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Effect of clozapine on the development and morphometry of *Sarcophaga ruficornis* Fabricius (Diptera: Sarcophagidae) and possible implications for forensic entomology

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Abstract:

The present study investigates the impact of clozapine on the development and morphometric parameters of *Sarcophaga ruficornis* Fabricius (Order Diptera, Family Sarcophagidae) among the different stages from egg to adult in Cairo, Egypt. Clozapine was administered in four different concentrations (25, 50, 75, and 100 mg/g), and its effect was compared to a control set. The results revealed a significant and concentration-dependent prolongation of the duration of the total life cycle. Moreover, clozapine influenced the duration of the various life stages and delayed the insect oviposition. Morphometric examination showed that larvae and pupae exposed to different concentrations of clozapine were significantly affected. Furthermore, clozapine-induced pupal mortality and morphological deformations, including incomplete adult emergence from pupae and abnormal wings in the newly emerged adults. These findings bring to light the adverse effects of clozapine on *S. ruficornis*, suggesting the presence of implications in the estimation of PMI in entomo-toxicological investigations.

Keywords: Forensic Entomology, Entomo-toxicology, Clozapine.

Introduction:

Entomo-toxicology is an emerging subject within forensic entomology evolved since the 1990s. It focuses on applying toxicological investigations of carrion-feeding insects as a reliable substrate instead of conventional toxicological analysis in advanced decomposition [1,2]. These insects were utilized for the identification of toxins and drugs within tissues, with the primary purpose of determining the minimum post-mortem interval (PMI) [3]. PMI was calculated by determining the age of the most

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advanced developmental stage of insects, that were found on the body when it was discovered [4].

Drugs, however, may have an impact on the growth rates of the breeding larvae where the presence of such toxicological substances in the corpse can accelerate or retard the rate of fly's development. As the growth rate is the basis for PMI assessment, this may lead to an over- or under-estimation of min. PMI [2]. Thus, understanding the influence of various classes of drugs and toxins on the developmental rates of numerous forensically relevant species could be useful before its use for PMI estimation to minimize the errors as much as possible [5,6].

Clozapine ($C_{18}H_{19}CIN_4$) is a typical anti-psychotic, neuroleptic drug belonging to the tricyclic dibenzodiazepine class. It was first prescribed in the 1970s for patients with schizophrenia, who did not respond to commonly used antipsychotics, or who had other psychotic disorders [7,8]. However, due to its potentially fatal side effects, its use has been restricted lately [9].

The recommended daily dosage of clozapine typically falls between 300 and 600 mg/day, with a maximum limit of 900 mg/day [10]. Even though the exact lethal dose of clozapine is unknown, a number of published reports indicate that most overdose deaths from the drug happen at doses higher than 2,000 mg. These cases are predominantly associated with central nervous system depression and in severe instances, may lead to death [11,12].

Its mode of action is assumed to be due to its interaction with dopaminergic and serotonergic neurotransmitter systems [13]. After oral administration, absorption occurred. Then in the liver, the cytochrome P450 system quickly metabolises it to polar metabolites, which include norclozapine, the main active metabolite that may cause adverse effects [14].

According to studies comparing the case fatality rate of different antipsychotics, clozapine was found to be much more toxic than other antipsychotics. It is reported as the most toxic in its class [15]. Severe poisoning caused by the ingestion of clozapine is a serious condition, with a reported mortality rate of 12% [11,16]. Each year, around two cases of clozapine intoxication are typically documented, and sadly, some of these cases lead to fatalities [8].

Overdose intoxication or suicidal attempts by clozapine were reported in many studies around the world [17-21]. In Egypt, Clozapine poisoning was documented in several cases [22-25]. Moreover, according to reports from the Tanta University Poison Control Unit, about 35% of admitted cases due to antipsychotic intoxication were due to clozapine [22]. Furthermore, the percentage of clozapine poisoning is estimated as 4.2 % of all poisoning cases admitted to the National Poisoning Center, Kasr Al Ainy Teaching Hospital in Cairo University for one year [26].

Based on the studies in the previous literature which highlighted the effects of several toxicological substances, clozapine has not been considered in any entomo-toxicological studies concerned with the insect's developmental rate except for the study of **Wang *et al.*, 2019** [27]. So, it is evident that further research is necessary to collect data on its effect on the life cycle and some developmental parameters of *Sarcophaga ruficornis* to reduce the possibility of errors arising during PMI estimation.

Materials and Methods:

1. Dose determination:

A 100-mg Clozapine tablet (manufactured by Multi-Apex Pharma S.A.E. – Egypt) marketed as clozapex 100 mg was applied. Tablets were grinded by using sterilized porcelain mortar and pestle. Four blind concentrations of clozapine were applied which were; 25 mg, 50 mg, 75 mg, and 100 mg.

2. Experimental design

2.1. Preparation of foodstuff:

A diet was prepared by mixing 250 g of fresh minced buffalo meat with 5 ml of distilled water to keep the bait hydrated during the study period. While the meat batches were still moistened, the previously prepared

drug concentrations were added to each meat batch separately. Then, it is mixed by hand for five minutes, followed by five minutes of mixing with a blender to ensure a uniform distribution of chemicals with the rearing medium in cleaned long-sided 450 ml plastic labeled pots [28].

For the clozapine spiked meat, concentrations of 0.1 mg/g, 0.2 mg/g, 0.3 mg/g, and 0.4 mg/g were reached by adding 25 mg, 50 mg, 75 mg, and 100 mg respectively to four separate batches of meat each of 250 g weight. They were labeled as (CLZ 25, CLZ 50, CLZ 75, and CLZ 100).

For every treatment, new gloves and mixing pots were used to prevent contamination, and the blender's head was flushed with boiling water and rinsed in 70% alcohol for 10 minutes in between usage.

In the control experiment, meat was mixed with distilled water instead of clozapine [29].

