

Article

Combined Aluminum Chloride and Lead Acetate Exposure Induces Anxiety-like Behavior, Cognitive Impairment, and Cholinergic–Oxidative Dysregulation in Adult Zebrafish: Optimization of a Dementia-like Model

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Featured Application

This zebrafish co-exposure paradigm (AlCl₃ + lead acetate) provides a practical platform for dose-finding and for screening neuroprotective candidates targeting anxiety, cognition, and cholinergic–oxidative pathways.

Abstract

Dementia-related neurotoxicity induced by environmental metals remains a public health concern, and experimentally tractable vertebrate models that integrate behavioral and molecular endpoints are needed. Here, we established an adult zebrafish (*Danio rerio*) co-exposure paradigm using aluminum chloride (AlCl₃; 0.75 and 1.50 μM) and lead acetate (AcPb; 0.26 and 0.53 μM) and assessed anxiety-like behavior and cognition together with cholinergic and oxidative stress biomarkers. In the Novel Tank Diving Test, co-exposure increased latency to the top zone and reduced vertical exploration (top/bottom time ratio), with enhanced freezing, while average speed and total distance were not significantly altered, indicating an anxiogenic phenotype without major locomotor impairment. In the Novel Approach Test, the high co-exposure condition (AlCl₃ 1.5 μM + AcPb 0.53 μM) produced the strongest avoidance of the inner zone and increased immobility, effects attenuated by mexazolam (10 μM). Cognitive performance was markedly impaired under combined exposure: in the Novel Object Recognition Test, the novel-object preference index decreased from 64.83% (control) to 35.75–36.73% (co-exposure; $p = 0.0016$), whereas galantamine improved preference (58.60%). In the Y-maze test, spontaneous alternation dropped from 78.22% (control) to ~42.90–44.94% in AlCl₃ 200 and both co-exposures ($p = 0.0048$), and time in the novel arm decreased from 26.09 s to 10.53–11.35 s ($p < 0.0001$). Biochemically, metals inhibited brain acetylcholinesterase, most strongly under high co-exposure (7.60 vs. 15.92 nmol ATCh/min/mg; ~52%), and induced severe oxidative stress, evidenced by decreased superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione, alongside increased malondialdehyde (MDA) and protein carbonyls (with MDA doubling to 14.90 vs. 7.04 nmol/mg). Pearson correlations supported coupling between the cholinergic/redox axis and novelty-related behaviors. Overall, this easy-to-



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implement zebrafish Al–Pb model provides a practical platform for mechanistic studies and in vivo screening of candidate neuroprotective agents.

Keywords: zebrafish; aluminum chloride; lead acetate; anxiety-like behavior; cognition; acetylcholinesterase; oxidative stress; dementia-like model

1. Introduction

Neurodegenerative disorders impose a substantial and increasing societal burden, with dementia—most commonly Alzheimer’s disease (AD)—as a major contributor [1–3]. For the applied scope of toxicology-oriented research, a key priority is the development of experimentally tractable vertebrate models that can reproduce measurable, dementia-relevant functional impairments and link those outcomes to mechanistic pathways that are amenable to intervention and screening [4,5]. Across diverse neurodegenerative phenotypes, convergent processes include mitochondrial dysfunction, impaired proteostasis, neuroimmune activation, and synaptic failure, with oxidative/nitrosative stress acting as a central amplifier of neuronal injury. In parallel, cholinergic dysfunction remains an important functional axis that can be quantified biochemically and targeted pharmacologically, providing a practical bridge between molecular injury and behavior [6–11].

Among potentially modifiable environmental factors, exposure to metals has attracted sustained interest because many metals can disrupt redox balance, protein folding, and neuroimmune pathways relevant to neurodegeneration [12,13]. Recent epidemiological syntheses support associations between heavy metal exposure and AD/ADRD, while also emphasizing study heterogeneity and the challenges of accurately assessing real-world exposure [14]. Importantly, exposures often occur as mixtures, and this reality motivates experimental paradigms that model combined burdens rather than isolated exposures. Aluminum (Al), one of the most abundant elements in the environment, can enter the body through water, diet, and occupational exposure. Although the causal role of Al in AD remains controversial, experimental evidence indicates that aluminum exposure can induce oxidative stress, weaken antioxidant defenses, and disrupt neuronal signaling, mechanistically intersecting processes relevant to A β and tau [15,16]. A recent meta-analysis of environmental Al exposure reports an observational association with AD risk, while emphasizing substantial heterogeneity and methodological limitations (e.g., exposure assessment and observational study designs) [15,16].

Lead (Pb) is a well-established neurotoxic heavy metal and remains a widespread environmental contaminant. Mechanistically, Pb can interfere with calcium-dependent signaling, impair mitochondrial function, and reduce antioxidant capacity [17]. More recently, the literature has highlighted transcriptional and epigenetic reprogramming as a plausible link between early-life or chronic exposures and later-life vulnerability to neurodegenerative phenotypes [18]. A recent systematic review focusing on the implications of Pb in the context of AD describes mechanisms by which Pb exposure may contribute to A β accumulation and tau hyperphosphorylation, converging on neuroinflammation and oxidative injury as upstream drivers [19].

Danio rerio (*D. rerio*, zebrafish) offers a practical vertebrate platform for such applied modeling, combining conserved neurotransmitter systems, robust behavioral phenotyping, and compatibility with medium-to-high throughput experimental designs [20,21]. Particularly for metal-induced neurotoxicity, *D. rerio* can integrate behavioral endpoints (e.g., novel tank, Y-maze, and novel object recognition) with biochemical parameters aligned to cholinergic function and oxidative stress. For lead, an adult zebrafish study showed that chronic

low-concentration exposure can impair memory and exploratory behaviors [22]. Moreover, exposure to heavy metals, including lead acetate (AcPb), has been associated with reduced AChE activity in the zebrafish brain and alterations in antioxidant competence, suggesting an intersection between cholinergic transmission and oxidative balance in this model [23].

Therefore, the objective of the present study was to establish and characterize an adult zebrafish co-exposure model using aluminum chloride (AlCl_3) and AcPb that integrates anxiety-like behavior, recognition and spatial memory deficits, and cholinergic–oxidative dysregulation within a single experimental framework. By combining multi-domain behavioral phenotyping with biochemical validation and pharmacological controls, this approach provides a practical mechanistically grounded system to benchmark chemically diverse agents—including bioactive compounds and extracts—for their potential to attenuate neurobehavioral impairment and normalize cholinergic and redox pathways. We hypothesized that co-exposure would elicit stronger and more integrated dementia-relevant phenotypes than single-metal exposures and that these effects would be sensitive to pharmacological benchmarking with mexazolam (anxiolytic control) and galantamine (cognitive/cholinergic control).

2. Materials and Methods

2.1. Animals and Husbandry

A total of 90 adult wild-type zebrafish (*D. rerio*) ~5 months old, were used in the experiments (body length ~4 cm; body weight ~0.5 g), with a balanced sex ratio (1:1). Fish were obtained from a licensed supplier and acclimated in the experimental facility for three weeks prior to exposure to minimize handling- and transfer-related variability [24]. During acclimation, animals were housed in 10 L tanks (10 fish/tank) containing conditioned tap water (aged for 7 days and treated with a commercial dechlorinating agent, AquaSafe[®], Tetra GmbH, Melle, Germany, according to the manufacturer's instructions). Water was renewed daily by replacing ~80% of the tank volume with freshly prepared conditioned water. Husbandry conditions were maintained constant throughout the study: temperature ~26–28 °C (maintained by a climate-controlled room; no in-tank heaters were used), pH 7.0–7.5 (within the commonly recommended range for this species), dissolved oxygen 8 ± 1 mg/L (continuous aeration), conductivity 1500–1600 $\mu\text{S}/\text{cm}$, and ammonium and nitrite concentrations < 0.001 mg/L. A 14/10 h light/dark photoperiod was applied [25,26].

Feeding was performed once daily using a flake diet [24]. To limit variability arising from husbandry procedures, all groups received the same amount of food; during feeding, the air pump was switched off for 10 min to allow uniform intake. Husbandry procedures were kept strictly constant throughout the experiment, given that factors such as maintenance regimen and housing conditions can sensitively influence outcomes in behavioral paradigms [27]. All procedures were conducted in accordance with the European framework for the protection of animals used for scientific purposes (Directive 2010/63/EU) and were approved by the Animal Research Ethics Committee of the Faculty of Biology, Alexandru Ioan Cuza University of Iași (approval number: BIO-UAIC-5583, dated 12 December 2024) [28].

2.2. Chemicals and Exposure Design

Working solutions of AlCl_3 and AcPb were prepared from 100 $\times$ stock solutions and added directly to the exposure tanks to reach the target nominal concentrations. For each nominal final concentration (100 and 200 $\mu\text{g}/\text{L}$), corresponding 100 $\times$ stock solutions (10,000 and 20,000 $\mu\text{g}/\text{L}$) were prepared and subsequently diluted 1:100 in the final exposure volume. In practice, for each 5 L tank, the 100 $\times$ stock was added in the appropriate volume to achieve the target final concentration (e.g., 50 mL of a 100 $\times$ stock for a 1:100 dilution in ~5 L), yielding nominal concentrations of 100 $\mu\text{g}/\text{L}$ (from the 10,000 $\mu\text{g}/\text{L}$ stock) and 200 $\mu\text{g}/\text{L}$ (from the 20,000 $\mu\text{g}/\text{L}$ stock). In the co-exposure groups, AlCl_3 and AcPb were added from

their dedicated stocks so that each compound reached its planned nominal concentration in the same final volume. To control dilution volume effects, the control group received an equivalent volume of vehicle (conditioned water). These doses are low-dose experimental neurotoxicity ranges, not acute toxicity, used to induce sublethal, chronic-like effects [29,30].

Mexazolam (anxiolytic positive control) was prepared as a concentrated stock in dimethyl sulfoxide (DMSO) and diluted in conditioned water immediately prior to use. Galantamine (GAL, cognitive/cholinergic positive control) was prepared in conditioned water; however, to control for any vehicle-related effects and ensure uniformity across conditions, DMSO was present at 1% (*v/v*) in all experimental groups, including the control group.

Exposures were conducted in a 5 L glass tank under static conditions (no water recirculation). Tanks were gently aerated and equipped with simple mechanical/biological filtration using inert sponge/foam media to support water quality. To avoid altering effective metal concentrations, no adsorbent filter media capable of binding metals (e.g., activated carbon or ion-exchange resins) were used during exposure. Tap water was used after being aged/conditioned for 7 days and then treated with a commercial water conditioner (AquaSafe, according to the manufacturer's instructions) prior to use. Water conditioning/dichlorination is considered necessary in laboratory fish husbandry because chlorine/chloramines in municipal water can be toxic to fish and must be neutralized before use [25]. No salts or other regulators were added to adjust conductivity/osmolality, as the conditioned tap water provided an adequate ionic background for zebrafish husbandry; conductivity was monitored and remained within 1500–1600 $\mu\text{S}/\text{cm}$.

Throughout the experiment, the exposure medium was renewed daily (complete replacement), and treatments were re-administered immediately after each water change to maintain exposure as constant as possible. This approach corresponds to a static renewal/semi-static regimen, used to minimize temporal concentration fluctuations (e.g., due to degradation, adsorption, or losses during filtration), and is supported by aquatic toxicology guidelines recommending renewal of exposure solutions at ~24 h intervals (depending on substance stability) to maintain target concentrations [31].

Administration of AlCl_3 / AcPb began 7 days before the first behavioral assessment (Figure 1). Thereafter, each behavioral test was performed at 48 h (two-day) intervals relative to the previous test, while exposure was maintained under the daily renewal and re-dosing regimen until completion of the testing battery. Behavioral testing was performed on the same individuals using a within-subject battery.

