

Effect of Diazepam and Ethanol on External Larval Morphology of *Chrysomya megacephala* (Diptera: Calliphoridae)

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ABSTRACT:

Chrysomya megacephala (Diptera: Calliphoridae) is a medically and forensically important blow fly, where larvae of *C. megacephala* are the most commonly studied insects (in relation to forensic science) that are associated with cadavers at crime scenes, especially the 3rd instar stage. Larvae of *C. megacephala* had been reared since hatching on freshly excised livers from rabbits (*Oryctolagus cuniculus*) that had been treated with three different doses each of diazepam, ethanol, or diazepam mixed with ethanol, to compare with the control group, fed with rabbit livers treated with only sterile saline. The external morphological changes, respiratory slits with bleb formation of the posterior spiracle and swollen anal pads, in the treated *C. megacephala* larvae could be clearly seen from the Scanning Electron Microscopy (SEM). Thus, diazepam and ethanol cause distinctive external morphological deformation in *C. megacephala* larvae which could identify the trace of both substances in late stage cadavers.

INTRODUCTION

Stress in modern life often leads some people to substance abuse from narcotics and prescription drugs, and in combination with ethanol for suicidal [1-3].

Diazepam is a drug prescribed for sleeplessness, anxiety, muscle spasms, and to provide sedation before medical procedures [4]. However, it has been reported that diazepam is more toxic than other benzodiazepines when overdosed [5, 6]. An alcohol beverage is simply any drink that contains ethanol, such as wine, beer, and spirits [7]. It is also one of the leading causes of death by poisoning [8-11]. Both diazepam and alcohol can damage the nervous system and liver, while the consumption of diazepam with alcohol has similar symptomatic effects, in terms of the heart rate and breathing, but is more potent. Indeed, the combination of these two substances can be very dangerous and more easily lead to an overdose [12].

Chrysomya megacephala is one of the forensically important blow fly species. It is endemic to Australia and the Pacific region, but during recent decades it has been distributed around the world, especially in Asia [13-17]. It is usually the first and main insect species to arrive at and colonize the cadaver after death and lays its eggs in the open areas of the body and wounds, with four stages of development (egg, larva or maggot, pupa and adult), of which only the larvae consume the body and then develop in or near the carcass. Thus, its biology, especially the morphological changes in the larvae, can be used to identify the time since death and, in some cases, the cause of death [18-22]. The larvae are easily collected and maintained in the laboratory for identification and

toxicological analyses, the results of which can be used to estimate the post-mortem interval and potential cause of death [23].

Sukontason et al. (2003) studied and described the larval morphology of *C. megacephala* collected from corpses using SEM because it provides excellent and detailed external morphology of the insects [24]. Only the first instar showed thick branches of the posterior spiracle hairs. Although these authors also described the external morphology of the second and third instar larvae, this described only the normal external morphology of *C. megacephala*. However, this research on the effects of diazepam, ethanol, and diazepam plus ethanol, examined the abnormal external morphological changes in *C. megacephala* larvae induced by these drugs using SEM. This is important in forensic science as the development of these insects is adversely affected by the presence of the drugs in the cadaver and does not follow a normal pattern, while, additionally, the characterization of these morphological abnormalities may provide clues to the cause of death of the cadaver.

MATERIALS AND METHODS

In total, 10 rabbit's livers were used for rearing the blow fly larvae. The fresh liver was collected from each of 10 male rabbits of approximately 3 kg weight [25, 26]. Rabbits (one per treatment) were treated with three doses of the respective drug (ethanol, diazepam, and ethanol mixed with diazepam) or just normal sterile saline (NSS; control). Drugs were administered by intravenous injection of the ear vein. The experimental groups were divided as follows; (i–iii) diazepam (Di) in 2.5 mL of NSS was administered at the LD₅₀ (9 mg/kg) [27], LD_{lowest} (3 mg/kg; no mortality) and LD₁₀₀ (45 mg/kg); (iv–vi) absolute ethanol (Et) at the LD_{lowest} (8 µL/kg), LD₅₀ (237 µL/kg) and LD₁₀₀ (700 µL/kg) [28–29]; (vii–ix) diazepam mixed with absolute ethanol (DiEt) at the same replete lethal doses for each compound and (x) 2.5 mL of NSS only as a control. The animal experimentation complied with the Chulalongkorn University Institutional Animal Care and Use Committee (IACUC) procedures, with animal use protocol and approval No. 1573018.

