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Effect of Diazepam and Ethanol on Internal Organs During Pupal Development of *Chrysomya megacephala* (Diptera: Calliphoridae) Using Micro-Computed Tomography

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Abstract

Rabbits were treated with three different doses of diazepam, ethanol or diazepam plus ethanol, and their liver was then removed after 1 h for rearing blowfly (Chrysomya megacephala) larvae. The pupae were sampled at three development ages (after cessation of eating) and, after preparation, were examined by micro-computed tomography using a Sky scan 1172 instrument to evaluate the effect of diazepam and ethanol on the development of the internal organs and tissues, such as brain, cavity of the body, muscles, yellow body, meconium, and, especially, the fat body. All the samples in the treatment groups showed abnormal body fat differentiation because metabolic activities were not completed, and these changes affected the functions of every internal system. The retarded differentiation of the fat bodies is an important result because these organs function as the liver in human, suggesting that toxin elimination from the blowfly's body and homeostatic maintenance of the hemolymph proteins, lipid and carbohydrates in each treatment group are abnormal. In the treatment groups, the effect of diazepam on the internal morphological changes of blowfly's pupae was more marked than that for ethanol.

Keywords: Forensic entomology; Entomotoxicology; Blowfly; Differentiation; Micro-computed tomography

Abbreviations: CNS: Central Nervous System; PMI: Post Mortem Interval; Micro CT: Micro Computed Tomography; Di: Diazepam; Et: Ethanol; DiEt: Diazepam mixed Ethanol; LD: Lethal Dose; AN: Antenna; AR: Archenteron; A-T: Abdomen-Thorax Division; BR: Brain; DVM: Dorso-Ventral Flight; FB: Fat Bodies; IM: Internal Mouthparts; ISM: Inter-Segmental Muscle; LGM: Leg Muscles; LHG: Larval Hindgut; LTT: Larval Tracheal Trunk; OM: Ommatidia (Eye); PV: Proventriculus; PT: Ptilinum; RC: Rectum; W: Wing Amg: Adult Midgut; CPS: Cephalopharyngeal; DLM: Dorsal Longitudinal Muscles; LHM: Larval Hypodermal Muscles; LSG: Larval Salivary Grands; ME: Meconium; RP: Rectal Pouch; YB: Yellow Body

Introduction

Nowadays, stress has become a major psychological problem among people of all ages and genders around the world. A stressful way of living can lead to the development of depression, anxiety and, potentially, suicide [1-3]. In recent decades, benzodiazepines have been used to treat anxiety, seizures, and insomnia. These man-made medicines cause mild to severe depression of nerves within the central nervous system (CNS) and sedation and are in common usage in adult men and women. Benzodiazepines are a potent, fast-acting and prescription only medication, but are used in overdose by many suicides, especially diazepam that is frequently co-imbibed with ethanol. Both diazepam and ethanol have some similar actions and work together to slow down the CNS, stop heart beating or breathing and, therefore, prevent oxygen reaching the brain leading to a loss of brain function and death [4-7]. Diazepam is a potent long-acting drug in the benzodiazepines class, and used as prescribed, it is believed to be safe and so is used extensively in the treatment of generalized anxiety and panic attacks, with or without agoraphobia and depression [8]. Diazepam is readily absorbed from the gastrointestinal tract, and is deposited in the blood, urine and even hair specimens [9]. However, in overdose diazepam can be lethal and has been shown to be relatively more toxic than other benzodiazepines [9-10]. From a forensic science point of view, little is known about

the effect of diazepam on the growth rate or morphological changes of dipteran species compared to the other drugs in the benzodiazepine group. Carvalho et al. [7] studied the effect of diazepam on the growth of the blowflies *Chrysomya albiceps and C. putoria* and reported a more rapid development rate when the larvae were fed on the tissues of rabbits that had been fed with diazepam compared to the control group [11].

Ethanol is contained in fermented beverages, such as wine, beer, and spirits, where sugar fermentation by yeast is the probably the oldest synthetic organic chemical produced by man [12]. Ethanol is euphorigenic, a CNS and respiratory depressant, and induces tolerance and physical dependence (addiction) [13]. Ethanol may be detected in the bodies of persons who have died regardless of the cause but has its highest incidences in deaths from violent circumstances [13-14]. It is also one of the leading causes of death by poisoning [15-18]. Detecting ethanol in the tissues of a cadaver can provide vital information about the circumstances of an individual's death. Mixing diazepam with ethanol damages the nervous and muscle systems, with the combined affect ranging from mildly uncomfortable to fatal, because both diazepam and ethanol act as depressants of the CNS and slow down the brain function, heart rate and breathing [6]. For several decades, forensic entomologists have used insects to detect drugs and other toxins in corpses when conventional matrices, such as the blood, urine and tissue, were no longer available [15,16,19,20]. As they feed on corpses, carrion insects accumulate drugs and their metabolites from the cadaver into their own bodies, and some of these chemicals are deposited in their internal organs. Therefore, analyzing insect tissues for traces of drugs or toxins and their metabolites has been developed for toxicological analysis [14,21,22].

