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Residues of chlorpyrifos in the environment induce resistance in *Aedes albopictus* by affecting its olfactory system and neurotoxicity

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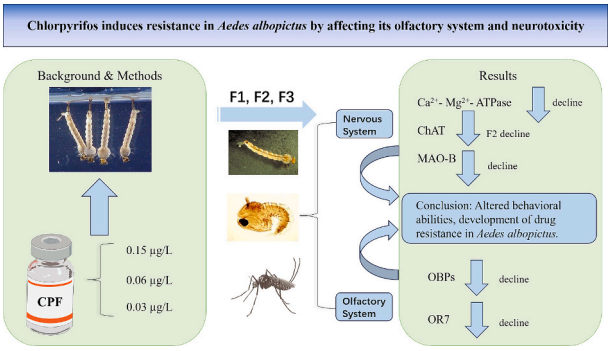
HIGHLIGHTS

- Chlorpyrifos affects the behavioral abilities of mosquitoes.
- Chlorpyrifos affects Ca^{2+}- Mg^{2+}-ATPase, ChAT activity, MAO-B expression in mosquitoes.
- Chlorpyrifos affects mosquito OBPs and OR7 expression.
- Mosquitoes develop resistance to pesticides under long-term stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Aedes albopictus, a virus-vector pest, is primarily controlled through the use of insecticides. In this study, we investigated the mechanisms of resistance in *Ae. albopictus* in terms of chlorpyrifos neurotoxicity to *Ae. albopictus* and its effects on the olfactory system. We assessed Ca^{2+}-Mg^{2+}-ATP levels, choline acetyltransferase (ChAT), Monoamine oxidase (MAO), odorant-binding proteins (OBPs), and olfactory receptor (OR7) gene expression in *Ae. albopictus* using various assays including Y-shaped tube experiments and DanioVision analysis to evaluate macromotor behavior. Our findings revealed that cumulative exposure to chlorpyrifos reduced the activity of neurotoxic Ca^{2+}-Mg^{2+}-ATPase and ChAT enzymes in *Ae. albopictus* to varying degrees, suppressed MAO-B enzyme expression, altered *OBPs* and *OR7* expression patterns, as well as affected evasive response, physical mobility, and cumulative locomotor time under chlorpyrifos stress conditions for *Ae. albopictus* individuals. Consequently, these changes led to decreased feeding ability, reproductive capacity, and avoidance behavior towards natural enemies in *Ae. albopictus* populations exposed to chlorpyrifos stressors over time. To adapt to unfavorable living environments, *Ae. albopictus* may develop certain tolerance mechanisms against organophosphorus pesticides. This study provides valuable insights for guiding rational insecticide usage or dosage adjustments targeting the nervous system of *Ae. albopictus*.

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1. Introduction

In recent years, significant advancements have been made in the investigation of the direct or indirect impacts on non-target organisms caused by various pesticides extensively employed for agricultural pest control. Organophosphorus pesticides represent a class of organic ester compounds comprising over 150 phosphorus atoms. Due to their potent toxicity and broad spectrum applications, they are globally utilized in agricultural production, encompassing insecticides like chlorpyrifos and dichlorvos, as well as herbicides such as glyphosate (Huang et al., 2020; Ding et al., 2019; Zhou et al., 2022a, 2022b). Chlorpyrifos (O, O-diethyl-O-3,5–6-trichloro-2-pyridyl-phosphorothioate, CPF), being an organophosphorus pesticide with a wide range of target organisms and persistent properties, finds extensive usage in rice, corn, and wheat cultivation (Gilani et al., 2016; Raj and Kumar, 2022). The excessive use of chlorpyrifos in agricultural practices not only leads to resistance development among target organisms but also adversely affects non-target organisms thereby causing irreversible damage to the ecosystem (Kumar et al., 2023; Elshikh et al., 2022).

Studies have demonstrated that neurotoxicity is the primary form of chlorpyrifos biotoxicity (Burke et al., 2017). Chlorpyrifos can inhibit insect acetylcholinesterase (AChE) activity, leading to an accumulation of the neurotransmitter acetylcholine (ACh), which causes persistent muscle irritation and ultimately paralyzing death (Meng et al., 2020; Toumi et al., 2015; Li et al., 2022). Additionally, chlorpyrifos can cause oxidative damage such as lipid peroxidation, reducing insect fat content and energy stores. This suggests that exposure to neurotoxic insecticides may potentially impact insects' avoidance behavior towards natural enemies, ability to find shelter, feeding patterns, respiration, locomotion and other key behaviors (Brackenbury, 2001; Janssens and Stoks, 2012). In response to selective pressure from insecticide exposure, insects adapt their behavior by employing avoidance strategies to minimize or avoid exposure to insecticides (Zalucki and Furlong, 2017; Kenis et al., 2023; Pavela et al., 2023).

The neurobehavior of animals is regulated by their nervous system, which serves as a comprehensive indicator of neurological damage in an organism (Ubaid et al., 2021). Ca^{2+}-Mg^{2+}-ATPase plays a crucial role in maintaining optimal intracellular concentrations of calcium and magnesium ions, facilitating action potential conduction in nerve cells, and contributing to neurological disorders in humans (Ji et al., 2009). Reduced activity of this enzyme may lead to abnormal distribution of calcium ions inside and outside the nerve membrane, resulting in intracellular calcium overload and subsequent memory impairment (Doulgeraki et al., 2002). Choline acetyltransferase (ChAT) and vesicular ACh transporter protein (VAChT) are responsible for mediating cholinergic transmission in the nervous system (Friedman et al., 2019). In the cellular environment, ChAT utilizes choline and acetyl coenzyme A to synthesize the neurotransmitter acetylcholine (Mille et al., 2021). In insects, deficiency of ChAT or VAChT prevents the synthesis or transport of ACh, leading to insufficient levels of this essential neurotransmitter for insect survival maintenance (Liu et al., 2022). Monoamine oxidase (MAO), a mitochondrial enzyme responsible for oxidative catabolism of endogenous monoamines and exogenous substances can be categorized into MAO-A and MAO-B based on its function. Among them, MAO-B exhibits higher selectivity towards tyramine and dopamine (Carradori and Silvestri, 2015; Rehuman et al., 2020). Selective inhibitors targeting MAO-B (MAO-BIs) have shown neuroprotective effects by enhancing cognition among patients with neurodegenerative diseases through increasing monoamine neurotransmitter levels within central nervous system (CNS) while reducing reactive oxygen species (ROS) formation (Paolino et al., 2023).

