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Chlorpyrifos-induced dopaminergic damage in *Drosophila melanogaster* assessed by gene expression, AChE assay, and negative geotaxis using a new feeding device

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ABSTRACT. We developed a new and simple feeding device for *Drosophila melanogaster*. In addition, we tested three negative geotaxis methods (measuring the percentage of the flies able to climb 8 cm in 8 s, measuring the distance climbed in 3 s, and measuring the distance climbed in 8 s). The flies were exposed to chlorpyrifos (CPF) using the new feeding device. Our results demonstrated that the three methods for measuring negative geotaxis could be used interchangeably with respect to the needs and conditions of the experiments; however, we recommend the 8 s method with PAST software because the other two methods were carried out using manual measurements. The use of this free software makes the process more accurate with no additional cost. We found that CPF caused impairment in locomotor activity, reduction in AChE activity, and disturbance of the dopaminergic pathways in *D. melanogaster*, suggesting that CPF toxicity is not confined to the cholinergic system. This study provides a new system to study neurodegenerative damage using a user-friendly and no-cost software for measuring climbing activity in *D. melanogaster*.

Key words: *Drosophila melanogaster*; Climbing assay; Negative geotaxis; PAST software; Chlorpyrifos; Feeding device

INTRODUCTION

The fruit fly *Drosophila melanogaster* is the closest invertebrate model organism to humans due to gene sequence similarity and conservation, as about 75% of established human disease genes are conserved in this fly (Pandey and Nichols, 2011). The fact that its brain is very simplified in comparison to the mammalian brain, while still maintaining considerable complexity with retained neurotransmitter systems make it a valuable and extensively used model for toxicological and environmental stress studies (Rand, 2010). In *D. melanogaster*, dopamine (DA) signaling modulates several vital behaviors similar to mammalian systems including locomotion, and drug response (Nichols, 2006; Cassar et al., 2015; Karam et al., 2020). A reduction in dopaminergic neurotransmission is known to interfere with behavior and lead to a reduction in locomotor activity (Sudati et al., 2013; Hanna et al., 2015). Like mammals, *D. melanogaster* can synthesize DA from tyrosine via two enzymatic steps. The first rate-limiting step is the conversion of tyrosine into l-3,4-dihydroxyphenylalanine (l-DOPA) by tyrosine hydroxylase (TH), encoded by the pale (ple) gene in *D. melanogaster*, followed by the second step where l-DOPA is converted to DA by decarboxylase enzyme, encoded by the DOPA decarboxylase gene (ddc) (Cichewicz et al., 2017; Karam et al., 2020). The synthesized DA binds with its receptors to exert a pharmacological and signal transduction activity and these receptors are encoded by *dop1r1*, *dop2r*, and *dopecr*. In *Drosophila's* brains, DA transporter (dat) terminates DA neurotransmission by rapid reuptake of DA back into the presynaptic terminal, where it is consequently degraded by DA N-acetyltransferase (aanat1) (Figueira et al., 2017).

Negative geotaxis (the tendency to climb against gravity) has been studied for more than a century as an intrinsic part of *D. melanogaster* locomotor behavior. It measures how rapidly a fly, as part of its innate escape response, is able to climb upwards after being tapped to the bottom of a vessel or a vial. Researchers have been using this escape reflex widely, taking advantage of this inherent trait of *D. melanogaster* when startled (Linderman et al., 2012; Taylor and Tuxworth, 2019). It is known that negative geotactic behavior declines in response to various factors, including genetic abnormalities, age, and environmental toxins (Ajjuri et al., 2014). Several studies have considered measuring negative geotaxis as one of the parameters to study locomotor behavior. In *D. melanogaster*, neuroprotective agents have been shown to counteract the decline in climbing activities after being exposed to neurotoxic agents (Khatri and Juvekar, 2016; Soares et al., 2017; Chaudhary and Dhande, 2018; Casu et al., 2020). Various approaches have been used by different laboratories for the measurement of negative geotaxis. A standard climbing test is performed by first putting a small population (e.g., 10) of age-matched flies into a graduated cylinder (or a plastic or glass tube, protected by a piece of parafilm or two vials taped facing each other to avoid fly escape). Flies are gently tapped down to the bottom of the cylinder, followed by measuring the climbing parameters such as the number of flies passing a line after a specified time interval or counting the flies that reached the top of the test apparatus (e.g.,14 cm) within 7 s (Soares et al., 2017), or as percent flies escaped beyond a minimum distance in a set time interval. Some studies, calculated the percent of flies escaped a distance of 6 cm in 60 s (Hosamani, 2009), 10 cm in 60 s (Chaudhary and Dhande, 2018), 10 cm in 20 s (Rao et al., 2016), 2 cm after 10 s (Barone et al., 2011), 5 cm in 20 s (Maitra et al., 2021), or by calculating the performance index (PI) defined as 1/2[(total number of flies (ntot) + numbers of flies at the top (ntop) - at the bottom (not))/ntot], where after 1