Chrysomya megacephala (Fabricius 1784), the oriental latrine fly, is one of the old-world blowfly species that was originally restricted in distribution to Australia and the Pacific, but during recent decades it has been spread around the world, especially in Asia [11,23-26]. This blowfly species is typically the primary species to arrive at the body after death, being attracted to the cadaver by the odour produced during the early stage of cadaver decomposition. Thus, it can be used to estimate the postmortem interval (PMI) [1,10,27-29]. Blowfly larvae pass through four developmental stages (egg, larva or maggot, pupa, and adult), of which the pupal development stage makes up to 50% of the total duration of the life cycle. Therefore, this stage is important in estimating the PMI [24,30-32]. From previous studies, the development time in the pupal stage of *C. megacephala* is 120-150 h, after which the pupae emerge as adults. The pre-pupae have soft cases that harden approximately 24 h after developing to the pupal stage [11, 29], but inside the hard pupal case the soft body is very easily damaged, and so age estimation of *C. megacephala* is best performed using micro-computed tomography (micro-CT).

Importantly, micro-CT scanning can be used to describe the blowfly pupa in detail without damaging the specimens. In the last few decades, many studies on the internal organs of various blowfly species have been reported, but no study on the effects of drugs on the morphological changes are available. Richards et al. [33] studied the morphological changes in the cyclorrhaphan fly *Calliphora vicina* (Diptera, Calliphoridae) at a greater temporal resolution using CT-Pro 2.1 (Nikon Metrology HMX ST 225 system), which revealed the external and internal morphological characters, and that the technique could be used to estimate a minimum PMI. Subsequently, Martin-Vega et al. [34] refined the available temporal resolution to 20% time-intervals of the intra-puparial period since these represented the intervals of the major morphological changes. Hall et al. [18] found the gas bubble during the metamorphosis of cyclorrhaphan flies, which demonstrated the value of the micro-CT technique to complement other methods for the study of developmental changes. Martin-Vega et al. [35,36] studied the internal morphological changes and estimated the age of cyclorrhaphan flies using micro-CT, where the results provided age-diagnostic qualitative characters for most of the 10% time-intervals of the total intra-puparial period. In this paper, evidence to support the use of micro-CT as a tool to describe the different internal morphological changes in the pupae of the blowfly *C. megacephala* when fed as larvae on diazepam-, ethanol-, or diazepam plus ethanol-tainted animal flesh is presented.

Materials and Methods

Preparation of animal models

Ten male rabbits (*Oryctolagus cuniculus*) of approximately 3 kg weight each were used as the animal model [6,37]. All rabbits received ethanol and/or diazepam in 2.5 mL of sterile saline solution by intravenous injection into the ear vein. Ten rabbits were divided into four major groups, each group comprised of three rabbits (except for only one in the control group IV) and treated with: (I) diazepam (Di) at 3, 9 or 45 mg/kg body weight (BW), which represent the LD_{lowest}, LD_{50} and LD_{100} dose [7]; (II) absolute ethanol (Et) at 8, 237 and 700 µL/kg BW or the LD_{lowest}, LD_{50} and LD_{100} [38,39]; (III) diazepam mixed with absolute ethanol (DiEt) at the same three doses as (I) and (II) above, and (IV) saline solution only as a control. Note that LD_{lowest} represents the lowest dose that some lethality is detected at, and LD50 and LD_{100} represent the dose that causes 50% and 100% mortality, respectively. Most of the rabbits died approximately 45 mins after drug treatment, especially at the respective LD_{100} doses, but, regardless, 1 h after the treatment each respective rabbit was euthanized in a carbon dioxide chamber and their liver was aseptically removed by autopsy. These procedures were performed in accordance with all ethical requirements for the use of animals in experimental research studies and complied with the Chulalongkorn University Institutional Animal Care and Use Committee (IACUC) procedures, with animal use protocol and approval No. 1573018.

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Stock maintenance

Adult blowflies were collected at Chulalongkorn University (Bangkok and Saraburi provinces, Thailand) using decaying organic matter and fish meat as baits. *Chrysomya megacephala* were separated from other species by the yellow colour of their sena and antennae, while the sexes of *C. megacephala* were separated by the length between the compound eyes [28]. Adults were kept and maintained in screen cages at 26 ± 2 °C, 60-70% relative humidity, and a 12-h photoperiod. Two circular panels, 100mm in diameter, were removed from the front of the cages for attaching nylon mesh stockings as the access sleeves of the cage interior and the imago flies were fed with a 1:2:1 (w/w/w) mixture of dry granular sugar: powder skimmed milk: brewer's yeast ad libitum for a week [17], with 500g minced pig's liver used as an oviposition medium. Blowflies were reared until the emergence of second-generation adult flies.