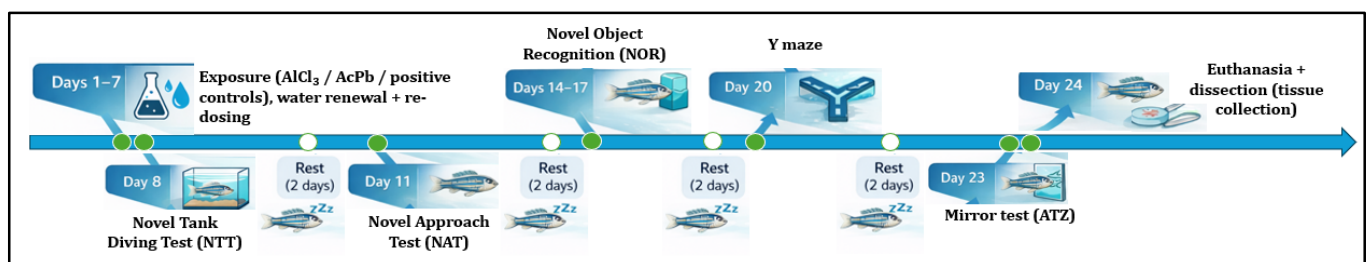


Figure 1. Experimental timeline of exposure and behavioral assessment in zebrafish. Fish were exposed to AlCl_3 , AcPb , or positive controls for 7 days (Days 1–7), with daily water replacement and re-dosing. Behavioral testing was then performed according to the following schedule: Novel Tank Diving Test (NTT, Day 8), Novel Approach Test (NAT, Day 11), Novel Object Recognition (NOR, Days 14–17; 4 consecutive days), Y-maze spontaneous alternation (Day 20), and Mirror test (ATZ, Day 23). All intervals between tests consisted of 2-day rest periods (Days 9–10, 12–13, 18–19, and 21–22). On Day 24, fish were euthanized, and tissues were collected for downstream analyses.

Nine experimental groups were used (Table 1) (e.g., number (n) = 10 fish/group): (1) Control; (2) AlCl_3 (0.75 μM); (3) AlCl_3 (1.50 μM); (4) AcPb (0.26 μM); (5) AcPb (0.53 μM); (6) AlCl_3 1.5 μM + AcPb (0.26 μM); (7) AlCl_3 (1.5 μM) + AcPb (0.53 μM); and (8) mexazolam (10 μM) as an anxiolytic positive control. A separate cohort included (9) galantamine (GAL, 2 μM) as a cognitive/cholinergic positive control, commonly used in zebrafish memory paradigms (e.g., Y-maze), including at 1 mg/L in the specialized literature [30,32].

Table 1. Experimental groups and nominal exposure concentrations.

Group	AlCl_3 (μM)	AcPb (μM)	Notes
Control	0	0	System water + 1% DMSO
AlCl_3	0.75	0	+1% DMSO
AlCl_3	1.50	0	+1% DMSO
AcPb	0	0.26	+1% DMSO
AcPb	0	0.53	+1% DMSO
AlCl_3 + AcPb	1.50	0.26	+1% DMSO
AlCl_3 + AcPb	1.50	0.53	+1% DMSO
Mexazolam	1.50	1.50	10 μM Mexazolam + 1% DMSO
GAL	1.50	1.50	2 μM GAL + 1% DMSO

Thus, across the entire study, there were nine distinct experimental conditions in total; however, each test battery included eight groups, with only one positive-control group applied per battery. Thus, nine experimental conditions were defined across the overall study, while each individual test battery included eight groups.

In line with ARRIVE 2.0 recommendations [33], fish were randomly allocated to experimental groups using a predefined randomization procedure (e.g., computer-generated random numbers). To minimize potential order effects, the sequence in which tanks/groups were run during behavioral testing was randomized across testing days. Behavioral testing and scoring were performed by trained experimenters blinded to treatment allocation using coded tank/video identifiers, and biochemical measurements were conducted on coded samples; statistical analyses were performed using blinded group codes, with decoding performed only after completion of data processing. Group size (n = 10 fish/group) was determined a priori based on prior experience with these paradigms and a power-based rationale (InVivoStat v4.0 (InVivoStat, London, UK) and an R-software v4.3.1 (R Foundation for Statistical Computing, Vienna, Austria)), ensuring adequate sensitivity to detect biologically meaningful differences in the primary outcomes [34].

2.3. Behavioral Testing

To evaluate the impact of AcPb and AlCl_3 on neurobehavioral function in adult *D. rerio*, a battery of ethological tests was applied to probe complementary behavioral domains, including anxiety-/stress-related indices, social reactivity/aggression, and cognitive performance. Testing was conducted during the light phase in a dedicated room with controlled illumination and noise, with each fish assessed individually. To reduce handling-related variability, transfer to the testing arena was performed rapidly and gently, and the testing order was kept constant and/or randomized across groups. Between trials, arenas were cleaned and refilled with fresh water to minimize chemical carry-over and residual olfactory/biological influences.

Behavior was video-recorded using a Logitech HD C922 Pro Stream camera (Full HD; Logitech, Lausanne, Switzerland) mounted above the arena and connected via USB to a laptop, and automated parameter quantification was performed with

ANY-maze® v7.2 (Stoelting Co., Wood Dale, IL, USA). To avoid confounding anxiety-like or cognitive alterations with potential global locomotor impairment, locomotor metrics such as total distance traveled and average velocity were systematically recorded and reported alongside each test's specific endpoints, enabling robust interpretation of behavioral effects in the context of overall activity levels.

2.3.1. Novel Tank Diving Test (NTT)

Novelty-induced diving anxiety was assessed using the NTT, a standardized zebrafish paradigm based on the initial tendency to remain in the lower portion of the tank and the gradual increase in exploration of the upper zone as anxiety diminishes. In line with configurations validated in the specialized literature, the NTT apparatus consisted of a ~2 L rectangular tank with dimensions of 16 (height) × 20 (length) × 5 (width) cm (Figure 2), filled with water to 15 cm, and virtually divided into two equal horizontal zones (lower vs. upper). The lateral and posterior walls were covered with an opaque white background to enhance contrast and reduce external visual cues [35].



Figure 2. Tank used for assessing anxiety-like behavior in adult zebrafish during the Novel Tank Diving Test (NTT).

Each fish was individually introduced into the tank and recorded for 6 min, and the ANY-maze® analysis included: time spent in the top/bottom zone, distance traveled in each zone, latency to the first entry into the top zone, and freezing/immobility indices (duration of episodes without displacement). In parallel, total distance traveled and average velocity were reported as locomotor control measures; representative trajectories were exported to illustrate the vertical exploration distribution (top vs. bottom), consistent with graphical representations of tracks and quantitative endpoints, in a manner like that described previously [36].

2.3.2. Novel Approach Test (NAT)

The NAT was used to assess novelty-induced anxiety (neophobia) in adult zebrafish, based on their tendency for thigmotaxis (predominant swimming near the tank walls) and avoidance of an unfamiliar object perceived as potentially threatening. The protocol was implemented in accordance with established descriptions of NAT in *D. rerio* [37], with minor adaptations of the arena geometry to the laboratory set-up [38]. In practice, each fish was individually introduced into a circular tank (20 cm in diameter), at the center of which a novel object was fixed (a multicolored LEGO® figurine LEGO System A/S, Billund, Denmark; Figure 3), attached to the bottom of the arena with silicone and kept fully submerged throughout the test. Recording was performed from a top-down perspective (camera mounted above the arena), and trajectory analysis was carried out using ANY-maze.

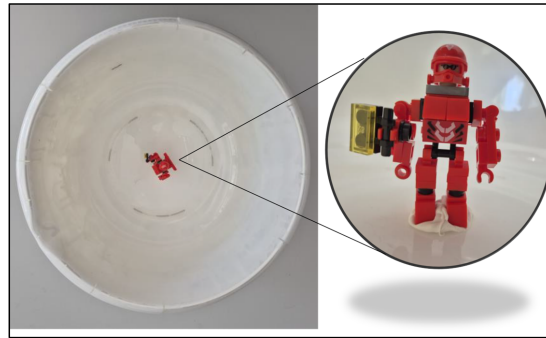


Figure 3. Tank used to assess anxiety-like behavior in adult zebrafish during the Novel Approach Test (NAT) and the figurine used as the anxiety-inducing novel stimulus in the NAT.

The arena was virtually divided into two zones: (a) a central zone defined as a 5 cm-diameter circle surrounding the novel object, and (b) a peripheral/marginal zone (the remainder of the arena). Each individual was recorded in a single 10 min session, and the quantified parameters included: time and distance spent in the central versus peripheral zone (greater center exploration indicating lower anxiety), latency to the first entry into the central zone (longer latencies indicating higher anxiety), immobility/freezing time, and locomotor control measures (total distance traveled and average velocity).

2.3.3. Novel Object Recognition (NOR)

The NOR test was used to assess recognition memory in zebrafish, based on their natural tendency to preferentially explore an unfamiliar object when it is presented together with a familiar object. The protocol was implemented in accordance with established descriptions for *D. rerio*, using a shallow water column (~4 cm) to restrict vertical swimming and enable accurate quantification of exploration from a top-down video recording [39].

The experimental arena consisted of a tank with a square base (top-view), placed on a white background to enhance contrast; to minimize external interference (reflections/visual cues), the setup was shielded with opaque material. The objects were two small rubber spherical toys of regular shape, fixed to the bottom of the tank with suction cups so that they remained fully submerged and stationary throughout the test (as illustrated in the figure: FO—familiar object; NO—novel object) (Figure 4).

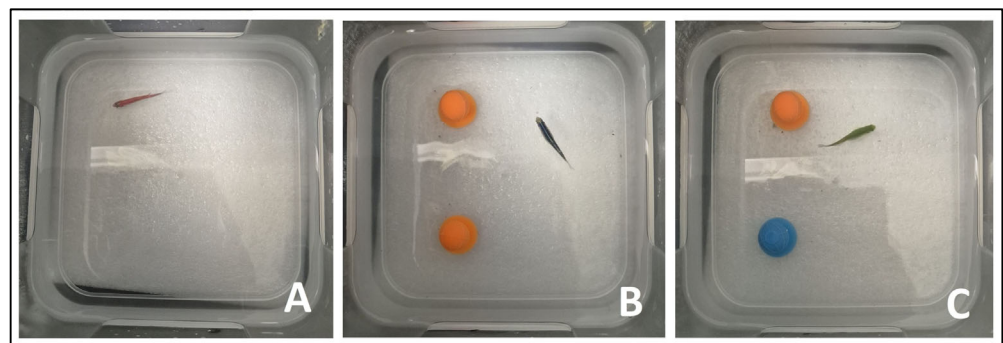


Figure 4. Stages of the Novel Object Recognition (NOR) test in *D. rerio*. (A) Habituation: the fish is introduced into the test arena in the absence of objects (two 5 min sessions) to allow familiarization with the environment. (B) Training/Familiarization: a 10 min exposure to two identical objects (orange spheres) fixed to the bottom of the arena to form a representation of the familiar object (FO). (C) Testing: a 10 min exposure during which one familiar object remains unchanged (orange), while the other is replaced with a novel object (NO, blue sphere); preference is quantified as the time spent in the novel-object exploration zone relative to the total exploration time of both objects. Images are shown from a top-down view, consistent with automated analysis in ANY-maze.

Testing comprised three phases: (i) habituation in the absence of objects (two 5 min sessions to familiarize the fish with the arena); (ii) training/familiarization (10 min) in the presence of two identical objects (both orange) to form a representation of the familiar object; and (iii) testing (10 min), in which one object was replaced with a novel object (blue), while the other remained familiar. The arena floor was virtually divided into three regions: the familiar object exploration zone (FO), the novel object exploration zone (NO), and the remaining area (XO). Recordings were obtained with a camera mounted above the arena, and automated analysis was performed in ANY-maze (zone-based tracking), in a manner similar to that described previously [36]. The primary endpoint was novel-object preference, expressed as a percentage:

$$Preference\%(NO) = \frac{T(NO)}{T(NO) + T(FO)} \times 100$$

where T (NO) and T (FO) represent the time spent in the NO and FO zones, respectively (the XO zone was used to describe overall exploration but was not included in the preference calculation).

2.3.4. Y-Maze Spontaneous Alternation

Spatial memory was assessed using the Y-maze test, which exploits the natural tendency of zebrafish to explore novelty. The maze was constructed from a glass tank with three arms (each arm: 25 cm length $\times$ 8 cm width $\times$ 15 cm height) arranged at 120° (Figure 5). For spatial orientation, visual cues (distinct geometric shapes) were placed on the external walls of each arm, while the remaining walls were made opaque/masked to reduce external stimuli; the bottom was kept white to enhance contrast and facilitate video tracking. The maze was filled with water from the holding tank/system water to a volume sufficient to fully cover the visual cues and ensure uniform exploration [40].