The olfactory function of insects plays a pivotal role in regulating various biological behaviors, including the recognition of target hosts, mates, and oviposition sites (Renou and Anton, 2020a, 2020b; Cassau and Krieger, 2021) Odor molecules from the natural environment permeate the insect's olfactory pores and form complexes with odor-binding proteins (OBPs) (Stengl and Funk, 2013). These complexes subsequently interact with olfactory receptors (ORs), eliciting changes in action potentials (Ai et al., 2022; Wheelwright et al., 2021; Wicher and Miazzi, 2021). Olfactory sensors exhibit diverse morphologies primarily distributed in the antennae, maxillary whiskers, and rostrum. Within this intricate olfactory system framework, OBPs and ORs play crucial roles (Guidobaldi et al., 2014). Insect antennal OBPs are small spherical water-soluble acidic proteins that are widely distributed in lymphatic fluid. Their interaction with odor molecules represents the initial biochemical step in insect's perception of external odors (Shukla et al., 2023). OBPs significantly contribute to mating selection and host preference during oviposition by acting as solubilizers, transporter proteins, ligand filters while also mediating activation of ORs for insect olfaction purposes (Paula et al., 2018; Zhou et al., 2022a, 2022b). The physiological function of insect ORs encompasses two key aspects: specific recognition of structurally similar odorant molecules belonging to a particular type or class and conversion of extracellular chemical signals into electrical signals transmitted to the central nervous system. During this process, these signals undergo processing and integration ultimately leading to physiological and behavioral responses in insects (Leal, 2013). Upon entering the olfactory apparatus through the stomata, odor molecules bind to ORs located on the dendritic surface of neurons under the synergistic influence of odor-binding proteins (OBPs). This binding event initiates cellular G-protein-coupled signaling pathways, subsequently activating membrane ion channels and ultimately inducing changes in membrane potential (Wei et al., 2020; Li et al., 2020a, 2020b; Sims et al., 2022).

Ae. albopictus, commonly known as the Asian tiger mosquito, belongs to the genus *Aedes* in the family Mosquitidae and serves as a significant vector insect (Jiang, 2022). The larvae of *Ae. albopictus* inhabit various aquatic environments, including both artificial water bodies such as catch basins, broken glass bottles, and discarded motor vehicle tires (Gao et al., 2018), as well as natural habitats like tree holes and rivers (Ma et al., 2016). During the field investigation, larvae of *Ae. albopictus* were collected from rivers (Keqiao Lizhu River, Shaoxing) and streams (Kaihua Gutianshan Nature Reserve, Quzhou)(Unpublished data). These water sources are mainly rainwater, tap water and groundwater (Mutshekwa et al., 2022; Ayllón et al., 2018). Although not directly targeted by chlorpyrifos, excessive use of this pesticide for crop protection can lead to environmental contamination through leaching into soil or water streams via rainfall (Bhende et al., 2022; Hossain et al., 2015). Studies have revealed elevated levels of chlorpyrifos in seawater, rivers, groundwater, and even rainwater exceeding permissible limits in certain countries (Sumon et al., 2018). Consequently, *Ae. albopictus* larvae inevitably face prolonged exposure to residual chlorpyrifos within their environment which increases their risk of developing resistance (Wang et al., 2022).

In this study, we investigated alterations in locomotor behavior and neurobehavioral-related enzyme activities (Ca^{2+}-Mg^{2+}-ATPase, ChAT), as well as the expression levels of *MAO-B*, *OBPs*, and *OR7* genes in different generations of *Ae. albopictus* exposed to long-term chlorpyrifos stress by simulating low concentrations of chlorpyrifos residues in an aquatic environment. This study provides evidence that prolonged exposure to chlorpyrifos impacts larval locomotor behavior in *Ae. albopictus* and induces resistance through adjustments in its own biochemical indices, thereby influencing the control of *Ae. albopictus*.

2. Materials and methods

2.1. Subjects and breeding process

The sensitive strain of *Ae. albopictus* was obtained from Sun Yat-sen University (Guangdong Province, China) and reared in a fully automated ACGII artificial climate chamber (QiuShi, Zhejiang, China). The chamber maintained an ambient temperature of 26–28 °C, a relative humidity of 70 % - 85 %, and a photoperiod of 14 L:10 D. The rearing methods for

Ae. albopictus were based on the study conducted by Zhang et al. (Zhang et al., 2023).

2.2. Ae. albopictus stress treatment

This experiment simulated the chronic behavioral response of *Ae. albopictus* larvae to sublethal concentrations of chlorpyrifos solution, following the WHO harmonized larval dip method (Fang et al., 2018). In accordance with the national standard GB/T 602–2002, a concentration of 25 g/L chlorpyrifos was selected in the pre-experiment. It was dissolved in acetone and then diluted with double-distilled water to create chlorpyrifos mother solutions with concentration gradients of 75 mg/L, 30 mg/L, and 15 mg/L for configuring the working solution. The sensitive strains of *Ae. albopictus* larvae had a 24-h LC_{50} value for chlorpyrifos at 1.5 μg/L. Based on this LC_{50} value, concentrations were further diluted to achieve ratios of 1/10, 1/25, and 1/50 for long-term stress experiments on *Ae. albopictus* using chlorpyrifos solutions at concentrations of 0.15 μg/L, 0.06 μg/L, and 0.03 μg/L respectively; acetone dilution served as the control group. After hatching, larvae were placed into syringes containing three different concentrations of chlorpyrifos treatment groups or acetone control group; treatment solutions were changed daily. Upon pupation, larvae from each treatment group were transferred into mosquito cages until they emerged as adult mosquitoes; their eggs were collected for subsequent generations' stress treatments. In this experiment, third-instar larvae, pupae, and adult mosquitoes of sensitive strains (F1-F3) of *Ae. albopictus* were used as experimental materials.

2.3. Determination of Ae. albopictus Ca^{2+}-Mg^{2+}-ATPase and Choline Acetyltransferase (ChAT) enzyme activity

We collected 40 third-instar larvae, 10 pupae, and 12 female mosquitoes from each tube and recorded the weight of each tube before and after sampling to determine sample weight. The samples were diluted with saline at a ratio of mass (mg) to dilution solution (μL) of 1:19, ground in a crusher for 6 min, and then ultrasonically crushed for 30 min to obtain the primary enzyme solution. ChAT was determined using an enzyme activity test kit (Jiancheng, Nanjing, China). Please refer to the instructions provided with the kit for details on conducting the enzyme activity assay.