Sampling of pupae

In each experiment, 20 pairs of second-generation adult flies were randomly selected and moved into an empty cage. They were allowed to lay eggs on a 50 g portion of minced liver from the respective treated rabbit, where numerous eggs were found 24 h later. The minced rabbit's liver with eggs was removed from the cage and placed in a small round plastic container (5-cm diameter and 4-cm height) and these were then placed in a larger round plastic container (8-cm diameter and 12-cm height) containing 3:1 (v/v) autoclaved soil: sand to a depth of approximately 4cm and covered by a soft, tiny-hole fine-mesh tulle net. The blowfly eggs hatched into larvae and fed on the minced rabbit's liver until they developed into 3rd instar larvae. Third instar larvae were then randomly selected and placed into a separate small round plastic container with autoclaved 3:1 (v/v) soil: sand for each experiment. These larvae were checked for pupation after 12 h. When the larvae stopped feeding (developed to pre-pupae) the time was set as 0 h. Four pupae from each experiment were removed during the first (S1: 24 h), second (S2: 72 h) and third (S3: 120 h) pupal stage [11] and placed directly into Boudin's solution for 48 h for fixing. The last stage (S3) was the final stage of development before emergence to adulthood. All the above stages were maintained at 26 ± 2 °C, 60-70% relative humidity, and a 12-h photoperiod.

5.4. Micro-CT scanning

The Pupae from each experiment were stained to 7 d by immersing in 0.5 M iodine in an aqueous solution before scanning [36-38]. After staining, each group was washed and immersed in 80% (v/v) ethanol for 24 h, before being stored in clean 80% (v/v) ethanol prior to micro-CT scanning. Each specimen was wrapped in paraffin film (Parafilm® M; Bemis Company, Inc.) containing 80% (v/v) ethanol and placed in a semi-microcuvette and sealed with paraffin film. The cuvette was put into the chamber and scanned in a Skyscan 1172 scanner operated at 70 kV, 114 µA and 8 W for an 885ms exposure. The resulting projections were reconstructed with a voxel size of 6.8 µm using the NRrecon reconstruction software and visualized with the Dataviewer program (Indiana University School of Medicine Micro Computed Tomography Center, Indiana, US).

Results

Morphological changes in the developing pupae

Development of the different parts (tissues) of the pupae is summarized in Tables 1-3 for S1-3, respectively, and representative micro-CT images are shown in Figures 1-4 for the control, Di, Et and DiEt groups, respectively.

Control group.

At 24-h pupal development (S1), the pupal bodies of the control group (Figure 1A) could easily be separated into the three main body regions of head, thorax, and abdomen. Fat bodies (FB) were dispersed equally in the head, thorax and abdomen and had a normal shape and size, being comprised of a mass of whitish cells in thin layers of one or two cells thickness. The developing wings (w) could be recognized as halteres, which were visible under the general wing development. Legs and segmentations (LGM) could also be recognized. Eyes (om) and mouthparts (IM) were discernable, and the antennae (an) were visible. The medulla regions of the brain (BR) and larval tracheal trunks (LTT) could be discerned. Only the larval hindgut (LHg) was evident in all the groups (except for $EtLD_{50}$). At 72-h pupal development (S2), the control group (Figure 1B and Table 2) showed fewer fat bodies compared to at S1, but the fat bodies were larger. Leg segmentations were distinctive compared to at S1, and the wings were further developed with the costa vein clearly discernable. The outer borders of the eyes were completely visible.

Flight muscles were developed further, where longitudinal muscles were still mainly dorsal and running almost the entire length of the thorax and the dorso-ventral muscle (DVM) pairs were present. Alimentary canal was discernable as a groove running the entire length of the sample and the archenteron (ar) was visible and moved to the posterior half of the abdomen, connected to anal and rectal regions. The brain was well developed and the proventriculus (PV) was present in this series. At 120-h pupal development (S3), the internal organs of the pupa in the control group (Fig. 1C and Table 3) were fully developed and ready to emerge as an adult. Mouthparts and antennae were fully melanised and legs and wings were developed. Outer eye regions were defined and with well-developed ommatidia along the entire outer edge, while the eyes were developed and connected to the brain by optical nerves. Ptilinum (Pt) was present at this stage and fully developed in adult. Fat bodies were present in the head lumen and filled in the abdomen, while the archenteron was well modified into the rectum (Rc). The thorax and the abdomen parts were separated by the abdomen-thorax (A-T) division, the midgut was present and inter-segmental muscles (ISM) were visible.