In each experiment, 20 pairs of adult blow flies, aged 3 days of the second generation were obtained from a laboratory colony at the Department of Biology, Faculty of Science, Chulalongkorn University, Thailand and moved into an empty cage. They were allowed to lay eggs on a 50 grams portion of minced liver from the respective treated rabbit at 26 ± 2 °C, with 60–70% relative humidity and a 12-h photoperiod. The blow fly eggs hatched into larvae and fed on the minced liver until they developed into 3rd instar larvae. Thirty of 3rd instar *C. megacephala* from each experiment were randomly selected and measured the length by Vernier caliper.

In each experiment, five samples of 3rd instar *C. megacephala* larvae were randomly selected and washed several times in NSS to remove any foreign materials that might obstruct the view of important structures during microscopic examination. All larval specimens were fixed in 2.5% (w/v) glutaraldehyde in phosphate buffered saline, pH 7.2 (PBS) at 4 °C for 24 h [20]. The samples were washed twice with 2.5% (w/v) glutaraldehyde in PBS and then dehydrated in 10%, 20%, 30%, 40%, 50%, 60%, and 70% (v/v) ethanol for 30 min each step, 70% (v/v) ethanol overnight, and in 95% (v/v) ethanol three times and finally preserved in absolute ethanol. Larvae were dried using a critical point dryer, coated with gold and then analyzed by SEM on a JEOL model JSM-6610LV microscope (Balzers Union SCD 040) [24].

RESULTS AND DISCUSSIONS

The statistical test indicated a significantly different among groups studied (C, Di, Et, DiEt) on 3rd instar variables analyzed: $F = 11.58$; $p < 0.001$. The mean values and SDs of larval length of different drugs and lethal dose are shown in Table 1. No correlation groups were observed in all treatment groups, which, the average larval length of control group and treatment groups were non-significant (Table 1).

Larvae from each of the nine treatment groups (LD_{lowest}, LD₅₀, and LD₁₀₀ doses for each of Di, Et and DiEt) were evaluated by comparing several aspects of the external morphology of the blow fly larvae to those from the control group using SEM analyses. The spiracular opening of the posterior part is the most distinctive feature for differentiating larval instars and can be used to identify the species of blow fly using the structure and number of the posterior spiracles. This is especially the case in the 3rd instar stage [30], where the size of the spiracles is increased dramatically and the morphological features are completely developed [31].

In the control group, the caudal segment or the posterior spiracular discs (psd) were located in a shallow cavity, and each posterior spiracle disc had spiracular slits or respiratory slits (rs) interspaced with fine branches of highly branched spiracular hairs, known as the peristigmatic tuft (pt) (Fig. 1A). The posterior view of the anal section in the control group showed a normal C-shape, centrally located with three respiratory slits, but the button (b) was concealed from the swollen wrinkled area (wa) (Fig. 1B). This agreed with a previous report on the morphology of *C. megacephala* larvae analyzed by SEM, in terms of the number of respiratory slits in each larval stage and the relative thickness of the branches of the posterior spiracular hairs [24]. In addition, the morphological changes of normal developing larvae and pupae of the calliphorid fly (*Calliphora vicina*), the anal segment or posterior part (spiracles and papillae) could be observed and used for identification to the species level [32].

