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Nickel exposure alters behavioral parameters in larval and adult zebrafish

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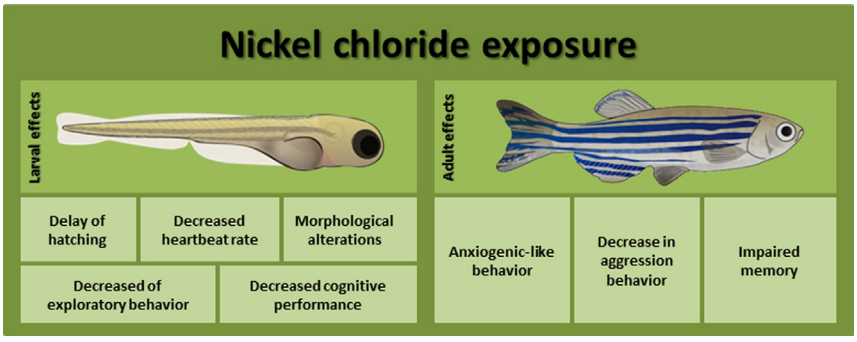
HIGHLIGHTS

- Acute and subchronic treatments with $NiCl_2$ induced increase in zebrafish body nickel levels.
- Delayed hatching and decreased heartbeat were observed in Ni- treated larvae.
- $NiCl_2$ treatment induced morphological alterations in zebrafish larvae.
- Changes in locomotor behavior were observed in Ni- treated larvae and adults.
- $NiCl_2$ caused a decrease in aggression and impaired memory in adult zebrafish.

GRAPHICAL ABSTRACT



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ABSTRACT

Nickel is a heavy metal that, at high concentrations, leads to environmental contamination and causes health problems. We evaluated the effects of $NiCl_2$ exposure on cognition and behavior in larval and adult zebrafish. Larval and adult zebrafish were exposed to $NiCl_2$ concentrations (0.025, 2.0, 5.0, and 15.0 mg/L) or water (control) in two treatment regimens: acute and subchronic. Larvae were exposed to $NiCl_2$ for 2 h (acute treatment: 5-day-old larvae treated for 2 h, tested after treatment) or 11 days (subchronic treatment: 11-day-old larvae treated since fertilization, tested at 5, 8 and 11 days post-fertilization, dpf). Adults were exposed for 12 h (acute treatment) or 96 h (subchronic treatment) and were tested after the treatment period. In both regimens, exposed zebrafish showed concentration-dependent increases in body nickel levels compared with controls. For larvae, delayed hatching, decreased heart rate and morphological alterations were observed in subchronically treated zebrafish. Larvae from subchronic treatment tested at 5 dpf decrease distance and mean speed at a low concentration (0.025 mg/L) and increased at higher concentrations (5.0 and 15.0 mg/L). Subchronic treated larvae decrease locomotion at 15.0 mg/L at 8 and 11 dpf, whereas decreased escape responses to an aversive stimulus was observed at 2.0, 5.0 and 15.0 mg/L in all developmental stages. For adults, the exploratory behavior test showed that subchronic nickel exposure induced anxiogenic-like behavior and decrease aggression, whereas impaired memory was observed in both treatments. These results indicate that exposure to nickel in early life stages of zebrafish leads to morphological alterations, avoidance response impairment and locomotor deficits whereas acute and subchronic exposure in adults resulst in anxiogenic effects, impaired memory and decreased aggressive behavior.

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These effects may be associated to neurotoxic actions of nickel and suggest this metal may influence animals' physiology in doses that do not necessarily impact their survival.

1. Introduction

Nickel is an abundant metal in the earth's crust. The concentrations of nickel have increased over the last few years as a result of metal exploitation and industrial development. Owing to the unique physical and chemical properties of nickel, its alloys are used extensively in the manufacturing of jewelry, medical implants, stainless steel and batteries, as well as in the plating industry and agricultural activities (Alsop and Wood, 2011; Denkhaus and Salnikow, 2002; Muñoz and Costa, 2012; Zhou et al., 2008). As a result, nickel extraction and use (processing and recycling) have led to increased levels of this metal in biogeochemical cycles and, consequently, environmental contamination and occupational exposure, since nickel pollution occurs in water, soil and air (Muñoz and Costa, 2012; Wu et al., 2016). Importantly, metals can bioaccumulate in living organisms, magnify in the food chain, and threaten human health. Various harmful effects of nickel exposure have been reported in humans as a result of occupational and environmental exposure, including contact dermatitis, respiratory diseases, renal and cardiovascular injuries, serious and irreversible neurological damage, abnormal fetal development and infertility and immunodeficiency (Guo et al., 2015; Marchetti, 2014; McDermott et al., 2015, Muñoz and Costa, 2012; Saito et al., 2016; Zhou et al., 2008).

Recently, the potential deleterious effects of nickel have drawn more and more attention owing the wide occurrence of nickel pollution in aquatic systems. Metals can enter aquatic systems naturally by slow leaching from soil/rock into the water; this route usually produces only a low concentration of nickel causing no serious deleterious effects on the health of organisms (Zhou et al., 2008). Although some metallic compounds can be strongly absorbed onto suspended particles and sediments, they can be released into the water under suitable conditions (as solubility, pH and oxidation-reduction potential), leading to further contamination of aquatic environments and high toxicity to organisms (Zhao et al., 2009; Zhou et al., 2008). Many countries have reported high levels of nickel in lakes and rivers (Gissi Stauber et al., 2016; Li et al., 2017; Niu et al., 2015; Villanueva and Botello, 1998; Skerfving et al., 1999; Wang et al., 2016) and several alterations have been observed in aquatic animals, particularly fishes. Reported effects of nickel exposure in fishes include morphological alterations such as skin abnormalities, modifications in gill and muscle development, and molecular changes, such as increased oxidative stress and apoptosis and changes in gene expression. Increased mortality, delayed hatching and decreased locomotor activity have also been reported (Defo et al., 2014; Hussainzada et al., 2014; Kienle et al., 2008, 2009; Ku et al., 2015; Scheil and Köhler, 2009; Zheng et al., 2014).

Larval and adult zebrafish have been widely used in research to assess toxicological effects (Bailey et al., 2013; Capiotti et al., 2011; Hill et al., 2005; Lutte et al., 2015; Nery et al., 2014; Nishimura et al., 2015; Siebel et al., 2011). Among the advantages of zebrafish as a research model are their small size, simple maintenance facilities, external fertilization, rapid development, transparent embryos, ease of genetic and other experimental manipulations, highly conserved genes and similarity to other vertebrates including humans in terms of physiological responses (Howe et al., 2013; Kalueff et al., 2014; Lele and Krone, 1996; Stewart et al., 2014). These features, along with the fact that fish accumulate chemicals by direct exposure to products in the water and indirectly through the food chain in the ecosystem (Powers, 1989), contribute to the usefulness of this species for toxicological studies.

In this sense, considering the growing concentration of nickel on aquatic environment and the possible deleterious effects on physiology and development of aquatic species, the present study analyzed the toxicological effects of $NiCl_2$ acute and subchronic exposure regimens in larval and adult stages of zebrafish (*Danio rerio*) to evaluate the impact in aquatic environments. This metal's effects on developmental parameters, avoidance response, and exploratory behavior in larvae were investigated, along with locomotor activity, social and aggressive behavior and long-term memory in adult zebrafish.