To keep the foodstuff moist, a few droplets of water were dispersed daily. Filter papers were placed on the top of the substrate in pots to prevent flies from direct contact with the meat and dying, it was also used as a platform for flies oviposition [30].

Each pot was placed in a relatively wide-mouthed plastic dish filled with a 2 cm-deep layer of sand to facilitate the normal pupation of the post-feeding larvae after wandering from the pots.

All were maintained separately in labeled rearing cages and housed under controlled temperature at 34 ºC (S.D. = 0.41ºC), and 14:10 light: dark photoperiod. A small bowl of water was placed at one corner of each cage to increase humidity as much as possible [31]. Each experiment (treated or control) was replicated three times.

2.2. Maintenance of insects:

Before the beginning of experiments, adult flies were supplied with meat blood as a protein source to enable the female's ovarian maturation [32], then the experiments were started on June 8, 2023.

Five males and five females obtained from the pure laboratory-stock cultures were introduced to each cage and were allowed to oviposit on substrates spiked with different concentrations of toxins. Continuous observations have occurred to record egg laying and note the times of oviposition. As soon as the females laid eggs, all adults were removed from cages and the time of eggs hatching was recorded.

To start the experimental colonies, the first larval instars were allowed to complete their life cycle upon the previously prepared baits in pots, covered with organza mesh for ventilation and secured with rubber bands. The duration of each growth stage (first instar, second instar, and third larval instars, post-feeding larvae, time of pupation, and the adult eclosion time) was carefully recorded in control and treated experiments at least once a day.

After pupation in the sand, meat pots were replaced with a small petri dish provided with a piece of cotton soaked with a sugary solution which was required for the emerging adults. After the emergence of adults, the Petri dishes containing pupae were maintained and housed in cages for three weeks after the last adult was observed to check for any additional instances of adult emergence [33].

3. Measuring morphological parameters:

Measurements were compared in three stages. The first was for the peak feeding third larval stage, the second was for puparia, and the last was after adult emergence. The procedures for sample collection and preservation were completed following the previously established standards for forensic entomology [34].

3.1. Larval measurements

Twenty larvae from each of the 18 rearing cages were randomly sampled (a total of 60 larvae for each experiment). The collected larvae of each experiment were divided into two groups; 30 larvae were utilized to measure development based on an increase in overall length and width, while the remaining 30

were used as an indicator of the developmental rate based on an increase in body weight.

3.1.1. Length and width measurement

Prior to measurement of length and width, the larvae were fixed by immersing in small vessels filled with boiling water for 60 seconds, then thoroughly rinsed with distilled water for 30 seconds, larvae were then dried on tissue paper to remove substrate residues. Larvae were then stored in 70% ethanol for preservation. This process causes the larvae to immediately stretch and stop enzyme activity, enabling maximum extension of the larva for more precise measurement [35].

The larval measurements were performed after 24 hours of preservation with a 0.1 mm calibration ruler of Tobo USB 2.0 digital microscope according to the methods used by **George *et al.* (2009)** [36] (Fig. 1), where larvae were examined laterally, and their lengths were measured by the distance from the most posterior abdominal segment to the most distal point of the head. The larval width was determined across the point where the fifth and sixth segments met.

3.1.2. Body weight measurement

The larvae used to measure weight were prepared by freezing, then thawed, rinsed, and dried with tissue paper according to **Singh *et al.* (2016)** [37]. After that, they were weighed to the closest 0.1 mg using an A&D HR-200 Analytical balance.

3.2. Puparia measurements

Puparia (without killing) were viewed ventrally, 30 samples were measured for length and width, and the other 30 were weighed. After measurements, puparia were returned to their pots to eclose. Puparial length was detected from the most anterior to the most posterior points of the body, and width was measured along the anterior margin of the posterior spinal band of the 5th body segment (Fig. 2).

3.3. Adults measurements

Thirty adults who emerged from the same measured puparia were killed and the wings were taken off and measured from the base to the distal end (Fig. 3).

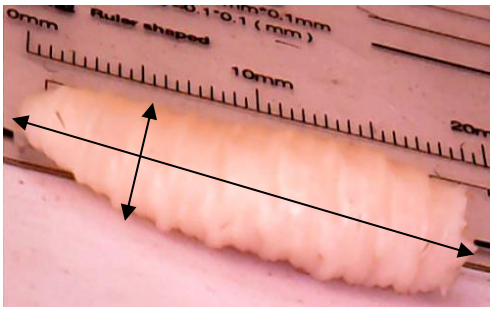

Fig. 1: The measurements of body length and width of larvae.

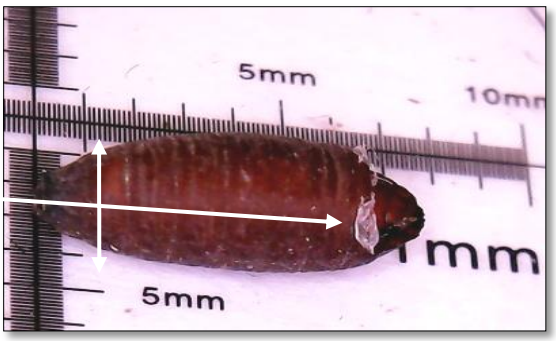

Fig. 2: The measurements of length and width of puparia.

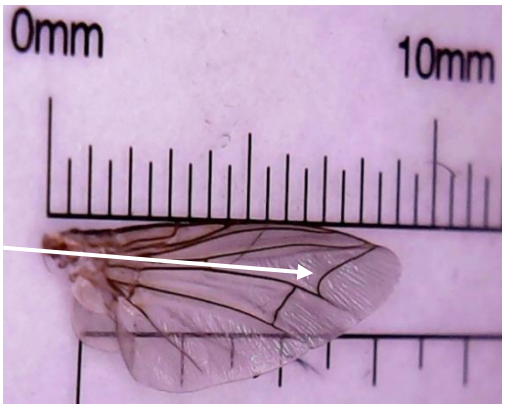

Fig. 3: The measurement of the length of fly's wing.

