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Hazardous effects of octopamine receptor agonists on altering metabolism-related genes and behavior of *Drosophila melanogaster*

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Abstract

Recent reports demonstrate that octopamine receptor (OR) agonists such as formamidine pesticides cause reproductive and developmental toxicity through endocrine disrupting effects in both humans and animals. Herein, we studied the effects of different sublethal concentrations of OR agonists, Amitraz and Chlordimeform, on growth, development, and reproduction of *D. melanogaster* from a genotype perspective view. As a result, the sublethal concentrations for both OR agonists delayed the developmental time including pupation and eclosion. It significantly reduced the lifespan, eclosion rate, and production of eggs. The mRNA expression of genes relevant for development and metabolism was significantly changed after exposure to sublethal concentrations of both OR agonists. Octopamine receptor in mushroom bodies (*Oamb*), trehalase enzyme (*Treh*), hemocyte proliferation (*RyR*), and immune response (*IM4*) genes were upregulated whereas, trehalose sugar (*Tret1-1*), mixed function oxidase enzyme (*Cyp9f2*), lifespan (*Atg7*), male mating behavior (*Ple*), female fertility (*Ddc*), and lipid metabolism (*Sxe2*) genes were downregulated. These results support the conclusion that OR agonists activate the octopamine receptor in *D. melanogaster* leading to an increase of trehalase enzyme activity and degradation of trehalose sugar into free glucose which results in rapid energy exhaustion, hyperexcitation, and disturbing of the octopaminergic system in *D. melanogaster.*

Graphical Abstract

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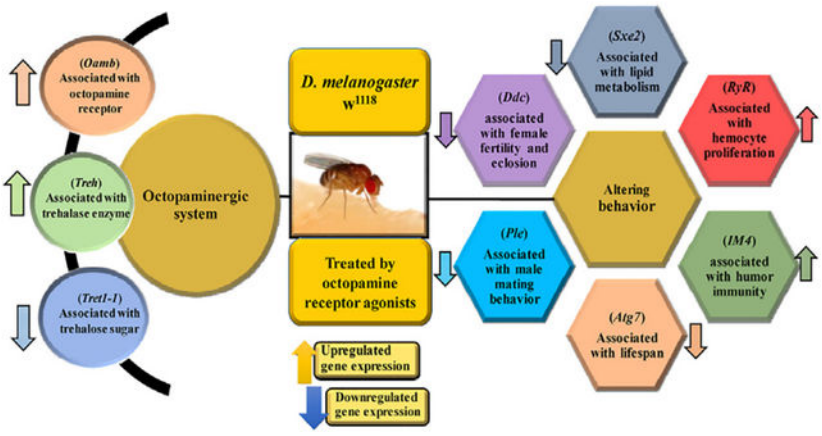
Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Mohamed Ahmed Ibrahim Ahmed: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Project administration, Resources, Software, Validation, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Christoph Franz Adam Vogel:** Funding acquisition, Methodology, Project administration, Resources, Software, Validation, Supervision, Visualization, Writing - original draft, Writing - review & editing.



Keywords

Octopamine receptor agonists (OR agonists); *Drosophila melanogaster*; Sublethal toxicity; Formamidines; Pesticides

1. Introduction

Pesticides play a vital role in controlling pests in agricultural crops and public health. The significance of pesticides in terms of secure food production and negative outputs are important issues (Ahmed and Othman, 2020; Mandi et al., 2019). For instance, pesticide resistance, contamination problems, declination of honeybees and pollination, and potential health effects are an increasing global problem. Regarding the public health awareness and rise of environmental health risks, the prevention of hazardous pesticides is indicated and the substitution with safe pesticides is needed (Zhang et al., 2018).

Formamidines, a group of pesticides that have various mechanisms of action, are used to control animal and crop pests worldwide (Ahmed and Matsumura, 2012; Kita et al., 2017; Gao et al., 2019). Interestingly, formamidines have a unique mode of action serving as agonists on octopamine receptors (ORs) in mosquitoes, locusts, cockroaches, and mites (Ahmed et al., 2015; Monteiro et al., 2019). Formamidines disrupt monoamine-mediated production of cyclic adenosine monophosphate (cAMP) and induce adverse behavioral changes in treated insects, which are considered the most important feature of their insecticidal actions (Ahmed et al., 2015; Kita et al., 2017).

Formamidine pesticides are quickly absorbed, distributed, metabolized, and eliminated; they are harmful to human health, particularly to children, through accidental exposure. Ingestion is considered the most common pathway of exposure, whereas in a small number of cases percutaneous exposure was reported (Dhooria and Agarwal, 2016; Moyano et al., 2019).

