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Dichlorvos alters morphology and behavior in zebrafish (*Danio rerio*) larvae☆

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ABSTRACT

Dichlorvos (2,2-dichlorovinyl-dimethylphosphate), an organophosphorus pesticide used for indoor insect and livestock parasite control, is among the most common commercially available pesticides. However, there are significant concerns over its toxicity, especially due to its relative stability in water, soil, and air. Zebrafish, an important developmental model, has been used for studying the effects of toxic compounds. The aim of this study was to evaluate the exposure to dichlorvos at early life stages (1 h postfertilization - 7 days postfertilization) in the zebrafish and its toxicological effects during the development, through morphological (7 days postfertilization), locomotor and social behavior analysis (7, 14, 30, 70, and 120 days postfertilization). Dichlorvos (1, 5, and 10 mg/L) exposure reduced the body length and heartbeat rate at 7 days postfertilization (dpf), as well as the surface area of the eyes (5 and 10 mg/L). The avoidance behavior test showed a significant decrease in escape responses at 7 (1, 5, and 10 mg/L) and 14 (5 and 10 mg/L) dpf zebrafish. The evaluation of larval exploratory behavior showed a reduction in distance traveled, mean speed (1, 5, and 10 mg/L) and time mobile (10 mg/L) between control and dichlorvos groups. In addition, the analysis performed on adult animals showed that the changes in distance traveled and mean speed remained reduced in 30 (1, 5, and 10 mg/L) and 70 dpf (5 and 10 mg/L), recovering values similar to the control at 120 dpf. The social behavior of zebrafish was not altered by exposure to dichlorvos in the early stages of development. Thus, the exposure to organophosphorus compounds at early stages of development induces an increased susceptibility to behavioral and neuronal changes that could be associated with several neurodegenerative diseases.

1. Introduction

The central nervous system (CNS) has sophisticated functions such as the ability to control behavior, learning, speech, memory, social and routine activities (Bjørling-Poulsen et al., 2008; Julvez and Grandjean, 2009). The primitive human brain develops into a complex organ, during prenatal life, from the ectodermal cells of the embryo. To achieve its full development, connections must be made along precise paths aimed at communication between cells. Thus, the brain becomes an organ with billions of highly specialized and interconnected cells, such as neurons, astrocytes and glial cells (Bjørling-Poulsen et al., 2008; Rice and Barone, 2000; Rodier, 1995). The time for these processes to occur must be rigid and controlled in order to achieve the correct sequence and timing in each stage of development. If these processes are interrupted or inhibited in any of these stages, the consequences may be permanent (Bjørling-Poulsen et al., 2008; Rice and Barone, 2000).

Neurotoxic agents can cause brain damage, especially if exposure occurs during the early developmental period (Grandjean and Landrigan, 2006). Among the documented agents are industrial chemicals, such as pesticides, organic solvents or metals, which are commonly found in air, food, water or soil. In addition, smoking,

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alcohol, and some drugs are also considered neurotoxic agents (Huizink and Mulder, 2006; Julvez et al., 2007). If the development processes of the immature nervous system are impaired, the effects can be long-lasting and permanent, leading to teratogenicity, deficits in mental capacity and behavioral disorders (Andersen et al., 2000; Bellinger et al., 1987; Debes et al., 2006; Grandjean and Landrigan, 2006; Hu et al., 2006; Huizink and Mulder, 2006; Julvez et al., 2007). In social aspects, the consequences of neurotoxicity during development include increased probability of school failure, decreased economic productivity, and increased risk of antisocial behavior or sociopaths (Grandjean and Landrigan, 2006).