2. Materials and methods

2.1. Animals

Larval (0–11 days post-fertilization, dpf) and adult (6–7 months) wild-type zebrafish (*Danio rerio*) from the Tübingen background were used. Animals were obtained from our breeding colony and kept in automated recirculating systems (Zebtec, Tecniplast, Italy) with reverse-osmosis-filtered water equilibrated to reach the recommended temperature (28 °C ± 2 °C), pH (7.0–7.5), conductivity (300–700 μS), ammonia (< 0.02 mg/L), hardness (80–300 mg/L), nitrite (< 1 mg/L), nitrate (< 50 mg/L) and chloride (0 mg/L) levels for the species. The animals were maintained in a light/dark cycle of 14/10 h. Animals were fed with paramecium between 6 and 14 dpf of age and received commercial flakes (TetraMin Tropical Flake Fish®) three times a day supplemented with brine shrimp (Westerfield, 2000) after 14 dpf.

For breeding, the female and the males (1:2) were placed in breeding tanks (Tecniplast, Italy) overnight and separated by a transparent barrier. After the adults spawned, viable embryos were collected and transferred to sterile petri dishes, which were kept in an incubator at 28.5 °C on a light/dark cycle of 14/10 h. The mortality, hatching rates and general morphology of the embryos and larvae were monitored daily under an inverted stereomicroscope (Nikon, Melville, USA). Malformed or inactive embryos and larvae were included in the morphological and heartbeat evaluation, but only animals without morphological changes were used in subsequent experiments to ensure that the behavioral effects were not overestimated. All protocols were approved by the Institutional Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, permit number 15/00463).

2.2. Treatments

Nickel(II) chloride hexahydrate ($NiCl_2$ $6H_2O$) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in bottled spring water to prepare a stock solution of 1.5 g/L $NiCl_2$. The test solutions were prepared directly from this stock solution before use. Larvae and adults were subjected to two periods of treatment, acute (short-time exposure) and subchronic (long-time exposure), and were exposed to four different nominal concentrations of $NiCl_2$ (0.025, 2.0, 5.0, and 15.0 mg/L in water) or water (control group). The nickel concentrations were chosen based on CONAMA legislation (National Environment Council - regulation No. 357, March 25th, 2005), which recommends a maximum total nickel concentration of 0.025 mg/L in fresh water bodies and 2.0 mg/L in effluent water (CONAMA, 2005; CONAMA, 2011). The higher nickel concentrations (5.0 and 15.0 mg/L) were chosen according to Kienle et al. (2008, 2009), who based them on previous studies near industrial sites in unpolluted Canadian lakes and rivers.

2.2.1. Larvae acute treatment

For acute exposure, embryos were raised in petri dishes with bottled spring water (20 fertilized eggs per dish) up to an age of 5 dpf. At 5 dpf,

the larvae were exposed acutely (for 2 h) to $NiCl_2$ at different concentrations or water. After the treatment period, the animals were tested and euthanized.

2.2.2. Larvae subchronic treatment

Embryos were kept in petri dishes under the above-mentioned conditions up to an age of 11 dpf. The exposure to $NiCl_2$ started immediately after fertilization (≤ 1 h post fertilization, hpf) and ended at 11 dpf. The larvae were exposed to $NiCl_2$ solutions ($NiCl_2$ dissolved in water) or water with daily changes during the treatment period. Animals were tested at 5, 8 and 11 dpf and euthanized at 11 dpf.

2.2.3. Adult acute and subchronic treatment

Adult zebrafish, aged between 6 and 7 months, were exposed to $NiCl_2$ concentrations or water in 2-L aquariums (10 zebrafish per tank) for 12 h (acute treatment) or 96 h (subchronic treatment). In subchronic treatment, the $NiCl_2$ solutions were changed daily. After the treatment period, the animals were tested and euthanized.

2.3. Analysis of $NiCl_2$ levels by ICP-MS - larval and adult zebrafish

The levels of $NiCl_2$ in larvae and adult zebrafish were assessed by inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies®), according to the method described by Ashoka et al. (2009) with minor modifications at the end of all treatment periods. To obtain samples, we euthanized larvae and adults by hypothermic shock. $NiCl_2$ levels were analyzed in the whole bodies of the acute treated larvae and in the brains of adults and subcronically treated larvae, which were removed by dissection. Briefly, a pool of three brains ($n = 4$) of adult animals and 25 larvae ($n = 4$) were washed four times with bottled spring water. After being cleaned, the samples were digested with 0.3 mL of concentrated nitric acid (Suprapur/Merck, Darmstadt, Germany) and 0.2 mL of hydrogen peroxide (Merck, Darmstadt, Germany) in a glass tube and subsequently incubated for 2 h in a water bath at 85 °C, after which they were diluted to a volume of 5 mL with a 2% solution of HNO_3 (this step was modified from the procedure of Ashoka et al. 2009). The samples were then placed in the automatic sampler to be analyzed. The $NiCl_2$ calibration curve was linear in the range of 0.5–200 ppb (ng/mL), and the results were expressed in nanogram per sample (ng/larva or ng/brain).

2.4. Larvae analysis

2.4.1. Morphological evaluation

Morphological evaluation of $NiCl_2$ exposure was performed only for subchronically treated larvae ($n = 50$). Larvae were evaluated at 5, 8 and 11 dpf under a stereomicroscope (3×). The body length (μm), ocular distance (μm) and surface area of the eyes (μm^2) were measured after photographic registration with the software NIS-Elements D 3.2 for Windows, supplied by Nikon Instruments Inc. (Melville, USA). The body length was defined as the distance from the center of the eyes to the tip of the tail bud. The ocular distance was defined as the distance between the inner edges of the two eyes, and the size of the eyes was defined as the surface area of the eyes (Capiotti et al., 2011; Lutte et al., 2015).

2.4.2. Heartbeat rate

Animals from the subchronic treatment group had their heart rates monitored at 5, 8 and 11 dpf under the stereomicroscope. Treated larvae and controls were placed in petri dishes with bottled spring water, and their heart rates were monitored for 60 s ($n = 30$). For all procedures, water temperature was kept stable at 28 °C by a thermo-plate coupled to the stereomicroscope.

2.4.3. Exploratory behavior

For the acute treatment, larvae ($n = 30$) were analyzed at 5 dpf. In the subchronic exposure group, different zebrafish larvae were analyzed at 5, 8 and 11 dpf (n = 30). Animals were individually placed in a 24-well plates, each well being filled with 3 mL of bottled spring water, for a 5-min session of exploratory behavior analysis following 1 min acclimation (Colwill and Creton, 2011). The performance was video recorded for automated analysis by ANYmaze (Stoelting Co., Wood Dale, IL, USA), which is able to track the swimming activity of the animals at a rate of 30 positions per second. Total distance travelled (m), mean speed (m/s, ratio between distance travelled and time mobile) and time mobile (s) were considered the main parameters of exploration of a new environment. We also determined the absolute turn angle (°), which represents the change in direction of the center point of the animal between two consecutive samples and evaluates erratic movements.

2.4.4. Avoidance behavior

After the exploratory evaluation, larvae were placed in 6-well plate (5 larvae per well, $n = 30$) over an LCD monitor for avoidance-escape behavior from a visual stimulus (a 1.35 cm diameter red bouncing ball) (Pelkowski et al., 2011) for a 5-min session following 2 min of acclimation. The sessions were video recorded and analyzed by ANYmaze software. A red bouncing ball travelled from left to right over a straight 2 cm trajectory on the top half of the well area (stimulus area) which animals avoided by swimming to the other (non-stimulus) half of the well. The number of larvae in the non-stimulus area during the 5-min session was considered indicative of deficits in the avoidance response.