Drosophila melanogaster, first described by Meigen in 1830, is considered a standard model for toxicological studies of xenobiotics in insects (Koon and Chan, 2017; Zhang et al., 2018; Li et al., 2019). Importantly, the genome of *D. melanogaster* has been completely sequenced, and the structural and functional genome has been found to be comparable to

the human genome (Zhang et al., 2018; Liu et al., 2019). Interestingly, approximately 75% of the genes that are involved in the development of human diseases are evolutionarily conserved across animal species, including Drosophila (Cheng et al., 2018). In addition, *D. melanogaster* has a short lifecycle, is easy to maintain in culture in the laboratory, produces many eggs, and has a low number of chromosomes (only four pairs). These characteristics of *D. melanogaster* make them amenable to molecular analyses (Zhang et al., 2018; Anet et al., 2019; Liu et al., 2019).

The present work aims to evaluate the acute toxicity of OR agonists on third instar larvae and adults. We assessed the effects of sublethal concentrations of OR agonists on growth, development, and reproduction of *D. melanogaster*, and determined changes in gene expression relevant in development and metabolism.

2. Materials and methods

2.1. Insect material

D. melanogaster wild type (w^{1118}) were kindly provided by Dr. Joanna Chiu, the University of California Davis and used for all experiments. Flies were maintained at 25 °C at a relative humidity of 70% under a light/dark cycle of 12-h vials (50 ml) containing a standard potato based medium which was obtained from Carolina Biological Supply Co. (Burlington, NC, USA).

2.2. Chemicals

Chlordimeform (99.8%) and Amitraz (96.8%) were purchased from Sigmae–Aldrich Co. (St. Louis, MO, USA) (Table 1).

2.3. Acute toxicity assay

The acute toxicity assay was carried out as previously described (Li et al., 2019). AMZ and CDM were dissolved in acetone and mixed with food medium (1 g) per vial to obtain the final concentration. Initial tests indicated that the acetone residue in the food medium had no toxic effects. Therefore, controls received only acetone and were run at the same time with each series of tests. A 1000 μg/ml stock solution of AMZ and CDM was formulated with acetone. For the larvae acute toxicity assay, dilutions of 0 μg/ml, 0.03 μg/ml, 0.3 μg/ml, 3 μg/ml, 30 μg/ml, and 300 μg/ml in drosophila food for third instar larvae and 0 μg/ml, 0.06 μg/ml, 0.6 μg/ml, 6 μg/ml, 60 μg/ml, and 600 μg/ml in drosophila food for adults were carried out for both pesticides in the experiments. In all acute toxicity assays, 20 *D. melanogaster* at 4–8 h post-emerged were transferred to each vial, and the controls received acetone as the vehicle. At least three replicates were performed for each concentration. Each assay was conducted at 25 °C. Mortality percentage was recorded after 24-h of exposure. Each fly was considered dead when it was not able to move when touched with dissecting forceps. For toxicological assays, sublethal concentrations of AMZ and CDM (approximately one-tenth of the acute LC_{50} values of AMZ and CDM) were used for third instar larvae, at 12.07 μg/ml and 7.61 μg/ml and 37.75 μg/ml and 20.10 μg/ml for adults, respectively. These sublethal concentrations were applied to the lifespan and oviposition experiments.

2.4. Developmental effect assay

The life cycle assay was carried out according to (Podder and Roy, 2015; Li et al., 2019) with some modification. Here, one-tenth of the LC_{50} values of AMZ and CDM were used at 12.07 μg/ml and 7.61 μg/ml, respectively. The assays were performed in three replicates. In all experiments, 20 s-instar larvae were secluded from vials and transferred to new vials for both the treatment with pesticides and controls. Additionally, mean values for larval duration, pupal duration, adult eclosion duration, and the number of individuals were estimated. According to the lifespan assay, a total of 20 adults (males and females) were cultured independently in vials with food that contained one-tenth of the LC_{50} values of AMZ and CDM (37.75 μg/ml and 20.10 μg/ml, respectively). Food was modified every 10 days. The assays were accomplished in three replicates. Number of deaths was recorded every day until the last fly died.

2.5. RNA extraction and quantitative real-time reverse transcriptase PCR (qRT-PCR)

In order to get more insight into the role of OR agonists and their effects on the octopaminergic system and altering the behavior of *D. melanogaster*, adult flies (4–5 days old) were exposed for 24-h to two different sublethal concentrations of LC_{50} values; one-tenth (12.07 μg/ml and 7.61 μg/ml) and one-third (67.00 μg/ml and 125.82 μg/ml) of AMZ and CDM, respectively. Total RNA was isolated from adults using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA purity and concentration were determined using a NanoDrop Spectrophotometer (Thermo Scientific, USA). RNA was isolated using an RNA isolation kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA was converted to cDNA using Applied Biosystems' High-Capacity cDNA Reverse Transcription Kit (Foster City, CA). Gene-specific forward and reverse primers (0.2 μM; IDT, Coralville, IA), cDNA (2 μL/reaction), and SYBR Green nucleic acid staining dye (10 μL/reaction; Applied Biosystems) were used for qRT-PCR using a Light Cycler LC480 Instrument (Roche Diagnostics, Indianapolis, IN). Gene expression was assessed using the ΔΔ-Ct method and standardized to the expression of the housekeeping gene, actin. Gene primers were designed using Primer3 primer design software (Untergasser et al., 2012). Primer sequences used in this study are shown in Table 2. Genes that were analyzed in this study include *Actin, Oamb, Treh, Tret1-1, Cyp9f2, RyR, IM4, Atg7, Ple, Ddc*, and *Sxe2*. The intra-assay variability was <7%. Data was analyzed with the Light Cycler analysis software.