min, flies reached the top of the column and flies that remained at the bottom were counted separately (Coulom and Birman, 2004). These climbing tests are relatively easy to perform but have some limitations. First, it is hard to record the accurate time of fast climbers (video recording to be started before tapping the vials). Second, different experimenters will have considerably variable results, so the same person should perform the whole experiment while trying to keep a constant tapping force to avoid variabilities (Willenbrink et al., 2016). Third, it is tedious and unsuitable for high-throughput studies. Multiple protocols have been developed for analyzing *Drosophila's* climbing behavior to improve this assay such as the Rapid Iterative Negative Geotaxis (RING) assay, which allows high-throughput analysis over numerous flies at the same time (Ali et al., 2011; Gargano et al., 2005). Most of the previously mentioned methods measure the climbing behavior by scoring for a minimum distance climbed in fixed time duration (Aggarwal et al., 2019). In negative geotaxis assay, mutations in genes can lead to severe motor malfunctions in flies. Additionally, flies respond to the tapping by climbing rapidly at first, then slowing down the velocity. Similarly, arbitrary 'finish line' (e.g., 8 cm) and duration (e.g., 10 s), are expected to influence the velocity and percentage performance parameters. In such cases, computational tools using software to calculate a single datum (fly) from each vial as the average score of the flies inside the vial after each trial and thus the distribution of flies along with the vial height after tapping instead of climbing velocity or individual fly performance might provide more sensitive, valuable, and absolute measurements of flies' performance.

Chlorpyrifos (CPF), is a widely used organophosphate insecticide to control agriculture and domestic pests. The main target for CPF is the acetylcholinesterase enzyme (AChE). The inhibition of AChE results in the accumulation of acetylcholine in the synaptic cleft, causing hyperexcitation at the central nervous system and disturbance of normal physiological functioning. Further mechanism of CPF toxicity was reported to be due to the generation of reactive oxygen species (ROS) leading to an oxidative stress condition (Gupta et al., 2010; Rodrigues et al., 2019; Gomes et al., 2020). Chlorpyrifos has been shown to alter motor activity and to cause DA neurons damage in the midbrain substantia nigra (Zhang et al., 2011). Although CPF has been shown to induce significant locomotor deficits in *D. melanogaster*, nevertheless, this defect was linked to the AChE inhibition activity and oxidative stress that can be caused during organophosphates poisoning (Rodrigues et al., 2019; Gomes et al., 2020).

Here we present a new system to study neurodegenerative damage in *D. melanogaster* using a simple feeding device and an accurate method using free software for quantifying the climbing behavior to test the effects of CPF.

MATERIAL AND METHODS

Chemicals

Chlorpyrifos 95%, Acetylthiocholine iodide, 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) obtained from Sigma Aldrich (St. Louis, MO, USA), and dimethyl sulfoxide (DMSO; 99.9% Heiltropfen, Germany) were used in this study.

Fly stock, diet, and rearing

Drosophila melanogaster, wild-type flies (Oregon R strain) were obtained from a colony maintained in the Entomology Laboratory at the Biology Department, United Arab Emirates University, which was originally procured from the United States of America (Carolina Biological Supply, Burlington, NC). The flies were maintained and reared on instant *Drosophila* medium Formula 4-24® (Carolina Biological Supply, Burlington, NC). All experiments were carried out in a climatic chamber (LPGC-A10, Labtron, UK) under controlled temperature (23±1°C) and 12:12 h light/dark cycle.

Feeding device

The feeding device (Figure 1) was assembled from a plastic Fisherbrand™ *Drosophila* vial (FisherScientific, USA) and a cotton swab Sky Organics™, which was inserted into a hole made in a foam plug Fisherbrand™ *Drosophila* BuzzPlugs™ (FisherScientific, USA). The diameter of the hole was 2 mm, which was made using a thin metal nail; the hole was small enough to allow the swab to fit snugly.

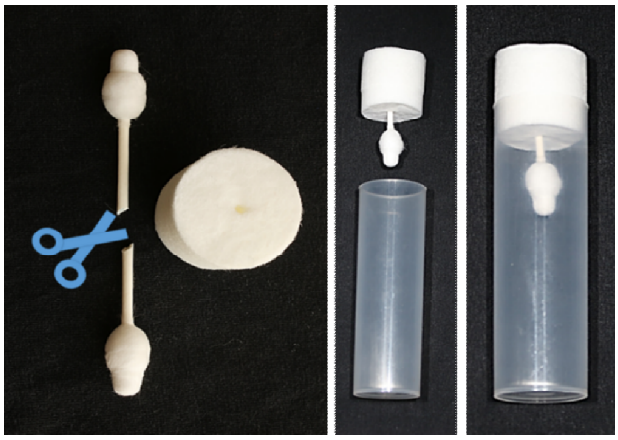

Figure 1. Cotton swab feeding device for *Drosophila melanogaster* adults. Left: one cotton swab was cut into two halves and a hole was made in a foam plug; Middle: one half of the cotton swab was inserted in a hole made in a foam plug; Right: the assembled feeding device.

Exposure to CPF for 24 h

Each experiment included three feeding devices for each treatment (CPF and control). The two treatments were 10% sucrose solution (control) containing DMSO (vehicle) and 10% sucrose solution containing 2 µM CPF. The CPF stock solution was prepared by dissolving CPF in DMSO and it was stored frozen at −20°C until usage. Deionized distilled water was used to prepare the 10% sucrose solution, which was used as a control after adding a DMSO amount equal to the one present in the CPF treatment. The DMSO concentration in the control (sucrose solution) did not cause any significant mortality or any disturbance in the locomotor activity compared to the sucrose alone (data not shown).

