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Chronic dimethomorph exposure induced behaviors abnormalities and cognitive performance alterations in adult zebrafish (*Danio rerio*)

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ABSTRACT

Dimethomorph is a systematic fungicide that inhibits sterol synthesis in fungi and unfortunately, there was only scarce data regarding its toxicity. Therefore, considering its extensive application in agriculture and its presence in food residues and the environment, its toxicities in non-target organisms, including aquatic animals, are required to be evaluated since they are sensitive indicators of ecological change. In this study, we evaluated the toxicities of dimethomorph after chronic exposure to adult zebrafish (*Danio rerio*) by conducting various behavioral assays, a passive avoidance test, and biochemical assays by ELISA. From the results, ~ 2 weeks exposure to dimethomorph caused lower locomotion, aggressiveness, and conspecific social interaction, and more robust predator avoidance behaviors. Furthermore, alterations in color preferences and short-term memory loss were also observed in the treated fish. In helping to elucidate the mechanism, the expression level of several important neurotransmitters in the brain tissue was measured. Interestingly, increment in several biomarkers, including serotonin, kisspeptin, epinephrine, norepinephrine, and dopamine was observed in the treated group along with a slight increase in other tested neurotransmitters, which were catalase, acetylcholine, and melatonin, which might play a role in the observed behavior alterations. Nevertheless, the results from the current study suggested possible alterations in the central nervous system by dimethomorph, and thus, consideration is required prior to the usage of this fungicide in the agricultural fields surrounding natural freshwater reservoirs.

1. Introduction

Pesticides are chemicals that are used in agriculture for the control of pests and weeds and are considered one of the major factors in the development of agricultural yield in the 20th century [2]. However, although pesticides enable users to achieve higher crop yields, at the same time, they could negatively affect the natural environment, including the soil environment, plant fertility, and sustainable productivity. Moreover, the extensive use of pesticides has often caused the development of pesticide resistance in insect pests, plant pathogens, and weeds, resulting in the need for several additional applications of the commonly used and different pesticides to maintain expected crop yields [68,83]. For example, pesticides used in agriculture are toxic to bees which are very important for pollination of crops including fruits and vegetables [25]. Meanwhile, in humans, pesticide such as dibromochloropropane (DBCP) was demonstrated to have a potency in suppressing spermatogenesis [5]. In the worst cases, these pesticides that are applied to crops would eventually end up in ground and surface water and once groundwater is contaminated, the pesticide residues could remain for long periods of time, resulting in the contamination of aquatic ecosystem and directly or indirectly effecting any organism that interacts with this environment.

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Unfortunately, although the neurotoxic effect of pesticides had been extensively studied, most of these studies focused on organophosphate or carbamate families while only relatively few studies investigated the neurotoxic effect of morpholine compounds. Dimethomorph (4-(3-(4-chlorophenyl)-3-(3,4 dimethoxy phenyl) acryloyl) morpholine), a cinnamic acid derivative and a member of the morpholine chemical family, is a fungicide that inhibits fungal cell wall synthesis and is commonly used in the process of agricultural production. For example, together with imidacloprid, it has been recommended and often found to be used in grape production in Taiwan [20]. Many studies have demonstrated the adverse effects of this pesticide on soil-borne organisms, including earthworms, and disturbances in microbial communities and some non-target microbial groups inhabiting soils [24,86]. Meanwhile, in rats, although dimethomorph has low oral toxicity in rats and is not an irritant to rabbit skin with minimal irritation to rabbit eyes, developmental exposure to this fungicide negatively impacted female reproductive development [16,36]. However, only a few studies that address its adverse effects on aquatic organisms, especially fish. A prior study had demonstrated that dimethomorph interfered with normal zebrafish embryo development while in combination with difenoconazole, it downregulated expression of mcm family genes, potentially disrupting DNA replication and cell cycle progression [32]. Moreover, in our previous experiment, dimethomorph was also found to increase the locomotion of zebrafish larvae during light-dark test [40]. Nevertheless, some studies have defined the toxicity of dimethomorph as relatively non-toxic when administered acutely to laboratory animals (Toxicity Category III) and low to moderate acute toxicity to freshwater fish, including zebrafish [58,91,95]. Despite this categorization, one has to keep in mind that those findings were based on the acute exposure of dimethomorph, thus, the more prolonged effect of this pesticide in aquatic animals, especially zebrafish, remains intriguing to be evaluated. The adverse effects of dimethomorph in various animal models from previous studies are summarized in Table 1.

Zebrafish have long been used as an animal model for biomedical research particularly in developmental, behavioral, neurotoxic, and genetic studies [59,96]. Given their genetic homology with most human genes, zebrafish are an excellent model for studying the health risks of pesticides in humans and the environment [38,39,48]. Furthermore, zebrafish also possess conserved neurological systems that are comparable to mammals with the large and variable behavioral repertoire of adult zebrafish [21,48]. In ecotoxicology studies, incorporating behavior analysis provides several advantages as it is an indicator of multiple levels of biological effects, among the most sensitive indicators of the impact of exposure, and is considered an early warning tool [28]. In addition, their small size, less costly maintenance, and relatively fast adaptation to a novel environment also make them a suitable animal model to assess the adverse effects of any toxicant, including pesticides [79].

Since we had found that dimethomorph could alter zebrafish larval behaviors in the previous study [40], here, we aimed to evaluate the adverse effects of dimethomorph in relatively low concentrations on adult zebrafish behaviors and cognitive performances after chronic exposure. Subsequently, the expression level of some important neurotransmitters in the fish brain was also measured to help in understanding the toxicity mechanism. We hypothesized that even in relatively low doses, dimethomorph could also alter adult zebrafish behaviors by compromising the expression of some neurotransmitters in the fish brain. The outline of this study is shown in Fig. 1.

Table 1
Adverse effects of dimethomorph in different multicellular organisms reported in previous studies.

Concentrations	Exposure Period	Species (stages)	Effects	Refs.
2–13.63 mg/L	24, 48, and 96 h	Zebrafish (*Danio rerio*) embryos	Developmental toxicity with increased deformity rate, lethality rate, hatching rate, and heart rate.	[32]
150; 300; and 600 μg/L	4 days	Duckweed (*Lemna minor* and *Spirodela polyrhiza*)	Decreased growth rate with inhibition up to 21 %	[27]
100 mg/kg	7, 14, and 28 days	Soil earthworm (*Eisenia fetida*)	significantly stimulated or inhibited SOD and CAT, indicating these concentrations could be toxic to earthworms.	[86]
60 and 180 mg/kg	6 and 16 days	Sprague Dawley (Crl: CD(SD)) female rats	Induced dose-related reductions to plasma corticosterone levels, indicating an impaired follicle growth	[16]
250; 500; and 1000 μg/L	96 and 168 h	*Lemna minor*	Growth inhibition with maximum inhibition reached was 45.5 ± 6.4 %	[57]

2. Material and methods

2.1. Fish Rearing

Wild-type adult zebrafish strains AB (4–6 months old) in a healthy condition were used in the present study. Prior to the exposure, the fish were maintained in a recirculating UV-filtered aquatic system at 27 ± 1 °C (pH 7.0–7.5) with a water conductivity maintained between 300 and 1500 μS and a 10/14-h dark/light cycle. Fish were fed twice daily with lab-grown brine shrimp and commercial dry food (Taiwan Hung Kuo Industrial Co., Ltd., Taipei, Taiwan), each once daily. The fish housing protocol was based on the previous study [11].