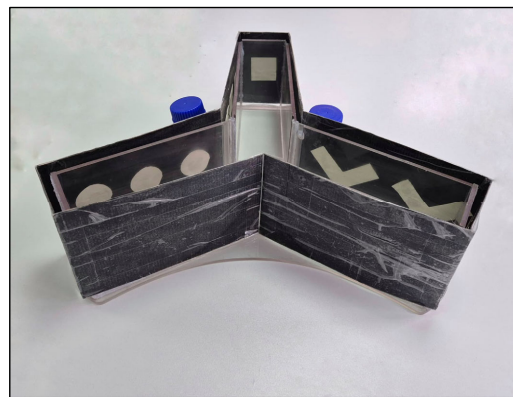


Figure 5. Y-maze apparatus used to assess spatial memory in adult zebrafish during the spontaneous alternation test. The maze consisted of three arms arranged at 120°, with distinct geometric visual cues on the external walls of each arm and masked opaque walls to reduce external stimuli.

The protocol comprised two trials: (i) a training phase (5 min), during which the fish had access to the start arm and the “other” arm, while the novel arm was blocked with a transparent divider; and (ii) a test phase (5 min), during which the fish was reintroduced through the start arm and allowed free access to all three arms. Arm assignment (start/novel/other) was randomized, and the neutral central zone was not included in the analysis. Recording was performed from a top-down perspective, and automated analysis was carried out in ANY-maze, quantifying: time spent in the novel arm, number of arm entries, spontaneous alternation (%) derived from the sequence of entries (commonly calculated as alternations/(total entries – 2) $\times$ 100), number of line crossings, and total distance traveled (locomotor control) [40,41].

2.3.5. Mirror Test (ATZ)

Social reactivity and impulse-like responding were assessed using the mirror test (mirror image stimulation), based on the fact that zebrafish respond to their reflection as if it were a conspecific, displaying approaches, contacts, and “biting/butting” episodes toward the mirror. The protocol followed the variant in which the fish is introduced into a tank that already contains the mirror (modification 2), described as a standard approach for quantifying mirror-biting responses [42]. In this experiment, the same tank as in the NTT was used, and a mirror was fixed with silicone to the inner surface of one lateral wall, sized to fit the wall. The tank was virtually divided into three regions, in accordance with testing guidelines: the mirror contact zone (0.5 cm from the mirror), the approach zone (an additional 2.5 cm beyond the contact zone, corresponding to the average length of an adult fish), and the peripheral zone (the remainder of the arena) [43].

Each fish was introduced individually (recommended from the end opposite the mirror to reduce transfer-stress-related confounding) and recorded for 3 min; analysis was performed in ANY-maze based on side-view video recordings, quantifying: time spent in each zone (peripheral/intermediate/contact), the number of mirror contacts/“bites” (mirror contacts/biting), and total distance traveled (locomotor control).

2.4. Biochemical Analyses

Following completion of the behavioral battery, zebrafish were euthanized by rapid cooling on ice. Whole brains were promptly excised, gently blotted, weighed, and processed on ice to minimize post-mortem enzymatic changes. Tissue was homogenized in ice-cold phosphate-based extraction buffer (0.1 M potassium phosphate, 1.15% KCl, pH 7.4; [1:10 *w/v*]) and clarified by centrifugation ([14,000 rpm/g], 10 min, 4 °C). The resulting supernatants were used for all biochemical determinations. Total protein content was measured by the Bradford method [43], and all endpoints were expressed relative to protein concentration (per mg protein). All reaction mixtures were prepared in 1.5 mL microcentrifuge tubes (Eppendorf-type) and, after the specified incubation and/or centrifugation steps, the reaction mixtures (or supernatants) were transferred to 1 cm pathlength disposable plastic cuvettes for absorbance measurements using a UV-Vis spectrophotometer (Beckman Coulter DU 730 Life Science, Beckman Coulter, Brea, CA, USA), as previously described [36].

Acetylcholinesterase (AChE; EC 3.1.1.7) activity was quantified using a DTNB-based colorimetric assay (Ellman method), with AChE iodide as substrate [44]. Briefly, the reaction mixture contained 0.25 M sodium phosphate buffer (pH 7.4), DTNB (1 mM), enzymatic extract, and a AChE iodide (5 mM) in a final volume of 1.475 mL (including the stop solution). The rate of thiocholine formation was monitored at 412 nm, and AChE activity was reported as nmol/min/mg protein. Antioxidant defense and oxidative injury were evaluated by quantifying superoxide dismutase (SOD; EC 1.15.1.1) [45], catalase (CAT; EC 1.11.1.6) [46], and glutathione peroxidase (GPx; EC 1.11.1.9) [47] activities, reduced glutathione (GSH) content [48], lipid peroxidation as malondialdehyde (MDA; TBARS) [49], and protein oxidation as protein carbonyls (PC; DNPH) [50]. SOD activity was assessed by inhibition of nitro blue tetrazolium (NBT) reduction in a riboflavin–light system [45]. Reaction mixtures contained 0.067 M potassium phosphate buffer (pH 7.8), EDTA (0.1 M), NBT (1.5 mM), riboflavin (0.12 mM), and enzymatic extract (omitted for blanks); after controlled light exposure, absorbance was read at 560 nm. SOD activity was expressed as units/mg protein, where one unit corresponds to 50% inhibition of maximal NBT reduction.

CAT activity was determined using the dichromate–acetic acid method [46] by incubating enzymatic extract with H₂O₂ substrate solution (0.16 M in phosphate buffer, pH 7.4) for 3 min; the reaction was stopped with potassium dichromate–glacial acetic acid reagent,

heated for 10 min at 95 °C, centrifuged (14,000 rpm, 5 min), and read at 570 nm. CAT activity was calculated from an H₂O₂ standard curve (0–80 µmol/mL) and expressed as µmol H₂O₂ decomposed/min/mg protein.

GPx activity was determined by monitoring enzyme-driven oxidation of GSH in the presence of H₂O₂ [47]. For GPx, the reaction mixture included enzymatic extract in 0.25 M sodium phosphate buffer (pH 7.4) with EDTA (25 mM) and NaN₃ (0.4 M); after pre-incubation (10 min, 37 °C), GSH (50 mM) and H₂O₂ (50 mM). After incubation under controlled conditions, the reaction was stopped with metaphosphoric acid, samples were centrifuged (14,000 rpm, 10 min), and residual GSH in the supernatant was quantified by DTNB at 412 nm. GPx activity was expressed as µmol GSH oxidized/min/mg protein. GSH levels were measured spectrophotometrically by DTNB at 412 nm [48].

Oxidative damage was evaluated at the lipid and protein levels. Lipid peroxidation was assessed as MDA equivalents using the TBARS assay [49]: homogenates were reacted with TBA reagent, heated for 1 h at 95 °C, cooled, centrifuged (14,000 rpm, 10 min), and the supernatant absorbance was read at 532 nm against a reagent blank. Protein oxidation was quantified as protein carbonyl content following DNPH derivatization [50]: proteins were precipitated with TCA, derivatized with DNPH, washed with ethanol:ethyl acetate (1:1), solubilized in guanidine hydrochloride, and absorbance was recorded at 370 nm; results were expressed as nmol DNPH/mg protein. For both assays, reagent blanks/controls were included, and measurements were performed in duplicate/triplicate when sample volume allowed.

For each assay, blanks and quality controls were included, and measurements were performed in duplicate/triplicate when sample volume allowed.

2.5. Statistical Analysis

Data are shown as individual values ($n = 10$ fish/group) and summarized as mean $\pm$ SD. All statistical analyses were performed in GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA). Between-group differences were primarily evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances using the Brown–Forsythe and Bartlett's tests.

To determine whether combined exposure produced effects exceeding simple additivity, planned 2×2 two-way ANOVAs were conducted with the factors Al exposure (present/absent) and Pb exposure (present/absent) on matched subsets comprising Control, AlCl₃ 1.50 µM, AcPb (0.26 or 0.53 µM), and their respective combinations. For these models, the Al $\times$ Pb interaction term was reported as the primary indicator of non-additive (interactive) effects. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Subsection Novel Tank Diving Test Reveals Anxiety-like Behavioral Alterations Induced by Al-Pb Co-Exposure

In the NTT (Figure 6), representative tracks (Figure 6A) suggested predominantly bottom-dwelling behavior and reduced vertical exploration, particularly under the co-exposure condition AlCl₃ 1.5 µM + AcPb. This observation was supported by quantitative analysis: latency to the first entry into the upper zone differed significantly among groups ($F(7.72) = 5.508$; $p < 0.0001$; Figure 6B) and was higher in the combined-exposure groups than in controls (AlCl₃ + AcPb 0.26 µM: $p = 0.0036$; AlCl₃ + AcPb 1.5 µM: $p = 0.0011$). In parallel, the top/bottom time ratio was significantly affected by treatment ($F(7.72) = 5.440$; $p < 0.0001$; Figure 6C), with co-exposure maintaining lower values compared with mexazolam (AlCl₃ + AcPb 0.26 µM: $p = 0.0319$; AlCl₃ + AcPb 0.53 µM: $p = 0.0040$), indicating a persistent preference for the lower zone.

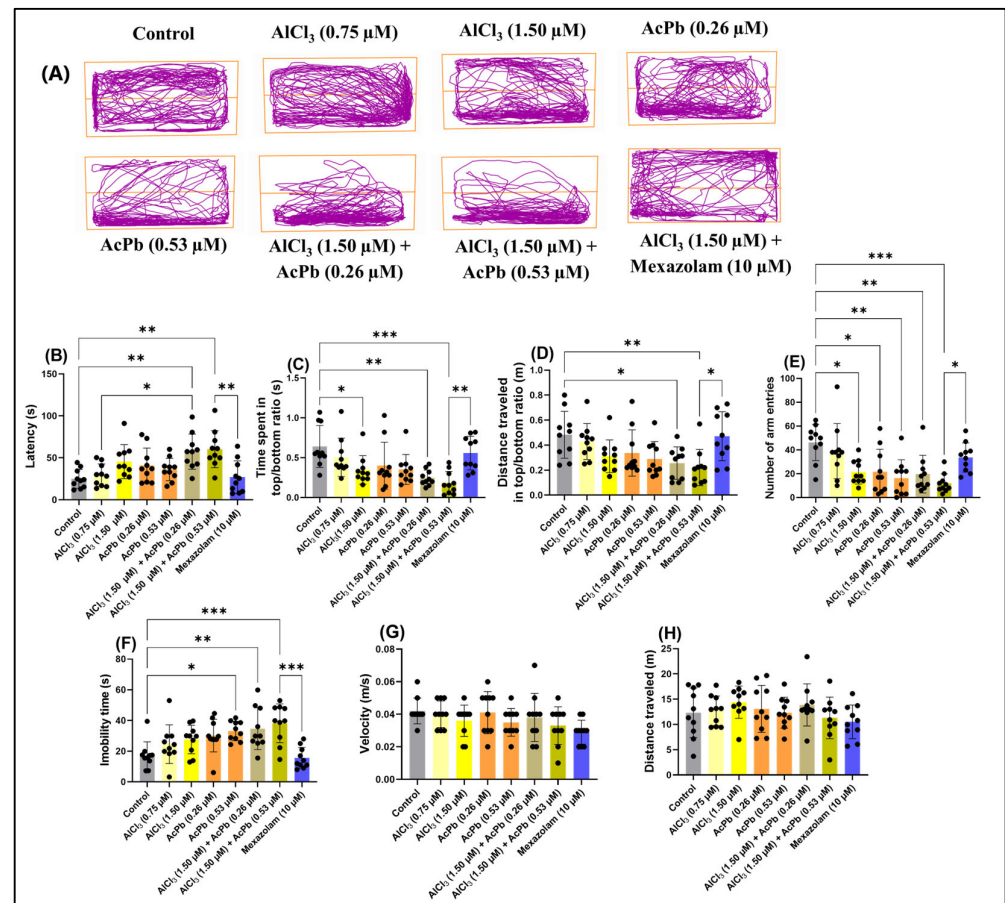


Figure 6. Effects of aluminum chloride (AlCl₃) and lead acetate (AcPb) exposure on anxiety-like behavior and locomotor activity in the Novel Tank Diving Test (NTT) in adult zebrafish. (A) Representative swimming tracks (ANY-maze) for each group during the trial (tank virtually divided into upper and lower halves). (B) Latency to the first entry into the top zone (s). (C) Top/bottom time ratio. (D) Top/bottom distance ratio. (E) Number of entries (transitions) between the virtual compartments. (F) Freezing/immobility time (s). (G) Mean velocity (m/s). (H) Total distance traveled (m). Groups: Control; AlCl₃ (0.75 and 1.50 µM); AcPb (0.26 and 0.53 µM); AlCl₃ (1.5 µM) + AcPb (0.26 or 0.53 µM); Mexazolam (10 µM). Data are presented as mean ± SD with individual data points overlaid (*n* = 10/group). Differences were analyzed by one-way ANOVA followed by Tukey's post hoc test; significance is indicated in the graph: * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

