heart, kidney, lung, and skeletal muscles contain drugs due to the presence of metabolic enzymes, such as mixed-function oxidase [36]. However, the present study's results contrast with those of Introna *et al*. [12], where morphine concentrations in *C. vicina* larvae were found to be similar to those in the human tissues used. In general, when larvae feed on drug-containing tissues, two processes can occur: bioaccumulation or excretion of the substance [11]. This study suggests that diazepam is involved in the metabolic processes affecting larval growth. Sadler *et al*. [37] proposed that drug-contaminated tissues consumed by larvae are stored in the crop until the pupal stage, which leads to increased absorption rates compared to elimination during the larval phase [37, 38].

In the present study, diazepam concentration was notably higher in the pupal stage compared to the larval stage. Although this did not influence the length of the pupal period, it could be attributed to the drug accumulating in the puparium's cuticle, without undergoing metabolic processing. Bourel *et al*. [39] suggested that the pupal cuticle functions as a storage organ, similar to adipocytes and pericardial nephrocytes. Anderson [40] also identified an unknown lipid layer beneath the pupal cuticle, responsible for storing substances like drugs. Miller *et al*. [41] observed that pupal cases are capable of retaining toxins, even after the adult insect has emerged and moved away from the carcass. The accumulation or elimination of toxins in insects may depend on the molecular weight of the substance. Bourel *et al*. [39] further explained that in larvae, water-soluble molecules are excreted from the hemolymph by malpighian tubules, while less-soluble molecules accumulate near pore canals in the cuticular matrix, with nephrocyte cells also storing these substances within their cytoplasm.

The study results confirmed that the diazepam concentration in adult flies was lower than in pupae, which is consistent with Bourel *et al*. [39] finding that small amounts of morphine could be extracted from dead adult *L. sericata* flies.

Conclusion

This investigation demonstrated that diazepam accelerates the growth of larvae but does not influence pupal development. Additionally, the study highlights a 12-hour discrepancy that should be considered when diazepam is present in the carcass. The findings also suggest that insects could serve as viable alternative samples for toxicological analysis, as diazepam was detected in insect specimens alongside carcass tissues.

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Conflict of Interest: None

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Ethics Statement: The study was approved by the University of Jeddah. The reference number for the ethics committee is not available.

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