The CPF concentration used in this study was selected based on a serial dilution bioassay study (data not shown). The median lethal concentration (LC50) values for different CPF concentrations were calculated using the AAT Bioquest® online calculator (https://www.aatbio.com/tools/lc50-calculator). Each cotton bud of the swab was moistened with 700 µL of 10% sucrose solution of the appropriate treatment. Age synchronized adult (4-6 days old) male flies (n = 40) were segregated under brief cold anesthesia and transferred into each feeding device. All treatments were done in triplicates and each experiment was repeated three times using new vials to avoid contamination. The mortality in the control was zero and thus we did not need to correct the mortality of the treatment groups.

Assessing fly survival

After introducing the flies into the feeding devices, the mortality was recorded after 24 h and expressed as the percentage of surviving flies compared to the control. Flies, incapable of coordinated movement, after gentle touching with a thin paintbrush, were considered dead. Surviving flies were then subjected to negative geotaxis, molecular, and biochemical assays.

Negative geotaxis assay

Flies were collected using a manual aspirator and transferred to a climbing apparatus after which the two plastic Fisherbrand™ *Drosophila* vials (FisherScientific, USA) (length, 9.5 cm; diameter, 1.5 cm) were vertically joined by Scotch® transparent tape (3M Stationary Products Division, MN, USA) facing each other and the openings of the vials were perfectly aligned. The flies were allowed to acclimatize for 30 min while the tubes were kept vertically. The climbed distance was calculated by three different methods (Figure 2). Each negative geotaxis experiment was repeated five times (technical replicates) at 1 min intervals using the same insects. The experiment was repeated three times using three different groups of insects (biological replicates).

In the first calculation method, flies were gently tapped down to the bottom of the vial and the number of flies that can climb beyond the 8-cm line, which was marked above the bottom of the lower vial within 8 s (Figure 2A) was captured using a digital camera [Sony Cyber-shot DSC-TX30 18.2 mega pixels camera (Tokyo, Japan)]. Six tubes were recorded at one time under normal white fluorescent light. The number of flies that passed the 8-cm mark was recorded as a percentage of the total flies (Feany and Bender, 2000; Chaudhuri et al., 2007; Ali et al., 2011). The second calculation method was similar to the RING assay (Gargano et al. 2005). The average height climbed by individual flies during 3 s after induction of negative geotaxis was captured using a digital camera (Figure 2B). In the third method, flies were gently tapped down to the bottom of the vial, and they were allowed to ascend the walls of the vials for 8 s (Figure 2C). The distance climbed by each fly was captured using a digital camera. In the second and third methods, we used the PAST Software (https://www.nhm.uio.no/english/research/resources/past/index.html) to accurately and uniformly calculate the distance climbed by each fly in the images captured by the digital camera. Briefly, we stopped the video at the set time interval for each test and we captured the screen. Then the image was opened in the PAST software and the climbed

distances were measured by selecting the "Geometry" tab and then selecting the "Measure on image" option from the dropdown menu. We placed the cursor on each fly in the image and clicked once, and after finishing we copied the Y values from the software and used them in the negative geotaxis analyses. The setup presented in the current study allowed the use of the same flies in the three negative geotaxis calculation methods, which eliminated any variability that might result from using three different groups of flies or conducting three different tests using the same flies. We achieved this by recording the climbing behavior of the flies for 20 s and later played the recordings and stopped the video at the required interval for each method and measured the distance climbed by each fly.

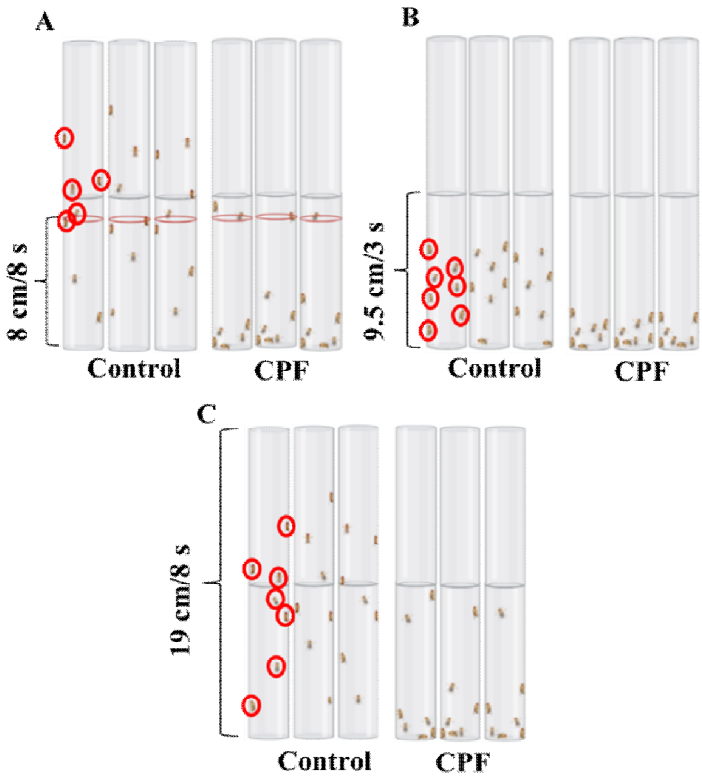

Figure 2. Schematic representation of the three negative geotaxis assays of *Drosophila melanogaster* adult male flies. (A) Measuring % of flies climbed 8 cm in 8 s. (B) Measuring average distance climbed by the flies in 3 s. (C) Measuring average distance climbed by the flies in 8 s.