2.5. Adult analysis

2.5.1. Novel tank test

Adult exploration was evaluated at the end of acute and subchronic treatment ($n = 30$). Animals were placed individually in experimental tanks (30 cm long × 15 cm high × 10 cm wide) with water. After 60 s of habituation, the locomotion and exploratory patterns of the fish were recorded for 5 min for subsequent analysis with the software EthoVision XT (version 11.5, Noldus) at rate of 15 positions per second (Altenhofen et al., 2017a,b). The following behavioral parameters were analyzed: distance travelled (m), velocity (m/s), time mobile (s) and time spent in each tank zone (bottom vs. upper levels) (s). The time spent in each tank zone was considered an indicator of anxiety-like behavior, since when introduced into a new environment zebrafish tend to spend more time at the bottom of the tank, until gradually moving to the upper zone after a few minutes (Levin et al., 2007).

2.5.2. Inhibitory avoidance task

To assess whether $NiCl_2$ could impair long-term memory in adult animals, we carried out an inhibitory avoidance test after 12 h (acute treatment) or 96 h (subchronic treatment) of exposure ($n = 12$) (Blank et al., 2009). There were two sessions, training and test, with a 24-h interval between them. In each session, animals were placed individually in an experimental aquarium (18 cm long × 9 cm wide × 7 cm high) with water, divided by a guillotine door into two compartments of equal size, one black (right side) and one white (left side). During the training session, the animal was placed in the white compartment with the door closed for one minute for habituation and environment recognition. After this period, the division was lifted. Once the animal crossed into the black side of the tank, the guillotine door was closed again, and an electric shock pulse of 3 ± 0.2 V was applied for five seconds. The animal was then removed from the apparatus and returned to its housing tank with only water for 24 h until the test session, which consisted of the same protocol as the training session, but without the electric shock. The latency to enter the black compartment during

each session was measured, and the expected increase in the test session was used as an index of memory retention.

2.5.3. Social interaction

The zebrafish is a schooling fish that may exhibit preference for its conspecifics. Adult social interaction was evaluated at the end of acute and subchronic treatments ($n = 30$). Briefly, fish were individually placed in an experimental tank, with water (30 cm × 15 cm × 10 cm, length × height × width). On one side of the experimental tank, an empty fish tank was placed; on the other side, a tank of identical size held 15 zebrafish, which were designated the "stimulus fish". The fish undergoing evaluation was allowed to acclimate to the experimental tank for a 30 s period, after which its behavior was video recorded over a period of 5 min for subsequent analysis with EthoVision XT (15 positions per second) according to Gerlai et al. (2000). To quantify fish social behavior and innate preference for conspecifics in detriment of the empty tank, the experimental tank was virtually divided into two equal halves: a "stimulus zone" closer to the "stimulus tank" where the conspecific school was and the other remaining half closer to the empty tank. The amount of time the experimental fish spent in each zone was measured during 5 min.

2.5.4. Aggression test

Adult aggressive behavior was evaluated at the end of acute and subchronic treatment ($n = 30$). The mirror test was used to measure aggression according to the procedure described by Gerlai et al. (2000). Each fish was individually placed in an experimental tank (30 cm × 15 cm × 10 cm, length × height × width). A mirror (45 cm × 38 cm) was placed at the side of the tank at an angle of 22.5° to the back wall of the tank so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away. Thus, when a test fish swam to the left side of the tank, their mirror image appeared closer to them. After 1 min of acclimation period, a 5-min session was recorded for subsequent quantification of aggressiveness with EthoVision XT (15 positions per second). Virtual vertical lines divided the tank into four equal sections and allowed the number of entries to each section made by the fish to be counted. Entry to the left-most segment indicated preference for proximity to the "opponent", whereas entry to the right most segments implied avoidance. The frequency of entries, the amount of time and circle swimming movement of the experimental fish spent in each segment was measured.

2.6. Statistical analysis

Nickel levels in tissue were evaluated by one-way ANOVA followed by a post hoc Tukey test. Data were expressed as the mean ± standard deviation (S.D). Survival and hatching rates throughout the 11 experimental days of subchronic treatment were analyzed by a Kaplan-Meier test. Data from heart rate and morphological evaluation were evaluated by one-way ANOVA followed by post hoc Tukey test. Pearson's correlation was performed for morphological measures, considering as factors body length, ocular distance and surface area of the eyes. Positive correlation was defined as $r^2 > 0.850$, and negative correlation as $r^2 > -0.850$. Data on exploratory and avoidance behavior in larvae were analyzed by two-way ANOVA (with age and concentration as factors) followed by a Bonferroni post hoc test and are expressed as the mean ± standard error of the mean (S.E.M). Behavior data from adults were analyzed with one-way ANOVA followed by Tukey test and are expressed as the mean ± S.E.M. Inhibitory avoidance training and test latencies within each group were compared by the Wilcoxon matched pairs test. Latencies of multiple groups were compared by the Kruskal-Wallis and Mann-Whitney *U* tests. For all comparisons, the level of significance was defined as $p \leq 0.05$.

3. Results

3.1. $NiCl_2$ levels after $NiCl_2$ exposure - larval and adult zebrafish

Ni levels were estimated to evaluate the deposition of this metal in the larvae and adults and its subsequent accumulation after the exposure. After acute exposure, larvae ($F_{(4,13)} = 7.572$, $p = 0.002$) and adults ($F_{(4,14)} = 5.467$, $p = 0.007$) treated at a concentration of 15.0 mg/L had significantly higher levels of Ni than the control groups (Fig. 1a and c). After subchronic treatment at a concentration of 2.0, 5.0, and 15.0 mg/L ($F_{(4,13)} = 40.33$; $p < 0.0001$), larvae showed elevated Ni levels in the brain compared with the water control group (Fig. 1b). Adults subjected to subchronic treatment presented higher levels of Ni at doses of 2.0, 5.0 and 15.0 mg/L ($F_{(4,14)} = 36.00$; $p < 0.0001$) than the control group (Fig. 1d). Ni levels from adult animals exposed to 0.025 mg/L $NiCl_2$ in acute and subchronic regimen did not differ from those of water controls, and this concentration did not alter the locomotor parameters tested (Supplementary Fig. 1). Thus, this $NiCl_2$ concentration was eliminated from subsequent behavioral analyses of adult zebrafish. Considering that Brazilian legislation allows this lower concentration (0.025 mg/L $NiCl_2$) in the water and its developmental effects have not been investigated in detail, we administered this dose in acute and subchronic treatments in the larvae to explore its potential effects at early life stages.

3.2. $NiCl_2$ chloride exposure in zebrafish larvae

3.2.1. Survival and hatching rates

The $NiCl_2$ exposure did not alter the survival rate of treated animals compared to control group in both acute and subchronic treatment regimens (Supplementary Fig. 2). However, a significant delay in hatching was observed in subchronically treated larvae at concentrations of 5.0 mg/L and 15.0 mg/L ($p < 0.0001$). Larvae from all groups started to hatch at 48 hpf. While 100% of animals in the control group, 0.025 mg/L and 2.0 mg/L concentrations hatched within72 hpf, only portions of animals exposed to the two higher doses hatched within this period (5.0 mg/L = 60%; 15.0 mg/L = 35.7%). The group exposed to a concentration of 5.0 mg/L finished hatching at 96 hpf, whereas the group subchronically treated with 15.0 mg/L only reached 100% of animals ecloded at 120 hpf (Fig. 2).