Humankind has practiced agriculture for over ten thousand years; however, the intensive use of agrochemicals to control crop pests and diseases has existed for just over half a century. The main and best-known class of agricultural pesticides comprises organophosphates, which exert a variety of adverse effects on health and the environment (Coggon, 2002; Kachaiyaphum et al., 2010; Peter et al., 2010). This class acts by inhibiting acetylcholinesterase, causing accumulation of acetylcholine and hyperstimulating the cholinergic receptors, triggering an acute cholinergic crisis, able to induce convulsions, alteration in the ventilation, and metabolic imbalance (Bajgar, 2004; Barthold and Schier, 2005; Jokanović, 2009). Dichlorvos is a highly toxic insecticide belonging to the organophosphates family. It is used in the control of insects on rice, cereals, fruits, vegetables, soy, and cotton, and is effective against aphids, mites, caterpillars, triplets, whiteflies, among others (Binukumar et al., 2012). This organophosphate causes intoxication through of inhalation, cutaneous absorption or ingestion, being persistent of degradation under natural environmental conditions, and may be deposited in the fruit or water (Ning et al., 2012). Rats chronically exposed to high concentration of dichlorvos, when treated orally and intraperitoneally, showed liver dysfunctions and somatic mutations (Pletsa et al., 1999; Binukumar et al., 2010a). In addition, the chronic exposure to dichlorvos induced nigrostriatal neurodegeneration, oxidative damage and significant behavioral impairments (Binukumar et al., 2010b).

Other important concern related to the frequent application of pesticides, such as organophosphates, is their accumulation in groundwater, reaching sources of water and sediment, which may cause ecotoxicological effects (Leong et al., 2007; Luo et al., 2008; Watson et al., 2014). Studies using model organisms, such as zebrafish, to assess the toxicology of chemicals present in the aquatic ecosystem can provide data for environmental risk assessment (Bortolotto et al., 2014). In addition, late evaluation of exposure to organophosphates at the early stages of development is important to elucidate the risks that these agents may bring in the future. Thus, in this study, we assessed the toxicological effects of dichlorvos exposure during zebrafish development, through the analysis of morphological measures and locomotor and social abilities of this species.

2. Materials and methods

2.1. Dichlorvos exposure

Adult wild-type zebrafish (*Danio rerio*), maintained in recirculating systems (Zebtec, Tecniplast, Italy), were mated at a ratio of 1:2 for female and males (Westerfield, 2000). The fertilized eggs were placed in Petri dishes (30 eggs per dish), and exposed to dichlorvos (2,2-Dichlorovinyl dimethyl phosphate; PESTANAL®, Sigma-Aldrich, St. Louis, MO) at the concentrations of 0 (control group), 1, 5, and 10 mg/L (Şişman, 2010; Zhang et al., 2010) for seven days (from 1-h post fertilization (hpf) to 7 dpf). After the exposure, the larvae were maintained in 3 L-aquariums with water, in a density of one animal per 60 mL (Fig. 1) until 30 dpf, when the density change to one animal per 200 mL until 120 dpf. Throughout the development, the animals were subject to different toxicological analyses. All details about zebrafish maintenance and experimental protocols are provided in the Supporting Information (SI Text). This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado - SISGEN (Protocol No. A3B073D).

2.2. Morphological analyses

The toxicological effects of dichlorvos were observed by alteration on morphology and heartbeat rate in 7 dpf larvae (Altenhofen et al., 2017a; Nabinger et al., 2018). Furthermore, the LC50 was determined in animals during dichlorvos exposure.

2.3. Behavioral analyses

The exploratory behavior of the larvae was based on Colwill and Creton (2011) and analyzed at 7 and 14 dpf ($n = 30$). After the analysis of exploratory behavior, the animals were placed in 6-well plate (five larvae per well, $n = 40$) over a LCD monitor for measuring cognitive ability and avoidance responses to a visual stimulus (a 1.35 cm diameter red bouncing ball) (Pelkowski et al., 2011). The locomotor behavior of the adult animals was observed at 30, 70, and 120 dpf ($n = 20$), and social preference and aggression were analyzed at 70 and 120 dpf ($n = 30$) (Levin et al., 2007; Gerlai et al., 2000).

2.4. Determination of acetylcholinesterase activity

The experiments were conducted using samples containing a pool of two adult brains and 10 larval brains. The animals were euthanized by hypothermal shock (Matthews and Varga, 2012; Wilson et al., 2009), and the protein was measured by the Coomassie blue method (Bradford, 1976). AChE activity was determined according to the method proposed by Ellman et al. (1961) with minor modifications. AChE activity was expressed as micromoles of thiocholine (SCh) that were released per hour per milligram of protein.

2.5. Statistical analysis

Differences in morphological measures, heartbeat rate, and behavioral assessment were evaluated by one-way ANOVA followed by a post-hoc Tukey's test, and the significance level was set at $p < 0.05$.