4. Statistical analysis

Welch ANOVA test for unequal variances and post-hoc Games-Howell test were performed to evaluate differences in the mean results among the treated groups in case of all clozapine experiments, except for the larval measurements; Kruskal-Wallis test for non-parametric data was applied with post-hoc Dunn's test to identify the different groups in case of the significant results. All data were investigated for normality using the Shapiro-Wilk test. The homogeneity of variance was checked using box plots and Levene's test. Resulted values were represented as means ± standard deviation (SD). All statistical analyses of the data were performed with version 26 of IBM SPSS Statistics (IBM Corp. 2022). IBM SPSS Statistics for Windows, version 26.0. IBM Corp, Armonk, NY, all analyses were considered statistically significant at $p < 0.05$.

Results:

This study shows that the presence of clozapine has a great effect on the growth of *Sarcophaga ruficornis* throughout all developmental stages from egg to adult as shown in Table 1.

1. Effect on the duration of a total life cycle:

Results revealed that clozapine had a non-negligible concentration-dependent effect on the life cycle of *S. ruficornis*, where the total developmental duration was increased positively with the drug concentration. The control colony had developed faster (362.44 ± 8.171 h, $F_{(4,145)}$ =3765.6, $p < 0.0001$) than those of the various concentrations of clozapine-treated colonies (371.45 ± 6.69 h, 391.51 ± 1.27 h, 449.25 ± 4.70 h and 511.68 ± 4.61 h for CLZ 25, CLZ 50, CLZ 75 and CLZ 100 colonies respectively). This means that the total developmental duration was slowed when the concentration of clozapine increased (Fig. 4).

2. Effects on the different life stages

In addition to changes in the total duration of the life cycle as a result of clozapine, the development time was significantly different with different dosages of clozapine for eggs ($F_{(4,145)}$ = 108425.54, $p < 0.0001$), first instars ($F_{(4,145)}$ = 799.48, $p < 0.0001$), second instars ($F_{(4,145)}$ = 8235.2, $p < 0.0001$), third instars ($F_{(4,145)}$ = 21425.37, $p < 0.0001$), post-feeding larvae ($F_{(4,145)}$ = 22790.72, $p < 0.0001$) and pupae ($F_{(4,145)}$ = 3921.55, $p < 0.0001$). Additionally, the time taken by females for oviposition among the different treated colonies showed significant differences when compared to the control ($F_{(4,145)}$ = 221376.39, $p < 0.0001$). CLZ100 treated colony consistently demonstrated the highest mean values which highlight the substantial impact of different treatments of clozapine on *S. ruficornis*. More details were demonstrated clearly with *F* and *df* values at $p < 0.0001$ in Table 2.

In clozapine treatments, the spiked substrates were not highly attractive to the gravid females so they showed a significant delay in oviposition, which was obviously concentration-dependent, except for the concentration of CLZ 25 colony (28.39 ± 0.28 h), the oviposition was more accelerated than control (59.94 ± 0.91 h). Therefore, the greatest delay in oviposition was observed in CLZ 100 culture (167.77 ± 0.84 h,~ 7 days); the females observed most of the time flying away from the foodstuff and many egg batches laid on the food substrate spiked with this high concentration were seen desiccated and unable to hatch and survive as well.

Table 1: The effect of clozapine on the duration (in hours) of the life cycle of *Sarcophaga ruficornis* from egg to adult.

Colony	Mean duration (hours ± SD)	
Control	362.44 ± 8.17	(~15.1 days)
Clozapine treated colonies:		
CLZ 25	371.45 ± 6.69	(~ 15. 47 days)
CLZ 50	391.51 ± 1.27	(~ 16.31 days)
CLZ 75	449.25 ± 4.70	(~ 18.72 days)
CLZ 100	511.68 ± 4.61	(~ 21.32 days)

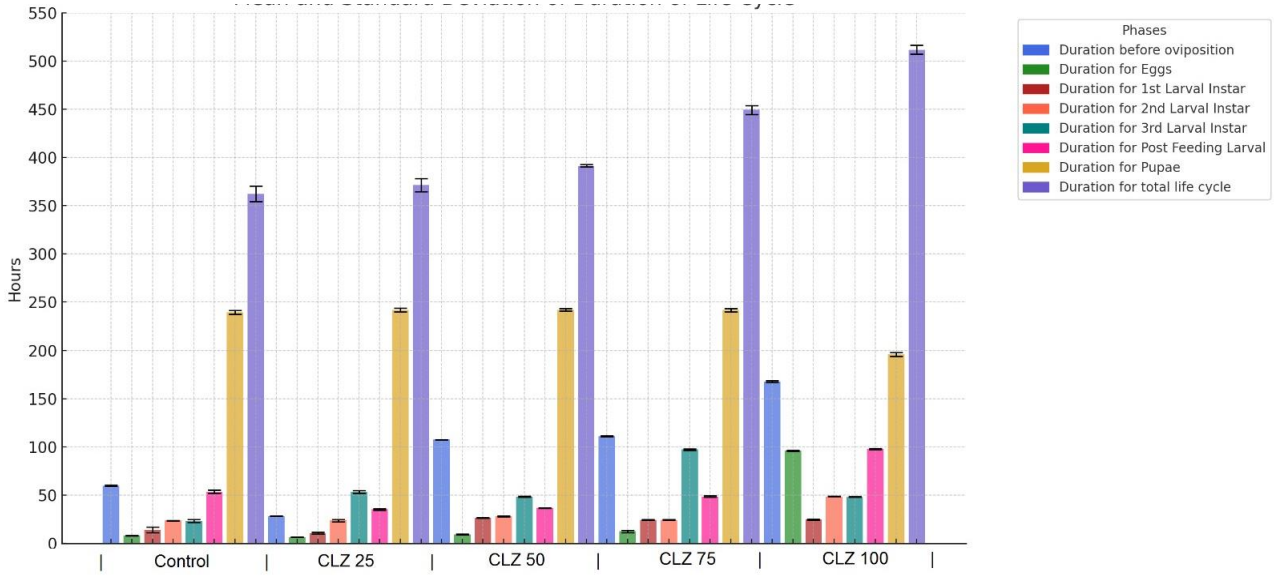

Fig. 4: Graphical representation of the duration of the different stages of *S. ruficornis* in the different concentrations in clozapine-treated (CLZ) and control colonies.