2.2. Dimethomorph exposure

Dimethomorph with 98–100 % purity was purchased in powder form (Aladdin, Shanghai, China) and dissolved in DMSO. Afterward, healthy zebrafish regardless of the gender ratio were randomly selected and separated into three groups. For the 1st batch of the experiment (Fig. 1), each group in each replicate consisted of ten fish and was put in a beaker glass filled with a 3 L of dimethomorph solution. Since this experiment was done in three biological replications, a total of 30 fish for each group was applied. Meanwhile, for the 2nd batch of the experiment, seven fish were applied for each group in each replicate and exposed to the dimethomorph in a 2 L solution. A total of 14 fish for each group was used for this batch of experiments since this experiment was conducted in two biological replications. Each group was exposed to either 0 (control), 0.5, or 1 ppm of dimethomorph solution. The control group (vehicle control) underwent identical experiment procedures including 0.02 % DMSO exposure without the presence of dimethomorph and DMSO in this concentration was demonstrated to be relatively safe for zebrafish behaviors since previous studies found that even in higher doses (0.05 % and 0.1 %), DMSO did not alter locomotion or anxiety-like behavior in zebrafish [43]. Meanwhile, the selected concentrations of dimethomorph were based on the previous reports regarding the availability of dimethomorph in nature. First, The French Institute for the Environment (IFEN) in 2006 recorded that the highest concentration of dimethomorph in surface water in France was 406 μg/L. Next, a prior study also found the initial concentrations of this fungicide in the soil in several cities in China in 2008 and 2009 ranged from 0.85 to 1.77 mg/kg [50]. In addition, these doses were also selected based on a prior study in *Lemna minor* that used 250, 500, and 1000 μg/L of dimethomorph as they considered that these doses are environmentally representative

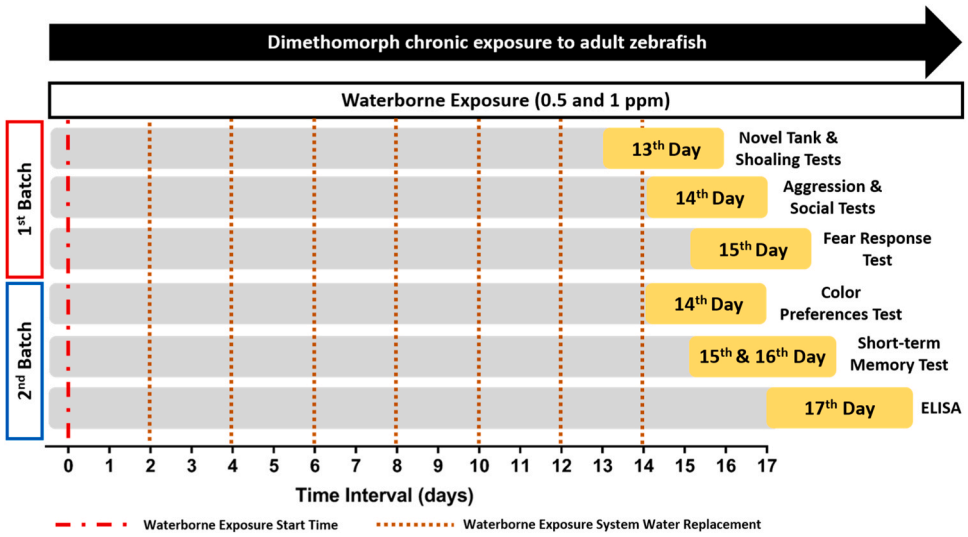

Fig. 1. Experimental design regarding the evaluation of dimethomorph chronic exposure in the relatively low concentrations (0.5 and 1 ppm) to adult zebrafish applied in the current study. The red line indicates the exposure start time while the brown lines indicate the schedule for the waterborne exposure system water replacement.

concentrations [57]. Furthermore, these doses were also applied considering the available toxicity data in several aquatic organisms, including rainbow trout fish (LC_{50} = 1.5–6.2 ppm), estuarine fish (LC_{50} = 11.3 ppm), and estuarine invertebrates (EC_{50} = 5.13 ppm for mollusks and 33 ppm for mysid shrimp) [31]. During the exposure period, aeration was provided in the exposure container to consistently provide oxygen to the fish. Every two days, the solution was refreshed to maintain the pH level (7.0–7.5), ammonia (< 2 ppm), nitrite (< 1 ppm), nitrate (< 40 ppm), and concentration of dimethomorph, and the beaker glass was cleaned to remove excessive foods that might cause some bacterial or fungal infections [7].

2.3. Multiple fish behavior analysis

The fish behaviors analysis was conducted on three consecutive days after the exposure. On 13 days post-exposure (dpe), the exposed fish underwent novel tank and shoaling tests while on the following day, aggressiveness and conspecific social interaction assays were carried out. Finally, in the 15 dpe, the fear response test was conducted as the last behavior test in this section. All of the behavior tests were conducted in an acrylic trapezoid tank (28 cm × 15.2 cm × 6 cm) filled with ~ 1.25 L of filtered water. During the novel tank test, the fish behavior responses to the new environment were recorded for one minute with a 5-min interval until 31 min. In the shoaling test, fish with a shoal size of three were put into the test tank and after 5 min of acclimation, their shoaling behaviors were recorded. Meanwhile, in the aggressiveness test, a mirror was put on one side of the test tank to evaluate the fish's behaviors toward their reflection after ~ 1 min of acclimation. Next, in the conspecific social interaction test, another zebrafish was put in the test tank together with the tested fish with a transparent separator between them. After they were acclimated for ~ 5 min, their interaction was recorded. Finally, by using a similar setting with a conspecific social interaction test, the fear response test utilized convict cichlid (*Amatitlania nigrofasciata*) that was separated by the transparent partition to elicit fear response in the tested fish after ~ 1 min of acclimation. The fish behaviors in every test, except the novel tank test, were recorded by using Canon EOS 600D (Canon Inc., Tokyo, Japan) with a 55–250 mm lens for 5 min. Afterward, the fish coordinates in every frame in each video were analyzed by using idTracker, and the output trajectories were used to calculate several important behavior endpoints by using Microsoft Excel [71]. The applied behavior assay protocol was based on the prior study and this experiment was done in triplicate [8].

2.4. Fish color preferences analysis

On the fourteenth day of exposure, the tested fish in each group were moved into an acrylic tank (21 × 21 × 10 cm) that was filled with ~ 1.5 L of filtered water. The test tank was equally divided into two compartments to observe the fish color preferences in two-color combinations from four colors (green, blue, yellow, and red) in a single attempt. During the test, the behaviors of the fish in a group of six were recorded for 30 min using a high-quality CCD camera (ONTOP, M2 module, Shenzhen, China) with a resolution of 3264 × 2448 pixels at a 30 fps frame rate. Afterward, to track the fish position during the test, UMAtracker was utilized [93]. This assay was conducted in two replications and followed the previously published method [77].

2.5. Short-term memory analysis

The short-term memory test was carried out on two consecutive days. The analysis utilized an acrylic tank (30 × 20 × 20 cm) that was filled with ~ 3 L of filtered water. The test tank was equally divided into two compartments (black and white colors) with a movable separator to separate the compartments. On the fifteenth day of exposure, the tested fish underwent several sessions, which were acclimation, familiarization, and training. During the acclimation session, the fish were individually placed in the tank to be habituated with the test tank for ~ 5 min. Later, in the familiarization session, the separator was placed between the compartments for ~ 1 min to prohibit the fish from swimming to the black compartment. Finally, the separator was taken out to allow the fish to swim to the dark compartment in the training session. However, in entering the dark compartment, a mild electric shock (25 V, 1 mA) would be administered at a maximum of three times at 5-s intervals. If the fish did not move to the white compartment within 5 min, they were manually put back into the white compartment and this session was repeated a maximum of three times with an acclimation process in between for ~ 1 min. On the sixteenth day, the fish was put into the white compartment and the time of the fish entering the dark compartment was recorded. This test was conducted according to the previous publication in two replications [75].