Similarly, the top/bottom distance ratio was significantly affected ($F(7.72) = 3.914$; $p = 0.0011$; Figure 6D), with the most relevant difference observed between the higher-dose co-exposure and mexazolam (AlCl₃ + AcPb 0.53 µM vs. mexazolam: $p = 0.0160$). In addition, the number of entries (transitions) between the virtual compartments differed significantly among groups ($F(7.72) = 6.023$; $p < 0.0001$; Figure 6E), and the AlCl₃ + AcPb (0.53 µM) combination showed fewer transitions than mexazolam ($p = 0.0465$), suggesting reduced exploration. The freezing component was also treatment-sensitive: immobility time increased significantly ($F(7.72) = 6.104$; $p < 0.0001$; Figure 6F), with clear differences versus mexazolam for AcPb 0.53 µM ($p = 0.0075$) and for both co-exposures (AlCl₃ + AcPb 0.26 µM: $p = 0.0026$; AlCl₃ + AcPb 0.53 µM: $p = 0.0002$). Importantly, mean velocity (Figure 6G) and total distance traveled (Figure 6H) did not differ significantly among groups ($p = 0.1031$ and $p = 0.3816$, respectively), supporting that the observed changes primarily reflect increased anxiety-like behavior and reduced vertical exploration rather than a global locomotor deficit.

3.2. Subsection Novel Approach Test Reveals Anxiety-like Behavioral

In the NAT (Figure 7), significant group differences were observed for latency to enter the inner zone (Figure 7B; one-way ANOVA: $F(7.72) = 3.573$, $p = 0.0024$). Co-exposure to $AlCl_3$ 1.50 μ M + AcPb 0.53 μ M was associated with the most pronounced delay in inner-zone exploration, being higher than in the AcPb 0.26 μ M and AcPb 0.53 μ M groups (both $p = 0.0479$) and than in the mexazolam 10 μ M group ($p = 0.0091$), suggesting an exacerbated anxiogenic response under higher-dose co-exposure. Consistently, time spent in the outer zone differs among groups (Figure 7C; $F(7.72) = 3.382$, $p = 0.0035$), with corresponding changes mirrored by time spent in the inner zone (Figure 7D; $F(7.72) = 3.382$, $p = 0.0035$), indicating a redistribution of exploration between the periphery and the center (thigmotaxis). Overall, the pattern in the figure indicates a stronger peripheral preference under co-exposure, whereas mexazolam counteracted this profile (relative to the high-dose co-exposure; $p = 0.0144$ for the relevant post hoc comparison).

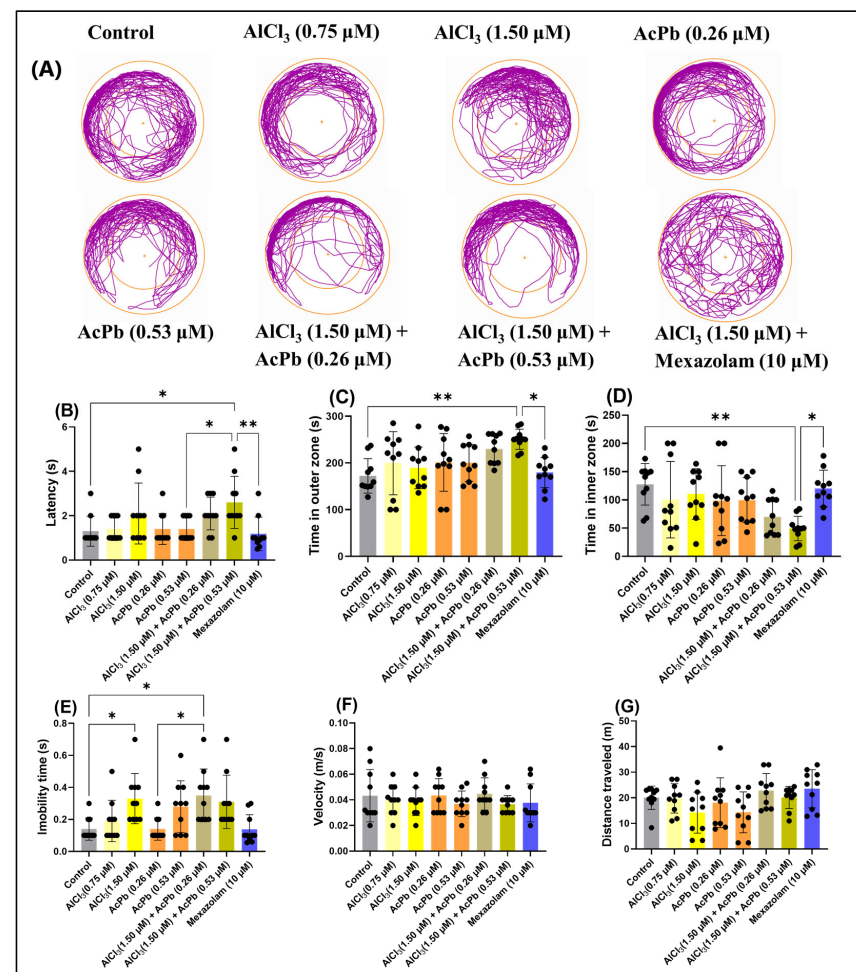


Figure 7. Novel Approach Test (NAT)- avoidance of a novel object (anxiety-like behavior). (A) Representative zebrafish swimming tracks in the circular arena during the NAT, with virtual delimitation of the central zone (around the object) and the peripheral/outer zone; the novel stimulus object was placed centrally. (B–G) Quantification of NAT parameters for the eight experimental groups (Control; $AlCl_3$ 0.75 μ M; $AlCl_3$ 1.50 μ M; AcPb 0.26 μ M; AcPb 0.53 μ M; $AlCl_3$ 0.75 μ M + AcPb 0.26 μ M; $AlCl_3$ 0.75 μ M + AcPb 0.53 μ M; Mexazolam 10 μ M): (B) latency to the first entry into the central zone; (C) time spent in the peripheral (outer) zone; (D) time spent in the central (inner) zone; (E) immobility (freezing); (F) total distance traveled; (G) mean velocity. Data are shown as individual values (dots) and mean $\pm$ SD (bars), $n = 10$ fish/group. Differences were analyzed by one-way ANOVA followed by Tukey's post hoc test; significance is indicated in the graph: * $p < 0.05$ and ** $p < 0.01$.

For immobility (Figure 7E), robust differences were detected among treatments ($F(7.72) = 4.827, p = 0.0002$). Compared with the Control group, AlCl_3 1.50 μM increased immobility ($p = 0.0398$), and co-exposure to AlCl_3 1.50 μM + AcPb 0.26 μM also showed higher immobility ($p = 0.0152$). Moreover, AlCl_3 1.50 μM exceeded the AcPb 0.26 μM group ($p = 0.0398$), and the AlCl_3 1.50 μM + AcPb 0.26 μM co-exposure was higher than AcPb 0.26 μM ($p = 0.0152$). Mexazolam reduced immobility relative to both AlCl_3 1.5 μM ($p = 0.0371$) and the AlCl_3 1.5 μM + AcPb 0.26 μM co-exposure ($p = 0.0141$), confirming the sensitivity of the test to an anxiolytic control. Regarding locomotor parameters, mean velocity did not differ among groups (Figure 7F; $F(7.72) = 0.5945, p = 0.7583$), and distance traveled showed only a modest overall effect (Figure 7G; $F(7.72) = 2.400, p = 0.0289$) without robust pairwise differences after Tukey correction, supporting that the observed changes in latency/thigmotaxis/immobility primarily reflect an anxiety-like component rather than major locomotor impairment.

3.3. Cognitive Performance in NOR

As shown in the representative swimming tracks and bar plots (NOR; Figure 8A), object-directed exploration (time spent in the familiar object zone, FO, and the novel object zone, NO) displayed a treatment-dependent pattern, supported by a significant interaction in the two-way ANOVA ($F(7.144) = 3.848, p = 0.0007; ***$). In contrast, neither the main effect of treatment (row factor, $p = 0.0961$) nor the overall difference between object zones (column factor, FO vs. NO; $p = 0.0897$) reached significance, indicating that the FO/NO balance varied across groups rather than following a uniform shift. Across all animals, mean exploration times were slightly higher in FO (30.19 s) than in NO (26.69 s), but this difference was not significant.

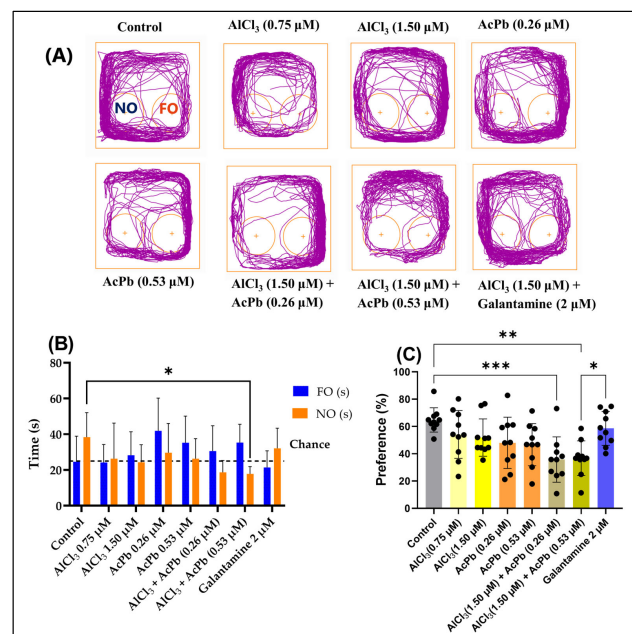


Figure 8. Novel Object Recognition (NOR) performance in zebrafish following exposure to AlCl_3 and/or lead acetate (AcPb), and the effect of galantamine (GAL). (A) Representative locomotor trajectories during the NOR test, highlighting exploration around the familiar object (FO) and novel object (NO) zones. (B) Time spent exploring FO and NO (s) for each experimental group. (C) Preference index (%) for the novel object (NO), calculated as NO exploration relative to total object exploration. Bars represent mean $\pm$ SD, with individual data points shown ($n = 10/\text{group}$). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

At the group level (cell means, seconds), controls showed higher NO than FO exploration (FO 24.61 vs. NO 38.40), whereas the combined exposure groups displayed a clear redistribution of exploration toward the familiar object zone (AlCl₃ + AcPb 0.26 µM: FO 30.58 vs. NO 18.64; AlCl₃ + AcPb 0.53 µM: FO 35.28 vs. NO 17.81), consistent with reduced novelty-directed behavior visible in the trajectories (Figure 8). Post hoc comparisons across cell means further supported this pattern, with NO time being significantly lower in AlCl₃ + AcPb (0.53 µM µg/L) compared with Control: NO (adjusted $p = 0.0412$). GAL shifted exploration toward a more “control-like” profile (FO 21.42 vs. NO 32.10), consistent with improved novelty-oriented behavior in the tracks (Figure 8B).

In line with the above, the preference index differed significantly among treatments (one-way ANOVA: $F(7,72) = 4.598$, $p = 0.0003$; ***; Figure 8). Preference was markedly reduced under combined exposures (AlCl₃ + AcPb 0.26 µM: 35.75; AlCl₃ + AcPb 0.53 µM: 36.73) versus Control (64.83), with significant pairwise differences (Control vs. AlCl₃ + AcPb 0.26 µM: $p = 0.0010$; Control vs. AlCl₃ + AcPb 0.53 µM: $p = 0.0016$). Importantly, GAL improved preference (58.60%) and was significantly higher than both combined exposure groups (AlCl₃ + AcPb 0.26 µM vs. GAL: $p = 0.0203$; AlCl₃ + AcPb 0.56 µM vs. GAL: $p = 0.0309$), while single toxicant groups (AlCl₃ or AcPb alone) did not differ significantly from Control in the post hoc analysis (Figure 8C).