3.2.2. Morphology

The potential teratogenic effects of subchronic exposure to $NiCl_2$ on larval morphology were evaluated at 5, 8 and 11 dpf. There were no visible morphological alterations in acutely treated animals at 5 dpf. The most prominent effects of subchronic exposure to $NiCl_2$ were reduced body length and reduced surface area of the eyes. At 5 dpf, animals treated subchronically at all treatment concentrations had shorter bodies than control animals ($F_{(4245)} = 21.94$; $p < 0.0001$) (Fig. 3a). The eye surface area was significantly decreased in groups treated with 2.0 mg/L, 5.0 mg/L and 15.0 mg/L in relation to controls ($F_{(4236)} = 3.703$; $p = 0.006$) (Fig. 3c). At 8 dpf, the decrease in body length remained significant at 5.0 mg/L and 15.0 mg/L ($F_{(4245)} = 3.426$; $p = 0.009$) (Figure b) whereas the surface area of the eyes showed no significant difference across groups at this time period (Fig. 3d). There were no differences in ocular distance (Fig. 3 e and 3 f). After 11 days, no changes were observed between the groups in any parameter (Supplementary Fig. 3). Additionally, there was no correlation between body length, ocular distance and surface area of the eyes.

3.2.3. Heartbeat rate

Larvae exposed to subchronic treatment with $NiCl_2$ showed a decreased heart rate over the 11 days of exposure. The measure was performed at 5, 8 and 11 dpf, and, at all these ages, we observed a significant difference in groups exposed to 2.0, 5.0 and 15.0 mg/L compared with the control group ($F_{(4145)} = 54.96$, $p < 0.0001$ at 5 dpf;

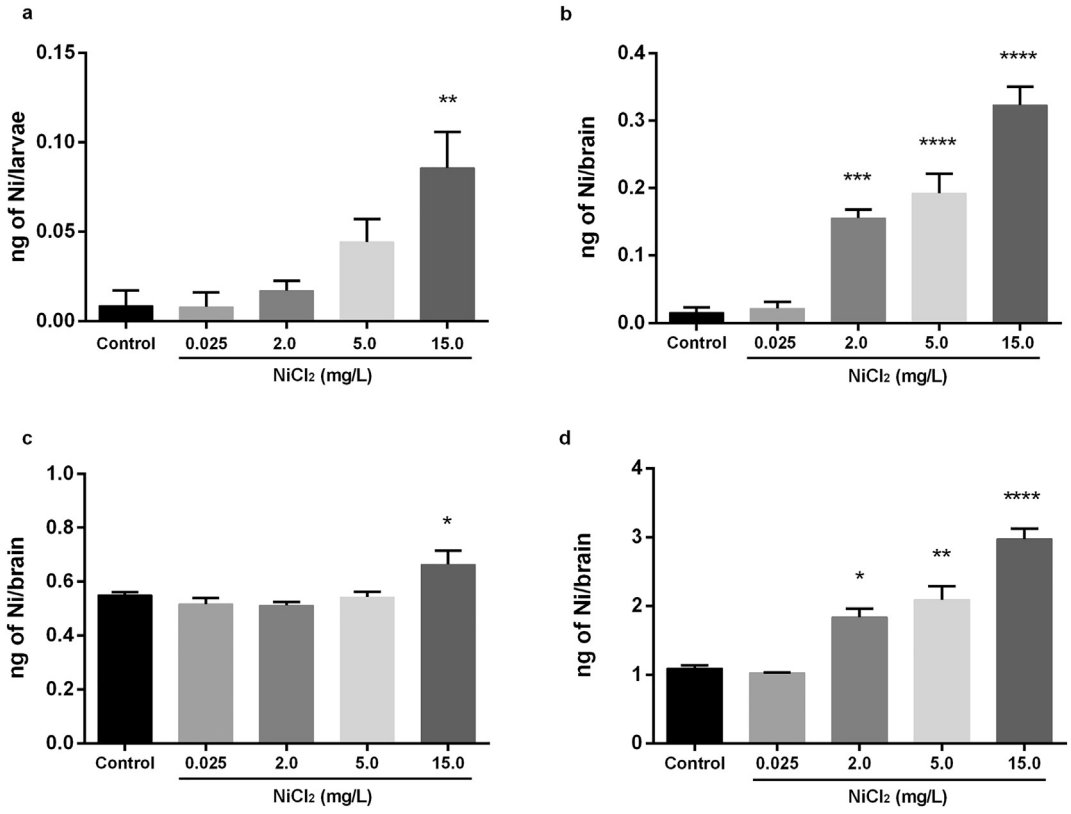

Fig. 1. Analysis of levels of $NiCl_2$ in zebrafish larvae and adult by ICP-MS. (a, b) larvae analysis, acute treatment (pool of 25 larvae, $n = 4$) and subchronic treatment, respectively; (c, d) adults analysis, acute treatment and subchronic treatment, respectively (pool of 3 brain, n = 4). Bars represent mean ± S.D. One-way ANOVA was used, followed by a post-hoc Tukey's test. * represents significant difference $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ in relation to control.

$F_{(4145)} = 33.46$, $p < 0.0001$ at 8 dpf; $F_{(4145)} = 52.60$, $p < 0.0001$ at 11 dpf) (Fig. 4a, b, c).

3.2.4. Exploratory behavior

In acutely treated animals, individual exploratory evaluation showed no significant differences between groups (Supplementary Fig. 4). However, $NiCl_2$ exposure induced significant differences at subchronically treated animals in relation to controls at each evaluation time. The distance travelled showed a significant decrease in animals exposed to 0.025 mg/L and a significant increase in the 5.0 mg/L and 15.0 mg/L groups compared with controls at 5 dpf. At 8 and 11 dpf, the decrease observed in the 0.025 mg/L group remained significant, and significant decreases in distance travelled were also observed in the 15.0 mg/L group at these time points (Treatment: $F_{(4431)} = 29.41$; $p < 0.0001$. Age: $F_{(2431)} = 34.31$; $p < 0.0001$. Interaction: $F_{(8431)} = 15.54$; $p < 0.0001$) (Fig. 5a).

When average speed was compared, we observed significant effects of $NiCl_2$ exposure at 5 dpf. There was a decrease in mean speed in the 0.025 mg/L group and an increase in 5.0 mg/L and 15.0 mg/L groups when compared with the controls. At 8 and 11 dpf there were no statistical differences between treated animals and controls, but we observed differences between the days analyzed (Treatment: $F_{(4431)} = 21.37$; $p < 0.0001$. Age: $F_{(2431)} = 13.96$; $p < 0.0001$. Interaction: $F_{(8431)} = 5.150$; $p < 0.0001$) (Fig. 5b). The analysis of mobile time during the 5 min session showed a significant decrease at 8 and 11 dpf in the15.0 mg/L group (Treatment: $F_{(4431)} = 48.91$; $p < 0.0001$. Age: $F_{(2431)} = 19.29$; $p < 0.0001$. Interaction: $F_{(8431)} = 11.95$; $p < 0.0001$) (Fig. 5 c).