2.6. Brain tissue preparation, total protein estimation, and neurotransmitters determination

On the seventeenth day of exposure, the fish were euthanized by

immersion in ice water, and their brain tissues were collected. Later, every brain tissue from three to four fish was combined to prepare an independent homogenate in a 1.5 ml Eppendorf tube, and phosphate buffer saline (PBS) at pH 7.2 was added to each sample with the amount according to the weight of the homogenate [37,69]. Afterward, the homogenates were homogenized by using a bullet blender (Next Advance, Inc., Troy, NY, USA) for 15 min and the samples were centrifuged at 15000 rpm at 4 °C for 15 min, followed by transferring out the obtained supernatant into a new Eppendorf tube and stored at -80 °C for further analysis. In determining the concentration of total protein of the samples, the BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA) was used and a microplate reader (Multiskan GO, Thermo Fisher Scientific, and Waltham, MA, USA) at 562 nm was utilized to analyze the color formation. Several essential neurotransmitter levels, which were serotonin (5-HT), acetylcholine (ACh), catalase (CAT), and kisspeptin (KISS), in the brain of the tested zebrafish, were measured by ELISA by following the provided protocol from the manufacturer (Zgenebio Inc., Taipei, Taiwan). The absorbance of the final mixture was analyzed using the same microplate reader at 450 nm wavelength. This experiment was performed in two replicates with a total number of twelve to fourteen fish per group.

2.7. Statistical analysis

All of the statistical analyses and the graphs were conducted and made, respectively, by GraphPad Prism software (GraphPad Software version 8, La Jolla, CA, USA). All of the data are expressed either as mean with SEM or median with quartiles. In analyzing the behavior data, either two-way ANOVA with Geisser-Greenhouse correction or Kruskal-Wallis test, a non-parametric test, was used since it did not require a normal data distribution while for ELISA data, one-way ANOVA, a parametric test, was applied to evaluate the statistical differences between the control and each treated group. The statistical differences between the groups are indicated either with "*" ($p < 0.05$), "**" ($p < 0.01$), "***" ($p < 0.001$), and "****" ($p < 0.0001$).

3. Results

3.1. Dimethomorph reduced locomotion and altered exploratory behaviors of zebrafish

To evaluate the behavior responses of the fish in a novel environment that could be indicated by their locomotor activity and exploratory behaviors, a novel tank test was conducted. Generally, zebrafish prefer to stay at the bottom of the tank with low locomotion due to exposure to a new environment during the first 5–10 min of exposure to the novel tank with a gradual increase of the locomotion and exploratory behavior to the top portion of the tank, which usually indicates a decrement in their anxiety level, as shown by the control group in the current results [15]. After analysis, chronic exposure of dimethomorph was demonstrated to cause a low locomotion activity in the zebrafish. Interestingly, this alteration only occurred in the low-concentration group as shown by statistically lower average speed and swimming time movement ratio with higher freezing time movement ratio than the untreated group (Fig. 2A–C). However, since there was a significant *P* value for interaction in rapid movement time ratio, the effect of the treatment differed depending on the time and the difference of the rapid movement time ratio to the control group was larger in 1 ppm group (mean difference = 0.5557) than for 0.5 ppm (mean difference = 0.1872) (Fig. 2D). This statistical significant of interaction indicates that the other two *P* values are insignificant, thus, the dataset of this behavior endpoint did not further analyzed by Dunnett's multiple comparison test. Meanwhile, in terms of the exploratory behaviors, chronic exposure to dimethomorph in both doses was found to alter these behaviors in zebrafish. Even though no statistical difference was found in the time in top duration and latency to enter the top endpoints, both treated groups showed a decrease in their number of entries to the top, indicating the less vertical transition of the treated fish compared to the control group (Fig. 2E, G, H). In addition, statistical significant results for interaction were also observed in the total distance traveled at the top. Similar to the rapid movement time ratio, a significant *P* value of interaction was also found after a statistical analysis applied in its dataset, indicating that the effect of the treatment was not the same at each timepoint with the difference of this endpoint to the control group was larger in 0.5 ppm group (mean difference = 30.73) than for 1 ppm (mean difference = − 17.10) (Fig. 2F). Thus, the following comparison test was not applied to this dataset. To sum up, while the low concentration of dimethomorph could reduce fish locomotion, dimethomorph in both concentrations was also demonstrated to alter their exploratory behaviors during the novel tank test.

3.2. Dimethomorph altered aggressiveness, fear response, and conspecific social interaction behaviors of zebrafish

To conduct a deeper study in evaluating the chronic effect of dimethomorph in fish behaviors, various behavior tests were carried out, which were aggressiveness, fear response, and conspecific social interaction tests. First, mirror biting assay is an efficient and simple method to analyze the aggressive behavior of fish by measuring their interaction with their reflections from the mirror. From the results, chronic exposure to dimethomorph in both concentrations reduced the intensity of fish interaction with their reflection, which was indicated by statistically lower mirror biting time and longest duration in the mirror side percentages compared to the untreated group (Fig. 3A&B). Furthermore, similar results were also found in the conspecific social interaction test. Zebrafish are well known for their social behaviors since they encourage fish during threats presence and increase fish swimming efficiency [72]. Here, dimethomorph in both doses decreased the interaction time of the tested fish with their conspecific, showed by statistically lower conspecific interaction time percentage and longest duration on the separator side, and higher average distance to the separator (Fig. 3E–G). However, despite its effect on the fish's conspecific social interaction, dimethomorph did not cause any significant effect on fish shoaling behaviors, one of the most zebrafish common social behaviors that is usually shown by the tendency of fish to swim in a group, which was indicated by the similar level of all shoaling behavior endpoints between both treated and control groups (Fig. S1A–D) [70]. Meanwhile, a slight alteration was observed in the fish fear response behavior, the innate behavior of zebrafish when they engage in threats, such as predators. However, this effect was only observed in the high-concentration group with a relatively low magnitude as shown by statistically lower approaching predator time percentage with similar average distance to the separator endpoints to the untreated group (Fig. 3C&D). Taken together, while acute exposure of dimethomorph in the given concentrations did not alter zebrafish shoaling behavior, it significantly affected the aggressiveness, fear response, and conspecific social interaction behaviors of fish.

3.3. Dimethomorph altered the color preferences of zebrafish in several color combinations

Since dimethomorph was shown to alter fish behaviors during the tests, it was intriguing to evaluate the adverse effect of this compound in altering the color preference of zebrafish, considering the well-known color preference of zebrafish, which is red > blue > green > yellow [77]. From the results, clear alterations were observed in three color combinations. First, in the green-blue color combination, the high dose group exhibited a tendency to the green compartment compared to the blue compartment unlike the untreated group (Fig. 4A). In another color combination, which was blue-yellow, abnormalities were also shown by both treatment groups that were indicated by the high preference of these treated groups to stay in the yellow color rather than the blue color