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Figure 1: Micro-CT images of C. megacephala pupae in the control group at the (A) S1, (B) S2 and (C) S3 pupal developmental stages.

Abbreviations: An = Antenna; Ar; Archenteron; A-T = Abdomen–Thorax Division; Br = Brain; DVM = Dorso-ventral Flight; Fb = Fat Bodies; Im = Internal Mouthparts; Ism = Inter - Segmental Muscle; LGM = Leg Muscles; Lhg = Larval Hindgut; Ltt = Larval Tracheal Trunk; Om = Ommatidia (Eye); Pv = Proventriculus; Pt = Ptilinum; Rc = Rectum; W = Wing.

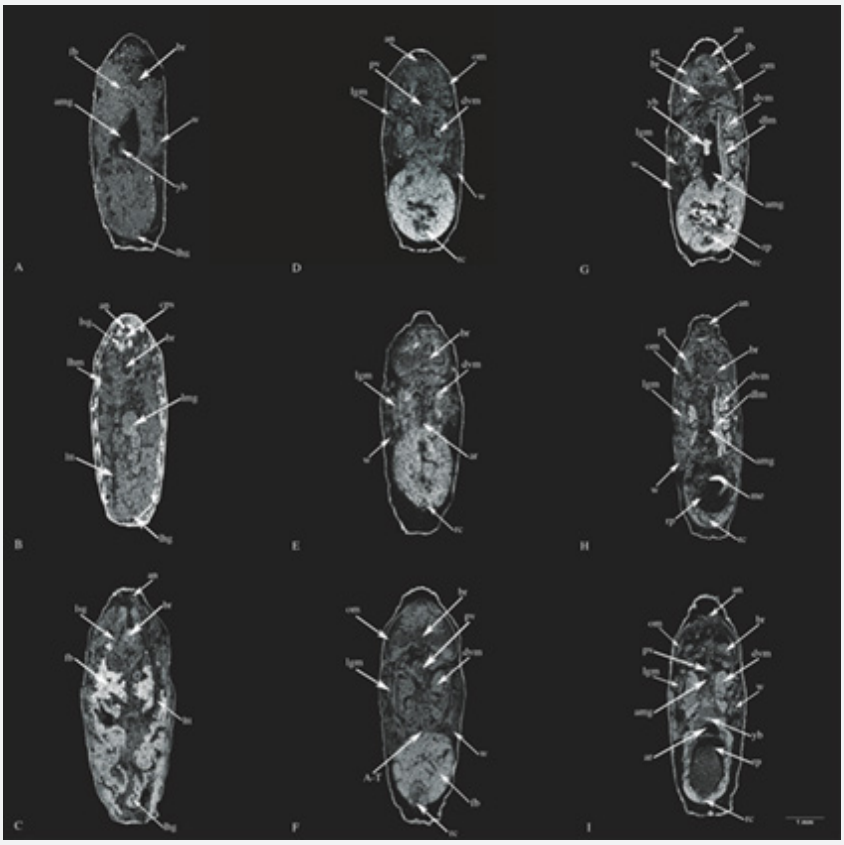

Figure 2: Micro-CT images of C. megacephala pupae at the (A, D, G) S1, (B, E, H) S2 and (C, F, I) S3 pupal development stages in the diazepam treated (Di) group at the (A, B, C) LDlowest, (D, E, F) LD50 and (G, H, I) LD100 doses.

Abbreviations not previously listed: AMG = Adult Midgut; CPS = Cephalopharyngeal; DLM = Dorsal Longitudinal Muscles; LHM = Larval Hypodermal Muscles; LSG = Larval Salivary Grands; Me = Meconium; RP = Rectal Pouch; YB = Yellow Body.

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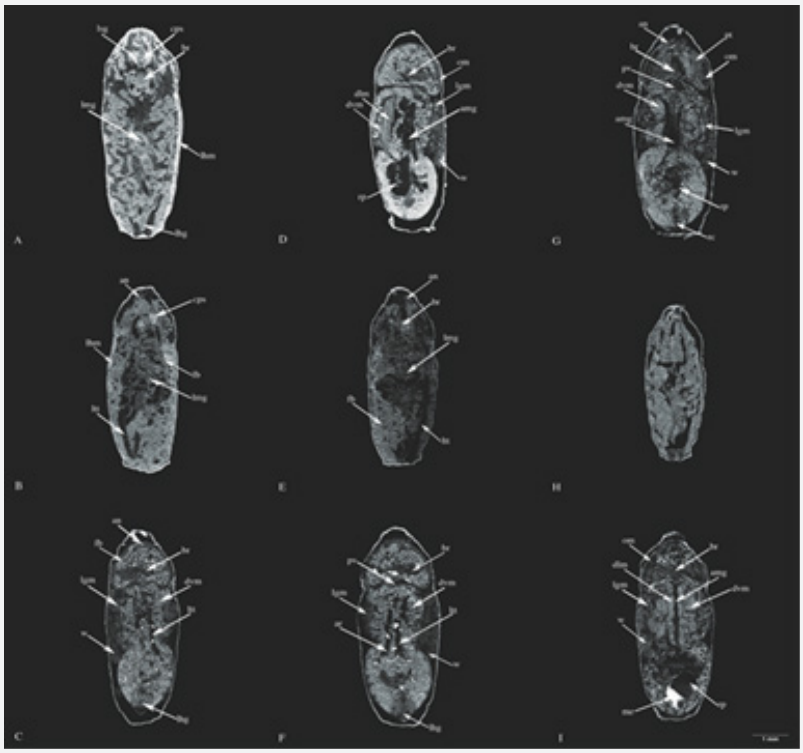