3.4. Cognitive Performance in Y Maze

For the number of arm entries, a global treatment effect was detected by one-way ANOVA ($F(7,72) = 2.171$, $p = 0.0469$). However, none of the pairwise comparisons remained significant after Tukey correction (all adjusted $p \geq 0.187$), in the context of variance heterogeneity (Brown–Forsythe $p = 0.0038$; Bartlett $p = 0.0003$), suggesting that this overall effect should be interpreted with caution (Figure 9B).

Locomotor activity quantified as distance traveled differed significantly among treatments ($F(7,72) = 3.472$, $p = 0.0029$) (Figure 9C). Post hoc testing indicated that the combined exposure at 200 µg/L (AlCl₃ + AcPb 0.53 µM) produced a greater distance traveled than AlCl₃ 1.50 µM alone (19.28 vs. 11.48 m; mean difference = 7.80 m; 95% CI 1.49–14.11; adjusted $p = 0.0057$) and then the AlCl₃ + AcPb 0.26 µM group (19.28 vs. 12.80 m; mean difference = 6.48 m; 95% CI 0.17–12.79; adjusted $p = 0.0399$) (Figure 9C). Consistently, mean velocity also showed a significant treatment effect ($F(7,72) = 2.893$, $p = 0.0101$), with AlCl₃ 1.50 µM exhibiting a lower velocity than the combined 200 µg/L exposure group (0.0300 vs. 0.0586 m/s; mean difference = −0.0286; 95% CI −0.0545 to −0.0027; adjusted $p = 0.0205$) (Figure 9D).

In contrast to these displacement-related changes, turn angle (°) showed a robust group effect ($F(7,72) = 4.182$, $p = 0.0006$), driven mainly by the combined exposure groups (Figure 9E). Specifically, the Control group exhibited a higher turning angle than AlCl₃ + AcPb 0.26 µM (41.020 vs. 26.449; adjusted $p = 0.0166$) and AlCl₃ + AcPb 0.53 µM (41.020 vs. 25.474; adjusted $p = 0.0081$). Moreover, AlCl₃ 0.75 µM differed from AlCl₃ + AcPb 0.53 µM (adjusted $p = 0.0381$), and both combined exposure groups had lower turning angles than GAL (AlCl₃ + AcPb 0.26 µM vs. GAL: adjusted $p = 0.0432$; AlCl₃ + AcPb 0.53 µM vs. GAL: adjusted $p = 0.0224$), suggesting a more “straight/less sinuous” exploration profile under co-exposure (Figure 9E).

Exploratory activity estimated by line crossings differed strongly among groups ($F(7,72) = 5.907$, $p < 0.0001$). AlCl₃ 1.50 µM showed reduced crossings compared with Control (28.46 vs. 49.06; adjusted $p = 0.0129$), AcPb 0.26 µM (28.46 vs. 51.68; adjusted $p = 0.0030$), and GAL (28.46 vs. 50.10; adjusted $p = 0.0073$). The AlCl₃ + AcPb 0.53 µM group also displayed fewer crossings than Control (29.26 vs. 49.06; adjusted $p = 0.0196$) and GAL (29.26 vs. 50.10; adjusted $p = 0.0114$). Additionally, AcPb 0.26 µM exceeded both combined

exposure groups (vs. AlCl_3 + AcPb 0.26 μM : adjusted $p = 0.0292$; vs. AlCl_3 + AcPb 0.53 μM : adjusted $p = 0.0047$) (Figure 9F).

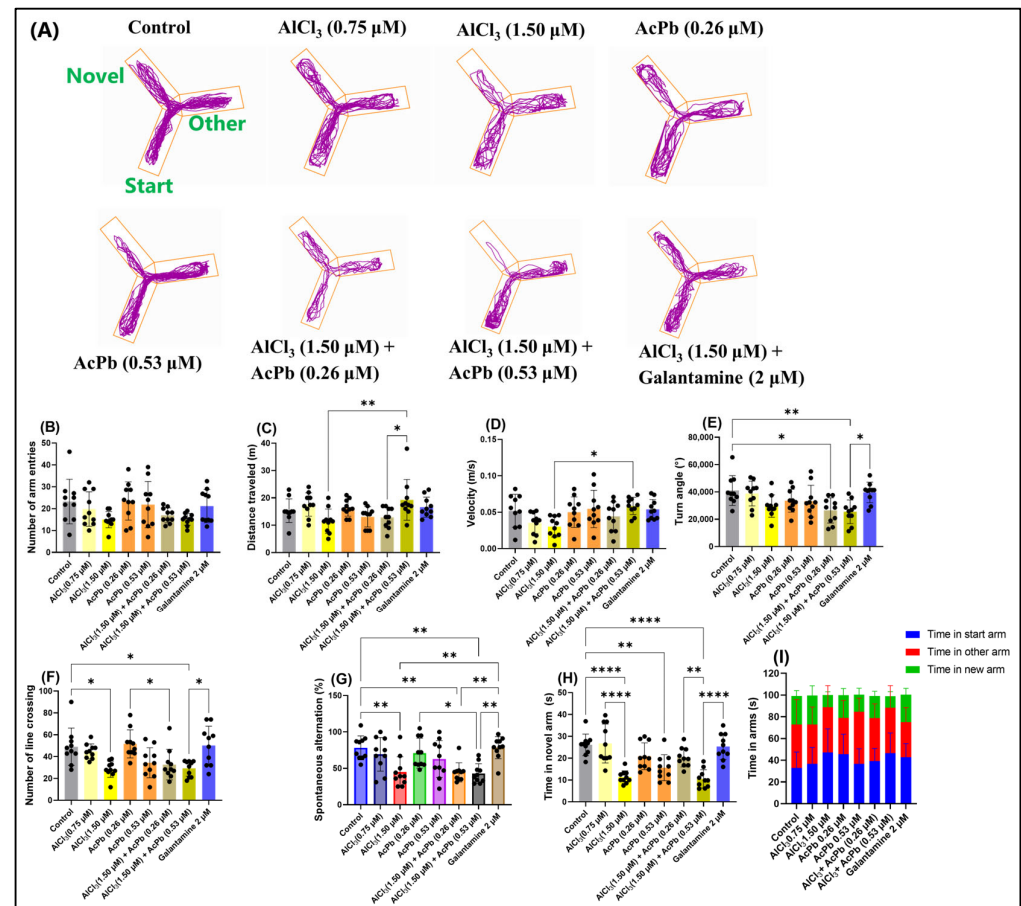


Figure 9. Y-maze performance and locomotor endpoints in zebrafish. (A) Representative locomotor trajectories during the Y-maze task; arms are labeled Start, Other, and Novel. (B–E) Locomotor/exploration metrics: number of arm entries (B), distance traveled (m) (C), average velocity (m/s) (D), and cumulative turn angle (°) (E). (F–H) Cognitive readouts during the test phase: line crossing (F), spontaneous alternation (%) (G), and time spent in the novel arm (s) (H). (I) Stacked bars showing the relative time distribution across Start, Other, and Novel arms. Bars represent group means $\pm$ SD with individual data points overlaid; statistical significance is indicated by brackets (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$).

Working memory performance, assessed by spontaneous alternation (%), was significantly influenced by treatment ($F(7,72) = 6.418$, $p < 0.0001$) (Figure 9G). Compared with Control (78.22%), alternation decreased markedly in AlCl_3 1.50 μM (44.94%; adjusted $p = 0.0048$), AlCl_3 + AcPb 0.26 μM (44.50%; adjusted $p = 0.0041$), and AlCl_3 + AcPb 0.53 μM (42.90%; adjusted $p = 0.0022$). Importantly, GAL maintained alternation at a level comparable with Control (78.40%) and differed significantly from AlCl_3 1.50 μM (adjusted $p = 0.0045$) and from both co-exposure groups (AlCl_3 + AcPb 0.26 μM vs. GAL: adjusted $p = 0.0038$; AlCl_3 + AcPb 0.53 μM vs. GAL: adjusted $p = 0.0020$). Moreover, AcPb 0.26 μM exceeded the 200 $\mu\text{g/L}$ co-exposure group (70.90 vs. 42.90%; adjusted $p = 0.0315$), indicating that higher-dose combined exposure was associated with the poorest alternation profile (Figure 9G).

Exploration of the novel arm, quantified as time spent in the novel arm (s), also showed a pronounced treatment effect ($F(7,72) = 13.03$, $p < 0.0001$) (Figure 9H). Relative to Control (26.09 s), novel-arm time was significantly reduced in AlCl_3 1.53 μM (11.35 s; adjusted $p < 0.0001$), AcPb 0.53 μM (15.65 s; adjusted $p = 0.0025$), and AlCl_3 + AcPb 0.53 μM (10.53 s; adjusted $p < 0.0001$). Several contrasts supported a dose-dependent impairment

amplified by co-exposure: AlCl_3 0.75 μM exceeded AlCl_3 0.75 μM (26.71 vs. 11.35 s; adjusted $p < 0.0001$) and exceeded $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (26.71 vs. 10.53 s; adjusted $p < 0.0001$). The $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM group also spent more time in the novel arm than $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (20.40 vs. 10.53 s; adjusted $p = 0.0052$). GAL maintained higher novel-arm exploration (25.30 s), differing significantly from AlCl_3 1.50 μM (adjusted $p < 0.0001$), AcPb 0.53 μM (adjusted $p = 0.0068$), and $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (adjusted $p < 0.0001$) (Figure 9H).

To capture time redistribution across arms (start/other/novel), two-way ANOVA revealed a significant interaction ($F(14.216) = 2.435$, $p = 0.0034$) and a strong main effect of arm (column factor: $F(2.216) = 56.77$, $p < 0.0001$) (Figure 9I), consistent with treatment-dependent changes in arm occupancy. Notably, the most affected groups showed a clear reduction in novel-arm occupancy accompanied by increased time in the start/other arms (e.g., AlCl_3 1.50 μM : start 47.30 s; other 41.50 s; novel 11.35 s; $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM : start 46.50 s; other 41.90 s; novel 10.53 s), supporting the one-way ANOVA results for novel-arm time.

Importantly, the Y-maze data (Figure 9) also highlight a potential locomotor confound: whereas AlCl_3 1.50 μM L reduced exploration (line crossings) and clearly impaired cognitive indices (alternation and novel-arm time), the $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM group displayed increased distance/velocity yet still showed poor cognitive performance. This pattern suggests that reduced alternation/novel-arm preference is not simply explained by hypoactivity but rather reflects a less efficient or more disorganized exploration strategy.

3.5. Internal Consistency of Novelty-Preference Endpoints

The heatmaps in Figure 10 illustrate the individual-level distribution of two variables associated with novelty preference for each experimental group (Figure 10A–C), highlighting inter-individual variability and treatment-related profile differences: (Figure 10B) T_{new} (time spent in the “new” component) and (Figure 10C) preference (preference index/parameter).

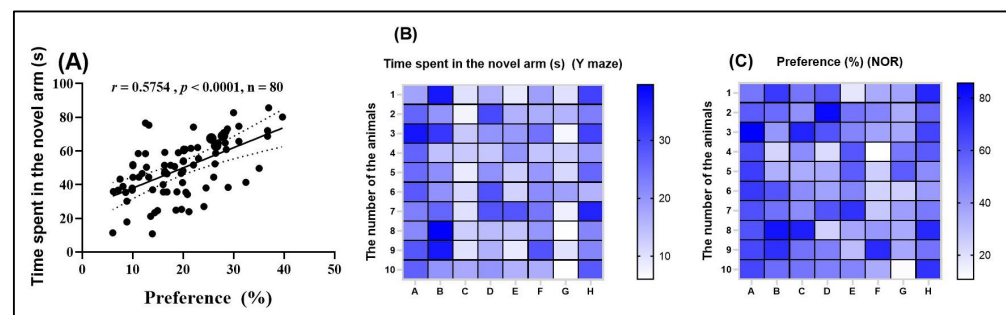


Figure 10. Heatmaps of individual-level responses for novelty-preference-related parameters across experimental groups (A–H). Each row represents one animal, and each column represents one group (A–H). Color intensity reflects response magnitude (higher values = darker shades). (A) Time spent in the novel arm (s) vs. Preference (%). (B) The number of animals vs. Time spent in the novel arm (Y-maze). (C) The number of animals vs. Preference (%) NOR). Correlations between endpoints were assessed using Pearson’s correlation, reporting the correlation coefficient r , the 95% confidence interval, and the p value.