2.4. Determination of Ae. albopictus monoamine oxidase (MAO-B), olfactory binding proteins (OBPs) and olfactory receptor (OR7) gene expression

Total RNA was extracted from previously prepared third-instar larvae, pupae, and adult mosquitoes using the Trizol method. The quality and concentration of RNA were assessed by agarose gel electrophoresis with a microspectrophotometer (Thermo, USA). cDNA was synthesized following the instructions of the Prime ScriptTM RT Reagent Kit (Haofeng, Hangzhou, China). *Ae. albopictus MAO-B*, *OBPs*, and *OR7* gene sequences were searched in the National Center for Biotechnology Information (NCBI) database. Specific primers (Table 1) were designed using Primer premier 5.0 software and synthesized by biologicals (Shangya, Hangzhou). Finally, real-time fluorescent quantitative PCR (qRT-PCR) was used to determine MAO-B gene expression in *Ae. albopictus* third-instar larvae, pupae and adult mosquitoes. The reaction mixture included cDNA (1 μL), forward and reverse primers (0.4 μL), SYBR TB Green dye (5 μL), and ddH_2O (3.2 μL). PCR conditions consisted of an initial denaturation step at 94 °C for 10 min followed by 30 cycles of amplification at 94 °C for 30 s; annealing at 50–60 °C for 30 s; extension at 72 °C for 45 s; and a final extension step at 72 °C for 10 min. The PCR products were identified by 1 % agarose gelelectrophoresis. *OBPs* and *OR7* genes were determined from the antennae of female mosquitoes 3–5 days after hatching of *Ae. albopictus* larvae, following the same procedure as for MAO-B genes.

2.5. Ae. albopictus behavioral tracking monitoring test

The zebrafish behavioral tracking system (Noldus) was utilized to select and separate third-instar larvae and pupae of *Ae. albopictus*, which were then placed in 24-well cell culture plates (Thermo) containing 1.2 mL of chlorpyrifos and acetone solution at corresponding concentrations. Subsequently, *Ae. albopictus* was photographed and recorded using the zebrafish behavioral tracking system (Noldus) for three repetitions, with each session lasting 30 min in a water bath maintained at a temperature range of 25–26 °C under white light conditions. Noldus software was employed to analyze various parameters including movement distance, movement speed, movement acceleration, cumulative movement time, activity level (body filling), and resting time.

2.6. Ae. albopictus Y-shaped tube avoidance experiment

The detection of drug resistance in experimental strains of *Ae. Albopictus* was conducted using the WHO-recommended method, which involved exposing adult mosquitoes to a membrane filter paper (Cheng et al., 2016; Fang et al., 2018). In this study, a Y-shaped tube setup was used, with one arm containing a filter paper soaked in 1 mL of chlorpyrifos at a concentration of 1.5 μg/L (experimental arm), and the other arm containing a filter paper soaked in 1 mL of pure water as a control. An air pump was turned on to facilitate airflow through activated carbon and purified water from both arms towards the main arm of the Y-shaped tube, creating an air circulation within the device for 10 min. Thirty female mosquitoes were then introduced into the main arm of the Y-shaped tube after being exposed to plumage for 3–5 days, allowing them to fly against the wind generated by the device for a duration of 30 min. The number of female mosquitoes recorded in both arms (experimental and control) was documented. Each group consisted of thirty female mosquitoes as an experimental repetition, and each concentration level of chlorpyrifos was repeated three times to analyze avoidance behavior exhibited by *Ae. albopictus* female mosquitoes.

2.7. Statistical analysis

The locomotor behavior indexes and enzyme activity of third-instar larvae, pupae, and adult mosquitoes of *Ae. albopictus* in F1, F2, and F3 generations were analyzed using IBM SPSS Statistics software. One-way ANOVA was employed to analyze the characteristics of resistance differences between experimental groups with different chlorpyrifos stress concentrations in different generations as well as between experimental and control groups in the same generation. Two-by-two comparisons were conducted using Tukey's method, and graphing was performed using GraphPad Prism Version 8.0.2 software.

Table 1
Primers for qRT-PCR.

Primer name	Forward primer(5′-3′)	Reverse primer(5′-3′)	GenBank
Actin	GCTACGTCGCCCTGCACTT	AGGAACGACGGCTGGAAGA	DQ657949.1
MAOB	GAACTCAGCGTGACGGATTG	CGGCACTAAGTTCCAGACGA	LOC109621484
OBPs	GGATGGAGCAGATAAAGAGC	TTACATGCGTTAAGAGCTTCC	XM_001657780.2
OR7	ATGCCGAACATTCGGCTGAT	TTATTTCAACTGCACCAACACCATG	NC_035109.1

3. Results

3.1. Changes in Ca^{2+}- Mg^{2+}-ATPase and Choline Acetyltransferase (ChAT) activities of Ae. albopictus under sub-lethal concentrations of chlorpyrifos stress

Under different concentrations of chlorpyrifos, the Ca^{2} + -Mg^{2+}-ATPase activity of *Ae. albo*pictus third-instar larvae, pupae, and adult mosquitoes from different generations (F1, F2, and F3) exhibited a significant decrease ($P < 0.001$) compared to the sensitive strains. Moreover, the inhibitory effect on enzyme activity became more pronounced with increasing chlorpyrifos concentration and persisted across generations (Fig. 1). Notably, no tolerance phenomenon was observed (Fig. 1). The cumulative stress toxicity increased with successive generations without any signs of tolerance development (Fig. 1). The ChAT activity in *Ae. albopictus* third-instar larvae mosquitoes from different generations under various concentrations of chlorpyrifos did not differ significantly from that of the sensitive strain in F1 but showed a significant decrease in both F2 and F3 (Fig. 2 A). Additionally, ChAT activity in pupae and adult mosquitoes displayed a notable decreasing trend compared to the sensitive strains; higher concentrations of chlorpyrifos resulted in more pronounced inhibition on ChAT activity. Furthermore, as generations progressed, persecution toxicity continued to accumulate without any apparent signs of tolerance at present (Fig. 2 BC).