Figure 3: Micro-CT images of C. megacephala pupae at the (A, D, G) S1, (B, E, H) S2 and (C, F, I) S3 pupal development stages in the ethanol treated (Et) group at the (A, B, C) LDlowest, (D, E, F) LD50 and (G, H, I) LD100 doses.

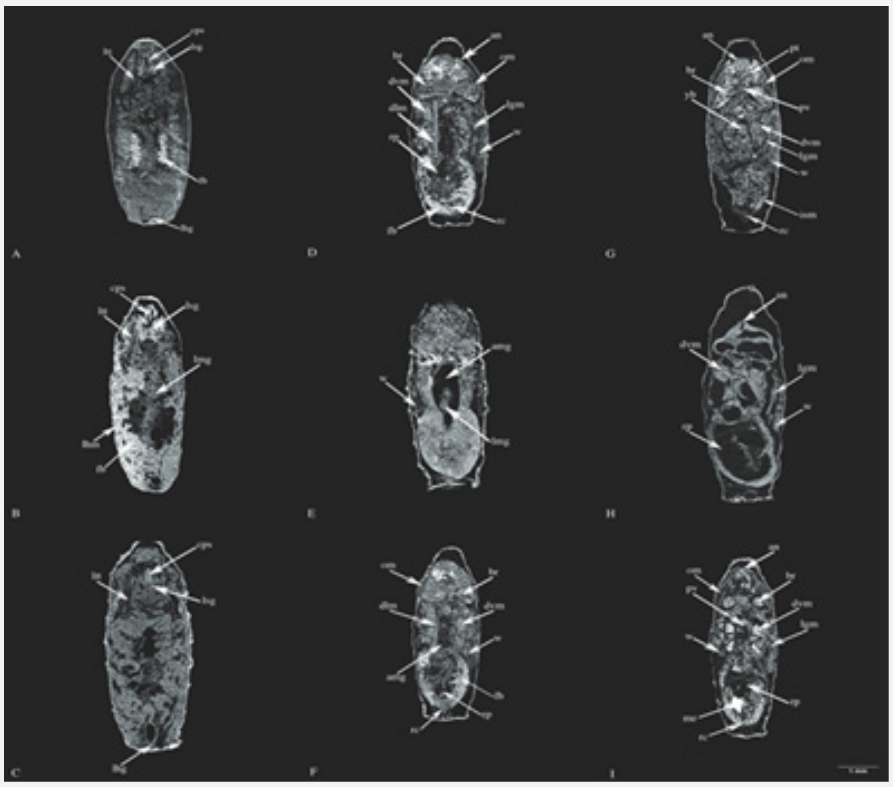

Figure 4: Micro-CT images of C. megacephala pupae at the (A, D, G) S1, (B, E, H) S2 and (C, F, I) S3 pupal development stages in the diazepam mixed ethanol treated (DiEt) group at the (A, B, C) LDlowest, (D, E, F) LD50 and (G, H, I) LD100 doses.

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Table 1: Chronology of the internal structural changes of *C. megacephala* pupae after 24 h development (S1), - = absent and + = present.