RT-qPCR

RNA was extracted from the flies of the control and the treated groups using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was measured by Quantus™ Fluorometer (Promega, Madison, USA). Real-Time Polymerase Chain Reaction was performed in triplicates and each reaction contained 50 ng of total RNA using Luna® Universal One-Step RT-qPCR Kit (New England Biolabs Inc., Ipswich, USA) in the

QuantStudio 5 Real-Time PCR system (Applied Biosystems). All kits were used as per the manufacturer's instructions. Relative transcript level was determined by the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). The following genes from the dopaminergic neurotransmission system were analyzed after exposing the flies to CPF: tyrosine hydroxylase (ple), DOPA decarboxylase (ddc), DA transporter (dat), D1-like receptor 1 (dop1r1), D2-like receptor (dop2r), DA/ecdysteroid receptor (dopecr), and DA N-acetyltransferase (aanat1). The glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene was used as the reference gene to normalize the expression levels (Table 1).

Table 1. Sequences of RT-qPCR Primers of the dopaminergic neurotransmission system.

No.	Primer	Sequence	Reference
1	ple	For 5'-AACACCGGATTCTCTCTCCG-3' Rev 5'-CTCGTGAATGGAGTCGGGCT-3'	Present study
2	ddc	For 5'-ACACAAATGGATGCTGGTGA-3' Rev 5'-AGAGGGTCCACATTGAACG-3'	Norry et al. 2009
3	dat	For 5'-GGTGCCCCTCTTCAAAGGAAT-3' Rev 5'-ATTACACGACGTCCAAGGCA-3'	Figueira et al., 2017
4	dop1r1	For 5'-ACGATGGCACAACGTTGACA-3' Rev 5'-GCACCGATAGGAAGATGCCA-3'	Figueira et al., 2017
5	dop2r	For 5'-CACAAGGCCTCGAAAAAGAA-3' Rev 5'-GCGAAACTCGGGATTGAATA-3'	Inagaki et al. 2012
6	dopecr	For 5'-AGGGTCCTGTGTGTACTGGT-3' Rev 5'-GCAAGAATTGTTGGCTTTTCCG-3'	Figueira et al., 2017
7	aanat1	For 5'-AACGAATCGGGCGAAAGTCT-3' Rev 5'-CGTTCAGGCGTGAAATTGGC-3'	Figueira et al., 2017
8	gapdh	For 5'-GCTCCTCAATGGTTTTTCCA-3' Rev 5'-ATGGAGATGATTCGCTTCGT-3'	Figueira et al., 2017

Measuring AChE activity

Acetylcholinesterase activity was evaluated using the method of Ellman et al. (1961). Samples from the treatment and control groups were homogenized as 1:100 [flies(mg)/ volume µl PBS (pH 7.4)+ protease inhibitor cocktail] and then centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was then used to determine AChE activity. The reaction mixture contained 80 µL of PBS (pH 7.4), 50 µL of 0.32 mM DTNB, 20 µL of the sample, and 50 µL of 10 mM acetylthiocholine. The change in absorbance was monitored at a wavelength of 405 nm (for 10 min) in a Platos-R-496-AMP AMEDA microplate reader (Labordiagnostik GmbH, Graz, Austria). To calculate the change in the activity of cholinesterase, the following formulas (Anjum et al., 2010) were applied:

$$\%\text{inhibition} = (\text{Control} - \text{Treatment}/\text{Control}) * 100 \quad \text{(Eq. 1)}$$

$$\text{Remaining activity} = 100 - \%\text{inhibition} \quad \text{(Eq. 2)}$$

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, CA, United States). A two-tailed Student's t-test was used to identify differences between the control group and the treatment groups. Gene expression data were analyzed using two-way ANOVA followed by Bonferroni post hoc test. Differences with $P < 0.05$ were considered statistically significant. The data were presented as mean ± standard error of the mean (SEM).

RESULTS

Effect of CPF on the survival of *D. melanogaster*

Exposure of adult male flies to 2 µM of CPF for 24 h caused a significant decrease in the percentage of surviving flies (64.2%) compared to the control (100 %) ($P = 0.0037$) (Figure 3).

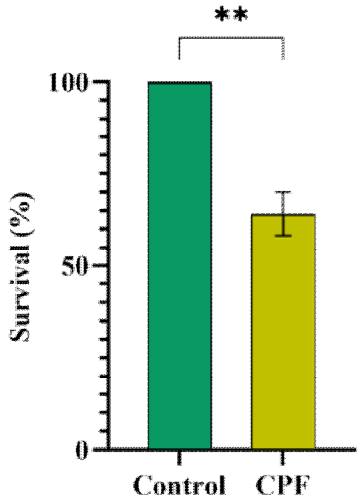

Figure 3. Effect of 2 µM CPF exposure on survival of *Drosophila melanogaster* adult male flies after 24 h. Bars represent the mean ± SEM of the average survival percentage of flies. Data were analyzed by a two-tailed Student's *t*-test. **P ≤ 0.005 indicates significant differences. CPF = Chlorpyrifos 2 µM, Control = DMSO 2 µM.