3.2. Generational changes in Ae. albopictus monoamine oxidase (MAO-B), olfactory binding proteins (OBPs), and olfactory receptor (OR7) gene expression under sublethal concentration of chlorpyrifos stress

Under different concentrations of chlorpyrifos stress, the expression of the MAO-B gene in third-instar larvae, pupae, and adult mosquitoes of *Ae. albopictus* was significantly reduced compared to the sensitive strain. This suggests that *MAO-B* gene expression is down-regulated under chlorpyrifos stress. When comparing different concentrations of chlorpyrifos stress, it can be observed that the *MAO-B* gene expression in *Ae. albopictus* exhibits an upward trend with increasing stress concentration, indicating a need for higher expression of the *MAO-B* gene to restore nerve conduction and improve locomotor ability in order to adapt to this adverse environment. Furthermore, there appears to be a weakening rather than a decrease in down-regulation of gene expression in the F3 generation at different ages, suggesting potential tolerance to chlorpyrifos by *Ae. albopictus* (Fig. 3). The *OBP* gene expression in the third-instar larvae of *Ae. albopictus* from the F1 and F3 generations under long-term stress of different concentrations of chlorpyrifos was significantly lower ($P < 0.01$) compared to that of the control group (Fig. 4 A). In all three generations, both pupae and adult mosquitoes showed significantly lower expression levels of the *OBP* gene compared to the control group ($P < 0.01$) (Fig. 4 BC). The changes in chlorpyrifos concentration did not have a significant impact on *OBP* gene expression, but there was an increase in expression with each successive generation under stress, suggesting that *Ae. albopictus* may have developed a certain degree of resistance to chlorpyrifos. Under long-term stress from different concentrations of chlorpyrifos, the expression level of *OR7* gene increased with each successive generation in third-instar larvae, pupae, and adult mosquitoes of *Ae. albopictus*, reaching its peak in the F2 generation before fluctuating and decreasing in the F3 generation (Fig. 5). Except for third-instar larvae and pupae from the F2 generation, there was a significant decrease in *OR7* gene expression in all other generations compared to the control group. The influence on chlorpyrifos concentration by *OR7* gene was not evident; however, it exhibited up-regulation with increasing toxicity accumulation until reaching a dramatic decrease as generational stress prolonged towards levels similar to those observed in the F1 generation, indicating potential tolerance development by *Ae. albopictus* mosquitoes towards chlorpyrifos.

3.3. Effects of sublethal concentration of chlorpyrifos on the motor behavior ability of Ae. albopictus

In the physical activity test conducted within a 30-min timeframe, significant differences in the physical activity of *Ae. albopictus* third-instar larvae and pupae were observed across generations ($P < 0.001$). Specifically, the F1 generation exhibited significantly weakened physical activity in third-instar larvae under higher concentrations of chlorpyrifos stress (Fig. 6 A). The overall trend for nymphs in each experimental group showed a significant increase in physical activity followed by a gradual decrease, eventually stabilizing with each generation of stress; however, there were no significant differences among the three experimental groups within the same generation (Fig. 6 B). In terms of cumulative movement time within a 30-min period, there was a highly significant difference observed for *Ae. albopictus* third-instar larvae across generations. The cumulative movement time of third-instar larvae was significantly shorter in the F1 generation under higher concentrations of chlorpyrifos stress; however, it increased in subsequent generations (F2 and F3) and approached levels similar to that of the acetone control group (Fig. 6 C). Similarly, for *Ae. albopictus* pupae, there were highly significant differences observed in cumulative movement time across generations. As generations progressed, the cumulative movement time decreased overall and then stabilized; however, no significant differences were found among the three experimental groups within each generation (Fig. 6 D).

The avoidance ability of *Ae. albopictus* adult mosquitoes exhibited significant differences between generations when subjected to long-term stress from varying concentrations of chlorpyrifos. In the F1 and F3 generations, adult mosquito avoidance was lower in all three experimental groups compared to the control group. In the F2 generation,

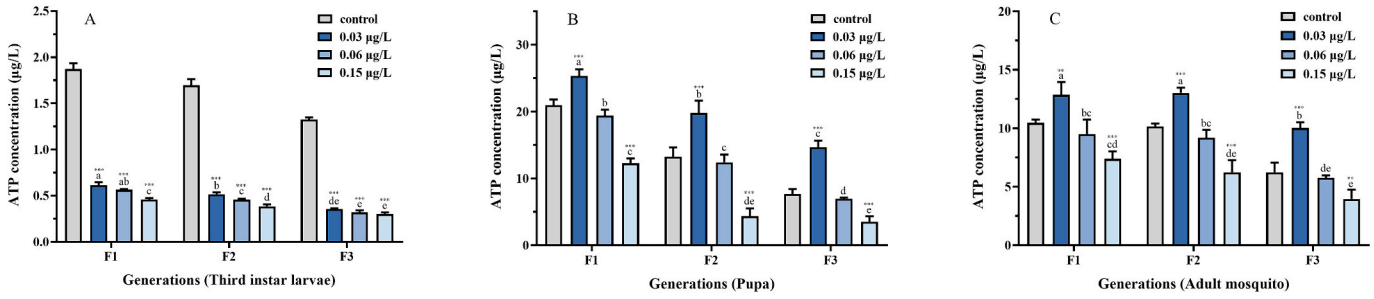

Fig. 1. Changes in Ca^{2+}-Mg^{2+}-ATPase activity in *Ae. albopictus* under different concentrations of chlorpyrifos stress were examined across generations. The chlorpyrifos concentrations tested included 0.03 μL/L, 0.06 μL/L, and 0.15 μL/L with acetone as a blank control. Enzyme activity was assessed using a kit. **A** Alterations in Ca^{2+}-Mg^{2+}-ATPase activities during different stages of third-instar larvae. **B** Variations in Ca^{2+}-Mg^{2+}-ATPase activities throughout pupal development. **C** Changes in Ca^{2+}-Mg^{2+}-ATPase activities among adult females at various stages. Three biological replicates and three technical replicates were performed. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

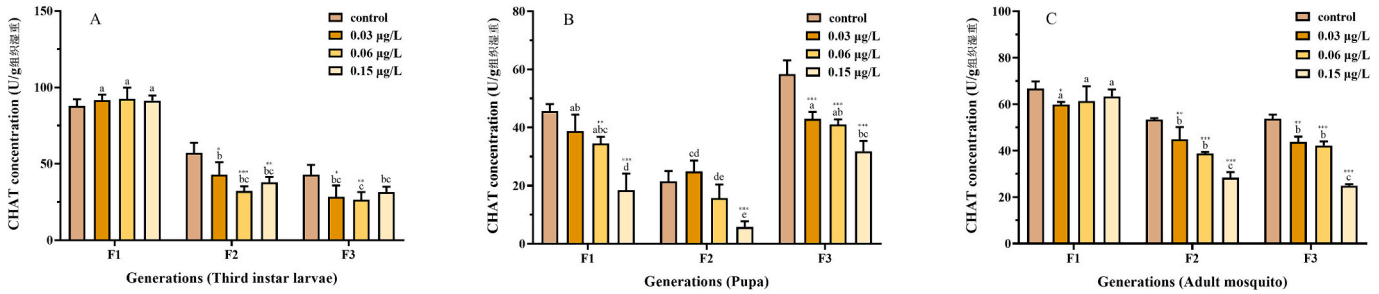

Fig. 2. Changes in choline acetyltransferase (ChAT) activity in *Ae. albopictus* under different concentrations of chlorpyrifos stress were examined across generations. The chlorpyrifos concentrations tested included 0.03 μL/L, 0.06 μL/L, and 0.15 μL/L with acetone as a blank control. The enzyme activity test was carried out using the kit. **A** Changes in ChAT activities were measured in third-instar larvae of *Ae. albopictus* at different developmental stages. **B** Changes in ChAT activities were measured in pupae of *Ae. albopictus* at different developmental stages. **C** Changes in ChAT activities were measured in adult female *Ae. albopictus* at different developmental stages. Three biological replicates and three technical replicates were performed. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