Table 2: Duration (h) of the different developmental stages of *S. ruficornis* reared on various concentrations of clozapine.

Treatment	Time before oviposition	Duration of egg	Duration of 1st larval instar	Duration of 2nd larval instar	Duration of 3rd larval instar	Duration of post-feeding larvae	Duration of pupae	Total life cycle duration
Control	59.94 ± 0.91a	8.09 ± 0.30 a	14.01 ± 2.81 a	23.81 ± 0.24 a	23.33 ± 1.35 a	53.64 ± 1.62 a	239.57 ± 1.87 a	362.44 ± 8.171a
CLZ 25	28.39 ± 0.28 a	6.41 ± 0.24 a	10.77 ± 1.12 a	23.63 ± 1.23 a	53.45 ± 1.59 b	35.40 ± 0.69 b	241.79 ± 1.91 a	371.45 ± 6.69 a
CLZ 50	107.52 ± 0.37 b	9.30 ± 0.59 b	26.51 ± 0.35 b	28.13 ± 0.59 b	48.56 ± 0.31 b	36.83 ± 0.26 b	242.18 ± 1.27 a	391.51 ± 1.27 b
CLZ 75	110.97 ± 0.44 b	12.49 ± 1.18 c	24.44 ± 0.29 b	24.23 ± 0.31 a	97.32 ± 0.62 c	48.75 ± 0.58 c	241.81 ± 1.76 a	449.25 ± 4.70 c
CLZ 100	167.77 ± 0.84 c	95.99 ± 0.45 d	24.88 ± 0.59 b	48.64 ± 0.30 c	48.30 ± 0.46 b	97.77 ± 0.87 d	196.09 ± 1.96 b	511.68 ± 4.61 d
F	221376.39	108425.54	799.48	8235.2	21425.37	22790.72	3921.55	3765.6
df	4,145	4,145	4,145	4,145	4,145	4,145	4,145	4,145

Means in the same column followed by different letters show significant effect of the different concentrations of clozapine according to Games-Howell post-hoc test ($p < 0.0001$).

3. Effects on the morphometric parameters

Results showed statistically significant differences among the control and the various clozapine treatments (CLZ 25, CLZ 50, CLZ 75, CLZ 100) where the larvae of control cultures attained the maximum length (21.54 ± 0.85 mm, $H_{(4)}$ =101.99, $p < 0.001$) compared to18.14 ± 0.79 mm, 18.58 ± 0.59 mm, 20.55 ± 1.29 mm and 18.81 ± 0.31 mm for the respective clozapine concentrations (CLZ 25, CLZ 50, CLZ 75 and CLZ 100). These results indicated that the various clozapine treatments reduce the length of the larvae and the significant differences among the treatment groups themselves, suggested varying impacts of different treatment levels. The larval width was disturbed due to the presence of clozapine, where the CLZ 75 colony shows a noticeably higher mean width (6.38 ± 0.12 mm, $H_{(4)}$ = 80.02, $p < 0.001$) compared to the control (5.92 ± 0.22 mm) and other groups of CLZ25 (5.65 ± 0.44 mm), CLZ 50 (5.76 ± 0.14 mm) and CLZ100 (5.97 ± 0.16 mm). Furthermore, the CLZ 75 treatment has a larvae of significantly heavier mean weight (210.55 ± 3.94 mg, $H_{(4)}$ = 135.57, p < 0.001) as compared to the control set (191.88 ± 6.12 mg) and CLZ 25, CLZ 50, CLZ 100 (163.62 ± 6.52 mg, 168.32 ± 1.89 mg and 145.77 ± 9.91 mg respectively).

Dunn's post-hoc analysis for the morphometric parameters (length, width, and weight) of the third instar larva of *S. ruficornis* showed significant variations among the different clozapine-treated colonies compared to the control. For length, significant decreases were noted in most comparisons except for CLZ 75 vs. control, CLZ 25 vs. CLZ 50, and CLZ 25 vs. CLZ 100. Despite width, significant differences were detected between CLZ 75 and all other treated groups, while other comparisons, such as control vs. CLZ 25 and CLZ 50, did not demonstrate significance. Regarding weight, the majority of pairwise comparisons revealed significant changes, especially when comparing CLZ 75 to other treatments; however, there was no significant difference between CLZ 75 and the control. Overall, these results suggest that clozapine exposure obviously affects larval development, with varying impacts across different concentration levels.

With regards to pupae (at day 5), since the assumption of homogeneity of variances was violated, we used Welch's ANOVA to determine if there are significant differences among the treatments for each morphological measurement. Results indicated significant differences among the various treatments for length ($F_{(4, 145)}$ = 207.9, p = 0.0001), width ($F_{(4, 145)}$ = 71.63, p = 0.0001), and weight ($F_{(4, 145)}$ = 2325.5, p = 0.0001). The CLZ 100 treated colony shows a lower mean pupal length (11.43 ± 0.25 mm) compared to control (12.78 ± 0.31 mm) and other clozapine concentrations (CLZ25 of 12.89 ± 0.24 mm, CLZ50 of12.45 ± 0.09 mm and CLZ75 of 12.23 ± 0.13 mm). Similar to length, the mean pupal width of the CLZ 100 treated colony is the lowest (4.98 ±0.06 mm) compared to control (5.33 ± 0.12 mm) and CLZ25, CLZ50 and CLZ 75 with means of 5.56 ± 0.15 mm, 5.34 ± 0.18 mm and5.20 ± 0.14 mm respectively. Regarding the pupal weight, also CLZ 100 treated group also has a smaller weight (86.73 ± 1.44 mg) compared to other treatments of CLZ 25, CLZ50, CLZ75 (104.23 ± 2.56 mg, 98.27 ± 0.43 mg, 95.26 ± 1.73 mg respectively) and the control group which has a significantly higher weight (126.54 ± 1.64 mg). Overall, the significant differences across the pupal stage indicate a substantial negative effect of clozapine on these parameters where the different treatment concentrations (CLZ25, CLZ50, CLZ 75, CLZ 100) show significantly lower pupal length, width, and weight compared to the control.