2.6. Statistical analysis

The corrected mortality was estimated according to Abbott's formula (Abbott, 1925). Acute toxicity assay data (LC_{50}, 95% CL values, slope, X^2, and g values) were calculated using IBM SPSS Statistics Desktop, V25 (SPSS, Inc., Chicago, IL). Toxicity index = [(LC_{50} value of the most toxic pesticide divided by the LC_{50} value of tested pesticide) × 100] for each stage. Tolerance ratio calculated by LC_{50} value for adults divided by the LC_{50} value for third instar larvae after 24-h exposure. Developmental effect and molecular biological assay data were analyzed by student *t-test*. Data represents mean fold induction ±SE relative to control group. Significance levels were considered when $p < 0.05$. Figures were generated using GraphPad Prism 6.01 software (San Diego, CA).

3. Results

3.1. Acute toxicity

The acute toxicity results are presented in Table 3. The LC_{50} values of AMZ and CDM on third instar larvae were 120.68 and 76.06 μg/ml, respectively, whereas the LC_{50} values on adults were 377.45 and 201.00 μg/ml, respectively, after 24-h of exposure. Further, the toxicity index data indicates that CDM was more efficient than AMZ on both third instar larvae and adults (1.59 and 1.88-fold, respectively (Table 4).

Based on the tolerance ratio values, the adults of *D. melanogaster* demonstrated a higher tolerance to AMZ and CDM than third instar larvae (3.13 and 2.64-fold, respectively) (Table 4).

3.2. Developmental effects of OR agonists

OR agonists prolonged the developmental time including pupation and eclosion of F1 *D. melanogaster* flies. The time required for pupa to become adults in AMZ and CDM treated groups was 10.00 ± 0.41 and 12.00 ± 0.82 days, respectively, compared to 6.00 ± 0.40 days for the control group (Fig. 1). The lifespan of adults after treatment with AMZ and CDM were 41.2 ± 1.73 and 38.7 ± 3.06, respectively, compared to 50.67 ± 2.08 in the control group (Fig. 2).

Pupation rate was not significantly affected by both AMZ and CDM using one-tenth of the semi-lethal concentrations (Fig. 3a). In contrast, eclosion rate was significantly inhibited (AMZ = 61.00 ± 2.90% and CDM = 49.00 ± 3.92%) compared to control (80.33 ± 2.52%) (Fig. 3b).

The number of eggs laid for each vial for AMZ treated flies was 28.67 ± 3.51, 14.67 ± 2.73 for CDM treated flies and 48.33 ± 3.06 in the control group (Fig. 4).

3.3. Effects of OR agonists on gene expression of D. melanogaster

Adult flies exposed to one-tenth and one-third of sublethal concentrations of OR agonists demonstrated significant effects on the selected genes (Fig. 5). Interestingly, the effect of CDM and AMZ on octopaminergic system was addressed. *Oamb*, associated with OR regulation, was stimulated significantly by both pesticides. The maximum expression of *Oamb* was induced by 7.14- and 5.24-fold at one-third of the sublethal concentration (Fig. 5a). *Treh*, regulates expression of the enzyme trehalase and was upregulated by 3.83- and 5.26-fold after treatment with one-tenth of CDM; and by 2.59 and 4.21-fold after exposure to one-tenth of AMZ (Fig. 5b). *Tret1-1*, associated with trehalose sugar, was significantly downregulated by one-tenth concentrations of CDM and AMZ by 8.33- and 3.70-fold compared to control (Fig. 5c). Level of the drosophila *Cyp9f2*, associated with P450 detoxification enzyme activity, was downregulated by treatment with one-tenth of sublethal concentrations of CDM and AMZ by 6.25- and 2.70-fold, respectively. Exposure to one-third of sublethal concentrations of CDM and AMZ led to 12.20- and 4.76-fold decrease of *Cyp9f2*, respectively (Fig. 5d). *RyR*, which controls hemocyte proliferation, was upregulated by CDM and AMZ, particularly at higher sublethal concentrations (4.93- and 4.17-fold,

respectively) (Fig. 5e). *IM4*, which is associated with humoral immunity, was found to be significantly induced after treatment with one-third sublethal concentrations of CDM (7.81-fold) and AMZ (6.44-fold) compared to the control (Fig. 5f). *Atg7* is an important factor to adjust the lifespan in *D. melanogaster* and was found to be downregulated by CDM (4.35- and 13.51-fold) and AMZ (2.44 and 5.56-fold) (Fig. 5g). Expression of *Ple*, which regulates mating behavior in males, was significantly downregulated by both pesticides at sublethal concentrations (CDM = 5.88 and 13.51) and (AMZ = 2.78 and 10.20-fold) (Fig. 5h). *Ddc*, which is associated with female fertility and eclosion, was downregulated particularly at one-third sublethal concentrations of CDM and AMZ by 6.25- and 5.26-fold, (Fig. 5i). Expression of *Sxe2*, involved in lipid metabolism, was suppressed by CDM and AMZ particularly at the higher concentration (7.69- and 3.57-fold, respectively) (Fig. 5j).