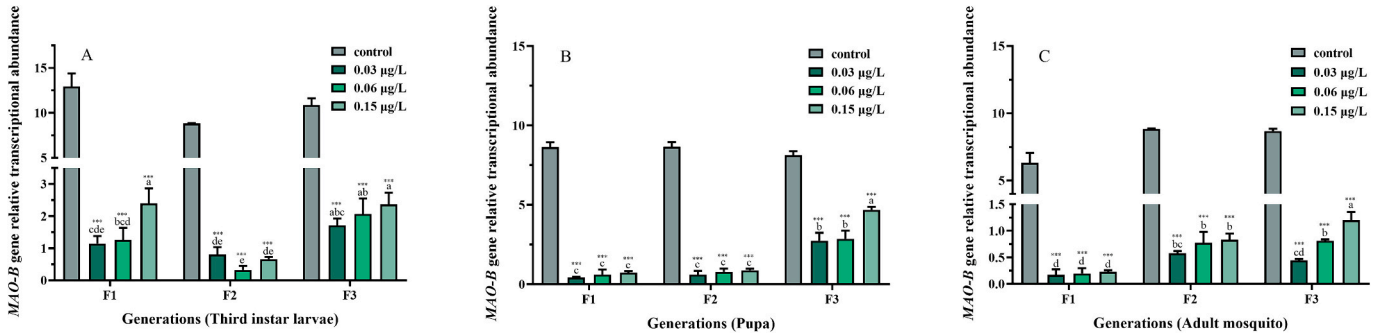

Fig. 3. Generational changes in *MAO-B* gene expression in *Ae. albopictus* under different concentrations of chlorpyrifos stress. Chlorpyrifos concentrations included 0.03 μL/L, 0.06 μL/L and 0.15 μL/L with acetone as a blank control. Real-time quantitative fluorescence PCR (qPCR) was used to detect the relative expression level of *MAO-B*. **A** Relative expression of *MAO-B* in third-instar larvae of *Ae. albopictus* at different stages. **B** Relative expression of *MAO-B* in pupae of *Ae. albopictus* at different stages. **C** Relative expression of *MAO-B* in adult of *Ae. albopictus* at different stages. Three biological replicates and 3 technical replicates were set. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

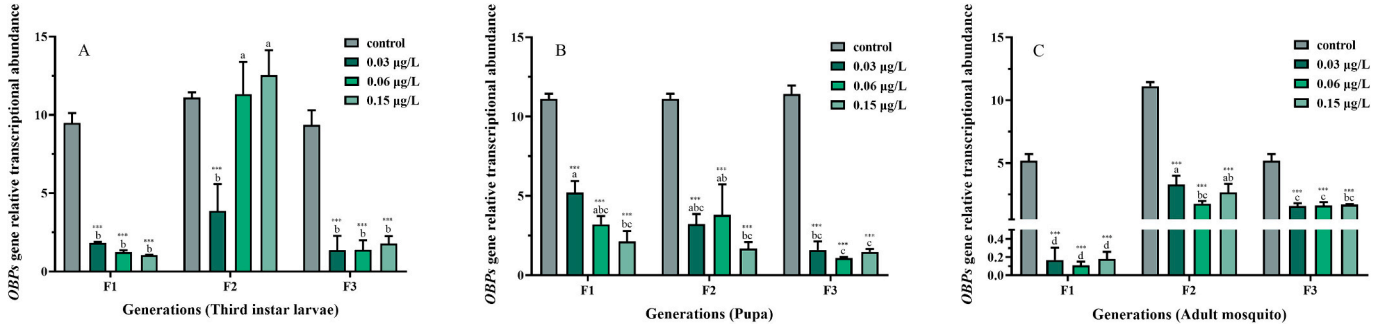

Fig. 4. Generational changes in *OBPs* gene expression in *Ae. albopictus* under different concentrations of chlorpyrifos stress. Chlorpyrifos concentrations included 0.03 μL/L, 0.06 μL/L and 0.15 μL/L with acetone as a blank control. Real-time quantitative fluorescence PCR (qPCR) was used to detect the relative expression level of *OBPs*. **A** Relative expression of *OBPs* in third-instar larvae of *Ae. albopictus* at different stages. **B** Relative expression of *OBPs* in pupae of *Ae. albopictus* at different stages. **C** Relative expression of *OBPs* in adult of *Ae. albopictus* at different stages. Three biological replicates and 3 technical replicates were set. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

there was no significant difference in mosquito avoidance between each experimental group and the F1 generation, while it decreased in the F3 generation. Across all three generations, higher concentrations of chlorpyrifos stress within each experimental group corresponded to increased levels of adult mosquito avoidance (Fig. 7).

4. Discussion

Research has demonstrated that organophosphorus pesticides exert toxic effects on the nervous system, internal organs, reproductive system, and immune system of living organisms. Among these effects, neurotoxicity is particularly significant (Wu et al., 2023; Durand et al., 2012). Currently, chemical control remains the primary approach for