Tissues and organs	Control	$DiLD_{lowest}$	$DiLD_{50}$	$DiLD_{100}$	$EtLD_{lowest}$	$EtLD_{50}$	$EtLD_{100}$	$DiEtLD_{lowest}$	$DiEtLD_{50}$	$DiEtLD_{100}$
Body regions	+	-	-	-	-	-	+	-	-	-
Antennae	+	-	+	+	-	+	+	-	-	-
Brain	+	+	+	+	+	-	+	-	-	-
Eyes	+	-	-	-	-	-	-	-	-	-
Mouthparts	+	-	-	-	-	-	-	-	-	-
Larva trachel trunk	+	-	+	+	-	+	+	+	+	+
Larva hindgut	+	+	+	+	+	-	+	+	-	+
Larval salivary glands	-	-	+	+	+	-	-	+	+	+
laval hypodermal muscles	-	-	+	-	+	+	-	-	-	-
Larvel midgut	-	-	+	-	+	+	-	-	-	-
Lega and segmentations	+	-	-	-	-	-	+	-	-	-
Fat body	+	+	+	+	+	+	+	+	+	+
Wings	+	+	-	-	-	-	+	-	-	-
Flight muscles (dvm and dlm)	-	-	-	-	-	-	+	-	-	-
Proventiculus	-	-	-	-	-	-	-	-	-	-
Cephalopharygeal	-	-	+	-	+	+	-	+	+	+
Ptilinum	-	-	--	-	-	-	-	-	-	-
Abdomen-thorax division	-	-	-	-	-	-	-	-	-	-
Inter-segmental muscle	-	-	-	-	-	-	-	-	-	-
Archenteron	-	-	-	-	-	-	-	-	-	-
Adult midgut	-	+	-	-	-	-	-	-	-	-
Rectum	-	-	-	-	-	-	-	-	-	-
Rectal pouch	-	-	-	-	-	-	-	-	-	-
Yellow body	-	+	-	-	-	-	-	-	-	-
Meconium	-	-	-	-	-	-	-	-	-	-

Table 2: Chronology of the internal structural changes of *C. megacephala* pupae after 72 h development (S2), - = absent and + = present.

Tissues and organs	Control	$DiLD_{lowest}$	$DiLD_{50}$	$DiLD_{100}$	$EtLD_{lowest}$	$EtLD_{50}$	$EtLD_{100}$	$DiEtLD_{lowest}$	$DiEtLD_{50}$	$DiEtLD_{100}$
Body regions	+	+	+	+	+		+	+	-	+
Antennae	+	+	-	-	-	+	-	+	-	-
Brain	+	-	+	+	+	+	+	+	-	+
Eyes	+	+	-	+	+	-	-	+	-	+
Mouthparts	+	-	-	-	-	-	-	-	-	-
Larva trachel trunk	-	-	-	-	-	-	-	-	-	-
Larva hindgut	+	-	-	-	-	-	-	-	-	-
Larval salivary glands	-	-	-	-	-	-	-	-	-	-
laval hypodermal muscles	-	-	-	-	-	-	-	-	-	-
Larvel midgut					+	+		+	+	
Lega and segmentations	+	+	+	+	+		+	+	-	+
Fat body	+	+	+	+	+	+	+	+	-	+
Wings	+	+	+	+	+		+	+	+	+
Flight muscles (dvm and dlm)	+	+	+	+	+	-	+	+	-	+
Proventiculus	+	+	-	+	-	-	+	+	-	-

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Cephalopharygeal	-	-	-	-	-	-	-	-	-	-
Ptilinum	-	-	-	-	-	-	-	-	-	-
Abdomen-thorax division	-	-	-	+	-	-	-	-	-	-
Inter-segmental muscle	-	-	-	-	-	-	-	-	-	-
Archenteron	+	-	+	-	-	-	+	-	-	-
Adult midgut	-	-	-	-	-	-	-	+	+	+
Rectum	-	+	+	+	-	-	-	-	-	+
Rectal pouch	-	-	-	-	+	-	-	+	-	+
Yellow body	-	-	-	-	-	-	-	-	-	-
Meconium	-	-	-	-	-	-	-	-	-	-

Table 3: Chronology of the internal structural changes of *C. megacephala* pupae after 120 h development (S3), - = absent and + = present.

Tissues and organs	Control	$DiLD_{lowest}$	$DiLD_{50}$	$DiLD_{100}$	$EtLD_{lowest}$		$EtLD_{100}$	$DiEtLD_{lowest}$	$DiEtLD_{50}$	$DiEtLD_{100}$
Body regions	+	+	+	+	+		+	+	+	+
Antennae	+	+	+	+	+		-	+	+	+
Brain	+	+	+	+	+		+	+	-	+
Eyes	+	+	+	+	+		+	+	-	+
Mouthparts	-	-	-	-	-		-	-	-	-
Larva trachel trunk	-	-	-	-	-		-	-	-	-
Larva hindgut	-	-	-	-	-		-	-	-	-
Larval salivary glands	-	-	-	-	-		-	-	-	-
laval hypodermal muscles	-	-	-	-	-		-	-	-	-
Larvel midgut	-	-	-	-	-		-	-	-	-
Lega and segmentations	+	+	+	+	+		+	+	+	+
Fat body	+	+	+	+	+		+	-	-	-
Wings	+	+	+	+	+		+	+	+	+
Flight muscles(dvm and dlm)	+	+	+	+	+		+	+	+	+
Proventiculus	+	-	-	+	+		-	+	-	+
Cephalopharygeal	-	-	-	-	-		-	-	-	-
Ptilinum	+	+	+		+		-	+	-	-
Abdomen-thorax division	+	-	-	-	-		-	-	-	-
Inter-segmental muscle	+	-	-	-	-		-	+	-	-
Archenteron	-	-	-	+	-		-	-	-	-
Adult midgut		+	+	+	-		+	-	-	-
Rectum	+	+	+	+	+		-	-	-	-
Rectal pouch	-	+	+	+	+		+	+	+	+
Yellow body	-	+	+	-	-		-	+	-	-
Meconium	-	-	+	-	-		+	-	-	+