4. Discussion

Octopamine receptor agonists, such as formamidine compounds, were investigated in numerous studies based on their biological and pharmacological properties (Zhou et al., 2008; Del Pino et al., 2017; Meriç Turgut and Keskin, 2017; Gao et al., 2019; Monteiro et al., 2019). To date, few studies focus on dipterous insects. In particular, there is limited data that exits on the possible biochemical and molecular biological effects of OR agonists on *D. melanogaster*. In this study, we focused on the role of OR agonists in altered behavior and the effects on the octopaminergic system in *D. melanogaster*. Herein, CDM was more potent than AMZ on both third instar larvae and adults after 24-h of exposure. The results agree with our earlier observation (Ahmed and Matsumura, 2012) showing that CDM was more toxic than AMZ on fourth instar larvae of *Aedes aegypti* after 24, 48, and 72-h of exposure.

Furthermore, in our results, OR agonists significantly prolonged the developmental stages, reduced the lifespan, decreased the eclosion rate and number of eggs compared to the controls. In line with this finding, we found that formamidines addressed significant reduction of larval development of dipterous insects, *Aedes aegypti* and *D. melanogaster* (Ahmed et al., 2015). Further, Gruntenko et al. (2007) demonstrated a correlation between increased levels of octopamine and a decreased number of vitellogenic (stages 8–10) and mature (stage 14) oocytes in *Drosophila virilis*, as well as a decreased fecundity of *D. melanogaster* and *D. viriles*. In general, octopamine takes the roles of epinephrine and norepinephrine in insects (Ahmed et al., 2015; Li et al., 2016). It plays a critical role in the control of major metabolic parameters in *D. melanogaster*. Furthermore, it modulates numerous behavioral and physiological processes in *D. melanogaster*, for instance, starvation resistance, life span, and metabolic traits (Li et al., 2016).

In our study, we observed significant changes in the expression of genes critically involved in major physiological processes in *D. melanogaster* such as mixed function oxidase (MFO), defense mechanisms, lifespan, mating behavior, fertility and eclosion, sugar metabolism, and energy production. In this interim, the mRNA expression of *Oamb*, an octopamine receptor gene found in mushroom bodies (Zhou et al., 2012; Kim et al., 2013), was highly increased. In correlation with that, *Treh*, the gene associated with the enzyme trehalase, was upregulated while *Tret1-1*, a trehalose sugar regulated gene, was downregulated. This suggests that OR agonists increase trehalase activity, resulting in trehalose degradation into

free glucose in the blood; which may cause anorexia plus depletion of energy leading to acceleration of energy exhaustion in affected *D. melanogaster*. This finding is likely to be the reason of altering behavior of *D. melanogaster*, such as significant changes in developmental stages period, lifespan, eclosion rate, and egg production because of hyperexcitation-induced glucose release which consequently cause quick energy exhaustion. In supporting of this finding, we found that formamidines act as octopamine receptor agonists to cause depletion of trehalose/glucose storage, which eventually leads to the exhaustion of the affected *Aedes aegypti* mosquitoes (Ahmed et al., 2015). Further, an increase in the level of glucose and treholase in the hemolymph of American cockroach, *Periplaneta americana*, was observed after the injection of the American cockroach by CDM or octopamine itself (Ismail and Matsumura, 1991). However, it was found that the increase in free glucose levels could be related to the increase of trehalase activity. In addition, in the tobacco hornworm *Manduca sexta*, it was observed that CDM or its metabolic activation to DCDM, acts on the OR of the tobacco hornworm larvae, which cause an increase in hemolymph sugars that could be a biochemical cause for the CDM-induced antifeeding activity and anorexia in *Manduca sexta* (Ismail and Matsumura, 1992).

Interestingly, *Cyp9f2* is an age-regulated gene involved in oxidation-reduction processes (Zhu et al., 2013). Here, we found that the mRNA levels of *Cyp9f2* were significantly downregulated in the treated groups. Insufficient *Cyp9f2* activity may affect metabolism of OR agonists resulting in higher OR levels and increased toxicity. For that reason, OR agonists are considered potent tools to treat some pests based on their synergistic action when they are mixed with certain pesticides. Similar concentrations as applied in this study are found to be sublethal concentrations in combination with other key pesticides on different insect pests (Liu and Plapp, 1990; Prullage et al., 2011; Rodriguez-Vivas et al., 2013; Ahmed and Vogel, 2016). However, recent studies describe reproductive and developmental toxicity caused by OR agonists through endocrine disrupting effects in humans (Dhooria and Agarwal, 2016; Moyano et al., 2019).