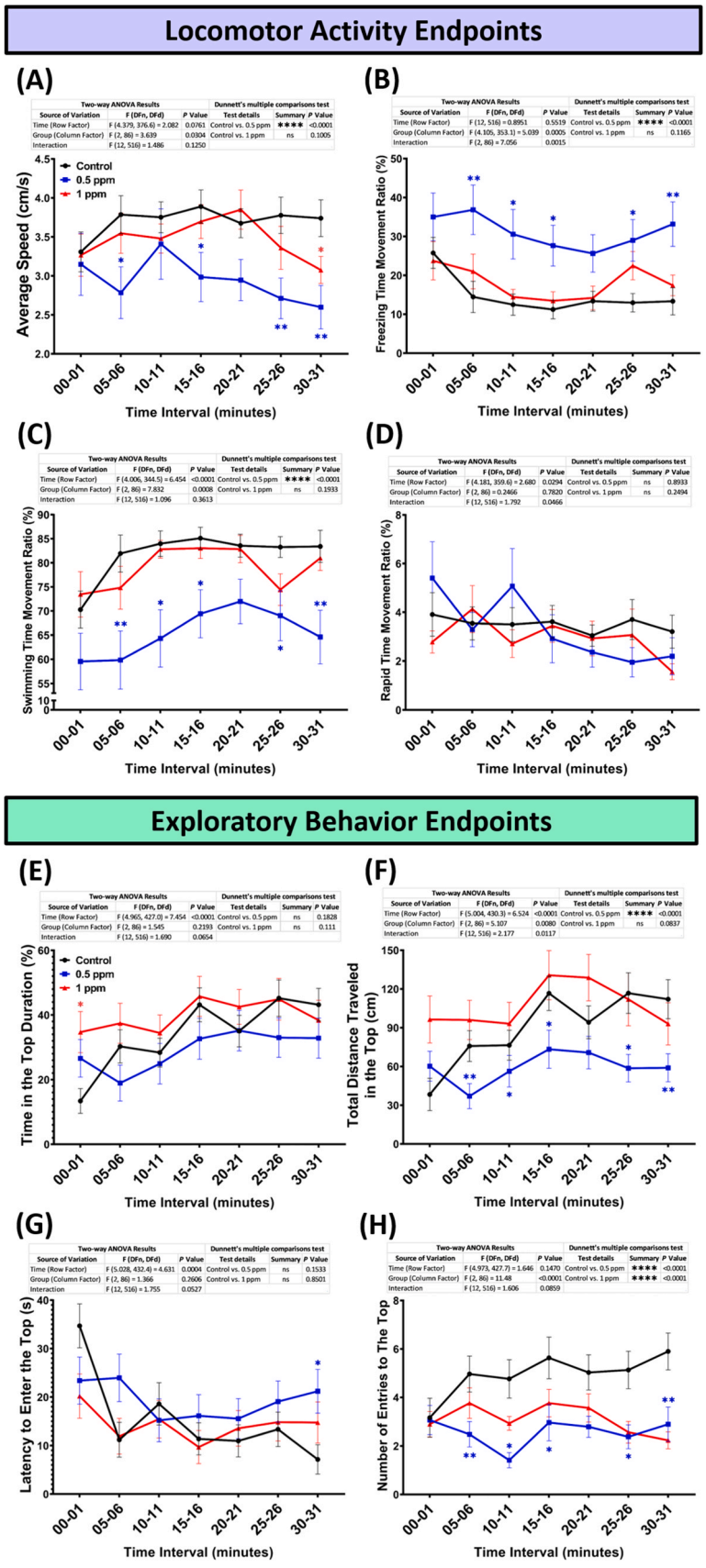

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Fig. 2. Result for locomotor activity endpoints consisting of **(A)** Average speed, **(B)** freezing time movement ratio, **(C)** swimming movement time ratio, and **(D)** rapid movement time ratio, and exploratory behavior endpoints comprised of **(E)** time in the top duration, **(F)** total distance traveled in the top, **(G)** the number of entries to the top, and **(H)** latency to enter the top behavior endpoints of zebrafish after exposure to dimethomorph at two different concentrations; 0.5 (blue) and 1 ppm (red) compared to the control (black). The data are presented as the mean with SEM. Two-way ANOVA with Geisser-Greenhouse correction was used to statistically challenge the results. Additionally, Dunnett's multiple comparison test was performed to observe the effect of each dimethomorph exposure compared to the control group (n control & 1 ppm = 30, n 0.5 ppm = 29; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

compartments (Fig. 4D). Finally, a significant alteration was also displayed during the test in the yellow-red combination. While the control group displayed a clear preference for the red color than the yellow color compartments, both of the treated groups did not show a similar color preference (Fig. 4F). Interestingly, while relatively normal color preferences were shown by both treated groups in the blue-red and green-red compartments (Fig. 4C&E), a less significant green color preference than yellow color was shown in the high dose group compared to the untreated group (Fig. 4B). Nevertheless, the chronic exposure of dimethomorph in the given concentrations altered the color preferences of zebrafish.

3.4. Dimethomorph slightly reduced the short-term memory of zebrafish

The various behavior alterations observed in the previous tests indicated that dimethomorph might already compromise the zebrafish brain, thus, a passive avoidance test was carried out to assess its neurotoxicity in the cognitive performance of zebrafish (Fig. 5A). Based on the results, a statistically higher latency in entering the dark chamber after the training session was shown by the control group, indicating that the fish in this group avoided the dark chamber as they were trained during the training session 24 h prior to the test session (Fig. 5B). However, different responses during the test session were displayed by both treated groups. Based on the statistical analysis result, these groups exhibited a statistically lower level of latency in entering the dark chamber compared to the control group, which might indicate that the treated fish possessed lower memory retention (Fig. 5B). To sum up, exposure to dimethomorph in relatively low doses induced short-term memory loss in adult zebrafish.

3.5. Dimethomorph slightly altered the expression level of several neurotransmitter biomarkers in fish brain tissue

Next, to help in elucidating the adverse effects of dimethomorph in zebrafish behaviors as mentioned in the results above, the expression level of several biomarkers, including serotonin, acetylcholine, catalase, kisspeptin, epinephrine, norepinephrine, dopamine, and melatonin from zebrafish brain tissues was measured by using ELISA. Interestingly, while statistically similar levels of catalase, acetylcholine, serotonin, and melatonin were observed between the control and treated groups (Table 2), a high level of kisspeptin, epinephrine, norepinephrine, and dopamine, was displayed by the treated groups, especially in the high-dose group, compared to the untreated group (Table 2), which might be related to the altered behaviors observed in the behavior tests mentioned above.

4. Discussions

The increase in pesticide usage for the enhancement of agricultural activity has become an essential part of agriculture. However, although they play a major role in achieving higher crop yields, they may also affect the non-target surrounding organisms and negatively affect the natural environment [73,90]. Here, the adverse effects of dimethomorph, one of the most commonly used fungicides, in an aquatic organism were evaluated by conducting a set of comprehensive behavior and biochemical tests. To the best of our knowledge, this is the first study to report the behavior and biochemical toxicities of dimethomorph in adult zebrafish. The current findings are important considering the lack of ecotoxicological data for fungicides on aquatic organisms given their frequency of use and the fact that most do not have specific modes of action, which makes them likely to be toxic to a wide range of organisms [53]. From the results, various behavior alterations, including low locomotion, abnormal exploratory behaviors, less aggressiveness, social interaction, altered fear response, and color preferences, were clearly shown in the treated fish, along with a short-term memory loss, indicating high toxicity of chronic exposure of this pesticide to the zebrafish even though in relatively low concentrations.

4.1. Dimethomorph in altering zebrafish locomotion and exploratory behaviors

First, the behavior alterations were observed in the treated fish during the novel tank test. Here, the treated fish, especially in the low concentration, exhibited a lower locomotion than the untreated fish during the whole session of the test. This result is plausible given that a prior study stated that generally, biopesticides, which fulfill the criteria of an endocrine disruptor, including dimethomorph, could cause harmful effects on the nervous system development in aquatic organisms and this neurotoxicity is responsible for changes in locomotor behavior and an alteration in the regulation of the dopaminergic systems which might also be reflected in the present study by the observed slightly abnormal dopamine level that might indicate a motor impairment related to dopaminergic blockage function [45,46,66,92]. Interestingly, a similar phenomenon was also observed in zebrafish larvae after being exposed to imazalil (IMZ), another fungicide that also can act as both androgen receptor antagonists and steroid synthesis inhibitors as dimethomorph [16]. After being treated with IMZ at 0.3 ppm, decrements in locomotor behaviors were observed, which might have resulted from impaired ACh-mediated neurotransmission in the larvae since a significantly lower mRNA level of AChE was found later in the study [42]. Considering the role of AChE in catalyzing the breakdown of ACh, although it did not reach a statistically different level, the slightly higher ACh level shown in the current study might also play a role in the observed behavior alterations during the novel tank test [13]. Furthermore, the lower locomotion in the treated fish might also have caused them to be reluctant to transitionally swim in the top and bottom portions of the test tank, eventually affecting their exploratory behaviors as shown in the results. Additionally, these abnormalities might also be due to the sedative effect of dimethomorph that was previously demonstrated in mice since, sedation can lead to decreased motility and disturbed behavior as shown in tolylfluanid, an amide fungicide, in a prior study [36,88]. While it does not reach a statistical difference, a substantially higher level of catalase is also worth noting, considering the role of catalase in preventing oxidative stress and cellular damage, which might also hint that there is an increased exposure of reactive oxygen species (ROS) after exposure of dimethomorph, as mentioned in a prior study regarding the pesticides exposure in stimulating the formation of ROS in many aquatic organisms [41,61,82]. Finally, the present study also found more pronounced decrements in locomotion in the low concentration group compared to the high dose group that might indicate non-monotonic dose-response (NMDR) relationship of dimethomorph in the context of locomotion reduction in zebrafish. While the observed relationship is subjected to be further studied in future, a similar phenomenon has been demonstrated in chlorothatonil, another fungicide, and other agrochemicals [76,80]. Based on their studies, NMDR can be caused by multiple mechanism that affect responses