In all nine treatment groups, the posterior end was deformed with abnormal development processes (Fig. 2). In the LD₅₀-treated larvae (Fig. 2B, E, H), the posterior part of the larvae was smaller than in the LD₁₀₀ and LD_{lowest} groups, especially in the ethanol-treated groups (Fig. 2E), where the EtLD₅₀ group was the smallest. The effect of ethanol (Et; Fig. 2D, E, F) on the size and morphology of the posterior part of the blow fly larvae could be distinctly observed when compared to those treated with diazepam plus ethanol (DiEt; Fig. 2G-I) or diazepam (Di; Fig. 2A-C). The peritreme in each treatment group is shown in Fig. 3, where the posterior spiracles were deformed with swollen spiracles in the EtLD₅₀ and EtLD₁₀₀ groups (Fig. 3E, F). Respiratory slits were destroyed with many bleb formations on the slits in the DiLD_{lowest} group (Fig. 3A), although there were fewer blebs on the slits in DiEtLD_{lowest}, DiEtLD₅₀, and DiEtLD₁₀₀ groups (Fig. 3G-I). However, the peristigmatic tufts were less branched in the DiLD_{lowest}, DiEtLD₅₀, and EtLD₅₀ groups than in the other treatment groups, which was related to the size of the posterior part of the blow fly larvae (Figs. 2B, E, H and 3B, E, H).

The bottoms were clearly visible in all the treatment groups except for the EtLD₅₀, EtLD₁₀₀, and DiEtLD₅₀ groups (Fig. 3E, F, I). The wrinkled areas in the LD_{lowest} and DiEt groups showed a normal formation similar to that in the control groups (Fig. 3A, D, G-I), but the wrinkled areas were swollen in the EtLD₅₀ and EtLD₁₀₀ groups (Fig. 3E, F), while in the DiLD₅₀ and DiLD₁₀₀ groups the wrinkled areas were flattened more than in the other groups (Fig. 3B, C). Previous work showed that codeine phosphate affected the size and posterior part of 3rd instar larvae of *Chrysomya albiceps*, where a deformed posterior spiracle with bleb formation on its slits was formed [33]. Therefore, the results from this study seemingly agreed with the results of Shaheen and Fathy on the effect of codeine phosphate on the changes in external morphology in blow fly larvae [33].

Ubero-Pascal et al. (2012) reported that the blow fly's anal protuberance (ap) was clearly visible in the 3rd instar as a semicircular shape with conically shaped anal pads (pd) [32], which is the same as in the control group of this study (Fig. 1C). However, larvae in the treatment groups had swollen anal protuberances (ap) (Fig. 4A-H), except for DiEtLD₁₀₀ (Fig. 4I). Moreover, the base of the anal pads (pd) in the larvae from the LD₅₀ treatment and one of the EtLD₁₀₀ larvae had a reversed triangle and swollen appearance (Fig. 4B, E, F, H), while the anal opening areas (ao) were not completely developed in the EtLD₅₀ and

EtLD₁₀₀ groups (Fig. 4E, F). However, the external organs of the larvae in each treatment are summarized in Table 2.

In conclusion, the posterior part of the blow fly larva is used to identify the species, especially in the 3rd instar larval stage. This study showed that Di, Et, and DiEt treatments at all three doses each had an effect on the size of the *C. megacephala* larvae and differentiation of the anal parts, with larvae in the LD₅₀ treatments being smaller than in the other treatments for Di, Et, and DiEt. Differentiation of the anal parts of *C. megacephala* larvae from the Et-treated groups changed more than in the DiEt- and Di-treated groups. Given that diazepam and ethanol have similar actions on a range of other organs, such as the brain, liver and fat cells in the insect, this suggests that the combination of both drugs could increase organ damage. All the Di, Et, and DiEt treatments effected the size and differentiation of the blow fly's posterior parts. Differentiation of the larva's organs was clearly seen by SEM analysis. Therefore, this preliminary study is of benefit for future research, especially for the identification of fly larvae at the species level and developmental stage (for cadaver age) in forensic science investigations.