3. Results

The LC50 values after 24–168 h were 62.4–21.5 mg/L, respectively (Table S1). Morphological alterations were analyzed in larvae exposed to dichlorvos during initial phases of developmental (1 hpf - 7 dpf, Fig. 2a). The results showed that the dichlorvos exposure caused a reduction in body length of 7 dpf larvae in all concentrations tested (1, 5, and 10 mg/L) ($F_{(3,236)} = 44.8$, $R^2 = 0.3628$, $p < 0.0001$; Fig. 2b). However, there were no differences in ocular distance between the control and the dichlorvos-treated groups ($F_{(3,236)} = 1$, $R^2 = 0.01313$, $p = 0.4$; Fig. 2c). On the other hand, the surface area at 7 dpf animals also was reduced at 5 and 10 mg/L, but there was no difference in concentration of 1 mg/L when compared to the control group ($F_{(3,236)} = 44.3$, $R^2 = 0.3603$, $p < 0.0001$; Fig. 2d). The results of teratogenic effects on cardiac system demonstrated a decrease in heartbeat rate of larvae (7 dpf) exposed to dichlorvos at all concentrations analyzed ($F_{(3,116)} = 196$,

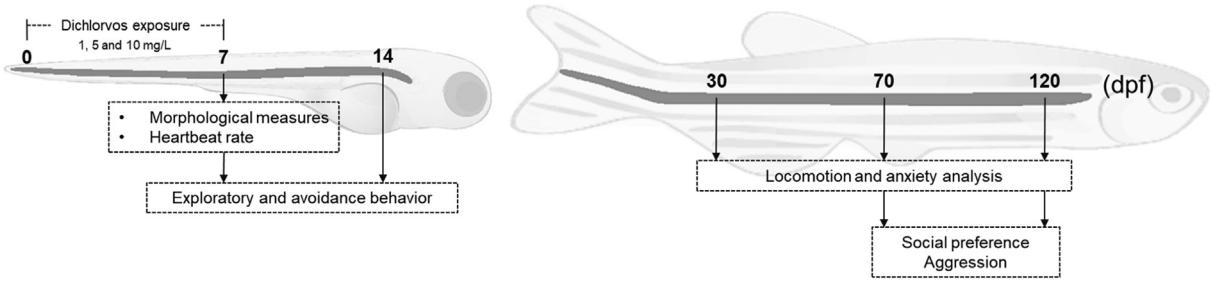

Fig. 1. Timeline of the experimental design. Zebrafish larvae were exposed to water (control group) or dichlorvos at concentrations of 1, 5 and 10 mg/L. The exposure occurred from 1 hpf to 7 dpf. At 7 dpf, morphological measures and heartbeat rate were analyzed as well as exploratory and avoidance behavior at 7 and 14 dpf. Locomotion and anxiety analysis were performed at 30, 70 and 120 dpf. Moreover, social preference and aggression were analyzed at 70 and 120 dpf.