Upregulation of *RyR* after treatment with OR agonists may result in increased renewal and the production of the cell cycle system to conserve the normal concentration of hemocytes. This mechanism prolongs durability to face the interference from external impacts of OR agonists. This finding agrees with results of previous studies (George and Ambrose, 2004; Rajak et al., 2017; Li et al., 2019) demonstrating that the total hemocyte count (THC) was increased when Drosophila were treated with pesticides.

In addition, *IM4*, a gene involved in immune function and defense in drosophila (Verleyen et al., 2006; Sackton et al., 2010), was significantly induced after exposure to OR agonists indicating a strong response to stress induced by OR agonists. *Atg7*, which is a life cycle-related gene in drosophila, was downregulated resulting in reduction of lifespan. A similar study has shown that loss of *Atg7* lead to a shorter lifespan in Drosophila (Juhász et al., 2007).

Furthermore, *Ple*, a gene related to male mating behavior (Liu et al., 2009; Li et al., 2019), was downregulated by OR agonists. It is likely that OR agonists reduce male reproductive system functions and affect mating behavior leading to a reduced number of eggs. In this

regard, *Ddc*, which play a role in female fertility, was downregulated by OR agonists resulting in reduction of oviposition probably based on the depletion of molting and eclosion process in *D. melanogaster* (McCrady and Tolin, 1994; Li et al., 2019). *Sxe2*, which is associated with fat metabolism, was downregulated, this may result in a reduction of body fat and energy storage in *D. melanogaster* (Fujii and Amrein, 2002).

Numerous studies have emphasized the effects of OR agonists on altering biological activities in other insects (Waliwitiya et al., 2010; Ahmed et al., 2015). In male hoverflies (*Eristalis* spp.), CDM affected the early, elementary motion detectors-associated contrast gain reduction and temporal frequency dependently. However, the neuron membrane conductance of *Eristalis* horizontal system neurons could be modulated and vanished by CDM-induced during and after visual stimulation. Thus, physical activity is mainly affected by motion adaptation presynaptically of lobula plate tangential cells in the insect optic ganglia (De Haan et al., 2012). Further, chemical communication in relation to courtship and mating was disrupted by sublethal doses of CDM even though it was a poor candidate for attracticide formulations among tested insecticides because males avoided contact with this insecticide (Haynes et al., 1986). The effects of a sublethal dose (1% mortality) of CDM affected the chemical communication of cabbage loopers, *Trichoplusia ni*, plus, decreased the mating success of males. In the same study, oviposition and egg hatching were also affected by CDM. Mated females treated with CDM laid significantly fewer eggs than acetone-treated females in the control group. In addition, hatchability of eggs laid by mated female *T. ni* treated with CDM was significantly lower than for eggs laid by control females (Clark and Haynes, 1992). Waliwitiya et al. (2010) observed that Desmethylchlordimeform, the demethylated CDM analogue, prevented the wing beating within 60 min and produced a profile of constant but lower frequency flight muscle impulses in the blowfly, *Phaenicia sericata*. Hiripi et al. (1999) revealed that the formamidines had a potent antifeeding effect when injected into the locust, *Locusta migratoria migratorioides*. The toxic effect of octopaminergic compounds on other organisms' behavior has been investigated as well. Brooker et al. (2011) examined four octopaminergic compounds, octopamine hydrochloride, clonidine hydrochloride, AMZ, and CDM on the parasite genus *Gyrodactylus*. All four tested compounds inhibited the movements of the parasite. Study by Meriç Turgut and Keskin (2017) on rainbow trout, *Oncorhynchus mykiss*, found that sublethal exposure of 0.84 mg/L AMZ resulted in upregulation of thyroid hormone receptor genes for muscle and liver, respectively, in a tissue-manner resulting in emerged an endocrine interaction between AMZ based formulation and thyroid hormone homeostasis. In summary, the findings of the current study show a strong correlation of the acute and developmental effects mediated by the OR agonists AMZ and CDM with the altered expression pattern of genes critically involved in these processes.

5. Conclusion

This study provides evidence that OR agonists trigger various sublethal effects on development, lifespan, eclosion, and reproduction. The changes of these phenotypes are likely due to the significant effects on the expression of genes, which are important regulators of these processes. The results contribute a solid reference for the effects of OR agonists on *D. melanogaster*. Further investigation is needed in order to better

understand behaviors such as locomotion, aggression, grooming, and olfactory response. Such information may lead to a more thorough understanding of the toxicological, physiological, and molecular biological mechanism of OR agonists on *D. melanogaster*. This work is upholding the use of *D. melanogaster* as a standard indicator for the toxicological assessment of OR agonists.