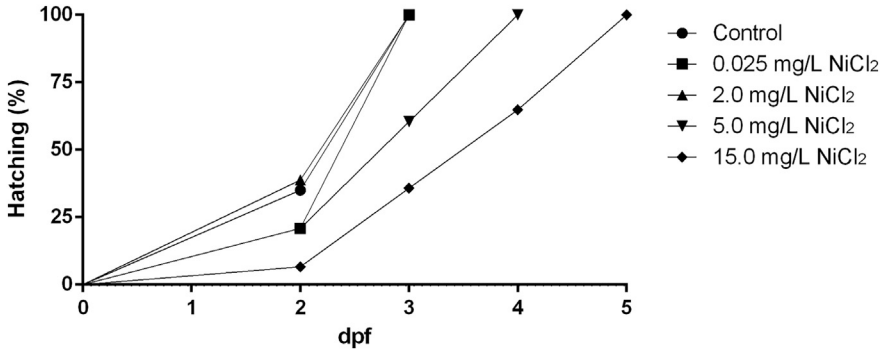

Fig. 2. Percentage of hatching in subchronic treatment compared with the control group ($p \leq 0.0001$). Data are showed as percentage of hatchings ($n = 150$ embryos).

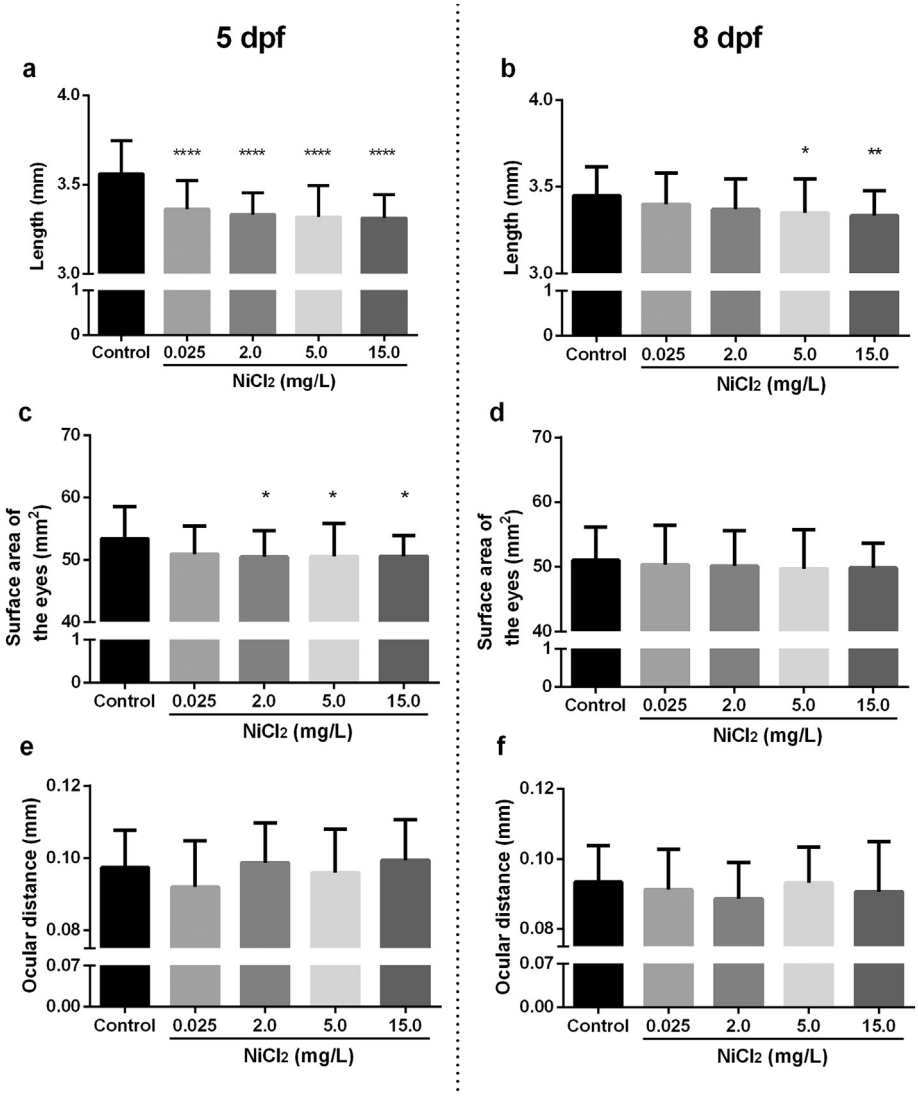

Fig. 3. Effects of subchronic exposure to $NiCl_2$ on morphological parameters of zebrafish larvae. Mean body length (μm) at 5 (a) and 8 dpf (b); mean surface area of eyes (μm²) at 5 (c) and 8 dpf (d); and mean ocular distance (μm) at 5 (e) and 8 dpf (f). Data are showed as mean ± S.E.M ($n = 50$). One-way ANOVA was used, followed by post-hoc Tukey's test. * represents significant difference at $p \leq 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$ in relation to control at the same developmental phase.

The absolute body turn angle during movement was increased in animals treated with 15.0 mg/L when compared with controls at 5 dpf. At 8 and 11 dpf analysis showed a significant decrease in the 0.025 mg/L group and an increase in the 15.0 mg/L group (Treatment: $F_{(4431)} = 17.00$; $p < 0.0001$. Age: $F_{(2431)} = 40.89$; $p < 0.0001$. Interaction: $F_{(8431)} = 12.90$; $p < 0.0001$) (Fig. 5d).

3.2.5. Avoidance behavior

There were no changes in on avoidance response after acute $NiCl_2$ treatment (Supplementary Fig. 4), but decreased in avoidance performance was observed in subchronically treated animals at the periods evaluated. When tested at 5 dpf, animals treated with 5.0 mg/L and 15.0 mg/L showed a diminished escape response when compared with

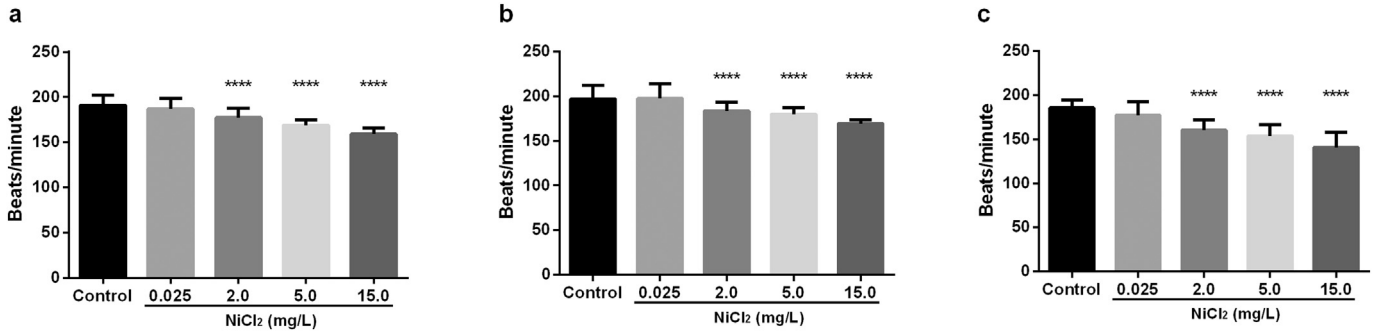

Fig. 4. Heartbeat rate of subchronic treated larvae measured at 5 (a), 8 (b) and 11 dpf (c). Data are expressed as mean ± S.D. ($n = 30$). One-way ANOVA was used, followed by post-hoc Tukey's test. **** represents significant difference at $p \leq 0.0001$ in relation to control.