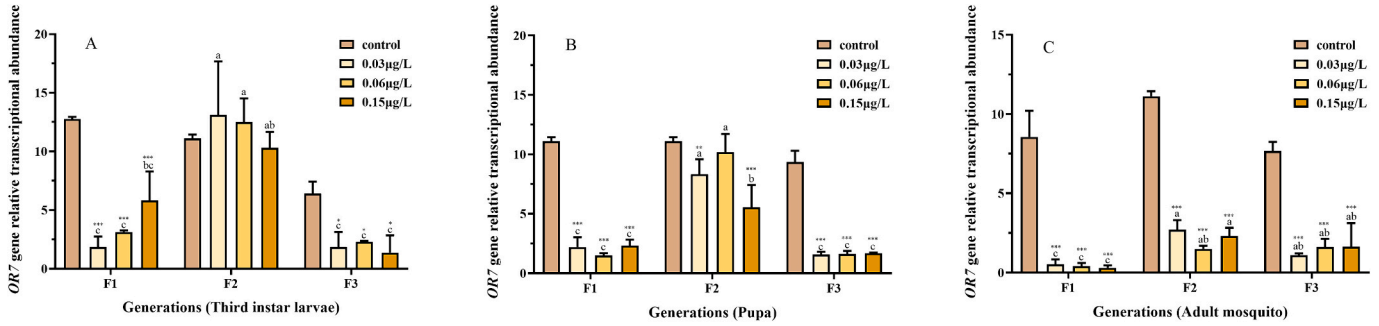

Fig. 5. Generational changes in *OR7* gene expression in *Ae. albopictus* under different concentrations of chlorpyrifos stress. Chlorpyrifos concentrations included 0.03 μL/L, 0.06 μL/L and 0.15 μL/L with acetone as a blank control. Real-time quantitative fluorescence PCR (qPCR) was used to detect the relative expression level of *OR7*. **A** Relative expression of *OR7* in third-instar larvae of *Ae. albopictus* at different stages. **B** Relative expression of *OR7* in pupae of *Ae. albopictus* at different stages. **C** Relative expression of *OR7* in adult of *Ae. albopictus* at different stages. Three biological replicates and 3 technical replicates were set. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

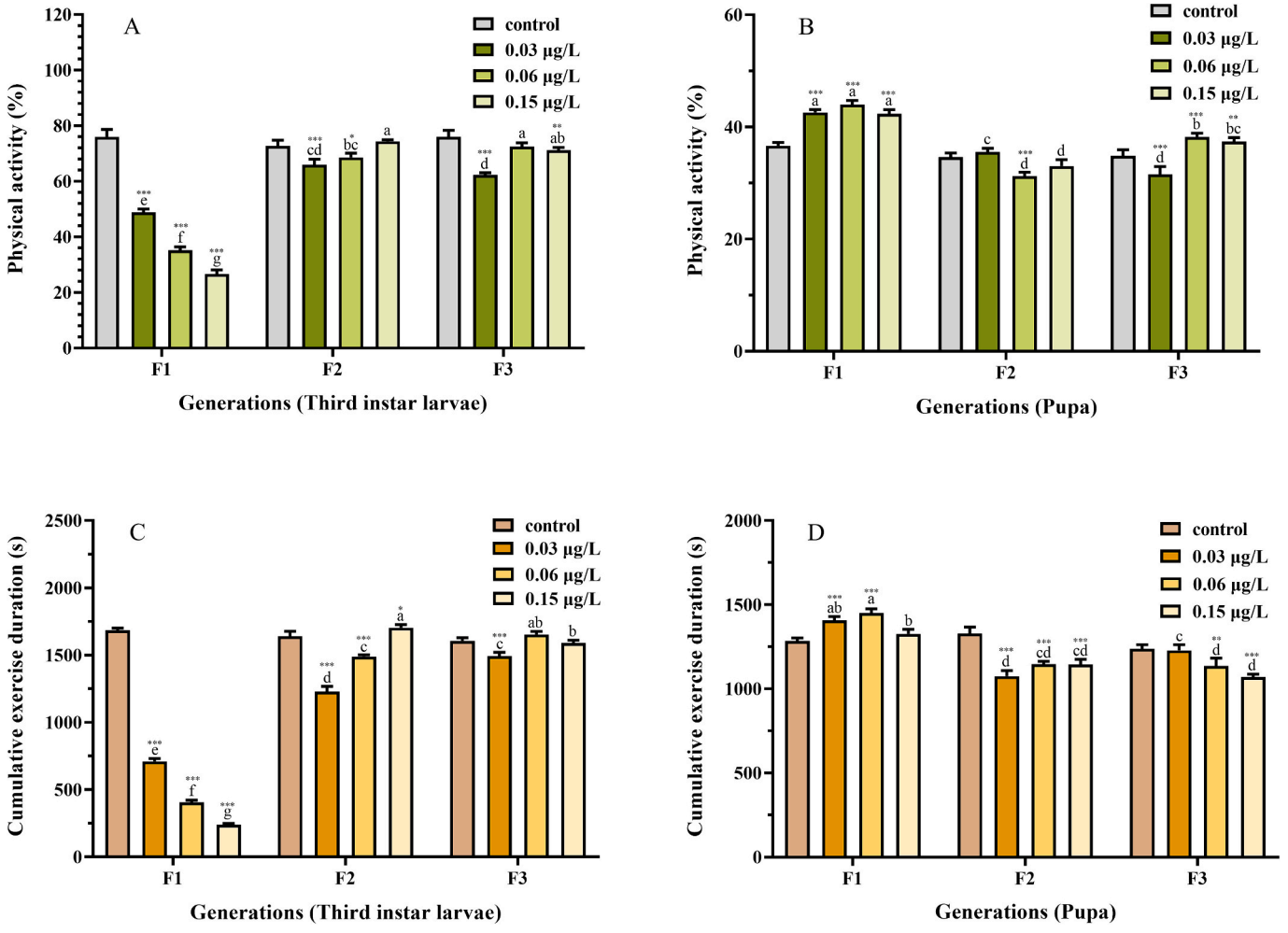

Fig. 6. Physical activity test results and Cumulative exercise duration in *Ae. albopictus* under three generations of chlorpyrifos stress. Chlorpyrifos concentrations included 0.03 μL/L, 0.06 μL/L and 0.15 μL/L with acetone as a blank control. *Ae. albopictus* was photographed and recorded with a zebrafish behavioral tracking system (Noldus) for 30 min in a water bath at 25–26 °C under white light for three repetitions. **AB** Physical activity test results in third-instar larvae and pupae of *Ae. albopictus* at different stages. **CD** Cumulative exercise duration results in third-instar larvae and pupae of *Ae. albopictus* at different stages. Three biological replicates and 3 technical replicates were set. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