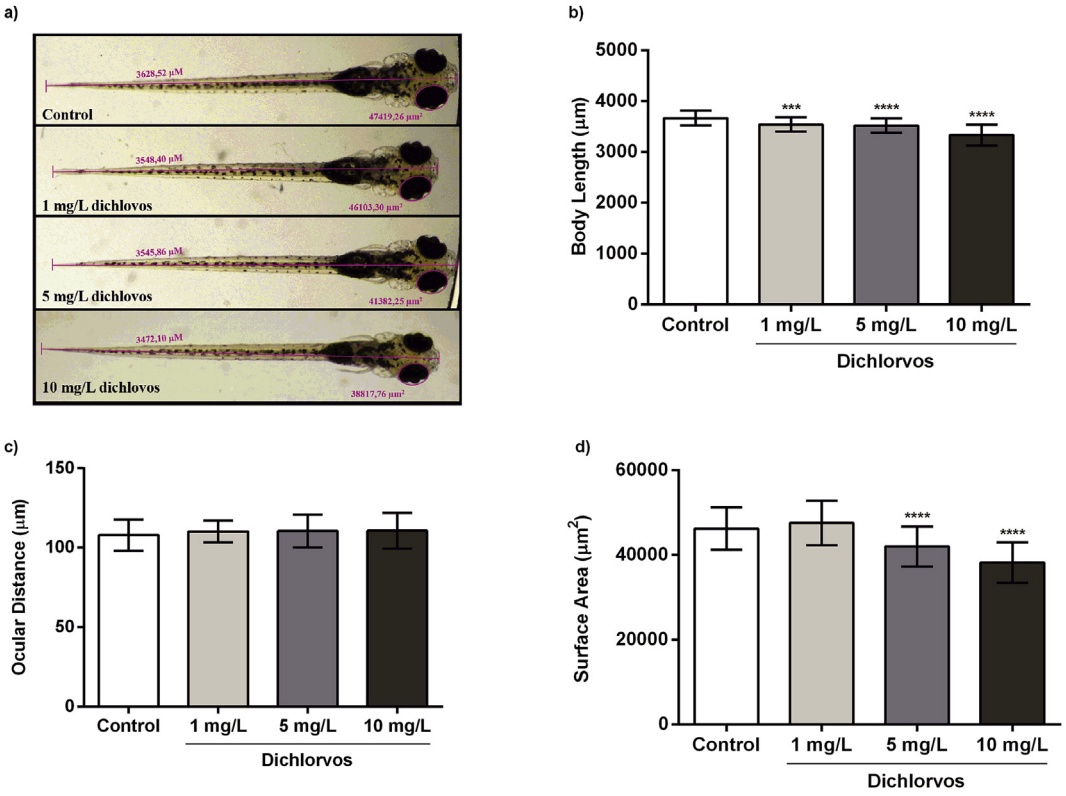

Fig. 2. Pictures (a) represent morphological alterations induced by dichlorvos exposure at 7 dpf, with scale showing the body length and surface area of the larvae control and exposed to concentrations of 1, 5 and 10 mg/L. Effects of dichlorvos exposure in early stages of development on body length (b), ocular distance (c) and surface area of the eyes (d) were evaluated in zebrafish larvae at 7 dpf. Data are expressed as the mean ± SD from 60 animals tested individually for each group and were analyzed by one-way ANOVA, followed by Tukey *post-hoc* test. ***$p < 0.001$, ****$p < 0.0001$.

$p < 0.0001$; Fig. 3).

The exploratory behavior of the larvae was evaluated at 7 and 14 dpf. Animals exposed to dichlorvos at 1, 5, and 10 mg/L displayed reduced traveled distances in relation to the control group at 7 and 14 dpf ($F_{(3,116)} = 58.1$ and $F_{(3,116)} = 12$, respectively; $p < 0.0001$; Fig. 4a). The parameter of mean speed was also decreased at 7 and 14 dpf in all dichlorvos groups ($F_{(3,116)} = 24.8$, $p < 0.0001$, $F_{(3,116)} = 6.8$, $p < 0.001$, respectively; Fig. 4b). Moreover, animals exposed to dichlorvos at concentration of 1 and 5 mg/L showed no significant difference in time mobile at both 7 and 14 dpf; however, 10 mg/L dichlorvos induced reduced this parameter at both ages ($F_{(3,116)} = 6.1$, $p < 0.001$ and $F_{(3,116)} = 3.7$, $p < 0.05$, respectively; Fig. 4c) when compared to the control group. The measure of time spent outside well area showed no significant difference in both 7 and 14 dpf between treated and control groups ($F_{(3,116)} = 0.5$, $p = 0.7$ and $F_{(3,116)} = 0.2$, $p = 0.9$, respectively; Fig. 4d).

Fig. 5 shows the ability of larvae to escape from an aversive stimulus. There was a decrease in the percentage of animals with escaping responses from an aversive stimulus in all dichlorvos concentrations tested at 7 dpf. It was seen that the animals exposed to the pesticide, remained about 60% of the time in the area without the stimulus whereas the control group persisted at 85% of the time. At 14 dpf, there was also a reduced percentage of animals that escape from the stimulus (75 and 70%) at dichlorvos concentrations of 5 and 10 mg/L, respectively, when compared to the control group (85%). However, no difference was observed in animals exposed to 1 mg/L dichlorvos (79%).