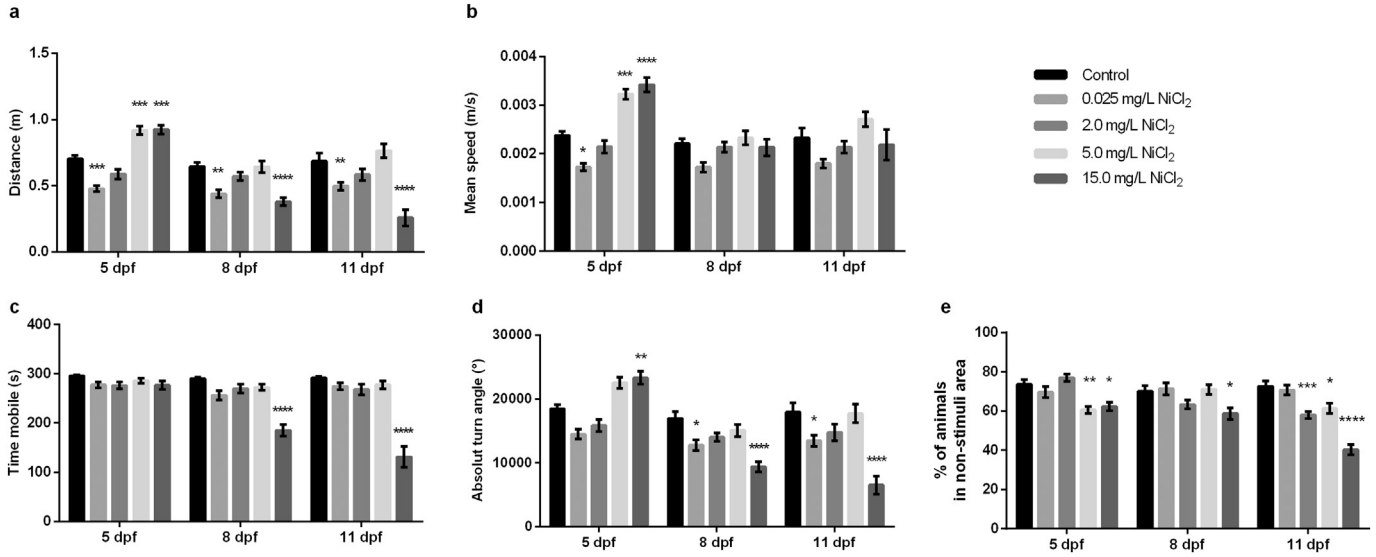

Fig. 5. Exploratory and avoidance behavior at 5, 8 and 11 dpf of larvae treated subchronically. Total distance travelled (a), mean speed (b), time mobile (c), absolute turn angle (d) and avoidance response (e) are analyzed. Data are expressed as the mean ± S.E.M. (n = 30). Two-way ANOVA was used, followed by Bonferroni post-hoc test. * represents significant difference at $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ in relation to control.

controls. At 8 dpf animals treated with 15.0 mg/L showed a significant deficit in escaping the bouncing ball whereas at 11 dpf this deficit was observed in animals treated with 2.0 mg/L, 5.0 mg/L and 15.0 mg/L when compared with the control group (Treatment: $F_{(4435)} = 24.91$; $p < 0.0001$. Age: $F_{(2435)} = 14.29$; $p < 0.0001$. Interaction: $F_{(8435)} = 7294$; $p < 0.0001$) (Fig. 5e).

3.3. Nickel(II) chloride exposure in zebrafish adults

3.3.1. Novel tank test

The swimming pattern of adult animals was analyzed after acute (12h) and subchronic (96 h) treatments. Individual evaluation showed no statistical differences between acutely treated groups (Supplementary

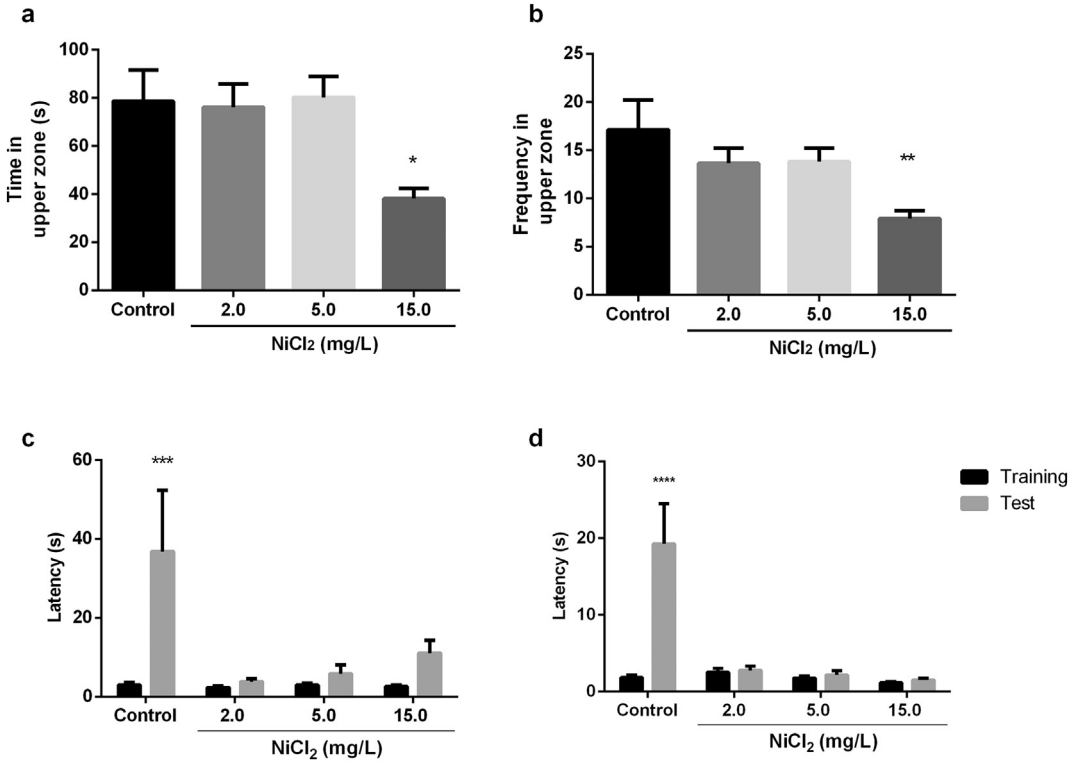

Fig. 6. Locomotor activity and avoidance task of $NiCl_2$-subchronic treated adult zebrafish. Time spent in the tank upper zone (a), frequency of entries in the upper zone (b) 96 h after treatment (n = 30). Avoidance task in acute treatment (c) and subchronic treatment (d) ($n = 12$). Data are presented as mean ± S.E.M; * represents significant difference at $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ indicate the differences between training and test sessions for each group compared. Locomotor activity was analyzed by One-way ANOVA, followed by Tukey test, and avoidance behavior via Wilcoxon matched pair test. No differences were found between training performances among all groups as evaluated by Kruskal–Wallis test.

Fig. 1). After subchronic treatment, however, animals from the 15.0 mg/L group showed a significant decrease in the time spent in the upper zone of the tank, remaining at the bottom longer ($F_{(3116)} = 3.972$; $p = 0.006$) (Fig. 6a, b).

3.3.2. Inhibitory avoidance task

$NiCl_2$ treatment at all concentrations tested (2.0, 5.0 and 15.0 mg/L) significantly impaired inhibitory avoidance long-term memory in both acute ($p \leq 0.001$) and subchronic ($p < 0.0001$) regimens (Fig. 6c, d).