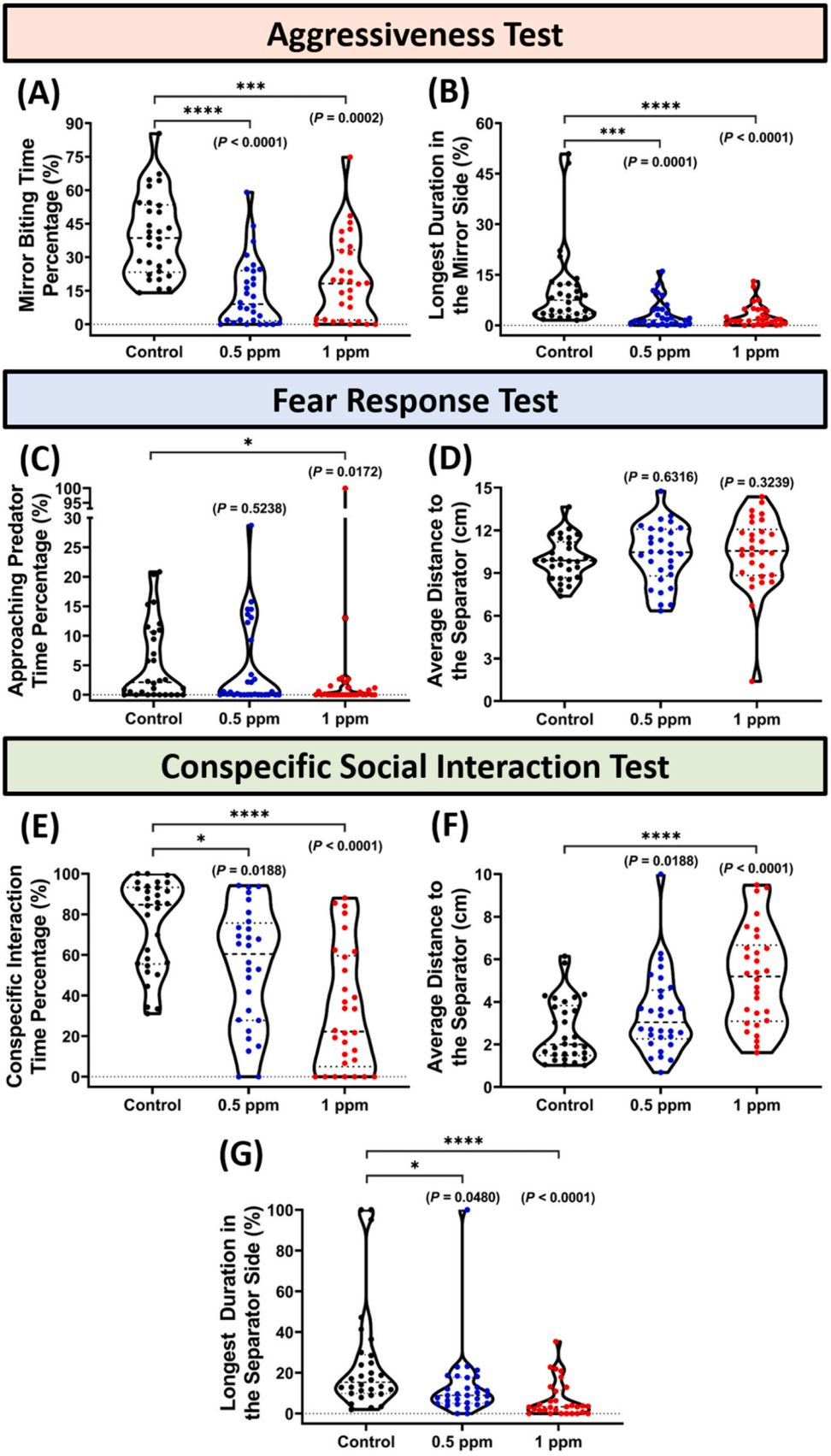

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Fig. 3. Result of zebrafish aggressiveness test consisting of **(A)** Mirror biting time percentage and **(B)** longest duration in the mirror side endpoints from the mirror biting test, fear responses test comprised of **(C)** approaching predator time percentage and **(D)** average distance to the predator's separator endpoints from predator avoidance test, and conspecific social interaction test including **(E)** conspecific interaction time percentage, **(F)** longest conspecific interaction percentage, and **(G)** average distance to the conspecific's separator endpoints from the social interaction test of zebrafish after dimethomorph exposure at two different concentrations: 0.5 (blue) and 1 (red) ppm in comparison to the control group. Data was presented in a violin plot, with a dashed line showing the median and a dotted line showing the interquartile. Kruskal-Wallis test continued with Dunn's multiple comparisons used to analyze the data ($n = 30$, except 0.5 ppm in Fig. 2E–G ($n = 28$); * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$).