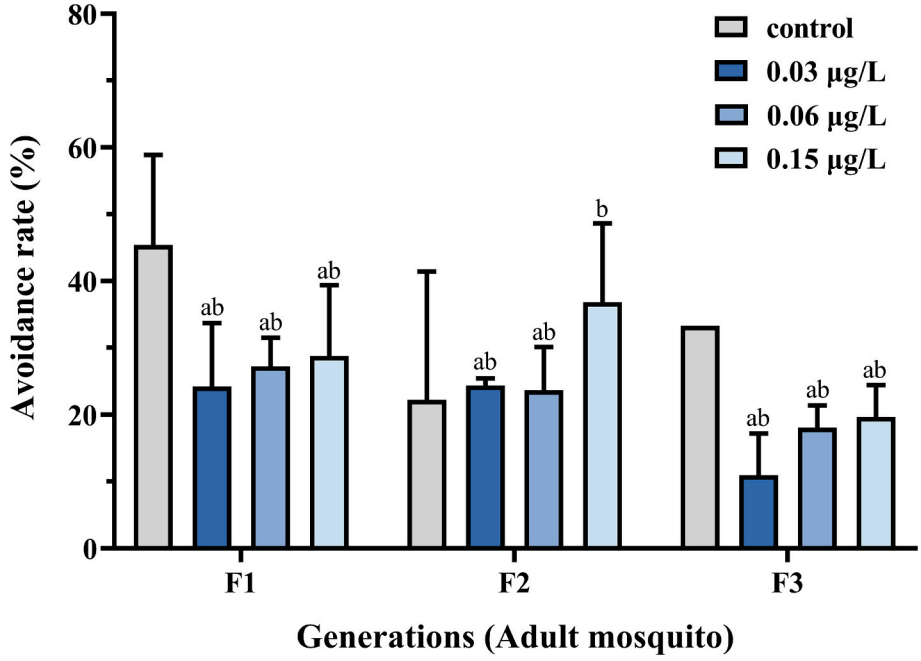

Fig. 7. The avoidance behavior of *Ae. albopictus* females was assessed using a Y-shaped tube assay, in which a filter paper containing 1 mL of 1.5 μg/L chlorpyrifos was placed in the experimental arm and a filter paper with 1 mL of pure water served as control in the other arm. Thirty female mosquitoes aged between 3 and 5 days were introduced into the main arm of the Y-tube and allowed to fly against the wind for 30 min while recording their distribution between the two arms. Each group of thirty females constituted an experimental replicate, and each concentration of chlorpyrifos was repeated three times to analyze mosquito avoidance. (One-way ANOVA followed by Tukey's test was used to determine significant differences between generations for each concentration within the experimental group; * indicates significant differences between the experimental group and the control group within the same generation, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

managing *Ae. albopictus*, employing pyrethroids. However, the excessive use of insecticides has led to the development of resistance (Naqqash et al., 2016; Zulfa et al., 2022). Additionally, unintended exposure to non-targeted insecticides in the environment can disrupt normal activities of *Ae. albopictus* and contribute to its resistance through various mechanisms over time, thereby increasing challenges in prevention and control efforts. Research findings indicate that *Anopheles sinensis* in Shanghai has already exhibited resistance against pyrethroids and organophosphorus insecticides (Fang et al., 2019). While *Culex tritaeniorhynchus* breeding in rice fields demonstrates high levels of resistance to organophosphorus insecticides due to prolonged exposure from pesticide application on rice crops (Wu et al., 2016).

Considering the impact of organophosphorus pesticides on biological neural systems, resistance development manifests at a microscopic level through alterations in neurotoxicity-related enzyme activity, gene expression within the olfactory system, and modified avoidance behavior. Voltage-gated sodium channels are the primary target for the neurotoxic effects of pyrethroids on insects, while voltage-gated calcium channels and voltage-gated chloride channels serve as secondary targets (Zhu et al., 2020). Currently, numerous studies on Ca^{2+}-Mg^{2+}-ATPase have been conducted both in China and abroad, primarily using rat models. These studies have focused on treating human epilepsy, heart failure, and other neurological disorders. However, there is a relative scarcity of research conducted on insects. In this experiment, we capitalized on the crucial role of Ca^{2+}-Mg^{2+}-ATPase in animal neural pathways and extended its investigation to the nervous system of insects to explore its interconnection with insect behavioral resistance. Measurements of Ca^{2+}-Mg^{2+}-ATPase activity in *Ae. albopictus* revealed a significant decrease in enzyme activity with increasing concentrations of chlorpyrifos stress. Regarding age groups, third-instar larvae group exhibited the lowest Ca^{2+}-Mg^{2+}-ATP enzyme activity among each generation, while the pupal group displayed the highest enzyme activity across generations. Across generations at identical ages and under similar chlorpyrifos treatment concentrations, Ca^{2+}-Mg^{2+}-ATP enzyme activity gradually decreased from F1 to F3; moreover, this decrease intensified with higher treatment concentrations—indicating that chlorpyrifos has a cumulative toxic effect on *Ae. albopictus*. Simultaneously, reduced Ca^{2+}-Mg^{2+} ATPase activity may lead to abnormal distribution of intracellular calcium ions inside and outside nerve membranes as well as intracellular calcium overload—a condition that could potentially impair memory function in *Ae. albopictus* and affect its behavior.

The enzyme ChAT is responsible for catalyzing the biosynthesis of ACh and is widely recognized as a reliable phenotypic marker for cholinergic neurons (Yasuyama and Salvaterra, 1999). In earthworms exposed to low doses of chlorpyrifos, an abnormal increase in brain ACh content was observed (Li et al., 2020a, 2020b). Consistent with the findings from the Ca^{2+}-Mg^{2+}-ATP enzyme activity assay, the results of the ChAT enzyme activity assay demonstrated a decline in ChAT enzyme activity concomitant with increasing concentrations of chlorpyrifos stress. Regarding age-related differences, the pupal group exhibited the lowest ChAT enzyme activity while relatively higher levels were observed in third-instar larvae across generations. In terms of generations, when comparing the results of experiments conducted at the same age and with the same concentration of chlorpyrifos treatment, we observed a gradual decrease in ChAT enzyme activity as generations progressed. Furthermore, it was noted that differences in ChAT enzyme activity among different concentrations of chlorpyrifos stress at the same age gradually diminished from the F1 generation to the F3 generation. This suggests that a weakened resistance may have developed in subsequent generations of *Ae. albopictus*, thereby reducing the toxic effects exerted by chlorpyrifos.