The Games-Howell post-hoc analysis revealed significant differences in the morphometric parameters of pupae of *S. ruficornis* among the various concentrations of clozapine colonies and the control one. Regarding pupal length, all comparisons were significantly different, with the

main differences noted between the control and CLZ 100 colonies. Pupal width also showed significant differences in most comparisons, except for the colonies of control vs. CLZ 50. For the pupal weight, all pairwise comparisons showed significance, with the most difference observed between the colonies of control and CLZ 100.

About the wings' length of the newly emerged adults, the average wing length of adults reared on baits treated with CLZ 100 was significantly shorter (7.683 ± 0.39 mm, $F_{(4, 70)} = 98.862$, $p < 0.000$) than that of control adults (8.3874 ± 0.139 mm) and those reared on CLZ 25 (8.7 ± 0.167 mm), CLZ 50 (8.533 ± 0.0588 mm), and CLZ 75 (8.117 ± 0.23 mm) treatments. The Games-Howell post-hoc test further confirmed significant differences in wing length across all *S. ruficornis* treatment groups when compared to the control. Notably, the highest concentration of clozapine (CLZ100) resulted in the most pronounced reduction in wing length, demonstrating a dose-dependent inhibitory effect of clozapine on adult wing development.

Overall, the morphometric parameters of *S. ruficornis* were affected greatly by the presence of clozapine in a concentration-dependent manner to some extent (Figs 5 - 7).

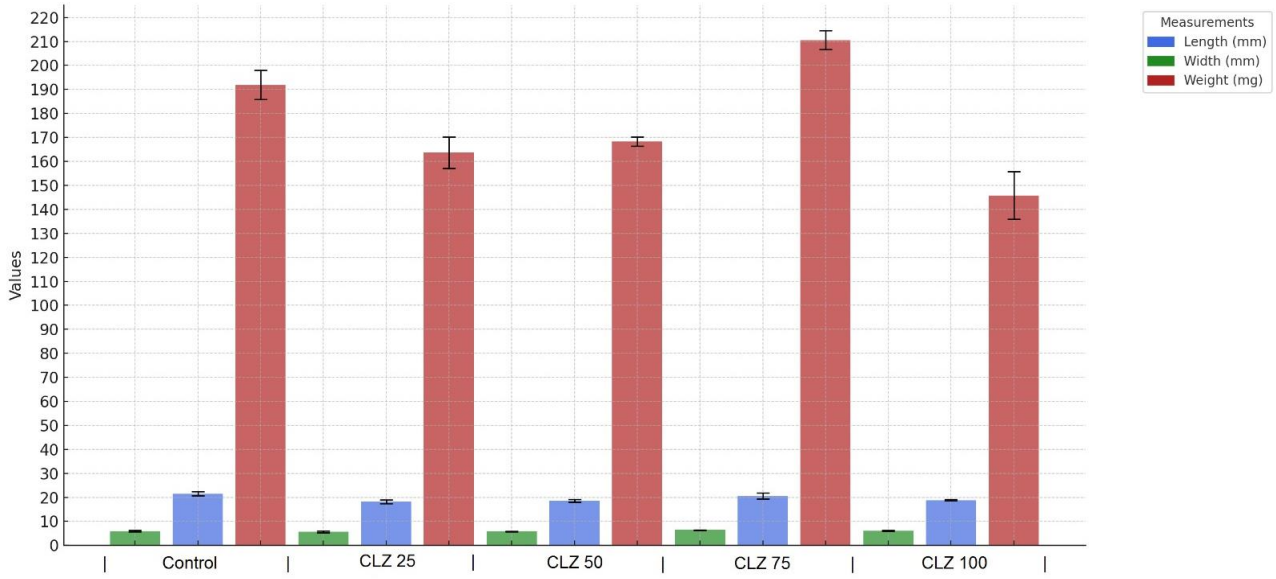

Fig. 5: Graphical representation of the morphometric measurements of length (mm), width (mm), and weight (mg) of *S. ruficornis* 3rd larval instars for each dataset (Control, CLZ25, CLZ50, CLZ75, and CLZ100).