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HIGHLIGHTS

- CDM is more potent than AMZ on both larvae and adults of *D. melanogaster.*
- *D. melanogaster* adults demonstrated a higher tolerance to AMZ and CDM than larvae.
- *Oamb, Treh*, *RyR*, and *IM4* genes were significantly upregulated.
- *Tret1-1, Cyp9f2, Atg7, Ple, Ddc*, and *Sxe2* genes were significantly downregulated.

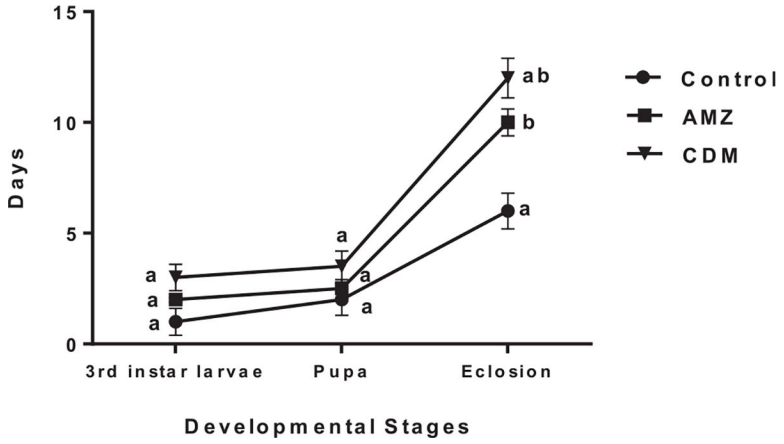


Fig. 1.

Effect of AMZ (12.07 μg/ml) and CDM (7.61 μg/ml) given in the feeding medium on development of second-instar larvae of wild type *D. melanogaster* the time required to become pupae and go through adult stage. Data represents the mean of fold induction ± SE relative to control groups. Different letters represent significant differences between experimental groups ($p < 0.05$).

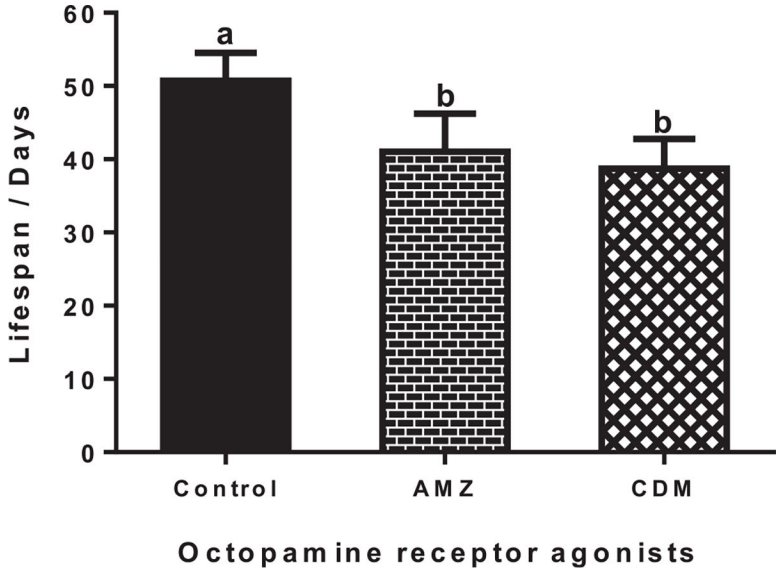


Fig. 2.

Effect of AMZ (37.75 μg/ml) and CDM (20.10 μg/ml) for observation of the lifespan of *D. melanogaster* adult flies. Data represents the mean of fold induction ± SE relative to control groups. Different letters represent significant differences between experimental groups ($p < 0.05$).

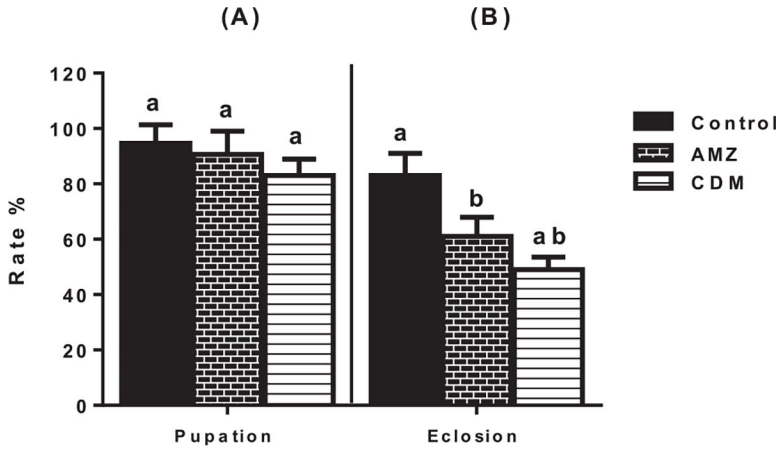


Fig. 3.