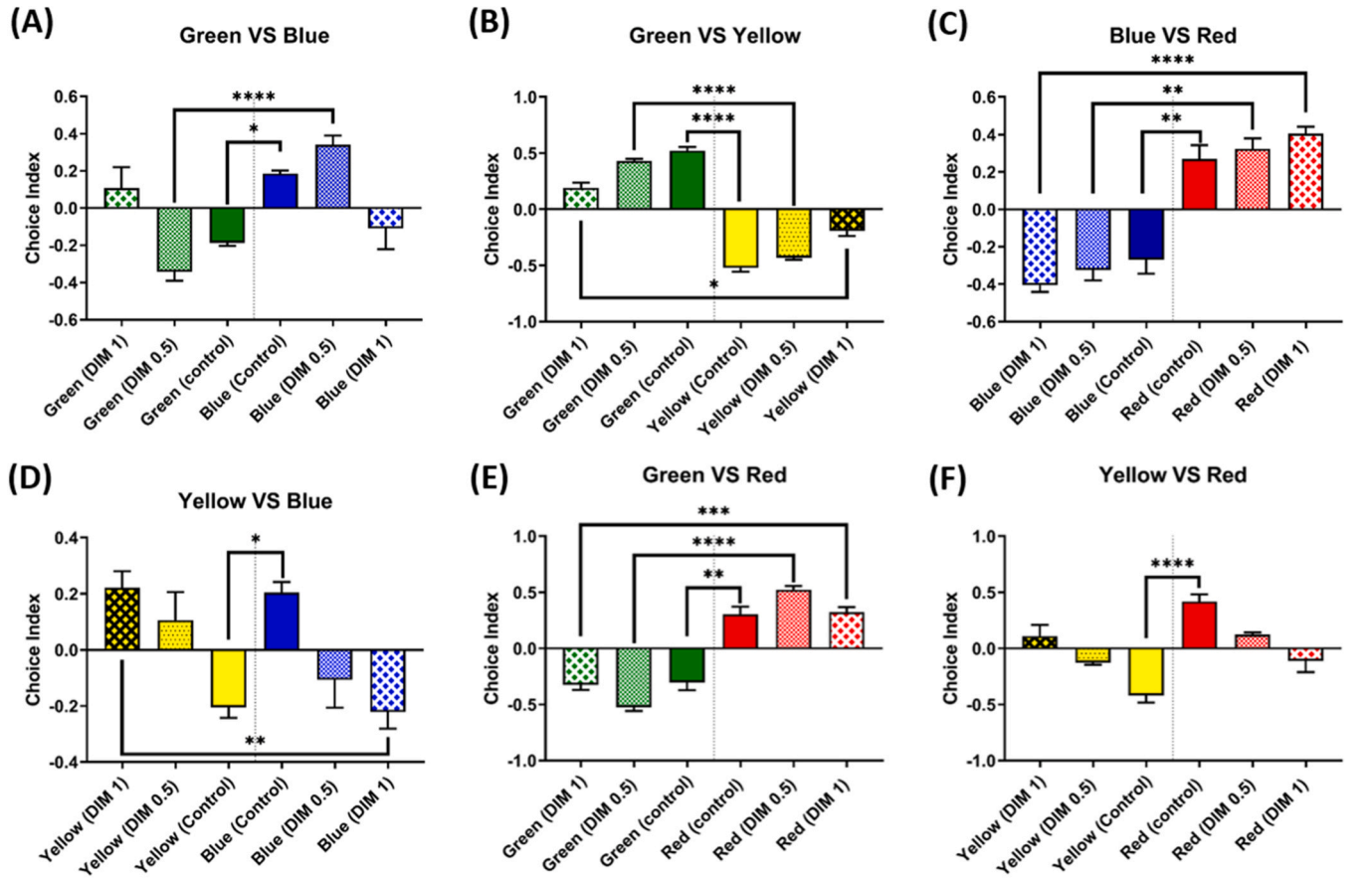

Fig. 4. Comparison of color preferences in zebrafish after being exposed to 0 (Control), 0.5 (DIM 0.5), and 1 ppm (DIM 1) of dimethomorph in four color combinations, including **(A)** green-blue, **(B)** green-yellow, **(C)** blue-red, **(D)** yellow- blue, **(E)** green-red, and **(F)** yellow-red. The data are expressed as mean with SEM and were analyzed by Kruskal-Wallis test continued with Dunn's multiple comparisons test ($n = 12$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

differently at different doses, or by endocrine disruption by substance interacting with the endocrine system, which might account for the current findings since as mentioned above, dimethomorph has been shown to possess endocrine disruption properties [10,87,9].

4.2. *Dimethomorph in affecting social behaviors of zebrafish*

Next, clear behavior alterations were also displayed by the treated fish, especially in the fish social-related behaviors. Here, after exposure, the treated fish exhibited a lower aggression level compared to the control. Interestingly, while in zebrafish, decreased aggressiveness was also observed after exposure to carbendazim, one of the commonest fungicides in agriculture [26], abnormalities in aggressive behavior were also observed in mice after exposure to triadimenol, another fungicide. Later, it was suggested that these alterations were occurred due to their effect on the central nervous system, which somewhat in line with the hypothesized current mechanism of dimethomorph's toxicity mentioned above [36]. The current behavior results were also similar to another prior study which showed that gestational exposure to chlorpyrifos, a pesticide, is associated with heightened levels of anxiety and reduced intensity of aggressive behaviors during lactation in female mice. In addition, delayed initiation of social investigation and a diminished response to social novelty were also displayed by the mice, where similar phenomena were also observed in the dimethomorph-treated fish during the conspecific social interaction assay [84,85]. Considering the well-known important roles of serotonin in many behavioral functions in vertebrates, from ingestive behavior to impulse control, an increase in serotonin neuronal activity and serotonin release, could increase in risk of developing anxiety and depression, [44,56]. Furthermore, anxiety, which could lead to impairment in exploratory and social behaviors, also might be induced by increased levels of dopamine and norepinephrine as demonstrated by a prior study in fish, which was observed in the present study [33,51]. Thus, Along with the transcriptional changes of nervous system-related genes, we hypothesized that the observed adverse effects in the social behaviors of the fish might also be related to the relatively significant increment in the norepinephrine and substantial increase in the serotonin and dopamine levels found in the current study [94]. Nevertheless, the current findings suggested that dimethomorph overall impairs the social competencies of zebrafish.

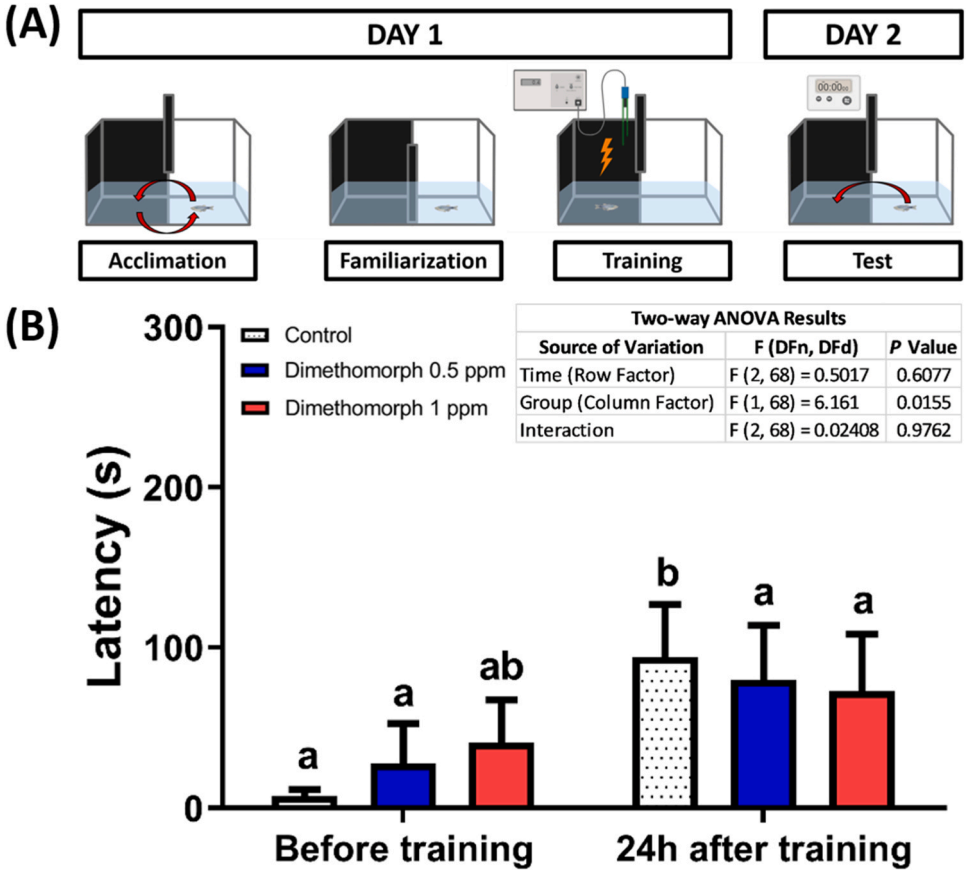

Fig. 5. **(A)** The schematic of the passive avoidance experimental protocol (created with BioRender.com) and **(B)** comparison of the fish latency after being exposed to 0 (Control), 0.5, and 1 ppm of dimethomorph to swim into the dark chamber at 24 h post-training sessions. The data are expressed as mean with SEM and were analyzed by Two-way ANOVA test continued with uncorrected Fisher's LSD test. Different letters (a and b) signify statistical differences ($P < 0.05$) (n control = 14, n 0.5 ppm = 12, n 1 ppm = 11).

