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Morphological and Molecular Alterations Induced by Lead in Embryos and Larvae of *Danio rerio*

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Abstract: Lead (Pb) is one of the most toxic and persistent elements and may adversely affect both humans and wildlife. Given the risks posed to humans, lead is listed among priority substances of public health importance worldwide. In fish, available studies deal with high doses, and the potential hazard of Pb at low concentrations is largely unknown. Given its well-demonstrated translational value for human toxicity research, we used zebrafish as a model species. Embryos were exposed to two environmentally relevant concentrations of lead (2.5 and 5 µg/L) from 6 h post-fertilization and analyzed after 48, 96, and 144 h. The morphological abnormality arose after 48 h, and the incidence and intensity were dose and time dependent. Spinal and tail deformities were the most frequently detected alterations. Pb also modulated the expression of genes involved in the toxicological responses (*sod* and *mt*), thus demonstrating that zebrafish's early stages are able to mount an adaptive response. Moreover, *ldh* and *β-catenin* were significantly upregulated in all groups, whereas *wnt3* expression was increased in the high concentration group. Our results confirm that zebrafish embryos and larvae are valuable early warning indicators of pollution and may play a major role in ecosystems and human health monitoring.

Keywords: lead; zebrafish; early developmental stages; morpho-functional modifications

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1. Introduction

Heavy metals are naturally found in the earth's crust, and some are essential in biological systems at very low concentrations fig [1]. These elements are considered toxic when their presence exceeds the vital dose, and heavy metal pollution is currently considered one of the most serious environmental issues [2–6].

Lead, among the most common heavy metals, is ubiquitous in the environment and can be found in traces in soils, plants, and water [7,8], although it has no recognized physiological role in living organisms. For its physical and chemical properties, lead has known many applications, and has been used by humans since prehistoric eras. However, following the industrial revolution, intensive anthropogenic activities, including industrial and agricultural practices, resulted in an increased and uncontrolled discharge of lead in all environmental comparts.

Lead is considered one of the most toxic and persistent elements and adversely affect humans and wildlife. Water, food, and air are key routes for human exposure in emergent nations, but lead poisoning also occurs in developed countries such as the United States. Given the risks posed to humans, lead is listed among priority substances of public health importance worldwide [4,5,9]. It is also one of the top three toxic pollutants of environmental concern [10,11], occupying second place in the Priority List of Hazardous Substances [7,12–14].

Once released into the environment, lead can enter aquatic habitats due to direct input, atmospheric deposition, erosion, or agricultural and industrial wastewater. Currently, lead pollution is so pervasive that globally many aquatic bodies are contaminated [15], and lead concentration in surface waters ranges from 0.05 μg/L to 566.2 mg/L worldwide [9,16–18].

In fish, lead can be absorbed from water, food, or sediment, but regardless of the route of entry into the body shows a high tendency to bioaccumulation in various fish tissues (including gills, liver, skin, and muscles), depending on environmental factors, species-specific features, concentration, and time of exposure [8,19,20]. From literature, it is known that in fish, embryos and larvae are the most sensitive life stages, and they can therefore serve as an early indicator of pollution even at low levels of exposure, which also applies to Pb and other heavy metals [21–23]. Nevertheless, Pb-induced alterations during development are largely overlooked, and most of the available studies have been conducted on adults. Pb was reported to induce several detrimental effects in adult fish, including neurotoxicity [8,24,25], alteration of liver metabolism [26,27], oxidative stress induction [28–30], immunological and haematological dysfunctions [22,31–34] and alteration of gills and liver histology [32,35–38].

Here we used as model species zebrafish (*Danio rerio*), a well-acknowledged tool widely used in environmental and human health risk assessment, as proven by the growing number of studies using this organism in the past few decades [39]. Its small body size, short reproductive cycle, high fecundity, and overall easy maintenance and husbandry make zebrafish an ideal model for environmental studies; on the other hand, its high genetic homology with humans provide a powerful basis for human toxicity research and increases the translational value of this model [40–42]. Notably, the early stages of development offer a valuable tool for in vivo ecotoxicological studies and allow the extrapolation of results to other vertebrate species, including humans.

World Health Organisation (WHO) and Food and Agriculture Organisation of the United Nations (FAO) have set high standard levels of daily Pb intake as acceptable (up to 7 μg/kg body weight); however, no such guideline is given for infants and children, who are relatively more sensitive. An accurate assessment of human risk and human uptake of Pb is made difficult due to the unavailability of data. It has been recently highlighted that the potential hazard of Pb below a certain threshold dose (i.e., 100 μg/L) remains largely under-investigated, and even very low Pb concentrations, claimed as harmless, may represent a risk for human health [7,9].