Locomotor changes were verified in animals at 30, 70, and 120 dpf exposed to dichlorvos during initial phases of developmental (1 hpf - 7 dpf). The animals exposed to this insecticide at all

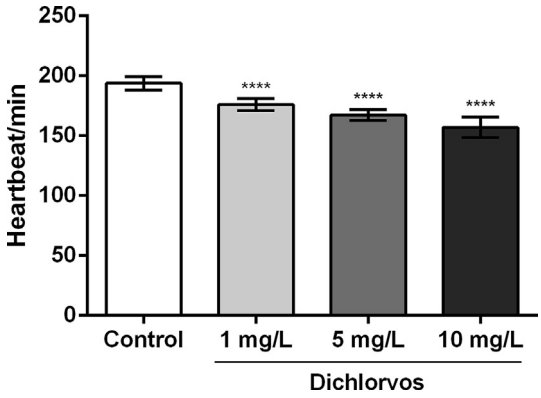

Fig. 3. Effects of dichlorvos exposure in early stages of developmental on heartbeat rate was evaluated in zebrafish larvae at 7 dpf. Data are expressed as the mean ± SD from 30 animals tested individually for each group and were analyzed by one-way ANOVA, followed by Tukey *post-hoc* test. $^{****}p < 0.0001$.

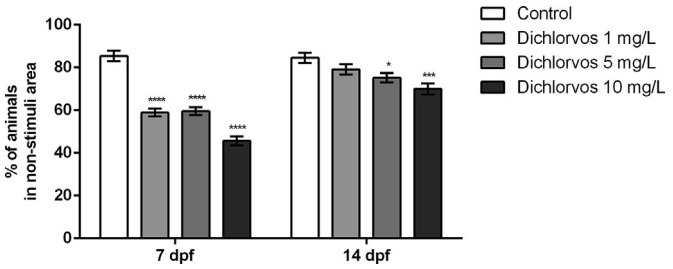

Fig. 5. Avoidance behavior of dichlorvos treated and control groups in early stages of development. Zebrafish larvae were evaluated at 7 and 14 dpf. Data are expressed as the percentage mean ± SEM from 40 animals tested individually for each group and were analyzed by one-way ANOVA, followed by Tukey *post-hoc* test. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

concentrations displayed decreased distance traveled (Fig. 6a) and mean speed (Fig. 6b) at 30 dpf ($F_{(3,76)} = 46.8$ and $F_{(3,76)} = 47.2$, $p < 0.0001$; respectively). However, only the higher dichlorvos doses (5 and 10 mg/L) were able to reduce distance traveled and mean speed at 70 dpf ($F_{(3,76)} = 17.5$ and $F_{(3,75)} = 13.8$, $p < 0.0001$; respectively). At 120 dpf, there was no significant difference between treated animals and control group at distance traveled and mean speed measures ($F_{(3,76)} = 1.472$, $p = 0.2$ and $F_{(3,76)} = 2$, $p = 0.1$; respectively). The time mobile (Fig. 6c) and time spent in bottom zone (Fig. 6d) did not present significant changes between treated and control groups analyzed at 30 ($F_{(3,76)} = 2$, $p = 0.1$ and $F_{(3,76)} = 0.5$, $p = 0.7$; respectively), 70 ($F_{(3,76)} = 2.4$, $p = 0.1$ and $F_{(3,76)} = 3.6$, $p = 0.1$; respectively), and 120 dpf ($F_{(3,76)} = 0.9$, $p = 0.5$ and $F_{(3,76)} = 2.5$, $p = 0.2$; respectively).

Social preference and aggression were assessed only at 70 and 120 dpf, because the social and aggressive behavior of zebrafish is not completely developed at 7, 14, and 30 dpf (Buske and Gerlai, 2012). Dichlorvos exposure during initial phases of development (1 hpf - 7 dpf) was not able to alter these parameters at 70 ($F_{(3,76)} = 1.3$, $p = 0.3$ and $F_{(3,76)} = 0.4$, $p = 0.8$; respectively) and 120 dpf ($F_{(3,76)} = 2.9$, $p = 0.1$ and $F_{(3,76)} = 3.3$, $p = 0.1$; respectively) (Table S2 and Fig S1).