Pearson correlation analysis (Figure 10A) performed on individual values indicated a moderate-to-strong positive association between time spent in the novel arm and preference ($r = 0.5754$; 95% CI: 0.4072–0.7059; $p < 0.0001$; $n = 80$). This result suggests that individuals spending more time in the “new” component also tend to show higher preference values, supporting convergence between these two indicators of novelty-oriented behavior.

3.6. Assessment of Behavior in the ATZ

In the ATZ paradigm, analysis of spatial distribution and interaction components with the “zone of interest” revealed a differentiated profile across AlCl_3 , AcPb , and their combinations (Figure 11; $n = 10/\text{group}$). For time spent in the outer zone (Figure 11B), no

significant differences were observed among groups (one-way ANOVA: $F(7,72) = 1.130$, $p = 0.3542$), indicating that overall peripheral preference was comparable across treatments (Tukey: all comparisons ns).

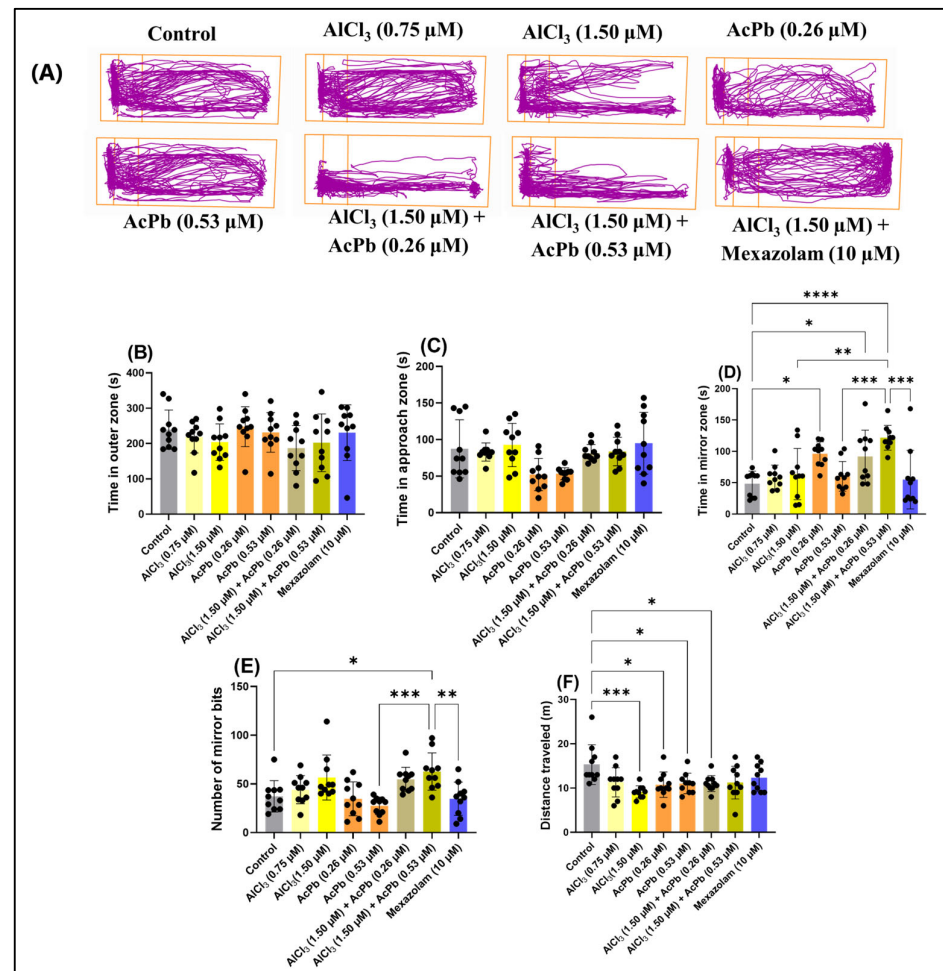


Figure 11. (A) Representative swimming trajectories in the ATZ arena, illustrating the spatial distribution of exploration. (B–F) Quantitative ATZ behavioral parameters (including time spent in the outer zone and additional exploration/locomotor indices): (B) time in outer zone (s). (C) time in approach zone (s). (D) time in mirror zone (s). (E) number of mirror bits. Bars represent the mean ($\pm$ SD), and dots represent individual values for each animal within the group. Bars represent group means $\pm$ SD with individual data points overlaid; statistical significance is indicated by brackets (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and **** $p < 0.0001$).

In contrast, time spent in the approach zone varied significantly among treatments (one-way ANOVA: $F(7,72) = 4.050$, $p = 0.0009$) (Figure 11C). Tukey post hoc testing showed that both AcPb groups displayed lower values than $AlCl_3$ 1.50 μM ($AlCl_3$ 1.50 μM vs. AcPb 0.26 μM : $p = 0.0196$; $AlCl_3$ 1.50 μM vs. AcPb 0.53 μM : $p = 0.0254$) and than mexazolam 10 μM (AcPb 0.26 μM vs. mexazolam: $p = 0.0106$; AcPb 0.53 μM vs. mexazolam: $p = 0.0140$). Descriptively, AcPb groups showed a mean approach-zone time of ~ 52 – 53 s compared with ~ 93 – 95 s for $AlCl_3$ 1.50 μM and mexazolam, suggesting reduced approach-directed exploration under lead acetate exposure. For time spent in the mirror zone (Figure 11D), a significant overall treatment effect was also detected (ANOVA: $F(7,72) = 6.895$, $p < 0.0001$), confirming group differences.

Consistently, the number of mirror bites (Figure 11E) was significantly influenced by treatment (ANOVA: $F(7,72) = 5.770$, $p < 0.0001$), with a prominent profile under combined exposure. Specifically, the $AlCl_3$ + AcPb (0.53 μM) group displayed more

mirror bites than Control ($p = 0.0212$), AcPb 0.26 μM ($p = 0.0075$), AcPb 0.53 μM ($p = 0.0003$), and mexazolam ($p = 0.0076$), suggesting an enhanced agonistic/reactive response under higher-dose co-exposure. In addition, AlCl_3 1.50 μM induced more mirror bites than AcPb 0.53 μM ($p = 0.0047$), and AlCl_3 + AcPb (0.26 μM) exceeded AcPb 0.53 μM ($p = 0.0109$), supporting that the AlCl_3 component (alone or in combination) contributes to increased reactivity in the mirror test (Figure 11E).

Regarding total distance traveled (Figure 11F), a significant treatment effect was observed (ANOVA: $F(7,72) = 3.401$, $p = 0.0034$). Compared with Control (mean ~ 15.3 m), distance traveled was lower in AlCl_3 1.50 μM ($p = 0.0005$), AcPb 0.26 μM ($p = 0.0261$), AcPb 0.53 μM ($p = 0.0462$), and AlCl_3 + AcPb (0.26 μM) ($p = 0.0444$), indicating reduced global locomotor activity across several exposure conditions, without consistent differences among toxicant groups themselves (Tukey: mostly ns).

3.7. Individual-Level Visualization of Behavioral Parameters and Correlations Between Endpoints

The heatmaps (Figure 12) illustrate the individual-level distribution of three behavioral endpoints for each treatment group (Figure 12D,F), with each row representing one animal (1–10) and color intensity indicating response magnitude. Overall, this representation highlights inter-individual variability and allows visual appraisal of between-group trends for: (Figure 12D) time spent in the top/bottom zone (s), (Figure 12E) time spent in the inner zone (s), and (Figure 12F) number of mirror bites.

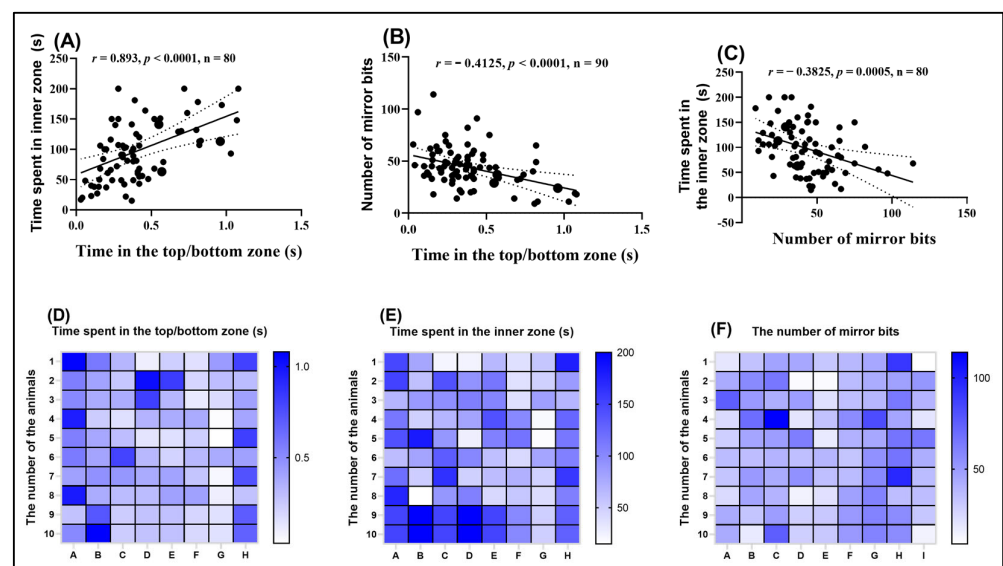


Figure 12. Correlations between endpoints were assessed using Pearson’s correlation on individual values, reporting the correlation coefficient r , the 95% confidence interval, and the p value: (A) time spent in inner zone (s) vs. time in the top/bottom zone (s); (B) number of mirror bites vs. time in the top/bottom zone (s); (C) time spent in inner zone (s) vs. number of mirror bites. Heatmaps of individual-level behavioral responses for each experimental group (D–F). Each row represents one animal (1–10), and each column represents one group (D–F). Color intensity reflects response magnitude (higher values = darker shades). (D) Time spent in the top/bottom zone (s). (E) Time spent in the inner zone (s). (F) Number of mirror bites.

Pearson correlation analysis performed on individual data revealed significant associations among these parameters. Time spent in the top zone correlated positively, with moderate strength, with time spent in the inner zone ($r = 0.4893$; 95% CI: 0.3020–0.6402; $p < 0.0001$; $n = 80$) (Figure 12A), suggesting that individuals who explore the top zone more also tend to spend more time in the central/inner zone. In contrast, top-zone time correlated negatively with the number of mirror bites ($r = -0.4125$; 95% CI: -0.5709 to

-0.2246 ; $p < 0.0001$; $n = 90$) (Figure 12B), indicating that stronger exploratory behavior is associated with reduced aggressive/social responding in the mirror test. Mirror bites also showed a negative correlation with inner-zone time ($r = -0.3825$; 95% CI: -0.5555 to -0.1777 ; $p = 0.0005$; $n = 80$) (Figure 12C), supporting an inverse relationship between mirror-directed responding and central-zone exploration.

3.8. Marker Cholinergic (AChE Activity)

A significant treatment effect was observed on acetylcholinesterase (AChE) activity (one-way ANOVA: $F(7,40) = 11.24$, $p < 0.0001$; Figure 13A). Compared with the control group (15.92 nmol ATCh/min/mg protein), AChE activity was significantly reduced following exposure to AlCl_3 (0.75 μM : 10.70; $p = 0.0010$; 1.50 μM : 9.71; $p < 0.0001$) and AcPb (0.26 μM : 12.24; $p = 0.0437$; 0.53 μM : 8.31; $p < 0.0001$). Co-exposure further intensified the inhibition ($\text{AlCl}_3 + \text{AcPb}$ 0.26 μM : 9.00; $p < 0.0001$; $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM : 7.60; $p < 0.0001$), with the lowest AChE activity recorded under the higher-concentration co-exposure ($\approx -52\%$ vs. control). As expected, GAL (2 μM) decreased AChE activity relative to control (11.90; $p = 0.0199$), and the $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM group remained significantly lower than GAL ($p = 0.0103$), suggesting a more pronounced AChE suppression under combined metal exposure (Figure 13A).