Table 2
The levels of several biomarkers, including catalase (CAT), acetylcholine (ACh), 5-HT (serotonin), kisspeptin (KISS), epinephrine (EPI), norepinephrine (NE), and dopamine (DA) in zebrafish brain tissues after being exposed to 0.5 and 1 ppm of dimethomorph, compared to the control, measured using enzyme-linked immunosorbent assay (ELISA). Data are presented as mean with SD and were analyzed by one-way ANOVA followed with Dunnett's multiple comparisons test to evaluate the statistical differences between control group and each treatment group ($n = 4$; * $P < 0.05$).

Biomarkers(Unit)	Control	Dimethomorph			
		0.5 ppm		1 ppm	
	Concentration	Concentration	*P* value	Concentration	*P* value
CAT(CAT (U)/Total Protein (mg))	4.1 ± 0.9599	5.53 ± 0.6695	0.1007	5.231 ± 1.106	0.2020
ACh(ACh (ng)/Total Protein (mg))	39.23 ± 12.09	43.41 ± 6.336	0.8606	49.58 ± 17.60	0.4475
5-HT(5-HT (ng)/Total Protein (mg))	30.69 ± 4.203	39.68 ± 8.432	0.2548	45.18 ± 10.51	0.0586
KISS(KISS (ng)/Total Protein (mg))	5.128 ± 1.268	7.346 ± 1.303	0.0744	7.819 ± 1.403	0.0332 (*)
EPI(EPI (ng)/Total Protein (mg))	2.24 ± 0.5153	3.055 ± 0.6203	0.2068	3.749 ± 0.8493	0.0210 (*)
NE(NE (ng)/Total Protein (mg))	1.343 ± 0.3096	1.824 ± 0.2871	0.2033	2.244 ± 0.5416	0.0192 (*)
DA(DA (pg)/Total Protein (mg))	18.32 ± 3.475	23.57 ± 4.247	0.1338	26.95 ± 3.426	0.0176 (*)
MT(MT (pg)/Total Protein (mg))	11.23 ± 1.992	14.22 ± 1.969	0.3257	16.45 ± 4.498	0.0686

4.3. Dimethomorph in altering the fear response of zebrafish

Further, dimethomorph exposure, specifically in the high dose, also slightly caused a more robust fear response behavior in zebrafish toward the cichlid fish, which was indicated by the less time of the exposed fish to approach the stimulus's side. This finding suggested that dimethomorph could increase fear in zebrafish. In teleosts, behaviors such as flight, fear, avoidance, and anxiety are innate and mostly controlled by nervous system structures [55]. Initially, we suspected that the alterations in the fish fear response might be due to the observed changes in the expression level of kisspeptin in the fish brain since although this neuropeptide is well-known for its role in reproduction, it has been proven to subserve and additional role for fear modulation [65]. However, based on other prior studies, kisspeptin had been shown to decrease fear in fish via the serotonergic system, which contradicted the current results [22,60,65]. Therefore, a deeper investigation was conducted by calculating another behavior endpoint from the test. Later, it was found that the observed abnormal behaviors might be mainly due to the hypoactivity-like behaviors, which were indicated by significantly lower average speed, possessed by the treated fish and thus, causing the fish to infrequently swim in the tank, let alone approach the predator's side (Fig. S1E). We hypothesized that this phenomenon might be

reflected as their response to the stress caused by the dimethomorph exposure, which led to the release of catecholamines, including epinephrine and norepinephrine into the systemic circulation or either due to the sedative effects that could be elicited by dimethomorph as a result of its capability to alter the central nervous system of the fish. Regardless, the slightly higher level of kisspeptin could not compensate for the robust fear response behavior in zebrafish which was mainly due to the low locomotion of the fish that were treated with dimethomorph.

4.4. Dimethomorph in affecting zebrafish color preferences

Innate color preferences are one of the important elements in providing information on how to appropriately respond to environmental stimuli. As vertebrates, zebrafish possess eyes and retinas that are similar to humans and other vertebrates. They have been studied for their color preferences and the results showed that they have natural color preferences without any rewarded stimuli [67]. Here, while many studies have demonstrated different color preferences in zebrafish that might be due to many factors, including population origin and the used color intensity, the control fish displayed a strong preference toward red color, which is in agreement with a prior study [12,74], with overall color preference ranking of red > blue > green > yellow that also consistent with our previous findings [77]. However, after exposure to dimethomorph, their color preference ranking was slightly shifted. While overall they still preferred red color to blue, green, and yellow colors, they did not show any strong preference nor aversion to the other colors as the control did. To the best of our knowledge, this is the first study to evaluate the toxicity of fungicide, specifically dimethomorph, in the color preference of zebrafish. Nevertheless, several studies have addressed similar issues in other animal models. Almeida et al. found that exposure to sublethal doses of the fungicide mixture of thiophanate-methyl and chlorothanlonil changed the abilities of between yellow and blue in stingless bees (*Partamona helleri*) by impairing a bee's ability to discriminate wavelengths or by impairing their adaptive learning [3]. Meanwhile, the presence of chlorpyrifos, one of the most common waterborne pesticides encountered in coral reefs, may lead to a loss or reversal of visual lateralization during a critical step of coral reef fish life cycle which may be due to its neurotoxicity and endocrine disruption characteristics [14]. While further work is still required to determine the mechanisms of dimethomorph in altering the innate color preference of zebrafish, the present study highlighted its effects on the vision-mediated behavior of adult zebrafish which is important for animal survivability considering its association with the ability to help them in identifying food, locating mates, and avoiding toxic prey and predators [74].

4.5. Dimethomorph in reducing the short-term memory of zebrafish

Finally, our hypothesis suggesting the ability of dimethomorph to alter the nervous system in the fish brain is supported by the observed impaired cognitive performance of the treated fish in the passive avoidance test. In the present study, the treated fish showed a significantly reduced memory retention in avoiding the dark chamber than the untreated fish, which was similar to a significant impairment in zebrafish memory that was also observed after exposure to Roundup®, the most popular and widely used herbicide in the majority of the world [18]. Unfortunately, there is limited research that specifically examines the adverse effects of morpholine fungicides on the cognitive abilities of animals. However, previous findings suggested that morpholine derivatives can interact with various central nervous system targets, potentially influencing the cognitive functions of the target individuals [47]. Thus, we suspected that dimethomorph could inhibit acetylcholinesterase activity in the central and peripheral nervous system or by inhibition in the gene transcription that enables 'memory' protein creations as shown by an exposure to deltamethrin, a pyrethroid insecticide, in common carp (*Cyprinus carpio*) [29]. Meanwhile, based on the mice studies, while some of the findings indicated that the cognitive impairment may be closely linked to the Ach function deficient in the synapses, some results also implied that additional mechanisms are implicated as well, including the possibility of the role of oxidative stress in the reported cognitive deficits [4]. In addition, we also suspected that the abnormalities in the dopamine level may also play a role in the fish's cognitive deficit considering the involvement of dopaminergic cells in the central functions, including learning and memory, of the central nervous system [35]. This speculation was supported by a prior study that found an increment in dopamine levels in zebrafish brains with impaired acquisition and consolidation of spatial memory together with reduced locomotion after being chronically treated with paraquat, a toxic herbicide [17]. Nevertheless, the current results suggest that dimethomorph might induce general memory impairment in adult zebrafish.