Treatment groups

Morphological changes of the pupae (S1) are summarized in Tables 2-4 and representative micro-CT images are shown in Figures 2-4. In the LD_{lowest} groups (no mortality), $DiLD_{lowest}$, $EtLD_{lowest}$ and $DiEtLD_{lowest}$ (Figures 2A, 3A and 4A, respectively), the body regions of the developing flies in all three treatment groups were hardly recognizable. Fat bodies were smaller than in the control group and abnormally shaped, the wings were incompletely differentiated, medulla regions of the brain were visible, but the shape of the brain was abnormal. The adult midgut was recognizable only in the $DiLD_{lowest}$ group (Figure 2A). Results for the $DiLD_{50}$ group were like the $EtLD_{lowest}$, $EtLD_{50}$ and $DiEtLD_{50}$ groups (Figures 2B,3A&B,4B), where the internal morphology of the pupae still resembled that of the third instar larva. The larval

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hypodermal muscles (LHM) had not yet started to degenerate because some internal tissues were attached to the larval cuticle. Brain, larval salivary gland (LSG) and cephalopharyngeal (CPS) skeleton could be recognized and were positioned at approximately the same level in the head part (Figure 2B). Larval tracheal trunks, hindgut and midgut were visible. The shape and size of the fat bodies were abnormal in all three treatment doses and enlarged in both the $EtLD_{50}$ and $DiEtLD_{50}$ groups (Figures 3B, 4B), while the rest of the organs could not be identified (Figures 2B, 3A, 4B).

For the $DiLD_{100}$ group (Figure 2C), the differentiation of organs was faster than in the $DiLD_{50}$ group (Figure 2B), but the fat bodies were abnormal and could be detected in both the thorax and abdomen parts. Brain and salivary gland were visible in the same area. Antennae were visible but their position was higher than in the control group. The larval tracheal trunk and hindgut could be recognized. Most of the tissues and organs of $DiLD_{100}$ were like those in the $DiEtLD_{100}$ group (Figures 2C, 4C). In the $EtLD_{100}$ group, the developmental stage of the organs was faster than in the other groups except for the control group (Figures 1A, 3C). The body was divided into the head, thorax and abdomen, as in the control group. Some organs were visible, such as the brain, antennae, wings, segment of legs, larval tracheal trunk, larval hindgut, and fat body, but the shape and size of all organs were abnormal, including the brain, tracheal trunk, wings and legs (Figure 3C). For the treated groups at 72 h (S2), the results are summarized in Table 2 for all treatment groups. Development rate of the three different Di groups (LD_{lowest}, LD_{50} and LD_{100}; Figures 2D-F) were faster than the control group, especially for the $DiLD_{100}$. Differentiation of the organs was completed, same as in S3. The development rate of the $EtLD_{lowest}$ and $EtLD_{100}$ groups were like the control group, but for the $EtLD_{50}$ group no further development was detected (Figure 2E).

The development rate of the DiEt group was like both the Di and Et treatments. Six pupae of both the Et and the DiEt groups had a similar development rate in the LD_{lowest} and LD_{50} groups but were different in LD_{100} because the differentiation of the organs in the DiEt group was clearly completed, like in the Di group (Figures 4D-F, 5D-F). After 120 h (S3), the results for all nine treatment groups are summarized in Table 3. The differentiation of the organs in the $DiLD_{50}$ and $DiLD_{100}$ groups were more clearly visible than in the DiLD0 group (Figure 3G-I). In the DiLD0 and $DiLD_{100}$ groups, the yellow body was visible in the central part of the insect body (Figure 3G; I). The leg and wing muscles were clearly visible. The thorax and abdomen parts could not be separated, and the abdomen part was filled with abnormal shaped fat bodies. In the $DiLD_{50}$ group, part of the body was fused in the head, thorax and abdomen parts and the abdomen was filled with meconium (Figure 2H). The head, thorax and abdomen in the $DiLD_{100}$ group were clearly separated and the head part was larger than the thorax and abdomen (Figure 2I). Proventriculus was visible in the $DiLD_{100}$ group (Figure 2I). For the Et treatment, all the pupae in the $EtLD_{50}$ group were dead (Figure 3H). The visibility of the organs in the $EtLD_{lowest}$ and $EtLD_{100}$ groups was unclear. However, some organs could be defined, such as the antennae, eyes, brain, wings and associated muscles, legs and associated muscles, rectal pouch and rectum (Figure 3G, I). In the $EtLD_{100}$ group, the abdomen was filled with meconium, the same as in the $DiLD_{50}$ group (Figures 2H, 3I). In the DiEt treatment, the differentiation of the organs in $DiEtLD_{50}$ was not completed. However, the organs in LD_{lowest} and LD_{100} groups were clearly visible, while a yellow body was found in LD_{lowest}, like in the Di group (Figures 2G, 4G) and meconium was found in the LD_{100} group, like in the Di group (Figures 2I, 4I).