We have tested the AChE activity in 7 dpf larvae immediately after dichlorvos exposure (1 hpf - 7 dpf) and there were no

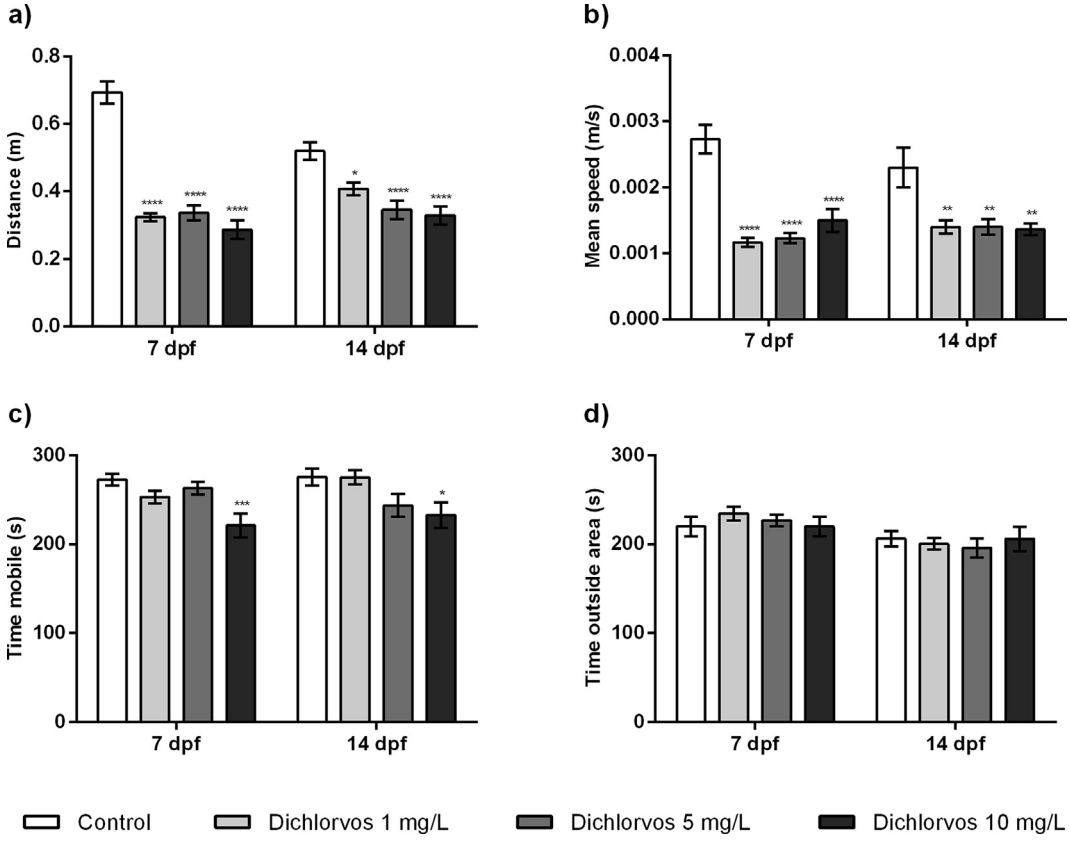

Fig. 4. Exploratory behavior of dichlorvos treated and control groups in early stages of developmental. Distance traveled (a), mean speed (b), time mobile (c) and time outside area (d) were evaluated at 7 and 14 dpf. Data are expressed as the mean ± SEM from 30 animals tested individually for each group and were analyzed by one-way ANOVA, followed by Tukey *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