3.3.3. Social interaction

The acute and subchronic treatments with $NiCl_2$ at all doses analyzed did not induce any social interaction deficit in zebrafish, and all treated animals presented the same preference for the stimulus area as their respective controls (Supplementary Fig. 5).

3.3.4. Aggression test

Subchronic $NiCl_2$ exposure significantly impacted aggression behavior as evaluated by the frequency of entries and time spent in the segment nearest to the mirror image. Our results showed that treated animals from all groups visited the segment nearest to the mirror fewer times ($F_{(3116)} = 4.080$; $p = 0.008$) (Fig. 7a), but no difference was observed in the time spent in this segment when compared with the control group (Fig. 7b). $NiCl_2$ treated animals (5.0 and 15.0 mg/L Ni) displayed less rotation movements than controls ($F_{(3,\ 116)} = 4.300$; $p = 0.006$) (Fig. 7c), reinforcing the $NiCl_2$-induced decrease in aggressive behavior.

4. Discussion

In the present study, we analyzed the effects of acute and prolonged exposure to $NiCl_2$ on larvae and adults zebrafish. We observed that $NiCl_2$ exposure caused morphological and physiological alterations, in subchronically treated larvae. Subchronic treatment for 11 dpf also impaired exploration of a new environment and induced significant avoidance response deficits in larvae tested at different time points during exposure. When adult animals were exposed for 96 h, $NiCl_2$ induced an anxiogenic effect and decreased aggression behavior. Importantly, $NiCl_2$ exposure in both acute and subchronic regimens impaired long-term memory formation for an aversive stimulus.

The World Health Organization (WHO) has established a maximum acceptable concentration of 0.025 mg/L Ni in water. In Brazil, the CONAMA set the control and maximum limits of contaminants in the water. According to this resolution, the maximum total concentration of Ni allowed is 0.025 mg/L in fresh water bodies and 2.0 mg/L in effluent water. However, a Brazilian study, that evaluated the effects of $NiCl_2$ exposure at concentrations of 0.025 mg/L $NiCl_2$, observed gill damage in tilapia (*Oreochromis niloticus*) (Marcato et al., 2014). In our study, zebrafish larvae treated at this same dose presented morphological and behavioral alterations. Furthermore, we observed changes in adult animals exposed to concentrations of 2.0 mg/L, including impaired memory formation and decreased aggressive behavior.

Zebrafish larvae hatch from the entire egg at 2–3 dpf and display a range of swimming, hunting, escape, and avoidance behaviors during the first week of development (Colwill and Creton, 2011). These parameters are altered by $NiCl_2$ exposure, which could significantly impair animals' survival capacity in a natural environment despite the lack of direct mortality effects. In the present study, in contrast with previous studies, embryos exposed to acute and subchronic treatments did not exhibit an increased mortality rate compared with controls (Kienle et al., 2008, 2009; Ku et al., 2015; Scheil and Köhler, 2009). The discrepancies observed between this and previous studies on survival may be attributable to variations in water parameters, such as pH, temperature and hardness, which alter the activity of $NiCl_2$, decreasing or increasing its toxicity (Scheil and Köhler, 2009; Scott and Sloman, 2004).

Importantly, we also observed that $NiCl_2$ caused a delay in hatching in subchronically treated animals, which could make them more vulnerable to predation, among other deleterious effects. In a similar study, Kienle et al. (2008) observed that embryos of the control group hatched within the first 96 hpf. In our study, as generally expected for this species, all animals from the control group and animals those treated with the 0.025 and 2.0 mg/L concentrations hatched within 72 hpf, while animals treated with higher concentrations (5.0 and 15.0 mg/L) took up to 96 hpf to complete the hatching process. The delay of hatching was also observed in other studies testing $NiCl_2$ exposure effects (Dave and Xiu, 1991; Scheil and Köhler, 2009; Scheil et al., 2010). The delay of hatching enzyme (chorionase, a metal-protease) may be attributable to effects of $NiCl_2$ upon the activity or secretion of this enzyme (Hagenmaier, 1974). Specifically, the mechanism of interaction between $NiCl_2$ and hatching enzyme is uncertain. Hatching time can be affected secondarily as a result of primary effects on developmental rate of the embryo or early larval stages (Rosenthal and Atderdiee, 1976), or toxicants may interfere with the hatching process directly (Dave and Xiu, 1991).

Our study also demonstrated a decrease in body length and surface area of the eyes at 5 and 8 dpf whereas only length was reduced at 8 dpf. By contrast, LeFauve and Connaughton (2017) did not observe changes in notochord length, eye diameter, or inter-ocular distance at 7, 9 or 11 dpf when the animals were exposed to nickel in the first 72 hpf. LeFauve and Connaughton (2017) tested lower nickel concentrations than we tested in our study. Therefore, it is reasonable to suggest that the morphological changes observed are due to the higher nickel concentrations tested. In addition, we tested longer exposure periods, which may have contributed to the morphological changes observed.

In humans, $NiCl_2$ exposure can affect several systems, including the cardiovascular system. Although $NiCl_2$ has not been associated with any subchronic diseases, this metal has potential cardiotoxic effects that may increase the risk of developing heart disease (Afridi et al.,

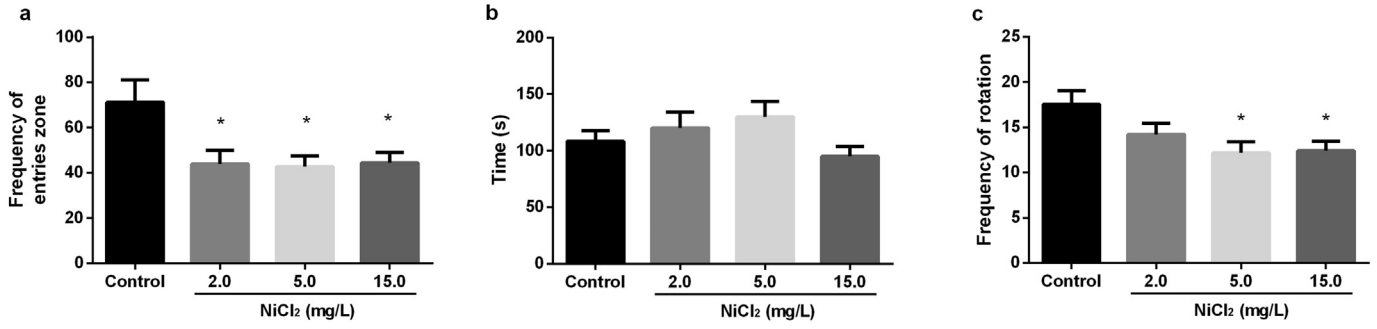

Fig. 7. Induced aggression deficits by $NiCl_2$-subchronic treatment in adult zebrafish. Frequency of entries (a) and time spent (b) in zone nearest to the mirror and frequency of rotation (c). Data are expressed as the mean ± S.E.M. (n = 30), and were analyzed by one-way ANOVA followed by Tukey test. * represents significant difference at $p \leq 0.05$ in relation to control.