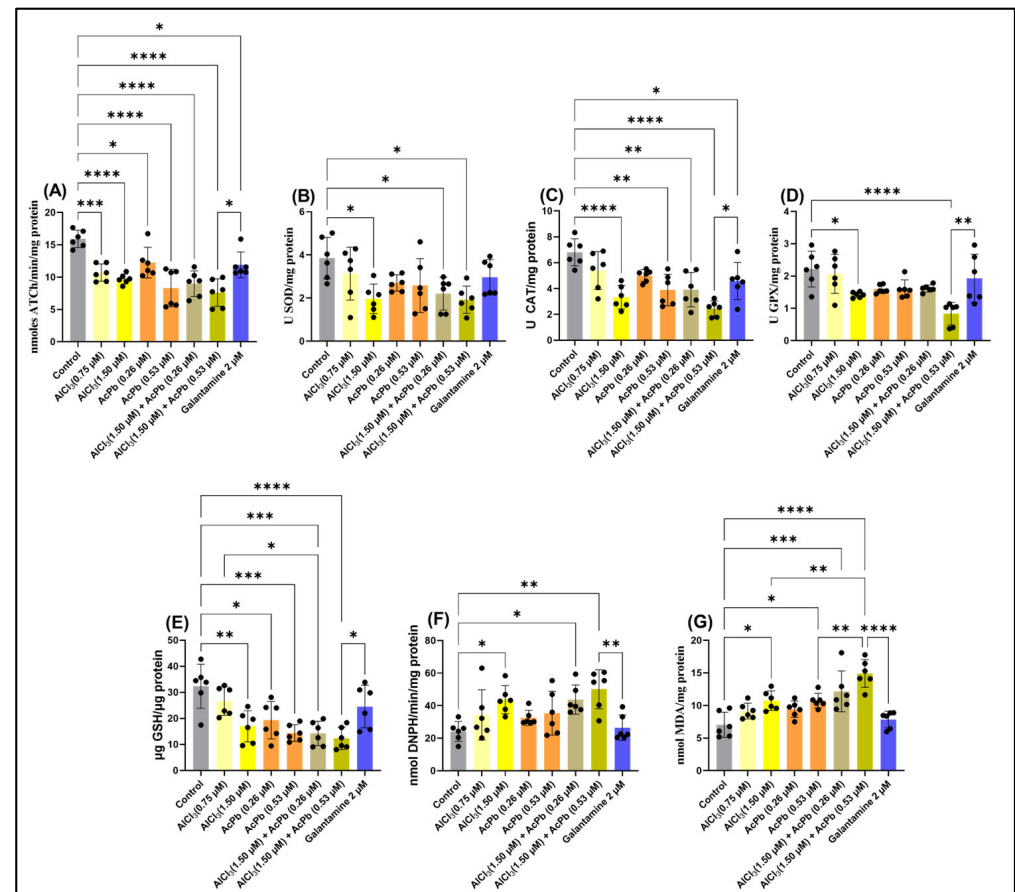


Figure 13. Effects of aluminum chloride (AlCl_3), lead acetate (AcPb), and their co-exposure on cholinergic activity and oxidative-stress biomarkers. (A) AChE activity (nmol ATCh/min/mg protein). (B) SOD activity (U SOD/mg protein). (C) CAT activity (U CAT/mg protein). (D) GPx activity (U GPx/mg protein). (E) GSH level ($\mu\text{g GSH}/\mu\text{g protein}$). (F) Protein carbonyls (PC) (nmol DNPH/min/mg protein). (G) MDA level (nmol MDA/mg protein). Data are presented as mean $\pm$ SD with individual values overlaid ($n = 6/\text{group}$). Statistics: one-way ANOVA followed by Tukey's test; significance is indicated in the figure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

3.9. Antioxidant Defense (SOD, CAT, GPX) and the Non-Enzymatic Antioxidant (GSH)

For SOD, one-way ANOVA indicated a significant overall treatment effect ($F(7,40) = 3.267$, $p = 0.0077$; Figure 13B). Post hoc testing revealed significant decreases mainly under the higher-impact conditions, namely AlCl_3 1.50 μM (1.97 vs. 3.86 in Control; $p = 0.0135$), $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM ($p = 0.0462$), and $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM ($p = 0.0109$), supporting a relevant reduction in the capacity to neutralize superoxide radicals under higher and/or combined exposure. A robust effect was also identified for CAT ($F(7,40) = 8.658$, $p < 0.0001$; Figure 13C). Compared with Control (6.80 U/mg protein), CAT activity was significantly reduced in AlCl_3 1.50 μM (3.33; $p < 0.0001$), AcPb 0.53 μM (3.89; $p = 0.0013$), $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM (3.91; $p = 0.0014$), and most prominently in $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (2.45; $p < 0.0001$), indicating marked impairment of peroxide detoxification under co-exposure. For GPX (Figure 13D), significant between-group differences were also present (as indicated by the significant ANOVA output), with the most evident decrease in $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (0.84 vs. 2.22 U/mg in Control; $p < 0.0001$), suggesting severe dysfunction of glutathione-dependent antioxidant activity under high co-exposure.

Consistent with the changes in antioxidant enzymes, GSH levels (Figure 13E) were significantly decreased under the most severe conditions. Relative to Control (32.34 μg GSH/ μg protein), GSH was reduced in AlCl_3 1.50 μM (16.96; $p = 0.0024$), AcPb 0.26 μM (19.30; $p = 0.0155$), AcPb 0.53 μM (14.36; $p = 0.0003$), $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM (14.24; $p = 0.0002$), and $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (12.34; $p < 0.0001$). Notably, the higher-concentration co-exposure produced the greatest depletion ($\approx -62\%$ vs. Control) and differed from GAL ($p = 0.0290$), supporting exhaustion of non-enzymatic antioxidant reserves in the presence of metals.

3.10. Oxidative Damage Markers (Protein Carbonyls and Malondialdehyde)

Protein oxidation, assessed as PC, showed a significant treatment effect ($F(7,40) = 4.602$, $p = 0.0008$; Figure 13F). PC values increased from 24.20 nmol DNPH/min/mg protein in the Control group to 43.67 in AlCl_3 1.50 μM ($p = 0.0395$) and 43.72 in $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM ($p = 0.0386$), reaching a maximum of 50.11 in $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM ($p = 0.0019$), indicating substantial oxidative protein damage under co-exposure. The high-dose co-exposure group also exceeded GAL ($p = 0.0058$).

Similarly, lipid peroxidation evaluated by MDA showed a strong overall effect ($F(7,40) = 11.46$, $p < 0.0001$; Figure 13G). Compared with Control (7.04 nmol/mg protein), MDA levels were significantly increased in AlCl_3 1.50 μM (10.75; $p = 0.0204$), AcPb 0.53 μM (10.75; $p = 0.0203$), $\text{AlCl}_3 + \text{AcPb}$ 0.26 μM (12.18; $p = 0.0004$), and $\text{AlCl}_3 + \text{AcPb}$ 0.53 μM (14.90; $p < 0.0001$). High-dose co-exposure more than doubled MDA relative to Control ($\approx +112\%$) and remained significantly higher than GAL ($p < 0.0001$), underscoring amplified membrane lipid damage under combined metal exposure.

3.11. Correlations Between Biochemical and Behavioral Parameters

Correlations between biochemistry and behavior indicated that disruptions along the cholinergic and redox axes are reflected in behavioral outcomes. AChE activity showed significant negative associations with time spent in the novel arm ($r = -0.2925$; 95% CI: -0.5324 to -0.009123 ; $p = 0.0437$; $n = 48$) (Figure 14A) and with preference (%) in the NOR test ($r = -0.2953$; 95% CI: -0.5345 to -0.01214 ; $p = 0.0416$; $n = 48$) (Figure 14B), suggesting that higher cholinesterase activity is associated with lower exploration/novelty-driven behavioral endpoints.

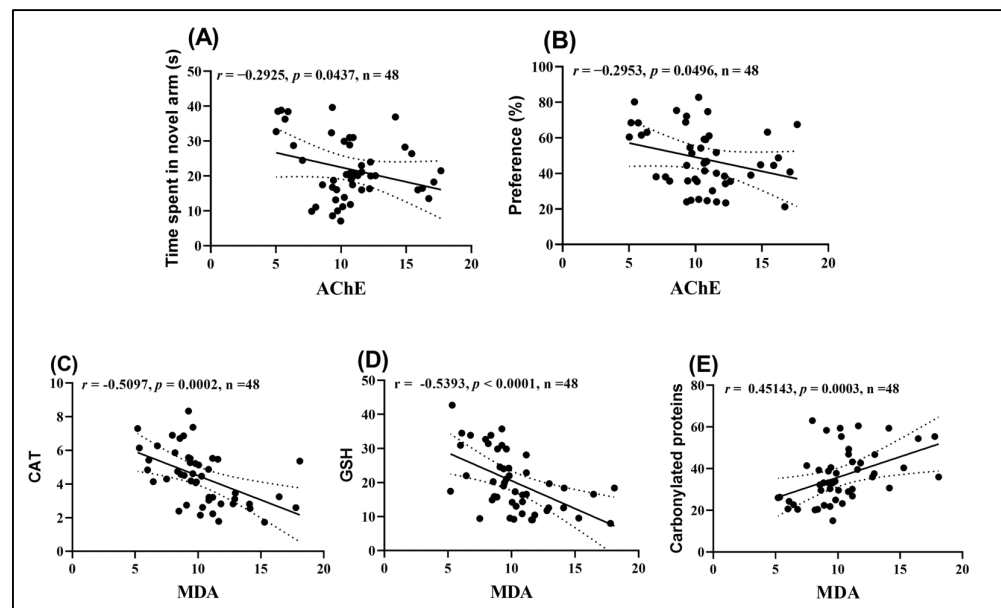


Figure 14. Pearson correlation analyses between biochemical biomarkers of the cholinergic axis/oxidative stress and behavioral endpoints, based on individual-level data (all experimental groups pooled). Each dot represents one individual; the linear regression line and Pearson's correlation coefficient (r) are shown, together with the p value (two-tailed test) and the 95% confidence interval for r (as reported by the software). (A) Correlation between AChE activity (nmol ATCh/min/mg protein) and the behavioral variable t . (B) Correlation between AChE and time spent in the novel arm (time in new arm, s). (C) Correlation between CAT activity (U CAT/mg protein) and MDA levels (nmol MDA/mg protein). (D) Correlation between MDA and time spent in the novel arm (time in new arm, s). (E) Correlation between protein carbonyls (PC) (nmol DNPH/min/mg protein) and MDA, highlighting co-variation between protein oxidation and lipid peroxidation. For each analysis, the number of XY pairs was $n = 48$.

Regarding oxidative stress, CAT showed a significant negative correlation with MDA ($r = -0.5097$; 95% CI: -0.6934 to -0.2637 ; $p = 0.0002$; $n = 48$) (Figure 14C), indicating an inverse relationship between enzymatic antioxidant capacity and lipid peroxidation. Consistently, MDA correlated negatively with GSH ($r = -0.5393$; 95% CI: -0.7141 to -0.3014 ; $p < 0.0001$; $n = 48$) (Figure 14D), suggesting that higher lipid peroxidation is associated with reduced exploration of the novel zone/novel element. In addition, PC correlated positively with MDA ($r = 0.4514$; 95% CI: 0.1919 – 0.6519 ; $p = 0.0013$; $n = 48$), supporting co-variation between protein oxidation and lipid peroxidation as a marker of global oxidative injury.

4. Discussion

This study demonstrates that co-exposure to AlCl_3 and AcPb induces, in adult zebrafish, an integrated phenotype of heightened anxiety and cognitive impairment, accompanied by cholinergic dysregulation and a robust oxidative-stress profile. The convergence between behavioral outcomes (NTT/NAT/NOR/Y-maze) and brain biomarkers (AChE, SOD/CAT/GPx, GSH, PC, MDA) supports the working hypothesis that metals—particularly when present as mixtures—engage shared molecular nodes (redox-mitochondria-neurotransmission), which translate into disrupted exploration, novelty processing, and working memory.