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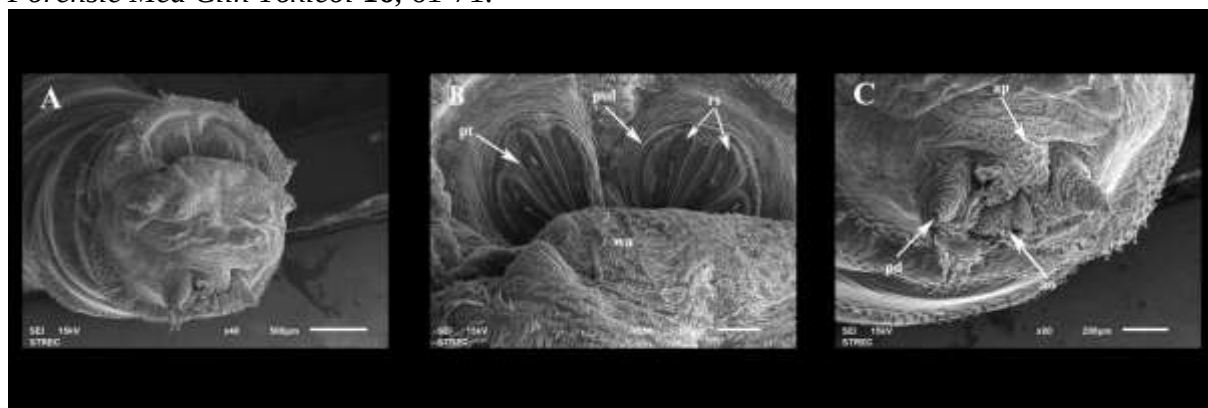


Fig. 1 Representative SEM images of the anal division morphology in 3rd instar larva of *C. megacephala* in the control group; (A) posterior view of anal division; (B) details of posterior spiracles and wrinkled area; and (C) detail of anal protuberance. (ap = anal protuberance; ao = anal opening; pd = anal pads; psd = posterior spiracular discs; pt = peristigmatic tuft; rs = respiratory slits; wa = wrinkled area).