MAO-B is capable of catalyzing the oxidative degradation of various monoamine neurotransmitters, such as dopamine (DA) and 5-hydroxytryptamine (5-HT), in vivo. Abnormal increases in activity or expression can lead to a decrease in neurotransmitter levels, resulting in oxidative damage to nerve cells (Zhou et al., 2019). Studies have demonstrated that exposure to low doses of chlorpyrifos inhibits the

secretion of 5-HT and DA in the brain of earthworms (Roczniak et al., 2015), leading not only to impairments in learning and memory but also affecting locomotor activity and slowing down movement. Under long-term stress of chlorpyrifos *MAO-B* gene expression was significantly lower in all age groups than in the control group. With increasing toxicity from chlorpyrifos exposure, *Ae. albopictus* developed a certain degree of tolerance towards it, as evidenced by increased expression levels observed among F3 generations compared with F2 generations across all age groups. The *MAO-B* gene expression on *Ae. albopictus* exhibited an upward trend with increasing stress concentrations, indicating that high expression levels are required for restoring nerve conduction and improving locomotor ability to adapt to this adverse environment under higher concentrations of chlorpyrifos; further highlighting how chlorpyrifos stress adversely affects the nervous system of *Ae. albopictus*.

Investigating the mechanisms by which insect sensory organs elicit behavioral responses in biological ecosystems or in the presence of other odor molecules can provide valuable insights into insect behaviors related to host recognition, interspecific interactions, and intraspecific communication. This knowledge can contribute to the development of effective strategies for pest management and protection of beneficial insects (Renou and Anton, 2020a, 2020b). Significantly higher expression of OBPs was found in resistant mosquito species of *Culex pipiens* in a study of drug-resistant burnout-causing Culex mosquitoes (Shen et al., 2022). The expression of *OBP13* in the head of the cabbage moth *P. xylostella* was significantly upregulated following chlorpyrifos treatment, indicating a close association between *OBP13* and the development of insecticide resistance (Bautista et al., 2009). The results regarding olfactory gene expression in *Ae. albopictus* demonstrated that OBPs' expression levels showed a noticeable decrease across all generations at different ages within the experimental group compared to those in the control group. Meanwhile, the expression of OBPs in the experimental group gradually increased with the increase of the time of chlorpyrifos stress, which indicated that long-term chlorpyrifos stress caused a gradual increase in the resilience of *Ae. albopictus*.

It has been demonstrated that numerous natural chemicals may elicit an avoidance response in insects through the olfactory system (Porciani et al., 2017). *Ae. aegypti* odorant receptor 31 (AaOr31) has been identified as an orthologous receptor (OR) to EBF, and it was found that Or31-mediated repulsion significantly synergizes with pyrethroid-induced activation of voltage-gated sodium channels (Liu et al., 2021). Activation of specific OR by bioallethrin contributes to the rejection of bioallethrin, potentially due to co-activation of OR and sodium channels (Valbon et al., 2022), indicating a correlation between OR expression and insect resistance mechanisms. Measurement of *Ae. albopictus' OR7* expression revealed that down-regulation was most pronounced in the adult mosquito group within each generation, with greater down-regulation observed in the F1 generation compared to the F3 generation during the same period; however, down-regulation of gene expression was minimal in the tritrophozoite group within each generation. In terms of generations, changes in chlorpyrifos concentration had a diminishing effect on *OR7* expression as generations increased, and at each concentration level, *OR7* expression remained relatively consistent among individuals from the F3 generation across all age groups. The impact of chlorpyrifos stress on *Ae. albopictus'* olfactory receptors exhibited an initial up-regulation followed by subsequent decrease with increasing toxicity accumulation, suggesting that high levels of *OR7* expression in all age groups during the F2 generation may have laid a foundation for *Ae. albopictus'* resistance against unfavorable environments.

Combined with the findings from *OBPs*, *OR7* measurements, and Y-shaped tube evasiveness experiments, the evasiveness of the experimental group was analyzed, revealing varying degrees of reduction compared to that of the control group. This is consistent with the results of He et al. (2019) in *Anopheles sinensis.* As the concentration of chlorpyrifos stress increased, there were alterations in the expression of *OBPs* and *OR7* genes, leading to an upward trend in mosquito avoidance behavior intensity. However, over successive generations, mosquitoes treated with the same chlorpyrifos concentration exhibited a gradual decline in avoidance. This suggests that olfactory system-related proteins play a crucial role in mediating behavioral resistance mechanisms against chlorpyrifos in *Ae. albopictus*.

In addition to affecting the ability of insects to avoid insecticides, chlorpyrifos significantly affects insect locomotion (Delnat et al., 2019). In this study, we utilized the Behavioral Trajectory Tracking System to analyze the behavioral patterns of third-instar larvae and pupae of *Ae. albopictus*. Our findings revealed that exposure to chlorpyrifos stress from the F1 generation inhibited cumulative locomotion time and physical mobility in third-instar larvae, with a more pronounced effect observed at higher stress concentrations. This is consistent with the findings of Adedara et al. in *Nauphoeta cinerea* (Adedara et al., 2016). As subsequent generations passed, these larvae gradually developed tolerance and regained their locomotor ability, leading to a gradual decrease in correlation between impaired locomotor ability and stress concentration. The cumulative movement time and physical activity of pupae did not exhibit significant inhibition in the F1 generation under chlorpyrifos stress; however, these parameters decreased in subsequent generations. It is postulated that this phenomenon can be attributed to the presence of a leathery protective layer on the pupae' surface, which transiently acts as a barrier against chlorpyrifos during the early stages of exposure. Furthermore, it is suggested that chlorpyrifos inhibits pupae locomotion ability and this impairment worsens with increasing toxicity accumulation.

5. Conclusions

Under prolonged exposure to chlorpyrifos, the activities of Ca^{2+}-Mg^{2+}-ATPase and choline acetyltransferase (ChAT), as well as the *MAO-B*, *OBPs*, *OR7* associated with the neural and olfactory systems of *Ae. albopictus* are altered, leading to a significant impact on the motor behavior of this species. In order to adapt to this unfavorable environment, *Ae. albopictus* inevitably adjusts relevant biochemical indices to enhance its own resistance against pesticides, thereby increasing the challenge for human management strategies relying on chemical methods. The objective of this study is to elucidate the molecular mechanism underlying resistance induced by chlorpyrifos residues in aquatic ecosystems, which will provide valuable insights for rational and appropriate insecticide use while also exploring novel approaches for managing insect neurotoxicity.

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CRediT authorship contribution statement

Yanrong Chen: Writing – original draft, Visualization, Methodology, Investigation. **Wen Li:** Visualization, Conceptualization. **Ruoyun Lan:** Writing – original draft. **Rufei Chen:** Investigation. **Jingchao Hu:** Data curation. **Chenyu Yang:** Visualization. **Ping Wang:** Data curation. **Bin Tang:** Writing – review & editing, Methodology. **Shigui Wang:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

All animal procedures have been approved by the Experimental Animal Ethics Committee (AEWC) of Hangzhou Normal University (Approval No. HSD20220103). Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172425.

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