4.6. Implications for future management of dimethomorph usage in the regulatory context

The present study highlighted the chronic exposure of dimethomorph in the various behaviors of adult zebrafish. In the wild, these behavioral changes can result in a decrement in their overall survivability rate. For instance, less aggression in fish can have negative consequences for fish survival and the overall health of fish populations since less aggressive species might be outcompeted by aggressive species, leading to changes in species composition and diversity within ecosystems [52,62]. Meanwhile, alterations in their social interaction and fear response can also affect their survivability considering affiliation with a social group facilitates foraging and sexual interaction, and less fear response increases the predation risk which consequently leads to a decrease or extinction of populations [64]. Moreover, considering the increased usage of pesticides nowadays and their long life since they are not degraded easily, their exposure to humans has also increased, and even a prior study considered that dimethomorph exposure to humans is not negligible [10]. Fungicide exposure could occur via various routes, including through drinking water, food since pesticides can enter the food chain and cause injury to human and animal health, and non-food exposure such as dermal contact and inhalation, resulting in serious health problems and lead to a number of pathological and disturbed biochemical processes [30,6,78]. Although the Environmental Protection Agency (EPA) reports indicate a low toxicity of dimethomorph to humans, there has been insufficient knowledge of its toxicity to humans, thus, its toxicity cannot be ignored as a prior study demonstrated its potential immunotoxicity in humans [49]. Therefore, these findings signify that, despite its important role in the economic production of a wide range of crops and vegetables, and becoming a large part of successful agriculture industries in many countries [1,19,34], the potential of dimethomorph's negative impacts, especially on aquatic ecosystems cannot be neglected. In addition, since it is impossible to completely restrict the usage of this fungicide due to economical and medical importance, the current findings also underscored the urgent need for comprehensive strategies to address the impact of dimethomorph by formulating evidence-based policies and implementation of effective measures that can reduce the entry of this fungicide into aquatic ecosystems and protect both aquatic life and human health from its detrimental effects on the future management of dimethomorph usage in horticultural crops, considering the risk to the environment posed by the use of fungicides compared to other types of agrochemicals, such as insecticides and herbicides, in horticultural production systems has received relatively little attention [89]. This strategy is also necessary to form a balance between controlling fungal disease risks to crops and protecting terrestrial and aquatic ecosystems, to still maintain the sustainability of horticultural production systems.

5. Conclusions

In summary, the chronic administration of dimethomorph in relatively low doses produced neurotoxic effects characterized by behavioral and cognitive performance changes in adult zebrafish (Fig. 6). These alterations might be related to the observed changes in the expression levels of several important biomarkers in the brain. However, the current study still has some limitations, including the limited biochemical results in explaining the whole mechanism of the observed dimethomorph toxicities, thus, further studies, including examining gene expression changes in key neural pathways to elucidate the underlying molecular or cellular mechanisms driving these alterations, such as oxidative stress, apoptosis, or inflammation, are required to address this matter. Furthermore, the following experiments are still required to elucidate the dose-dependency of dimethomorph behavior toxicities in zebrafish as hinted in the present study, and dose-response relationships across a range of dimethomorph concentrations to understand the threshold levels for toxicity and the severity of effects at varying concentrations. In addition, potential interactions between dimethomorph and other environmental factors also necessary to be assessed in the future, considering previous studies that demonstrated the effect of pH and temperature on the toxicity of pesticide captan and two common fungicides (tebuconazole and copper) to fungi and *Dapnia* spp., respectively [23,81], including its potency of synergistic effect with other contaminants as mentioned in a prior study that found the synergistic effects of the combination of dimethomorph with difenoconazole in embryonic zebrafish [32]. Finally, it is also intriguing to explore whether the observed behavioral changes are reversible after cessation of exposure since previous studies have demonstrated recovery of alterations in biochemical constituents caused by exposure to two organophosphate insecticides, which were chlorpyrifos and monocrotophos, on *Clarias batrachus*, and recovery of cytogenetic damage in *Anguilla anguilla* L that were exposed to a pyrethroid insecticide Decis® after the cessation of the exposure [54,63]. Nevertheless, this study reveals the potency of a serious ecological impact caused by dimethomorph for animal communities living in polluted water, thus, highlighting the importance of the decision-making consideration regarding the use and regulation of dimethomorph in achieving more sustainable aquatic ecosystems.

Ethics Approval

All experimental protocols and standard operation procedures regarding zebrafish were approved by the Committee for Animal Experimentation of the Chung Yuan Christian University (Number: CYCU107030, issue date 24 December 2017). All experimental guidelines were followed and procedures were performed in accordance with the guidelines for laboratory animals.

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Author Contributions

Heong-Ieng Wong and Chung-Der Hsiao designed the study. Gilbert Audira, Hsiu-Chao Chen, Wen-Wei Feng, Michael Edbert Suryanto, Ferry Saputra, and Kevin Adi Kurnia performed data analysis. Franelyne P. Casuga and Chih-Hsin Hung drafted the manuscript. Heong-Ieng Wong,

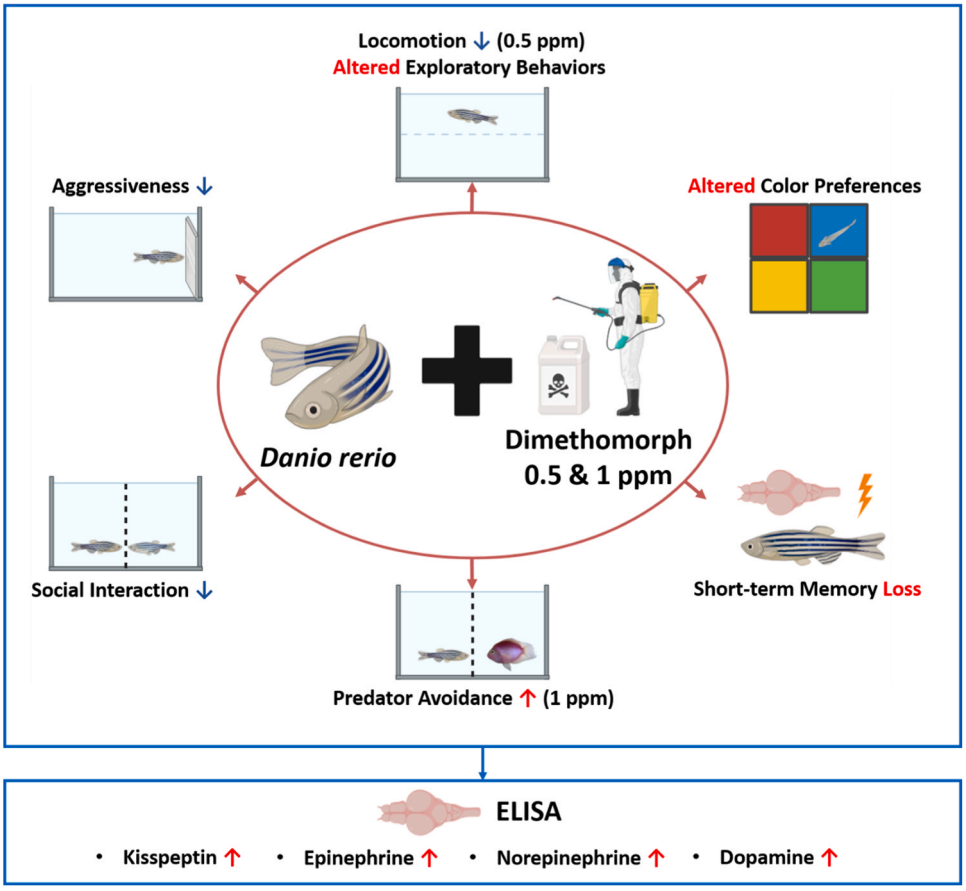

Fig. 6. Summary of the chronic toxicities of dimethomorph via waterborne exposure in adult zebrafish observed in the current study (↑: increased; ↓: decreased; created with BioRender.com).

Gilbert Audira, and Chung-Der Hsiao revised the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Casuga Franelyne P.: Validation, Resources. **Hsiao Chung-Der:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Saputra Ferry:** Visualization, Validation. **Kurnia Kevin Adi:** Visualization, Validation. **Suryanto Michael Edbert:** Visualization, Validation. **Chen Hsiu-Chao:** Methodology, Investigation. **Feng Wen-Wei:** Methodology, Investigation. **Wong Heong-Ieng:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Audira Gilbert:** Formal analysis, Data curation. **Hung Chih-Hsin:** Writing – original draft, 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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2025.101977.

Data availability

Data will be made available on request.

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