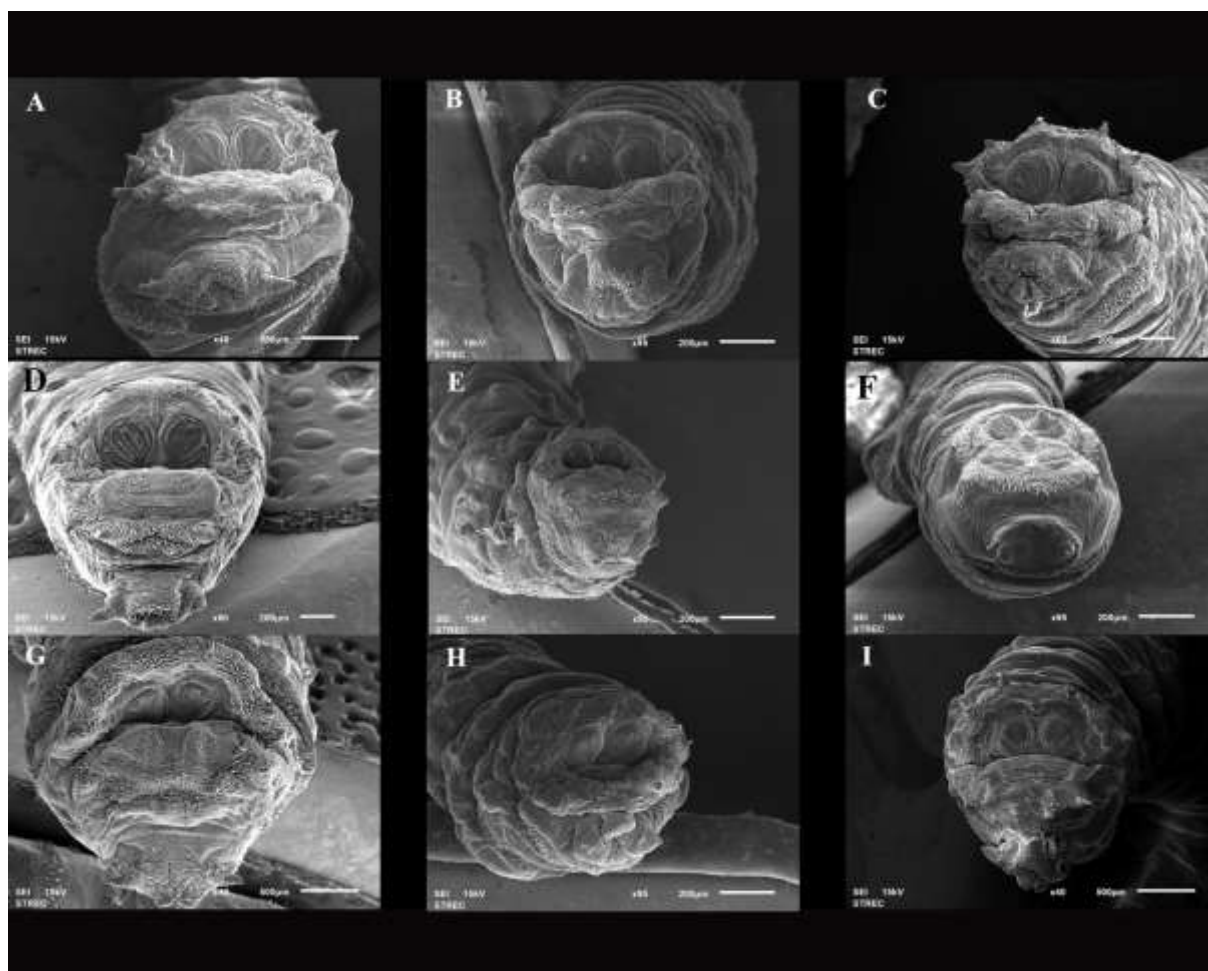


Fig. 2 Representative SEM images of the posterior view of the anal division in 3rd instar larva of *C. megacephala* in the (A) DiLD_{lowest}, (B) DiLD₅₀, (C) DiLD₁₀₀, (D) EtLD_{lowest}, (E) EtLD₅₀, (F) EtLD₁₀₀, (G) DiEtLD_{lowest}, (H) DiEtLD₅₀, and (I) DiEtLD₁₀₀ treatment groups.