Much more effort is needed to develop a biological early warning system, especially in aquatic ecosystems [9], also considering both direct Pb effects and its potential of biomagnification through the food chain.

In zebrafish embryos and larvae, available studies on Pb toxicity mainly focused on neurotoxicity, neurodevelopmental, and neurobehavior effects [40,43–48]. Surprisingly, morpho-functional investigations during the sensitive phase of development are scarce, and only a few studies have furnished an overall morphological evaluation [45,49–53]. Moreover, all available information on fish deals with high lead concentrations, and there is a lack of research on the adverse effects of lead at environmentally relevant concentrations [53].

On these bases, here we investigated for the first time the effects induced by two environmentally relevant concentrations of Pb (2.5 μg/L and 5 μg/L) in the zebrafish model. Moreover, starting from the assumption that fish embryos are especially susceptible to environmental contaminants during the first 24 h post-fertilization (hpf) [54], we selected for the experiment early development stages (starting from 6 hpf), evaluating the Pb toxic effects after 48, 96, and 144 h. Given the need to identify early, sensitive biomarkers of Pb exposure, we first investigated morphological alterations, recognized as one of the most straightforward tools to investigate the effects of contaminants in fish [55]. To allow an objective comparison of gross morphology deformity after Pb exposure, a morphological grading system was used.

Considering that the combination of morphological changes and transcriptional responses is recognized as valid and promising tools for studying ecotoxicological effects [23], in a second step, we also applied molecular biomarkers to have a more comprehensive overview of Pb-induced outcomes. In detail, we evaluated the expression pattern of some genes involved in the early developmental process (*Wnt* and *β-catenin*) to address the mechanism underlying the putative morphological modifications caused by Pb exposure. Since the mechanisms through which lead may exert its toxicity may be different and stage-specific, we evaluated the expression of several proteins known as valuable biomarkers of metal exposure in adult fish. To protect themselves against heavy metals, living organisms, including fish and mammals, activate several defense systems, including the metallothionein (MTs) modulation; these are low molecular weight proteins effective for detoxifying non-essential metals, and protecting cells from oxidative stress [35,56–62].

Incidence of oxidative stress resulting from exposure to Pb and heavy metal contamination is the most common response in both fish and mammalian models [51]; therefore, we investigated the expression of two markers of oxidative stress induction (superoxide dismutase 1-*sod1* and lactate dehydrogenase-*ldh*) during zebrafish development. The present study aims to elucidate the role of morphological studies for assessing sub-lethal Pb toxicity and provide a set of sensitive and early informative biomarkers for environmental risk assessment.

2. Materials and Methods

2.1. Test Substance

A stock solution of lead acetate [Pb $(CH_3COO)_2$, Sigma–Aldrich Chemical Co., St. Louis, MO, USA] (1000 μg/L) was prepared using distilled water. An appropriated stock solution was diluted in aged tap water to reach the following nominal concentrations: 2.5 μg/L and 5 μg/L, also referred to as the low and the high concentration, respectively. Low and high Pb concentrations correspond to 0.011% and 0.022% of the zebrafish embryos median lethal concentration at 96 h ($LC50_{96}$), respectively (Yin et al., 2017). These concentrations were chosen considering the worldwide environmental contamination of Pb in surface water and can be considered very low concentrations [9,16–18].

2.2. Zebrafish Husbandry and Maintenance

Healthy adults of both sexes of *Danio rerio* (approximately 6/8 months of age) were obtained from a local store. In the laboratory, animals were acclimatized in an aquarium containing 100 L of aged tap water for two weeks. During the acclimatization period, the animals were fed twice a day with commercial fish food and water parameters were daily monitored and kept constant (temperature 26 ± 0.5 °C, pH 7.3, conductivity 300 Ls/cm, dissolved oxygen 8 ± 1 mg/L, hardness 180 mg, 14 h light/10 h dark photoperiod).

Zebrafish embryos were obtained from the natural spawning of adult zebrafish. Briefly, adults male and females were collocated in standard breeding tanks with a 2:1 male-female ratio. The following day, embryos were collected, rinsed in water, and placed in a 26 ± 0.5 °C incubator in Petri dishes (density: 1 egg/mL) prefilled with aged tap water.

All experiments were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123, Strasbourg, 1985).

2.3. Exposure Conditions

According to a standard staging guideline, before the experiment started, embryos' developmental stage was determined under a stereomicroscope by distinctive morphological features [63]. Exposure started once embryos were at 6 hpf and was performed in Petri dishes (density: 1 egg/mL) prefilled with the appropriate Pb solution or untreated aged tap water (control). During the experiments, the solutions were renewed daily, according to Roy et al. (2015). The Petri dishes were placed in a temperature- and light-controlled incubator set to the same circadian rhythm used for adult zebrafish. After hatching and the

yolk sac regression, larvae were fed ad libitum with brine shrimp nauplii (*Artemia salina*). Two sets of experiments were conducted, and three independent replicates were performed for each.