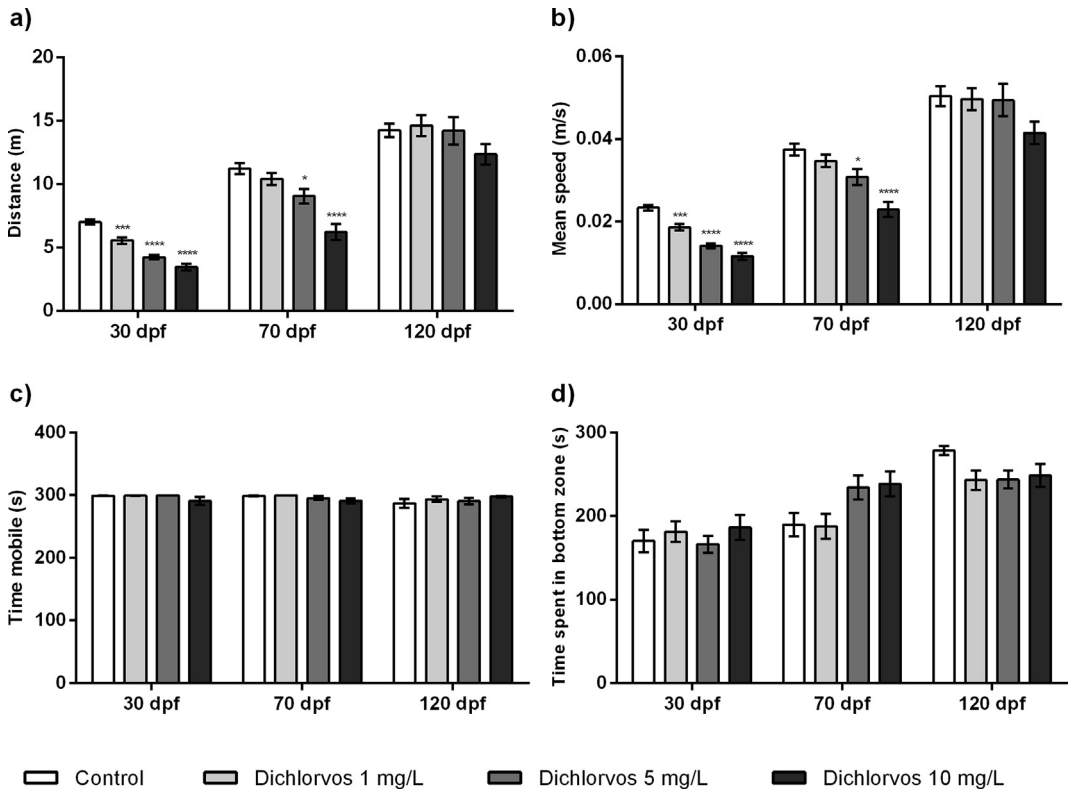

Fig. 6. Locomotor behavior of control and dichlorvos exposure in early stages of developmental performed in zebrafish adult. Distance traveled (a), mean speed (b), time mobile (c) and time spent in bottom zone (d) were evaluated at 30, 70 and 120 dpf. Data are expressed as the mean ± SEM from 20 animals tested individually for each group and were analyzed by one-way ANOVA, followed by Tukey *post-hoc* test. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

significant changes on AChE activity in the concentrations tested. AChE activity in 120 dpf adult zebrafish exposed to dichlorvos at early stages of the development were not altered (data not show).

4. Discussion

This study performed a behavioral screening at distinct developmental stages in zebrafish at 7, 14, 30, 70, and 120 dpf to establish the neurotoxicological effects induced by exposure to dichlorvos in the early stages of development (1 hpf - 7 dpf). Dichlorvos-exposed animals showed reduced body length at all concentrations tested (1, 5, and 10 mg/L), as well as decreased heartbeat rate. The two largest doses (5 and 10 g/L) were able to decrease the surface area of the eyes of the zebrafish larvae. Watson et al. (2014) verified the action of three organophosphates (chlorpyrifos, dichlorvos, and diazonon) on the zebrafish larval cardiac morphology and found a decrease in heart rate at 3 dpf after exposure to chlorpyrifos and dichlorvos at 100 μM concentration. In addition, diazonon, at the same concentration, produced cardiac edema and blood flow failure in 24 hpf larvae (Watson et al., 2014). Our findings demonstrate that, as observed for other toxic agents, dichlorvos exposure changed the heart rate and body size that may compromise the animal development.

Motor alterations have already been observed after exposure of zebrafish to toxic compounds (Altenhofen et al., 2017a,b; Lutte et al., 2015; Watson et al., 2014). This study investigated the ability of dichlorvos to alter the locomotor and cognitive parameters of the animal shortly after its exposure (7 dpf) and analyzed possible long-term effects caused by this insecticide (14, 30, 70, and 120 dpf). It was seen that immediately after the dichlorvos exposure, the 7 dpf larvae had a deficit in locomotor capacity, as well as one week after being removed from the exposure environment at 14 dpf. Other studies already showed that organophosphates may cause reduction of the swimming capacity of zebrafish larvae at 5 dpf (Richendrfer and Creton, 2015; Watson et al., 2014). Moreover, our study also observed if the exposure to dichlorvos, in the early stages of development, could modify the cognition of the animal. Thus, at 7 and 14 dpf, the zebrafish larval escape ability was evaluated through avoidance behavior task. Animals exposed to the insecticide had decreased escape capacity at all concentrations tested (1, 5, and 10 mg/L) in relation to the control group at 7 dpf. At 14 dpf, only the two highest doses (5 and 10 mg/L) induced this behavior. A study conducted with organophosphorus chlorpyrifos (0.001, 0.01, and 0.1 μM) showed that larvae exposed to 7 dpf did not change the preference in either edge well site (Richendrfer et al., 2012).