2011; Denkhaus and Salnikow, 2002; Leach et al., 1985). Previous studies reported $NiCl_2$-induced cardiac toxicity in rodents, including heart lesions and myocardial injury (Ilbäck et al., 1994; Lou et al., 2013; Magaye et al., 2014; Rubányi et al., 1983). In the present study, we observed that larvae subchronically treated with 2.0, 5.0, and 15.0 mg/L $NiCl_2$ showed decreased heart rates at 5, 8, and 11 dpf. The mechanisms underlying the cardiotoxicity of $NiCl_2$ are not fully elucidated, but could involve oxidative damage to cellular components, caused either due to the generation of reactive oxygen species (Osmancik et al., 2010) or by inactivation of the antioxidant defense system (Jia and Chen, 2008; Osmancik et al., 2010; Pan et al., 2010).

It has been reported that metals can alter swimming patterns of fish. Our study demonstrated that acute exposure to $NiCl_2$ did not alter behavior in the early stages of development, but subchronic exposure altered in a concentration-dependent manner the swimming behavior of zebrafish larvae at 5 dpf, reducing the distance travelled at a low concentration (0.025 mg/L) and increasing the same parameter at higher concentrations (5.0 and 15.0 mg/L). At 8 and 11 dpf, the activity of the fish decreased significantly at the highest dose (15.0 mg/L). This effect indicates that $NiCl_2$ is able to change the swimming pattern of the larvae at different time points. Moreover, a decrease in the movement time was observed at 8 and 11 dpf at a high dose (15.0 mg/L). This effect is in accordance with data from Kienle et al. (2008, 2009), who found that, in the same treatment period, $NiCl_2$ acute exposure decreased locomotor activity at concentrations of 7.5 mg/L and above (up to 15.0 mg/L) in subchronic treatment. In natural environments, decreased locomotor activity in fish might lead to increased downstream drift and/or predation risk, hence representing an ecologically relevant parameter for the health and survival of the species.

Additionally, our behavioral findings in larvae showed specific avoidance response deficits in animals treated subchronically. In this analysis, control animals avoided the visual aversive stimulus, swimming to the other non-stimuli area in a scenario that resembles that of escape from natural threats and predators. $NiCl_2$ impaired this response. We observed that $NiCl_2$ exposed-larvae did not escape the stimulus efficiently. At early test stages (5 and 8 dpf), only animals subchronically exposed to the higher concentrations were impaired (5.0 and 15.0 mg/L), but at 11 dpf all treated groups showed significant deficits that suggested a cumulative deleterious effect of $NiCl_2$ on avoidance responses. This is, to our knowledge, the first study demonstrating avoidance response deficits at this early life stage as a result of metal exposure, despite previous validation of the task to evaluate larval cognition (Colwill and Creton, 2011; Nery et al., 2014; Pelkowski et al., 2011). LeFauve and Connaughton (2017) demonstrated an impaired optomotor response at 7, 9 and 11 dpf in zebrafish exposed to nickel during the first 72 hpf. Then, it is no possible to exclude the explanation that the reduced aversive response observed in our study is due to a decrease in the optomotor response in nickel-treated larvae. Also, since the locomotor activity decreased this also might impact the aversive response.

In addition to morphological, physiological and behavioral changes in the early life stages of development, acute and prolonged exposure of adults to $NiCl_2$ also caused deleterious effects. Hussainzada et al. (2014), studying the effects of 24-h $NiCl_2$ exposure on adult male zebrafish, observed behavioral differences between controls and treated fish, described as sluggish swimming in the treated animals. In our study we did not observe such an effect of $NiCl_2$ on general locomotor parameters in acute or subchronic exposure regimens. However, we observed changes in anxiety, with anxiogenic-like behaviors after 96 h exposure to the 15.0 mg/L concentration. The evaluation of the anxiety-like status of fish has been previously proposed to assess behavioral effects of toxic substances (Maximino et al., 2010). Accordingly, studies have shown anxiogenic-like behavior in zebrafish and other fish species exposed to different metals and pollutants (Altenhofen et al., 2017a; Baldissarelli et al., 2012; Hallgren et al., 2011; Macaulay et al., 2015; Pereira et al., 2016; Rácz et al., 2012). Treated animals do not present alterations in other parameters, such as distance travelled, velocity and time mobile. However, we cannot exclude the hypothesis that the observed anxiety-like effect is due to animals might be experiencing insults to their health.

Memory is also negatively affected by $NiCl_2$ exposure. In the present study, acute and subchronic exposure leads to impaired long-term memory formation in adult animals. At all tested $NiCl_2$ concentrations, treated animals did not remember the previous day's training session as effectively as controls. Some metals have been described to cause deficits in learning and memory in zebrafish, including manganese, copper and lead, suggesting that neurotoxic effects of metals can impact cognition (Acosta Dda et al., 2016; Altenhofen et al., 2017a; Chen et al., 2012). When similar deficits were observed in the spatial memory of $NiCl_2$ treated mice, the authors suggested that the effects could be attributed to $NiCl_2$-induced oxidative phosphorylation and a resulting decreased in the energy supply of the brain (He et al., 2013).

The zebrafish is a highly social fish species that may exhibit preference for its conspecifics under certain circumstances. It has been reported that different metals may impact fish social behavior (Sloman et al., 2003a,b; Scott and Sloman, 2004; Weber and Ghorai, 2013). In the present study, we analyzed whether exposure to $NiCl_2$ could alter this conspecific preference, and we observed that neither acutely nor subchronically treated animals differed from controls in this regarding. However, subchronically treated animals showed decreased aggressive behavior during the mirror image test. Animals treated with all $NiCl_2$ concentrations made fewer visits to the segment nearest to the mirror than the controls, despite no difference in the time spent in this area. Additionally, there was a decrease in the frequency of circle swimming movement, a behavior commonly associated with attacking an opponent (mirror) (Oliveira et al., 2011; Teles and Oliveira, 2016). Aggression is an adaptive behavior that is essential for the establishment of social hierarchies, mating, and competition for food and territory (Brain, 1988). In this context, a certain level of aggression can be beneficial for the survival of an individual or species and fulfill a social function (Oliveira et al., 2011; Teles et al., 2013). A deficit in aggressive behavior may impair the survival of animals in natural environments regardless of the lack of effect on social behavior, reinforcing the deleterious effects of $NiCl_2$ exposure.

In our study, we evaluated effects in embryos, larvae and adult zebrafish in order to observe the nickel effects in all stages of development. Moreover, we performed a complete behavior analysis in larvae and adults. The results of this study are subject to certain limitations. We cannot exclude the fact that some of the effects observed after nickel exposure might be due to alterations in some pathophysiological and molecular processes, which were not analyzed in the present study. In fact, it is described that nickel exposure might lead to oxidative stress and mitochondrial dysfunction that might influence behavioral parameters tested (Xu, et al., 2010, 2015). Moreover, we observed some effects after nickel exposure, in a concentration (0.025 mg/L) that not induced an increase in nickel levels detected by ICP-MS in brain zebrafish. These effects might occur by some tissue-specific impact, which is not detectable by ICP-MS since we used a homogenate of brain larvae. Therefore it is not possible to exclude the possibility that nickel has been deposited in specific brain regions able to induce behavioral alterations. On the other hand, for the best of our knowledge, most of these parameters were not analyzed in previous studies.

In summary, our study demonstrated that $NiCl_2$ exposure induces morphological and physiological alterations in early life stages and behavioral changes in various developmental stages of zebrafish, possibly owing to toxic effects induced by this metal. Furthermore, these alterations may significantly impact the behavioral and physiological responses of zebrafish in natural habitats and suggest risks to other aquatic organisms and other vertebrates, since $NiCl_2$ may be bioaccumulated and magnified in the food chain.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.057.

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