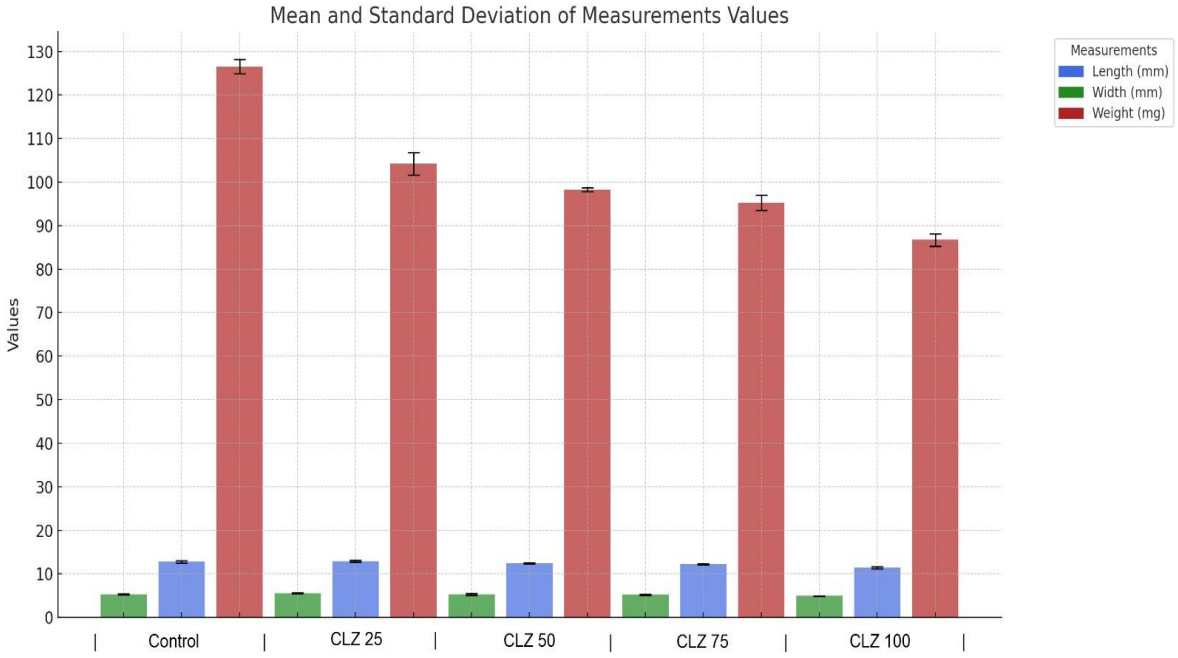

Fig. 6: Graphical representation of the morphometric measurements of length (mm), width (mm), and weight (mg) of *S. ruficornis* pupae for each dataset (control, CLZ25, CLZ50, CLZ75, and CLZ100).

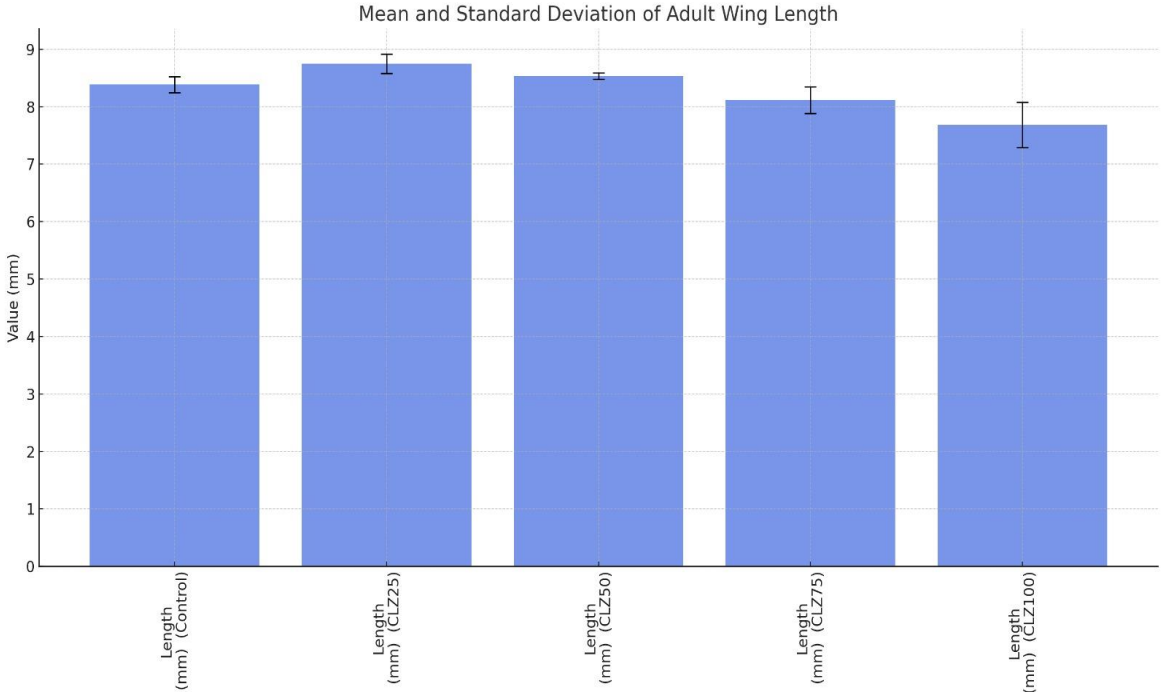

Fig. 7: Graphical representation of the morphometric measurements of length (mm) of the newly emerged adults of *S. ruficornis* pupae (at day 5) for each dataset (Control, CLZ25, CLZ50, CLZ75, and CLZ100).

4. Morphological abnormalities

Pupae in all treated cultures of the different clozapine concentrations exhibited higher mortality than that observed in control, particularly in the case of CLZ 100 concentration, where pupal mortality showed an expressive elevation resulting in a distinct reduction in the newly emerged adults. Furthermore, several adultoids in all treated cultures were with deformed bodies unable to survive and eclose completely from the puparia and remained attached to it (Fig. 8 A-D). It is worth mentioning that many emerged adults from all clozapine treatments showed morphological abnormalities; with wings unable to inflate or fully expand (Fig. 9 A-C) when compared to adults who emerged from the control set (Fig. 9 D).