Effects of AMZ (12.07 μg/ml) and CDM (7.61 μg/ml) on (A) pupation rate of third-instar larvae in terms of the rate of formation and (B) Eclosion rate of third-instar larvae in the control group and the group treated with sublethal concentration. Data represent the mean of fold induction ± SE relative to control groups. Different letters represent significant differences between experimental groups ($p < 0.05$).

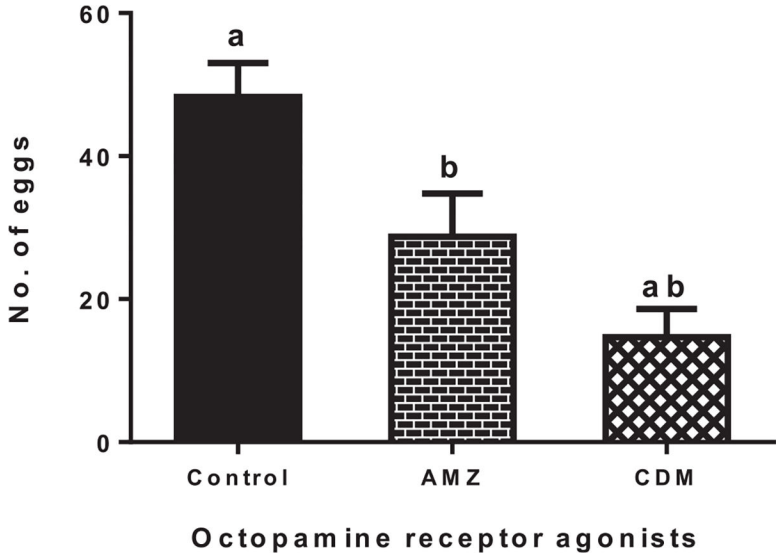


Fig. 4.

Effects of AMZ (37.75 μg/ml) and CDM (20.10 μg/ml) on egg production per vial after 24-h exposure. Data represent the mean of fold induction ± SE relative to control groups. Different letters represent significant differences between experimental groups ($p < 0.05$).

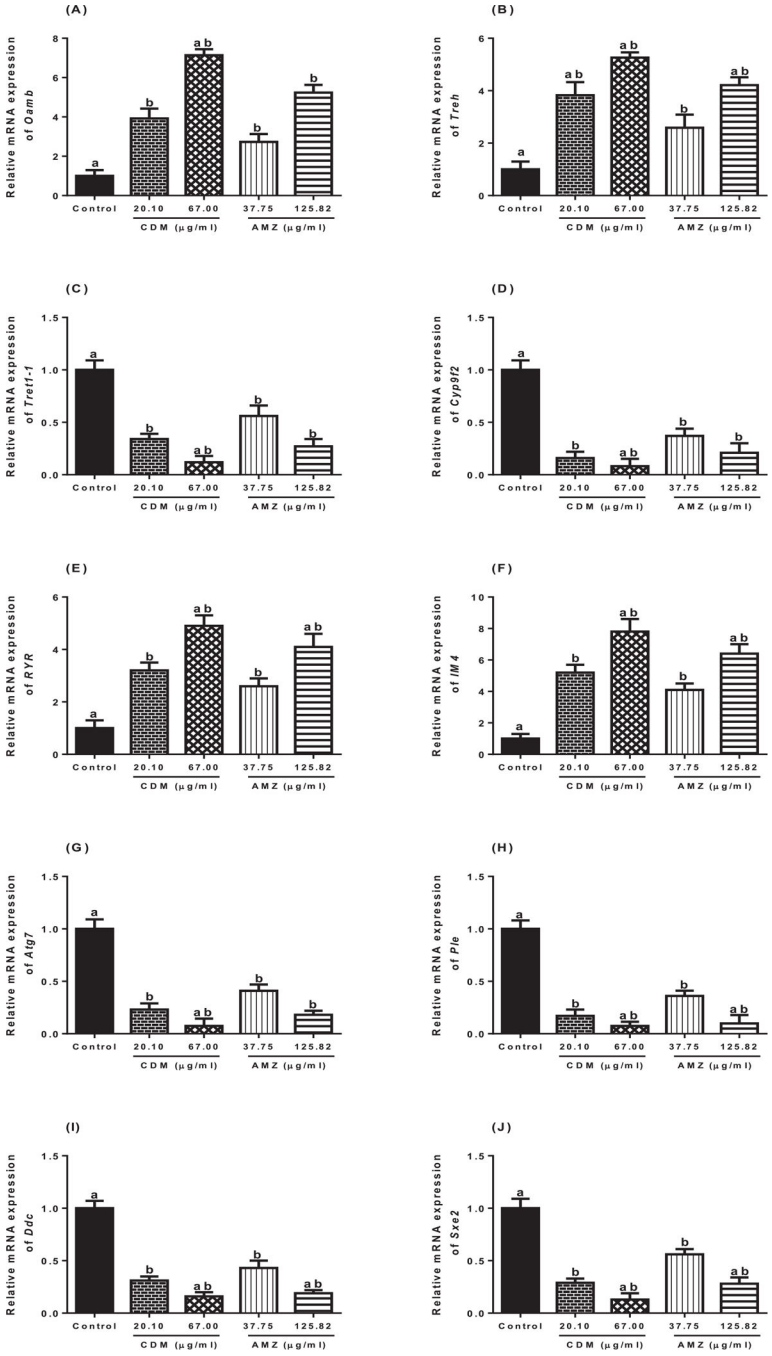
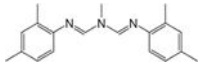
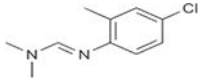


Fig. 5.