Locomotor performance of *D. melanogaster* exposed to CPF

We assessed the locomotor performance of adult male flies exposed to CPF by quantifying the climbing ability using three different negative geotaxis assays (measuring the percentage of flies able to climb 8 cm in 8 s, measuring the distance climbed in 3 s, and measuring the distance climbed in 8 s). The three methods revealed that the exposure to 2 µM CPF for 24 h caused significant severe locomotor impairment (decrease in climbing ability) ($P = 0.0015$, $P = 0.0027$, $P = 0.003$, respectively) (Figure 4). Among the untreated flies (control groups), 86.65% of the flies were able to pass the 8 cm within 8 s compared to 39.06% in the CPF-treated flies (Figure 4A). The average distance climbed by the same untreated flies was 30.70% and 65.41% of the vials in 3 and 8 s, respectively compared to 15.75% and 32.44% in CPF-treated flies during 3 and 8 s, respectively (Figure 4B and Figure 4C).

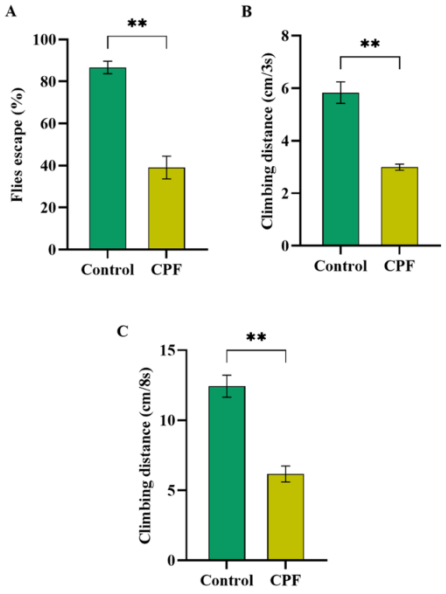

Figure 4. Effect of 2 µM CPF exposure for 24 h on climbing behavior of *Drosophila melanogaster* adult male flies determined by three negative geotaxis methods. (A) Measuring % of flies climbed 8 cm in 8 s. (B) Measuring average distance climbed by the flies in 3 s. (C) Measuring average distance climbed by the flies in 8 s. CPF = Chlorpyrifos 2 µM, Control = DMSO 2 µM.

Effect of CPF on the AChE activity

The AChE, which is the hallmark for organophosphate poisoning, was measured. The enzyme activity was significantly inhibited (P = 0.0017) in the flies exposed to CPF and these flies exhibited a 25.67% decrease in AChE compared to the control (Figure 5).

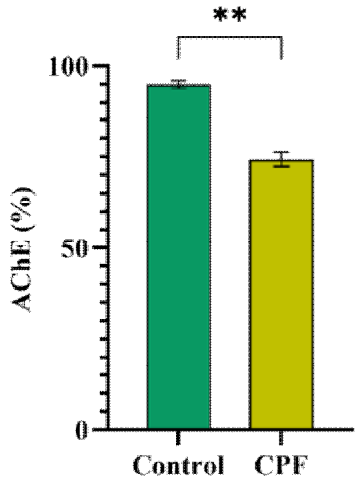

Figure 5. Acetylcholinesterase activity in *Drosophila melanogaster* adult male flies exposed to 2 µM for 24 h. Data were analyzed by a two-tailed Student's *t*-test. Results are expressed as a percentage of the control (mean ± SEM); **P ≤ 0.005 indicates significant differences compared to the CPF group. CPF = chlorpyrifos 2 µM, Control = DMSO 2 µM.

Effects of CPF on gene expression profile of dopaminergic system

We quantified the mRNA of seven genes responsible for DA biosynthesis, transportation, metabolism, and reception in total-RNA extracts. Flies exposed to 2 µM CPF for 24 h showed no statistically significant differences between the control and the treatment groups among all tested genes. However, flies exposed to CPF showed a decrease in the *ple* mRNA gene expression by 26.1% when compared to the control. An elevation in *ddc*, *dat*, *dop1r1*, *dop2r*, *dopecr*, and *aanat1* expression in flies exposed to CPF by 13.0, 38.31, 22.79, 27.77, 25.16, and 60.86%, respectively, was observed when compared with the control (Figure 6), and (Figure 7).

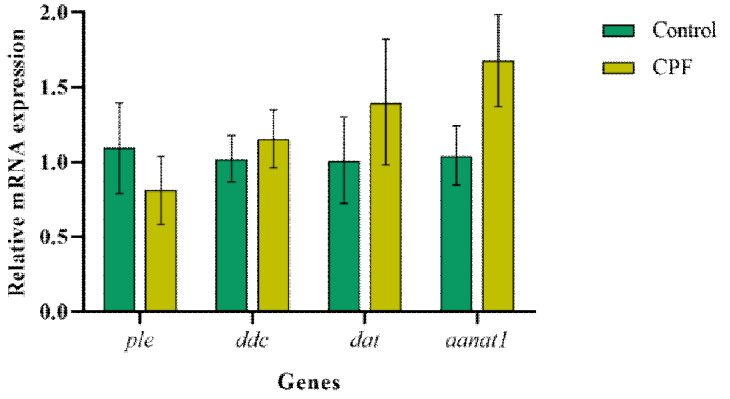

Figure 6. RT-qPCR gene expression of *ple*, *ddc*, *dat*, and *aanat1* in *Drosophila melanogaster* adult male flies exposed to 2 µM CPF. Results are expressed as mean fold change ± SEM relative to control flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. P ≥ 0.05 indicates no significant differences between control and CPF-treated flies. CPF = Chlorpyrifos 2 µM, Control = DMSO 2 µM.