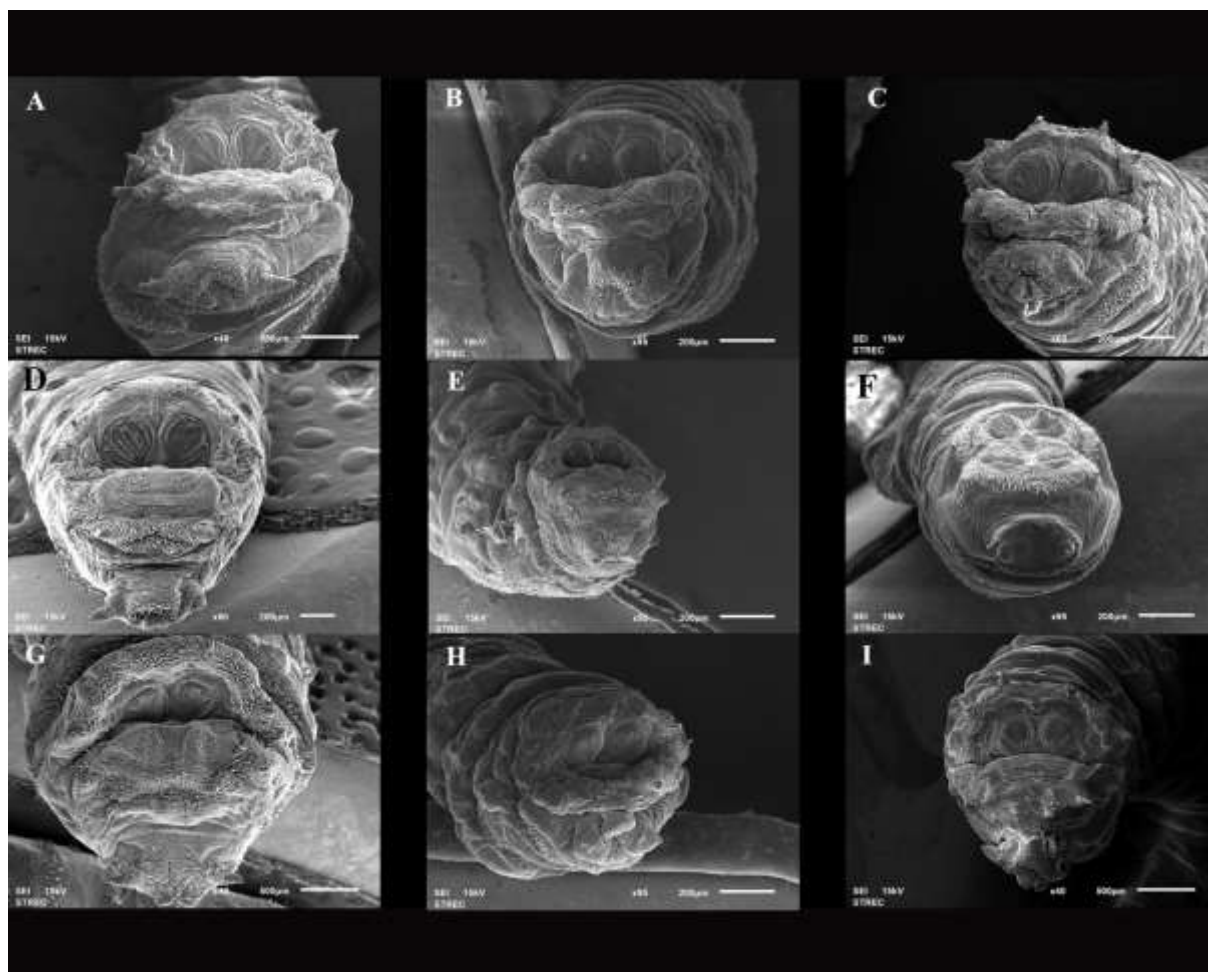


Fig. 3 Representative SEM images of the posterior spiracles and wrinkled area of anal division in 3rd instar larva of *C. megacephala* in the (A) DiLD_{lowest}, (B) DiLD₅₀, (C) DiLD₁₀₀, (D) EtLD_{lowest}, (E) EtLD₅₀, (F) EtLD₁₀₀, (G) DiEtLD_{lowest}, (H) DiEtLD₅₀, and (I) DiEtLD₁₀₀ treatment groups. (b = bottom; psd = posterior spiracular discs; pt = peristigmatic tuft; rs = respiratory slits; wa = wrinkled area).