More specifically, in the NTT, co-exposure amplified anxiety-like behavior, evidenced by bottom-dwelling, reduced vertical exploration, and increased freezing, with significantly longer latencies to first enter the upper zone and a lower top/bottom time ratio (Figure 6B,C). Importantly, mean speed and total distance did not differ significantly among

groups, indicating that the observed changes are predominantly anxiogenic rather than reflecting global hypoactivity (Figure 6G,H). This pattern is consistent with recent literature reporting that AlCl_3 exposure can elicit anxiety-like phenotypes in adult zebrafish in standardized behavioral paradigms, including the NTT, suggesting vulnerability of stress-related circuits to aluminum [42]. In the same vein, zebrafish pharmacological/toxic AD-like models built on the “nitro-oxidative stress + neuroinflammation + cholinergic dysfunction” framework frequently describe anxiety and exploratory disturbances as integral components of the phenotype [51].

In the NAT, the anxiogenic effect of combined exposure—particularly at the higher dose—was supported by an increased latency to enter the central zone and a redistribution of time toward the periphery (thigmotaxis), together with increased immobility (Figure 7B–E). Mexazolam consistently counteracted this signature, confirming the sensitivity of the test to an anxiolytic control. Conceptually, the robustness of the anxiety phenotype across two distinct paradigms (NTT and NAT) strengthens the interpretation that metal exposure disrupts novelty/threat reactivity through shared neurobiological mechanisms (e.g., oxidative stress, neuroinflammation, and neurotransmitter imbalance) [52].

In the NOR task, preference for the novel object decreased from ~64.8% in controls to ~35.8–36.7% in the co-exposure groups (Figure 8C), and time spent in the NO zone was significantly reduced in the higher-dose co-exposure compared with the control: NO condition. This profile indicates impaired novelty processing/recognition, consistent with disrupted recognition memory. Mechanistically, GAL increased preference (~58.6%) and was significantly higher than both co-exposure groups, supporting the involvement of the cholinergic axis in the observed cognitive phenotype.

From the perspective of the literature, zebrafish is increasingly used for rapid phenotyping of learning and memory disturbances, and NOR is considered a valid readout for recognition/novelty-seeking in pharmacologically or toxicologically induced cognitive-impairment paradigms [53]. Moreover, recent AD-like zebrafish models induced by aluminum (including AlCl_3 exposure) report cognitive deficits that co-occur with oxidative stress and cholinergic dysfunction, supporting the interpretation that reduced novelty preference reflects a functional neuronal deficit rather than a transient shift in exploratory behavior [51].

In the Y-maze, spontaneous alternation decreased markedly in the AlCl_3 1.50 μM group and in both co-exposure groups (~42.9–44.9% vs. 78.2% in controls), while time spent in the novel arm was strongly reduced (e.g., ~10.5–11.4 s in AlCl_3 1.50 μM and the 200 $\mu\text{g/L}$ co-exposure vs. 26.1 s in controls). GAL maintained alternation and novel-arm time at values comparable to controls, further supporting a key contribution of cholinergic function to working-memory performance.

An important interpretative point in this dataset is the dissociation between locomotor and cognitive indices: the higher-dose co-exposure increased distance/velocity (vs. AlCl_3 1.50 μM) yet preserved a severely impaired cognitive profile (low alternation and reduced “novel arm time”), suggesting a less efficient and/or disorganized exploration strategy (e.g., more “linear” trajectories with a reduced turning angle). This observation aligns with the concept that metal-induced cognitive impairment is not simply attributable to hypoactivity but rather reflects disruption of exploratory strategies and spatial-information integration. In the zebrafish learning/memory literature, the Y-maze is widely used precisely because it allows separation of cognitive outputs (alternation/novel-arm preference) from locomotor parameters, provided that appropriate controls are included [53].

In the ATZ (social reactivity and impulse-like responding in zebrafish), the absence of between-group differences in outer-zone time suggests that global peripheral preference (i.e., generalized thigmotaxis) was not the main driver of the behavioral phenotype. However, time spent in the approach zone was reduced in the AcPb-exposed groups, while mirror bites

increased most prominently under high-dose co-exposure (vs. control, vs. AcPb alone, and vs. mexazolam). From an interpretative standpoint, this combination is compatible with heightened agonistic reactivity and impaired regulation of responses to a social/competitive stimulus, particularly under conditions in which stress-related and executive-control networks may be compromised. Within a neurotoxicological framework, these shifts align with the hypothesis that metals can perturb neuromodulatory balance and stimulus reactivity through convergent mechanisms involving redox disruption, mitochondrial dysfunction, and Ca^{2+} -dependent signaling, thereby biasing behavior toward more impulsive and less adaptive response patterns when confronted with salient social cues [52].

The biochemical data provide a coherent molecular anchor for the behavioral phenotype. AChE decreased significantly across all toxic conditions, reaching its lowest level under the high-dose co-exposure (7.60 vs. 15.92 nmol ATCh/min/mg; $\approx -52\%$). Such an effect is well supported by the zebrafish toxicology literature: a classic study using zebrafish brain tissue showed that lead acetate can significantly reduce AChE activity, implicating disruption of cholinergic transmission as a proximal mechanism of metal neurotoxicity [23]. In addition, in more recent aluminum-induced AD-like zebrafish models, cholinergic dysfunction is frequently integrated into the causal chain underlying cognitive deficits and is often linked to redox imbalance and synaptic alterations, reinforcing the interpretation that the AChE inhibition observed here reflects functionally relevant neurochemical dysregulation rather than an isolated biochemical change [51].

Regarding the pooled correlations between AChE and “new arm time”, and between AChE and “preference”, the observed negative direction should be interpreted with caution, because pooled correlations may reflect both between-group differences and individual variability. These correlations should be interpreted cautiously, as pooled analyses may partly reflect group-level differences rather than within-group covariance. From a mechanistic standpoint, the central message remains that the cognitive phenotype co-occurs with a perturbed cholinergic status, and that GAL (an acetylcholinesterase inhibitor) normalizes behavior across multiple endpoints, suggesting that cholinergic balance is critical for performance in these tasks.

The biochemical dataset indicates a coherent redox response: decreases in SOD (e.g., 1.97 vs. 3.86), CAT (2.45 vs. 6.80), GPX (0.84 vs. 2.22), and depletion of GSH (12.34 vs. 32.34), occurring in parallel with increases in protein carbonyls (PC) (50.11 vs. 24.20) and MDA (14.90 vs. 7.04; $\approx +112\%$) under high-dose co-exposure. The internal correlations (CAT inversely correlated with MDA; PC positively correlated with MDA) further support that these markers reflect an integrated state of oxidative injury.

This overall pattern aligns well with the current literature positioning oxidative stress and mitochondrial dysfunction as central amplifiers of synaptic failure and neuronal vulnerability in neurodegeneration/AD [54]. Moreover, in AlCl_3 -induced AD-like zebrafish models, oxidative stress and neuroinflammation are repeatedly reported as major mechanisms, with direct impacts on behavior and cholinergic markers [51]. For Pb, there is strong evidence that exposure can produce memory deficits and persistent effects (including after early-life exposures), supporting the concept that lead may “program” long-term neuronal vulnerability—consistent with the cognitive phenotypes observed here, particularly at the higher dose and under co-exposure [55].

Our results consistently show that the most severe phenotypes are associated with co-exposure, which is highly relevant because real-world human exposures are rarely “single-metal.” In recent epidemiological work, metals assessed as mixtures have been linked to poorer cognitive performance and/or increased risk of cognitive decline/dementia, with some studies reporting more robust effects for mixture models than for individual metals [56]. In this context, an experimentally tractable in vivo mixture paradigm that

combines standardized behavioral outputs with biochemical anchors can provide a useful bridge between observational evidence and mechanistic validation.

Applied Perspective and Model Utility

From an applied-sciences standpoint, this adult zebrafish Al–Pb co-exposure paradigm can be used as a rapid *in vivo* platform for mixture hazard characterization and for prioritizing candidate interventions before validation in rodents [57,58]. Practical applications include: (a) screening antioxidants, nutraceutical formulations, and metal chelators for their ability to prevent or attenuate anxiety-like behavior and cognitive impairment [59]; (b) benchmarking cholinergic modulators using a functional phenotype that is demonstrably sensitive to pharmacological modulation [29]; and (c) testing interaction patterns (additive vs. non-additive effects) for environmentally relevant mixture scenarios [57,60]. Across the current battery, the most sensitive functional endpoints for screening include novelty-related cognitive measures (novel object preference; spontaneous alternation and novel-arm exploration in the Y-maze) and anxiety-like indices (bottom-dwelling/thigmotaxis and freezing in novelty-based paradigms), complemented by AChE activity and redox markers as biochemical anchors of intervention efficacy. Compared with rodent models, adult zebrafish offer practical advantages relevant to applied sciences, including reduced costs and time requirements, higher-throughput potential (especially for comparative screening of multiple candidates/doses), and alignment with 3R (replacement, reduction and refinement) principles [61]. Together, these features position the model as a pragmatic intermediate step between *in vitro* assays and later-stage mammalian studies for mixture neurotoxicity and neuroprotection screening [62–64].

An important limitation is the absence of histological and downstream molecular endpoints that would directly connect cholinergic dysfunction and oxidative stress to canonical AD-related processes (proteinopathies and neuroinflammation), as well as the lack of internal-dose confirmation for metals. Future work should incorporate quantification of metals in water and tissue, synaptic markers (e.g., PSD95, synaptophysin), antioxidant signaling pathways (e.g., Nrf2/HO-1), and A β /tau-related markers to enable multi-level validation and to facilitate the use of the Al–Pb paradigm for testing neuroprotective interventions *in vivo* [58,60,64].

By combining standardized behavioral tests with cholinergic and redox biomarkers, this Al–Pb co-exposure model provides a rapid, reproducible, and cost-effective platform for dementia-relevant phenotyping. In addition, it can serve as a screening system for evaluating biological or synthetic agents with potential neuropharmaceutical activity, enabling prioritization of multi-target candidates before validation in more complex and resource-intensive models.

5. Conclusions

Based on the obtained results, it can be concluded that co-exposure to aluminum chloride (AlCl₃) and lead acetate (AcPb) in adult zebrafish induces a coherent neurobehavioral and biochemical phenotype compatible with dementia-relevant neurotoxicity. Behaviorally, co-exposure produced a robust anxiogenic signature (increased bottom-dwelling, thigmotaxis, and freezing in the novel tank diving test/novel approach test) and a clear cognitive deficit, reflected by reduced novelty preference in the novel object recognition test and marked impairment of working memory and novel-arm exploration in the Y-maze—effects that cannot be explained simply by a global locomotor alteration. At the molecular level, the model revealed pronounced acetylcholinesterase inhibition and a severe oxidative-stress profile, characterized by decreased antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), depletion of reduced glutathione, and increased oxidative damage markers (protein

carbonyls and malondialdehyde), with a particularly strong amplification under high-dose co-exposure. Correlations between biomarkers and behavioral endpoints further support a functional link between the cholinergic/redox axis and cognitive performance, while the beneficial effects of galantamine across multiple behavioral parameters confirm the sensitivity of the paradigm to neuropharmacological intervention.

Overall, the proposed Al–Pb paradigm represents a practical, reproducible, and easy-to-implement vertebrate model for studying the functional consequences of mixed-metal exposure and for in vivo pre-screening of candidate neuroprotective interventions, including antioxidants, nutraceuticals, and chelators. Importantly, the findings support a dementia-like neurobehavioral phenotype driven by Alzheimer’s disease (AD) relevant mechanisms (cholinergic dysfunction and oxidative stress), rather than a model of full AD’s pathology.

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Abbreviations

The following abbreviations are used in this manuscript:

AChE	acetylcholinesterase
AcPb	lead acetate
AD	Alzheimer’s disease
AlCl ₃	aluminum chloride
ANOVA	analysis of variance
ATCh	acetylthiocholine
ATZ	aggression test in zebrafish (mirror test paradigm; approach/mirror/outer zones)
CAT	catalase
CI	confidence interval
GAL	galantamine
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-dithiobis(2-nitrobenzoic acid) (Ellman reagent)
EC	Enzyme Commission number

FO	familiar object
GPx	glutathione peroxidase
GSH	reduced glutathione
MDA	malondialdehyde
NAT	Novel Approach Test
NO	novel object
NOR	Novel Object Recognition
NTT	Novel Tank Test
PC	protein carbonyls
SD	standard deviation
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
UV-Vis	ultraviolet–visible (spectrophotometry)
XO	remaining area (non-object exploration zone)
Y-maze	Y-maze test (spatial working memory/spontaneous alternation)

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