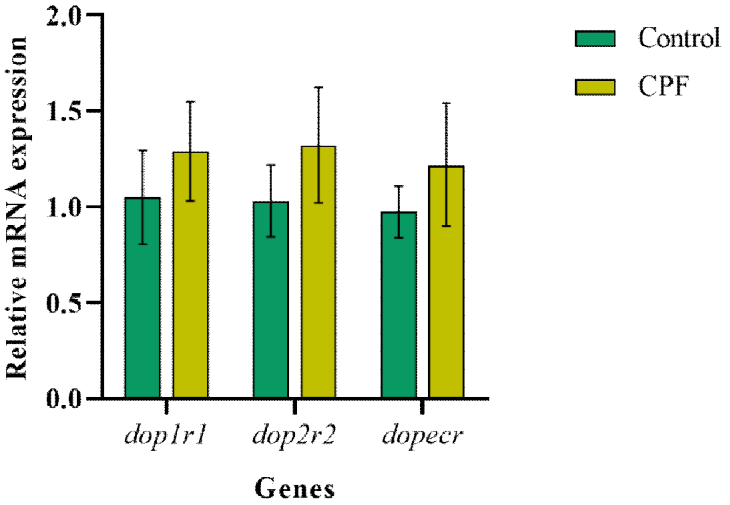

Figure 7. RT-qPCR gene expression of *dop1r1*, *dop2r*, and *dopecr* in *Drosophila melanogaster* adult male flies exposed to 2 µM CPF. Results are expressed as mean fold change ± SEM relative to control flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. P ≥ 0.05 indicates no significant differences between control and CPF-treated flies. CPF = Chlorpyrifos 2 µM, Control = DMSO 2 µM.

DISCUSSION

A growing number of studies have shown that exposure to CPF causes harmful effects on human and animal health. In the present study, exposure of *D. melanogaster* adult males to 2 μM CPF for 24 h was able to cause mortality, and the surviving flies exhibited severe locomotor deficits, with a concomitant decrease in AChE activity. Our findings regarding the effect of CPF on the survival and negative geotaxis of *D. melanogaster* are consistent with the findings of Rodrigues et al. (2019) and Gomes et al. (2020). The locomotor damage caused by CPF has been reported in several species, including aquatic organisms and rodents (Kavitha and Rao, 2008; Tilton et al., 2011; Zhang et al., 2011).

In many studies, the most common negative geotaxis method is a relative measurement by calculating the number of flies passing a line placed at a certain height (predefined distance) in the test vial. Although, this method is less time-consuming, it can be less accurate, especially when the treatment causes severe impairment in the locomotor activity. In such a case, biased results could be obtained as we might have no or very few flies that will be able to reach or pass the predefined distance. On the contrary, calculating the average distance covered by all flies individually in the test vial is an absolute measurement, which measures the average distance climbed by all flies and not just the ones that pass the predefined distance. Such a method is more accurate and more sensitive, especially when dealing with treatments that cause severe locomotor damage. Therefore, we suggest using the method that measures the distance climbed by the flies during 8 s using the PAST software when testing the negative geotactic behavior of *D. melanogaster*. Although other methods may use similar time or distance, but instead they measure the distance manually using a ruler inside or outside the assay vials (Barone and Bohmann, 2013), or using a graduated cylinder (Nichols et al., 2012; Madabattula et al., 2015). Such methods are not as accurate as using an image processing software because they are prone to human errors. In addition, some methods use image processing software, however, the software is either not free or not easy. For example, the software FreeClimber requires installing an Anaconda-based virtual environment as well as Python 3 virtual environment before using the software (Spierer et al., 2021). Overall, in the current study, we used free and user-friendly software to measure the climbed distance of individual flies after 8 s. The main advantages of this method are: (1) it is an absolute calculation because it takes into consideration all the flies and not just the ones that pass the predefined distance, (2) the measurement of the climbed distance is done precisely based on the number of pixels in the image and not visually by a ruler, (3) it uses a free software (PAST 4), which can be downloaded and used immediately, and (4) the new method requires fewer replications because it takes the distance data from all flies in the experiment (entire fly population).

To further understand other possible mechanisms of the locomotor impairment induced by CPF, we investigated its effect on the dopaminergic pathways. Here we show that exposure of *D. melanogaster* adult males to 2 μM CPF for 24 h was able to alter dopaminergic gene expression. Chlorpyrifos previously induced significant locomotor deficits in *D. melanogaster*; however, this defect was linked to the CPF main mode of action as an AChE inhibitor, in addition to the oxidative stress that can be caused during organophosphates poisoning (Rodrigues et al., 2019; Gomes et al., 2020). The effect of CPF on the TH expression in *D. melanogaster* has never been studied, however, it was reported that CPF caused a reduction in TH expression in rats which was accompanied by locomotion impairment (Zhang et al., 2011; Sheikh and Sheikh, 2020). In the current study, while CPF exhibited no statistically significant differences between the control and the treatment groups among all tested dopaminergic genes, nevertheless, we observed interesting trends in *ple* mRNA levels, which is the rate-limiting step

in the dopamine synthesis pathway in *D. melanogaster*. In addition, we observed a slight elevation in *ddc*, *dat*, *dop1r1*, *dop2r*, *dopecr*, and *aanat1* expression, indicating that the toxicity of CPF was not confined only to the cholinergic system, but also it exerts dopaminergic system toxicity. It is likely that there was no statistically significant difference due to the short duration of the exposure to the insecticide and apparently the 24 h were not long enough to cause severe disturbance in the dopaminergic system. Therefore, more research is required to test the effect of CPF long-term exposure on dopaminergic genes in *D. melanogaster* and to know how this can be linked to the behavioral deficits.