Gene Expression of (A) *Oamb*, (B) *Treh*, (C) *Tret1-1,* (D) *Cyp9f2*, (E) *RyR*, (F) *IM4*, (G) *Atg7*, (H) *Ple*, (I) *Ddc*, and (J) *Sxe2* of *D. melanogaster* exposed to different sublethal concentrations (one-tenth and one-third of LC_{50} values) of CDM (20.10 and 67.00 µg/ml) and AMZ (37.75 and 125.82 µg/ml). Data represent the mean of fold induction ± SE of mRNA expression relative to control groups. Control value was set as 1. Different letters represent significant differences between experimental groups ($p < 0.05$).

Table 1

Selected octopamine receptor agonists used in this study.

Common name	Group	Chemical	
		Name[a]	Structure
Amitraz	Formamidine	*N,N´*-[(Methylimino)dimethylidyne]di-2,4-xylidine	
Chlordimeform	Formamidine	*N´*-(4-chloro-2-methylphenyl)-*N,N*-dimethylmethanimidamide	

[a]The International Union of Pure and Applied Chemistry (IUPAC) name.

Table 2

Primer sequences used for qRT-PCR.

Gene Name	Forward Primer	Reverse Primer
Actin	5′-CAAAGCGCAAAAAGAACACA-3′	5′-AGAGGAGAGGGCGAGGTTAG-3′
Oamb	5′-GACGAGAGTGAACTGCACCA-3′	5′-TCCATGACCAGAGGATGACA-3′
Treh	5′-ATCTCCACTGTGGGTGAAGG-3′	5′-CGGGAGTGTTCAGGTTGTTT-3′
Tret1-1	5′-GCAAAGTGAAAGGCAGGAAG-3′	5′-CAGCGAGACACTCAGAGCAG-3′
Cyp9f2	5′-TCAAGGATCGCGAAAATACC-3′	5′-TTCATAGCATCGAGCACCAG-3′
RyR	5′-GCTGCACCATCTCTACGACA-3′	5′-CGGACAGCGGAACTCnTAG-3′
IM4	5′-ACCAACAACCACCAAACCTC-3′	5′-IGTTGGGTCAACATGGTTCT-3′
Atg7	5′-CATTCCGCrATAGGCACCAT-3′	5′-CGGCAAAGGAGAGAACAAAG-3′
Ple	5′-CAGACCAAACAAACCGTCCT-3′	5′-GACTCCGACTGCTCCTGTTC-3′
Ddc	5′-ACACAAATGGATGCTGGTGA-3′	5′-AAGTGGGATTTGCCAGTGAC-3′
Sxe2	5′-TATTCGGGAGACAGCAAACC-3′	5′-CCATAAACCTGCGGAGTGAT-3′

Table 3

Toxicity of octopamine receptor agonists on *D. melanogaster* larvae and adults after 24-h exposure.

Compounds	*n* [a]	After 24-h			
		LC_{50} [b] (95% CL)	Slope (±SE)	X^2 (*df*) [c]	g value [d]
On larvae					
Chlordimeform	360	76.06(17.12–90.96)	4.0 (±0.1)	0.3 (3)	0.07
Amitraz	360	120.68 (27.78–161.19)	4.1 (±0.1)	0.8 (3)	0.08
On adults					
Chlordimeform	360	201.00 (54.60–347.36)	4.8 (±0.1)	0.5 (3)	0.06
Amitraz	360	377.45 (79.48–499.32)	4.0 (±0.1)	0.1 (3)	0.09

[a] *n* = number of larvae or adults tested, including control.

[b] Concentration is expressed in mg/ml and the response determined after 24-h exposure.

[c] *df* = Degree of freedom.

[d] If g value < 0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid.

Table 4

Toxicity index and tolerance ratio of the third instar larvae and adults of *D. melanogaster* to octopamine receptor agonists after 24-h of exposure on basis of LC_{50} values.

Compounds	Toxicity index[a]		Tolerance ratio[b]
	Larvae	Adults	
Chlordimeform	100	100	2.64
Amitraz	63.03	53.25	3.13

[a] Toxicity index = [(LC_{50} value of the most toxic pesticide divided by the LC_{50} value of tested pesticide) × 100] for each stage.

[b] Tolerance ratio = LC_{50} value for adults divided by the LC_{50} value for third instar larvae after 24-h exposure.