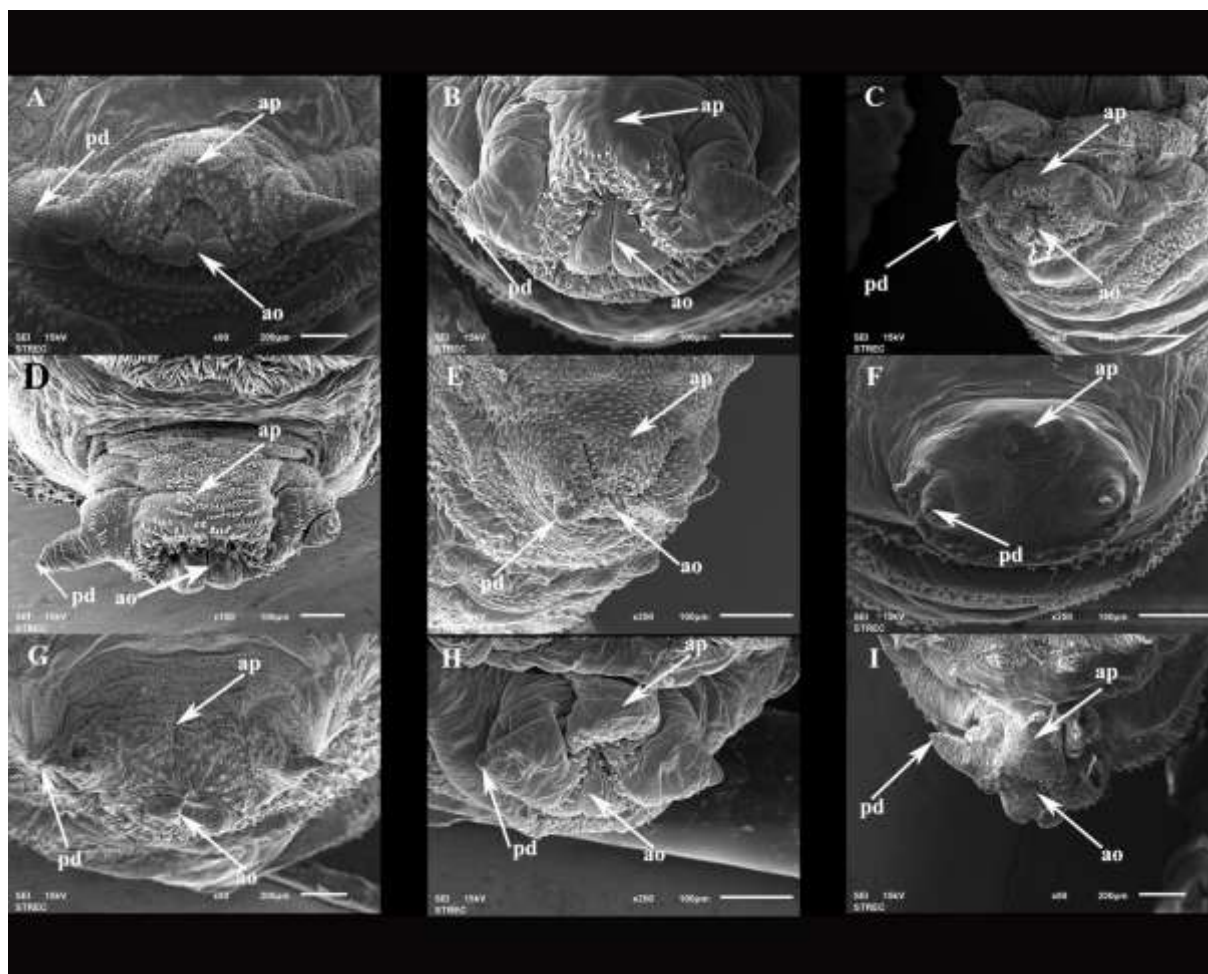


Fig. 4 Representative SEM images of the anal protuberance of the anal division in 3rd instar larva of *C. megacephala* in the (A) DiLD_{lowest}, (B) DiLD₅₀, (C) DiLD₁₀₀, (D) EtLD_{lowest}, (E) EtLD₅₀, (F) EtLD₁₀₀, (G) DiEtLD_{lowest}, (H) DiEtLD₅₀, and (I) DiEtLD₁₀₀ treatment groups. (ap = anal protuberance; ao = anal opening; pd = anal pads).

Table 1 Median lengths (mm) of *C. megacephala* larvae in the 3rd instars with diazepam and/ or ethanol.

Series (hour)	Control (a)	DiLD _{low} est (b)	DiLD ₅₀ (c)	DiLD ₁₀₀ (d)	EtLD _{low} est (e)	EtLD ₅₀ (f)	EtLD ₁₀₀ (g)	DiEtLD _{low} west (h)	DiEtLD ₅₀ (i)	DiEtLD ₁₀₀ (j)
	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)	Median (SD)
3 rd instar	14.5 4 (14.45) e	13.50 (13.67) cdeghij	13.32 (13.41) bdfghij	13.55 (13.59) bceghij	14.05 (14.05) abdgh	12.83 (12.86) ci	13.56 (13.58) bcdeghij	13.86 (13.95) bcdegj	12.91 (12.91) bcdfg	13.66 (13.59) bcdegh

*Median in a line followed by the same letters are not significantly different. Di, diazepam; Et, ethanol; DiEt, diazepam mixed ethanol; LD, Lethal dose

Table 2 Chronology of the external structural changes of *C. megacephala* 3rd larvae, - = abnormal and + = normal

Tissues and organs / Treatments	Control	DiLD _{lowest}	DiLD ₅₀	DiLD ₁₀₀	EtLD _{lowest}	EtLD ₅₀	EtLD ₁₀₀	DiEtLD _{lowest}	DiEtLD ₅₀	DiEtLD ₁₀₀
Anal opening (ao)	+	+	+	+	+	-	-	+	+	+
Anal pads (pd)	+	+	-	+	+	-	-	+	-	+
Anal protuberance (ap)	+	-	-	-	-	-	-	-	-	+
Buttom (bt)	+	+	+	+	+	-	-	+	-	+
Posterior spiracular discs (psd)	+	+	+	+	+	-	-	+	+	+
Peristigmatic tuft (pt)	+	-	+	+	+	-	+	+	-	+
Respiratory slits (rs)	+	-	+	+	+	+	+	-	-	-
Wrinkled area (wa)	+	+	-	-	+	-	-	+	+	+