CONCLUSIONS

Overall, one of the main findings of this study is the introduction of a new and simple feeding device for *D. melanogaster*, which can be assembled and used in any laboratory. This device enables the dispensing of toxic molecules to flies while feeding on a sucrose solution. There is no need to anesthetize the flies to change the food or the treatment because the soaked cotton bud is attached to the foam plug, which can be readily replaced with a fresh one after tapping down the flies gently. In addition, the findings of the present study showed that the three tested negative geotaxis assays were suitable for detecting changes in climbing behavior of the *D. melanogaster* adults. However, we recommend the 8 s method with the PAST software. Furthermore, this study provided a CPF concentration, which causes less than 50% mortality after 24 h of exposure. Together, the new cotton swab feeding device, the 8 s method using the PAST software, and the 2 µM CPF concentration provide a complete, simple, and fast system to study neurodegenerative damage in *D. melanogaster*.

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AUTHORS CONTRIBUTIONS

H.H. Abdulbaki performed the experiments, analyzed the data, and wrote the paper. M.A. Al-Deeb conceived and designed the study, analyzed the data, and helped write the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aggarwal A, Reichert H and VijayRaghavan K (2019). A locomotor assay reveals deficits in heterozygous Parkinson's disease model and proprioceptive mutants in adult *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 116: 24830-24839.

Ajjuri RR, Hall M, Reiter LT and O'Donnell JM (2014). Drosophila as a model for human movement disorders. In: Movement Disorders (Second Edition): Genetics and Models, Mark S. LeDoux ed. Academic Press.

Ali YO, Escala W, Ruan K and Zhai RG (2011). Assaying locomotor, learning, and memory deficits in *Drosophila* models of neurodegeneration. *J. Vis. Exp.* 49: e2504.

Anjum SI, Yousf MJ, Ayaz S and Siddiqui BS (2010). Toxicological evaluation of chlorpyrifos and Neem extract (Biosal B) against 3RD instars larvae of *Drosophila melanogaster*. *J. Anim. Plant Sci.* 20: 9-12.

Barone MC and Bohmann D (2013). Assessing neurodegenerative phenotypes in *Drosophila* dopaminergic neurons by climbing assays and whole brain immunostaining. *J. Vis. Exp.* 74: e50339.

Barone MC, Sykiotis GP and Bohmann D (2011). Genetic activation of Nrf2 signaling is sufficient to ameliorate neurodegenerative phenotypes in a *Drosophila* model of Parkinson's disease. *Dis. Model. Mech.* 4: 701-707.

Chaudhuri A, Bowling K, Funderburk C, Lawal H, et al. (2007). Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. *J. Neurosci.* 27: 2457-2467.

Chaudhary P and Dhande S (2018). Evaluation of Neuroprotective effect of medicinal plants in *Drosophila melanogaster* model. *Indian J. Pharm. Biol. Res.* 6: 9-12.

Casu MA, Mocci I, Isola R, Pisanu A, et al. (2020). Neuroprotection by the immunomodulatory drug pomalidomide in the *Drosophila* LRRK2WD40 genetic model of Parkinson's disease. *Front. Aging Neurosci.* 12: 31.

Cassar M, Issa AR, Riemensperger T, Petitgas C, et al. (2015). A dopamine receptor contributes to paraquat-induced neurotoxicity in *Drosophila*. *Hum. Mol. Genet.* 24: 197-212.

Cichewicz K, Garren EJ, Adiele C, Aso Y, et al. (2017). A new brain dopamine-deficient *Drosophila* and its pharmacological and genetic rescue. *Genes. Brain. Behav.* 16: 394-403.

Coulom H and Birman S (2004). Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. *J. Neurosci.* 24: 10993-10998.

Feany MB and Bender WW (2000). A *Drosophila* model of Parkinson's disease. *Nature*. 404: 394-398.

Figueira FH, de Quadros Oliveira N, de Aguiar LM, Escarrone AL, et al. (2017). Exposure to atrazine alters behaviour and disrupts the dopaminergic system in *Drosophila melanogaster*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 202: 94-102.

Gargano JW, Martin I, Bhandari P, and Grotewiel MS (2005). Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*. *Exp. Gerontol.* 40: 386-395.

Gomes KK, Macedo GE, Rodrigues NR, Ziech CC, et al. (2020). Croton campestris A. St.-Hill Methanolic Fraction in a Chlorpyrifos-Induced Toxicity Model in *Drosophila melanogaster*: Protective Role of Gallic Acid. *Oxid. Med. Cell. Longev.* 2020: 3960170.