Zebrafish locomotor patterns were analyzed at 30, 70, and 120 dpf after dichlorvos exposure at early stages of development to evaluate if locomotor changes were permanent. At 30 dpf, the animals showed a decrease in the distance traveled and mean speed at all concentrations tested (1, 5 and 10 mg/L). At 70 dpf, the distance traveled, and mean velocity remained altered only at higher doses (5 and 10 mg/L). Meanwhile, at 120 dpf, dichlorvos-exposed zebrafish had locomotion equal to the control in all parameters analyzed. Jantzen et al. (2016) showed that exposure up to 5 dpf to organofluorine compounds (2 μM) was able to reduce the distance traveled by zebrafish with 150 dpf; however, unlike our study, these compounds caused at long-term increased mean speed and anxiety in animals with 150 dpf. The changes in locomotor parameters possibly are not related to an inhibition of AChE activity once there are no changes in this enzyme activity after dichlorvos exposure in zebrafish larvae and adults.

The social preference and aggression parameters were evaluated at 70 and 120 dpf since the social behavior of zebrafish is not

developed in the early stages of development, acquiring shoaling behavior after 40 dpf (Buske and Gerlai, 2012; Dreosti et al., 2015). Our study observed that exposure to dichlorvos during the first stages of development did not alter the social preference and aggressive behavior at 70 and 120 dpf. In contrast to our findings, Jantzen et al. (2016) demonstrated that zebrafish larvae exposed to organofluorine compounds (2 μM) at 0 to 5 dpf presented increased aggressive behavior at 150 dpf.

A study conducted by Dambska and Maślińska (1988) showed that dichlorvos exposure in early stages of development (6th to 15th day of life) in rabbits was able to cause pathological changes in all neuroectodermal elements, such as cerebral and cerebellar cortex and corpus callosum evaluated by electron microscope. Furthermore, the administration of dichlorvos in guinea pigs between day 42 and 46 of gestation induced a severe reduction in brain weight in offsprings examined at birth (Mehl et al., 1994). Moreover, the chronic exposure to dichlorvos for 12 weeks causes microglial activation including induction of NADPH oxidase and a selective loss of dopaminergic neurons in rats (Binukumar et al., 2011). In addition, dichlorvos-exposed animals presented a loss of 60–80% of the nigral dopamine neurons and 60–70% reduction in striatal dopamine and tyrosine hydroxylase levels. These animals also exhibited α -synuclein and ubiquitin positive inclusions along with swollen, dystrophic neurites and mitochondrial abnormalities like decreased complex I and IV activities, increased mitochondrial size, axonal degeneration and presence of electron dense perinuclear cytoplasmic inclusions in the substantia nigra of rats (Binukumar et al., 2010b). Taken together, these results suggest that dichlorvos causes significant changes in the morphology of the CNS, which may persist throughout development. Our data showed that, after the dichlorvos removal, the treated animals at early stages of the development develop an adaptive mechanism, recovering the locomotor behavior at adult stage (120 dpf). Other possibility is a decrease in the dichlorvos neurotoxicity throughout the development, induced by neurogenesis and synaptic plasticity.

In summary, dichlorvos exposure, during the early stages of development, changed morphological and cardiovascular patterns at 7 dpf and decreased the locomotor and cognitive capacity at larvae of 7 and 14 dpf. During development, the motor parameters remained reduced (30 and 70 dpf), presenting a reversal at 120 dpf, when all the locomotor patterns of the treated animals are similar to the control group. Further studies are required to clarify the neurotransmission systems implicated in morphological and behavioral changes induced by dichlorvos exposure during the early stages of development.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.11.095.

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