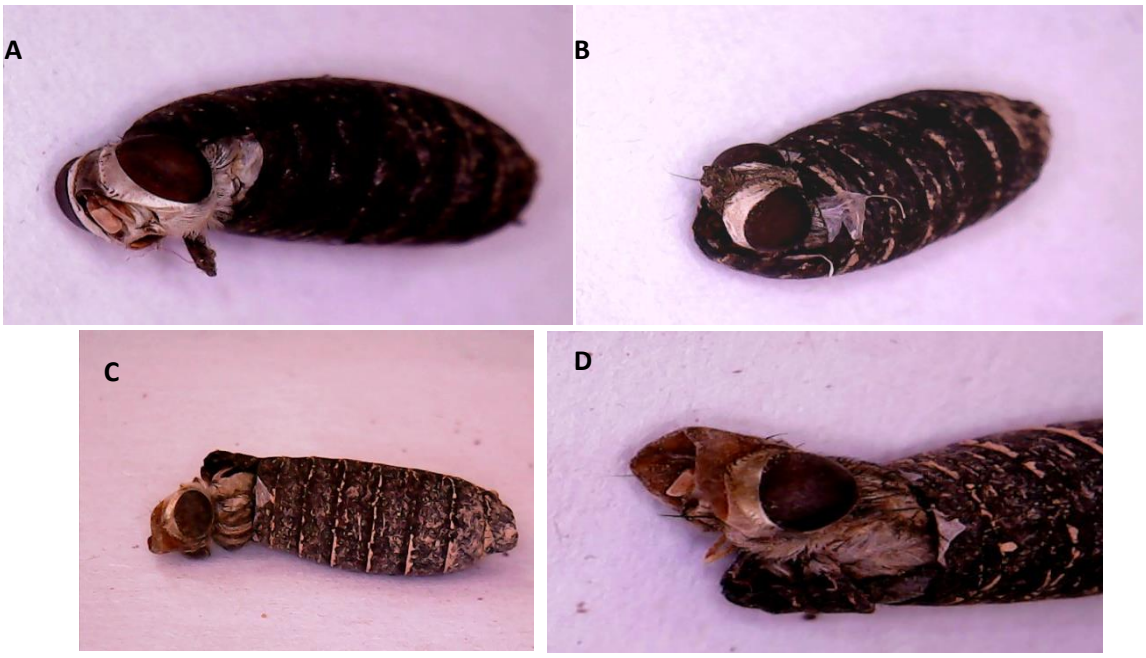

Fig. 8: Pupae of clozapine-treated colonies showing the failure of adults of *S. ruficornis* to emerge from pupae.

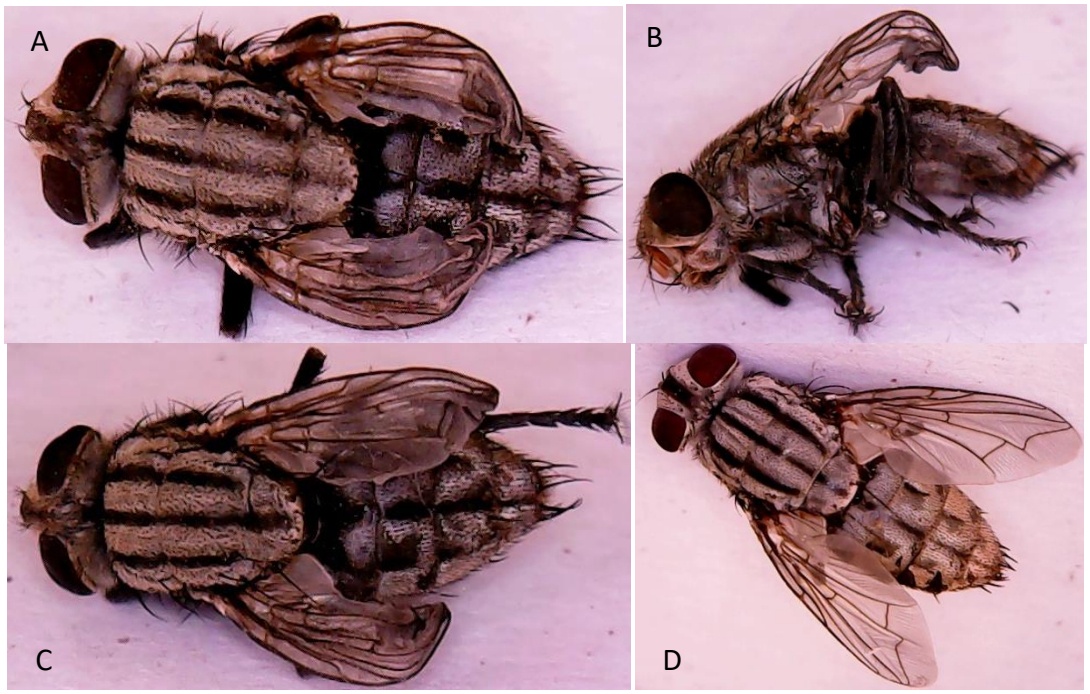

Fig. 9: Adult flies of *S. ruficornis*; A, B, and C showing deformation in wings of the emerged adults, D showing the normal fly emerged from the control colony.

Discussion:

Studies indicated that the presence of drugs and toxins in a person's body upon death can change the developmental rates of the necrophagous insects invading them, this difference was sufficient to alter PMI estimates [38,3,39] The work presented here demonstrated that the overall life cycle and the different morphological measurements of the different stages of *S. ruficornis* could be affected and prolonged significantly by the presence of clozapine in a dose-dependent manner.

The effect of the drugs and toxins on the necrophagous insects is known to depend on the toxin concentration [40]. Thus, in the present work, clozapine retards the total life cycle of *S. ruficornis* in colonies of higher clozapine concentrations than in lower ones. This is in line with the work of **Abd Al Galil *et al.* (2021)** [41], on *Sarcophaga peregrina*, *Sarcophaga dux*, and *Sarcophaga ruficornis* which were exposed to dimethoate.

According to the larval development it was prolonged in treated colonies as compared to control in a dosage-dependent way except at the highest dose of (CLZ 100) the larval duration decreased but still significantly longer than the control. This prolongation in larval duration indicates that clozapine may mimic the juvenile hormone's mechanism which inhibits the molting hormone (ecdysone) and accordingly inhibits the molting process causing the delay of the puparial stage [42]. Our results agreed with the results of **Wang *et al.* (2019)** [27] who observed that clozapine delayed the development of *Chrysomya megacephala* Fabricius (Calliphoride) larvae collected from the suicidal case of a woman who took clozapine. Results also agreed with that of **Musvasva *et al.* (2001)** [29] on *Sarcophaga tibialis* raised on baits spiked with hydrocortisone. Also, the larval duration of *Chrysomya albiceps* was prolonged as a result of morphine [34].