Gupta SC, Mishra M, Sharma A, Balaji TD, et al. (2010). Chlorpyrifos induces apoptosis and DNA damage in Drosophila through generation of reactive oxygen species. *Ecotoxicol. Environ. Saf.* 73: 1415-1423.

Hanna ME, Bednářová A, Rakshit K, Chaudhuri A, et al. (2015). Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*. *J. Insect Physiol.* 73: 11-19.

Hosamani R (2009). Neuroprotective efficacy of *Bacopa monnieri* against rotenone induced oxidative stress and neurotoxicity in *Drosophila melanogaster*. *Neurotoxicology.* 30: 977-985.

Inagaki HK, de-Leon SBT, Wong AM, Jagadish S, et al. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell*. 148: 583-595.

Karam CS, Jones SK and Javitch JA (2020). Come Fly with Me: An overview of dopamine receptors in *Drosophila melanogaster*. *Basic. Clin. Pharmacol. Toxicol.* 126: 56-65.

Kavitha P and Rao JV (2008). Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, Gambusia affinis. *Environ. Toxicol. Pharmacol.* 26: 192-198.

Khatri DK and Juvekar AR (2016). Abrogation of locomotor impairment in a rotenone-induced *Drosophila melanogaster* and zebrafish model of Parkinson's disease by ellagic acid and curcumin. *Int. J. Nutr. Pharmacol. Neurol. Dis.* 6: 90.

Linderman JA, Chambers MC, Gupta AS and Schneider DS (2012). Infection-related declines in chill coma recovery and negative geotaxis in *Drosophila melanogaster*. *PLoS One*. 7: e41907.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*. 25: 402-408.

Madabattula ST, Strautman JC, Bysice AM, O'Sullivan JA, et al. (2015). Quantitative analysis of climbing defects in a *Drosophila* model of neurodegenerative disorders. *J. Vis. Exp.* 100: e52741.

Maitra U, Harding T, Liang Q and Ciesla L (2021). GardeninA confers neuroprotection against environmental toxin in a *Drosophila* model of Parkinson's disease. *Commun. Biol.* 4: 1-13.

Nichols CD (2006). *Drosophila melanogaster* neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. *Pharmacol. Ther.* 112: 677-700.

Nichols CD, Becnel J and Pandey UB (2012). Methods to assay *Drosophila* behavior. *J. Vis. Exp.* 61: e3795.

Norry FM, Larsen PF, Liu Y and Loeschcke V (2009). Combined expression patterns of QTL-linked candidate genes best predict thermotolerance in *Drosophila melanogaster*. *J. Insect Physiol.* 55: 1050-1057.

Pandey UB and Nichols CD (2011). Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacol. Rev.* 63: 411-436.

Rand MD (2010). Drosophotoxicology: the growing potential for *Drosophila* in neurotoxicology. *Neurotoxicol. Teratol.* 32: 74-83.

Rao SV, Yenisetti SC and Rajini PS (2016). Evidence of neuroprotective effects of saffron and crocin in a *Drosophila* model of parkinsonism. *Neurotoxicology*. 52: 230-242.

Rodrigues NR, dos Santos Batista JE, de Souza LR, Martins IK, et al. (2019). Activation of p38MAPK and NRF2 signaling pathways in the toxicity induced by chlorpyrifos in *Drosophila melanogaster*: protective effects of *Psidium guajava pomífera* L. (Myrtaceae) hydroalcoholic extract. *Arab. J. Chem.* 12: 3490-3502.

Sheikh A and Sheikh K (2020). The expression change of glial fibrillary acidic protein and tyrosine hydroxylase in substantia nigra of the Wistar rats exposed to chlorpyrifos: a novel environmental risk factor for Parkinson's disease. *Exp. Brain Res.* 238: 2041-2051.

Soares JJ, Rodrigues DT, Gonçalves MB, Lemos MC, et al. (2017). Paraquat exposure-induced Parkinson's disease-like symptoms and oxidative stress in *Drosophila melanogaster*: Neuroprotective effect of *Bougainvillea glabra* Choisy. *Biomed. Pharmacother.* 95: 245-251.

Sudati JH, Vieira FA, Pavin SS, Dias GRM, et al. (2013). Valeriana officinalis attenuates the rotenone-induced toxicity in *Drosophila melanogaster*. *Neurotoxicology*. 37: 118-126.

Spierer AN, Yoon D, Zhu CT and Rand DM (2021). FreeClimber: automated quantification of climbing performance in *Drosophila*. *J. Exp. Biol.* 224: jeb229377.

Taylor MJ and Tuxworth RI (2019). Continuous tracking of startled *Drosophila* as an alternative to the negative geotaxis climbing assay. *J. Neurogenet.* 33: 190-198.

Tilton FA, Bammler TK and Gallagher EP (2011). Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. Comp. *Biochem. Physiol. C. Toxicol. Pharmacol.* 153: 9-16.

Willenbrink AM, Gronauer MK, Toebben LF, Kick DR, et al. (2016). The Hillary Climber trumps manual testing: an automatic system for studying *Drosophila* climbing. *J. Neurogenet.* 30: 205-211.

Zhang J, Zhao LL, Hu ZP, Zhou J, et al. (2011). Effects of low-dose chlorpyrifos exposure on dopaminergic neurons in the midbrain substantia nigra and neural behavioral development in neonatal rats. *Zhongguo Dang Dai Er Ke Za Zhi.* 13: 989-994.