Discussion

Internal morphological observation of the main organs and structure of *C. megacephala* pupae, as well as the internal morphological changes caused by the Di, Et and DiEt treatments, was evaluated using micro-CT scanning. The results from micro-CT scanning revealed similarities and differences when compared to previous studies in other species of calliphorid flies [18,33-36]. After 24-h pupal development (S1), the organs of the blowfly pupa in the control group had developed faster than in all the treatment groups, except for the $EtLD_{100}$ group. The size and shape of fat bodies, an essential tissue for storage and release of energy, in the treatment groups were changed. For the control group, our results were like the reported morpho-physiological changes in the fat body of Diptera [2], which have specific functions related to the stage of development. In the pupal stage, the fat body functions as storage for the proteins, lipids and carbohydrates required for the metamorphic development to imago and as supplies in the emerged imago [40]. In the control group, the regular shape of the fat body was globular [41], whereas the shape and the size of the fat bodies in the different treatment groups varied.

After 72-h pupal development (S2), the internal organs were well developed compared to in S1 in all cases except for in the $EtLD_{50}$ and $DiEtLD_{50}$ groups that still resembled their developmental stage at S1. The organs were clearly visible in both the control and the treatment groups (except for the $EtLD_{50}$ and $DiEtLD_{50}$ groups) and the larval hindgut had developed to the rectum in this series. After 120-h pupal development (S3), the differentiation of the organs in the control group pupae were clearly visible and fully developed, while the number of fat bodies was reduced as was their size compared to at S2. This agreed with a previous report [38] on the development of pupal organs by 90-100% of the total intra-puparial period using micro-CT visualization. However, the organs in all the treatment groups were irregularly shaped, including the antennae, brain, wings, legs and muscles. Moreover, the yellow body, the apoptotic larval midgut cells that can be observed when the adult midgut was closed, were found in $DiL\ D_{lowest}$, $DiLD_{100}$ and $DiEtL\ D_{lowest}$ in this series. In contrast, the yellow body was previously reported to be present in up to

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80% of the total intra-puparial period, but not beyond [38]. The meconium was found in the $DiLD_{50}$, $EtLD_{100}$ and $DiEtLD_{100}$ groups in S3, which is the waste products of metamorphosis, and is in accord with the reported finding of the meconium at the 80-100% total intra-puparial period [38].

Conclusion

Pupal development in the Di-treatment group was slowest and the LD_{50} had a larger effect on the organ's differentiation. Development of the Et-treated group was the fastest, but the LD_{50} Et and DiEt groups had a high total mortality, and the organs of the pupae were undifferentiated. The differentiation of organs in DiEt group depended on the administered dose. In the LD_{lowest} and LD_{50}, the toxicity of the Di and Et in the combined DiEt treatment were enhanced, yet apparently, the toxicity of the DiEt mixture was inhibited at the highest dose (LD_{100}). This may reflect changes in the level of ethanol or diazepam catabolic products resulting from metabolic inhibition or interactions, although this assumption requires validation. Regardless, consequently, the differentiation of organs in the LD_{100} group was faster and better than in the LD_{lowest} and LD_{50} groups. The Di, Et and DiEt treatments affected the pupal development time, size, organ development and development stage. The differentiation of the pupa's organs can be clearly seen by micro-CT. Therefore, this study confirmed the advantages of micro-CT in the study of internal morphological changes in fly pupae. Micro-CT is a less time-consuming and less invasive process, causing much less damage to the sample prior to analysis as it can be performed after simply peeling away the hardened and darkened pupal case. The inside of the opaque puparium can then reveal details of the pupa and can be used to study the internal changes inside the blowfly pupae to aid in forensic science investigations. However, the preliminary observations that ethanol, diazepam, and the combination of the two alter the development pattern in the flies in a compound and dose-dependent manner requires a more thorough evaluation (sample sizes, micro-CT time points and drug doses) as well as evaluation of causes (such as the level of liver catabolites) to be useful in forensic applications.

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