The puparial duration decreased compared to the control, with the colony exposed to CLZ 100 concentration exhibiting the shortest duration. This finding is consistent with the work of **Fouda *et al.* (2017)** [44] on *Chrysomya albiceps* exposed to malathion. However, these findings were in contrast with many studies on different drugs; the study of **Tahoun and Aouzied (2017)** [45] observed decreasing in larval duration and increasing in pupal duration of *Sarcophaga argyrostoma* reared on tissues treated with different concentrations of tramadol; study of **Goff *et al.* (1989)** [46] about the development of *Boettcherisca peregrina* (Diptera: Sarcophagidae) larvae reared on cocaine while no effect occurred in the pupal development; the studies of **Goff *et al.* (1992)** [47] and **Goff *et al.* (1997)** [48] on *Sarcophaga ruficornis* where methamphetamine and 3,4-methylenedioxymethamphetamine accelerated the larval growth.

Moreover, the highest dose of clozapine (CLZ 100) in the present study on *S. ruficornis* had the greatest pupal mortality which is compatible with the results of **Afifi *et al.* (2022)** [49] who noted that there is a clear dose response relationship between clonazepam dosage and pupal mortality in *Sarcophaga argyrostoma*, where higher concentrations of clonazepam lead to higher mortality. *Chrysomya albiceps* also demonstrate higher pupal mortality at the high concentration of nandrolone decanoate [50], while **Goff *et al.* (1989)** [46] observed no effect on the pupal mortality in *Boettcherisca peregrine* (Sarcophagidae) as a result of cocaine. However, the present results are opposite to that of **de Carvalho *et al.* (2012)** [51] where cocaine reduced the pupal mortality in *Chrysomya albiceps* and *Chrysomya putoria* (Calliphoridae).

In this study, clozapine delayed the egg laying of female flies of *S. ruficornis* in a concentration-dependent manner, a result that aligns with the findings of **Mahat *et al.* (2009)** [52] who reported a dose-dependent delay in oviposition of *Chrysomya megacephala* reared on carcasses poisoned with

malathion, **Gunatilake and Goff (1989)** [53] also noted the same results about the impact of malathion on the oviposition of *Chrysomya megacephala* and *Chrysomya rufifacies* which retard the initial oviposition by 1 - 3 days. Also, DEET (insect repellent) was found to cause a delay in egg laying in *Blaesoxipha plinthopyga* Wiedemann (Sarcophagidae) [54].

All treated cultures with varying clozapine concentrations showed some pupal mortality, particularly in the case of CLZ 100 treatment. Therefore, very few adults have emerged. Additionally, the newly formed adultoids with deformed bodies can't eclose completely and remain attached to the puparia. This observation may be due to interference with the eclosion hormone that is responsible for freeing adults from puparia [55,56], so the adult emergence was inhibited at the high levels of clozapine in the insect body. It is also noted that many emerged adults from all clozapine treatments showed morphological abnormalities in many body parts especially their wings which were unable to inflate and fully expand as occurred in the study of **Gaur and Kumar (2020)** [57] which may be interpreted by disturbance in the metamorphic changes due to interfering with the normal secretion of hormones resulting in developmental abnormalities [58].

Regarding the effects of clozapine on the morphometric parameters, results revealed that clozapine reduced the larval length in a dose-dependent manner which is consistent with the results of **Wang *et al.* (2019)** [27] who measured 3rd larval instars of *Chrysomya megacephala* collected from clozapine-suicidal case shorter than what would be expected for larvae reared under similar temperature conditions without exposure to clozapine and alcohol.

With regard to larval weight, clozapine reduced it except for the larvae of concentration of CLZ 75 culture which is heavier than control. These results completely differed from that of **Zou *et al.* (2013)** [59] where ketamine has an insignificant impact on the larval length and weight, while larvae of *Sarcophaga haemorrhoidalis* exposed to chlorpromazine have heavier bodies than unexposed ones [60]. In regard to the pupal morphometry, the presence of clozapine in high concentration resulted in the reduction of pupal length, width, and weight which is similar to observed in pupal measurements of *Chrysomya megacephala* and *Chrysomya saffranea* exposed to different concentrations of zolpidem in **Al-Shuraym *et al.* (2021)** [61] study. According to the work of **Souza *et al.* (2011)** [50], pupae of *Chrysomya megacephala*, *Chrysomya putoria*, and *Chrysomya albiceps* showed a great reduction in weight in high concentrations of nandrolone decanoate. Inversely, **Afifi *et al.* (2022)** [49] found that higher dosages of clonazepam significantly increased all measurements of both larvae and pupae of *Sarcophaga argyrostoma*.

Overall, the morphometric parameters of *S. ruficornis* were affected greatly by the presence of clozapine in a concentration-dependent manner to some extent. It is vital to focus on the complexity in comparing the obtained results on the impact of different drugs on different insect species. The lack of existing entomotoxicological studies regarding atypical antipsychotics like clozapine effects deters a more accurate interpretation of the current results.

The observed results emphasized the necessity for more data collection regarding drug effects on the entomological species of forensic interest because the effects of drugs are species-specific [62,63]. This need is particularly important for drugs like clozapine, which have been shown to impact the insects' development, leading to potential disturbances in PMI estimates. By expanding this area of research, the forensic field can develop more and be validated. This will improve forensic entomotoxicology's acceptability as reliable evidence in court and enhance its applicability in criminal investigations. Such development will help

forensic entomological experts estimate more accurate PMI and better understand the effect of various toxins on necrophagous insects.

Conclusion:

This study provides information that contributes to the understanding of how drugs such as clozapine affect insect development. Nevertheless, the effects of these drugs on the growth and development of necrophagous insects particularly flies have been paid little attention in forensic entomo-toxicological studies. The differential impacts on larval and pupal stages underscore the complexity of the effects of these chemicals. It has been shown that, despite the same species of flies, distinct effects can be produced by medications and toxicants belonging to the same class. Furthermore, a given toxin or medication may have different effects on two different species. Future research on clozapine on different forensically important species is required to ensure precise estimates of PMI.

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