Mortality and hatching rate—Embryo mortality and hatching rate were evaluated using a subsample of 30 embryos for each exposed group, including the control. The numbers of survived and hatched embryos were counted every day, and dead embryos were readily removed. Embryos/larvae viability (%) was assessed by observing the following parameters: egg coagulation, heartbeat, and spontaneous motility missing. In each treatment group, hatched larvae were counted, and the hatching rate (%) was determinate as follows: hatched numbers/total exposed numbers × 100.

Gross morphology, deformity incidence and quantitative real time PCR—For morphological analysis, animals (n = 90) were sampled at the selected time points: 48, 96, and 144 h corresponding to 54, 102, and 150 hpf. Prior to the morphological analyses, embryos/larvae were anesthetized in Tricaine methanesulphonate (MS-222) (200 mg/L) to inhibit movement. Observations were conducted using a stereomicroscope Optika SLX-3 equipped with a C-HP Camera (Optika Microscopes ITALY). All individuals were photographed and the obtained images were used to check the morphological malformations. Moreover, to evaluate the severity of morphological defects and allow reproducibility and comparison between the experimental and control groups, a morphological scoring system proposed by Herrmann [64] was used with some modifications. Briefly, observing the images acquired for each individual (dorsal and frontal view), the extent of each malformation was quantified by assigning a numerical value to each lesion. The grading system used was: 0 = no effect (−); 1 = weak (+); 2 = medium (++); 4 = strong (+++). The overall severity of deformities is expressed as the sum of the estimated values for each malformation. The obtained numerical scores for all experimental groups (including the control) were statistically compared. Cumulative deformity, defined as individuals with malformations among total observed individuals, and the deformity incidence, defined as the percentage of individuals with a specific malformation among total individuals, were recorded after 48, 96, and 144 h of exposure.

All photographed animals were promptly stored at -80 for subsequent real-time PCR analyses. Total RNA of all exposed groups and control group was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNAs quantity and quality were determined by spectrophotometry using the NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA), and for the cDNA synthesis, we used the High capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA, USA). The genes analysed were: superoxide dismutase 1 (*sod1*, accession number NM_131294.1), metal-regulatory transcription factor 1 (*mtf1* accession number NM_152981.1), wingless-type MMTV integration site family, member 3A (*wnt3a*, accession number NM_001007185.1), catenin beta-like 1 (*ctnnb1*, accession number NM_200866.1) and lactate dehydrogenase D (*ldhd*, accession number NM_199873.1). The samples were run in triplicate, and the output data were normalized against the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, accession number NM_001115114.1).

The amplification of cDNA was checked using TaqMan Gene Expression Assays. The Real-time PCR reactions (one cycle at 50 °C for 2 min, 95 °C for 10 min and 40 amplification cycles at 95 °C for 15 s, 60 °C for 1 min) were carried out, following the manufacturer's guidelines, in a Light Cycler (Applied Biosystems StepOn, Real-Time PCR System, Foster City, CA, USA). Each 20 µL reaction contained: 2 µL of cDNA, 10 µL of master mix (TaqMan Universal Master Mix II) (Applied Biosystems), 1 µL of assay mix (TaqMan Gene Expression Assay), and 7 µL of RNase-free H_2O. The reaction specificity was determined from the dissociation curve of the PCR product. Relative quantification of each gene expression level was normalized according to the average of the housekeeping gene expression levels. Relative mRNA expression of the gene was generated using the 2^{-DCt} method [65].

2.4. Statistical Analyses

All analyses were performed using Graph Pad Prism 8.00 (GraphPad Software Inc., San Diego, CA, USA) at a significance level of 0.05. Replicates were statistically compared for all endpoints using the Mann–Whitney test. Because no significant differences were found among the three replicates ($p > 0.05$), data were pooled for subsequent analyses. Survival analysis, using a Log-rank (Mantel–Cox) Test, was carried out to compare the survivals in the experimental conditions through the entire exposure period, and a Kaplan–Meier survival graph was used to illustrate cumulative survival. Chi-square and Fisher's exact tests were used to compare exposed groups to control groups with respect to hatching rate and deformity incidence. To test the level of significance of the grading system, two-way ANOVA followed by Tukey's multiple comparisons test was performed. Significant differences in the levels of gene transcripts between Pb-treated and control groups were compared using one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. The assumption of normality was tested with the Shapiro–Wilk test.

3. Results

3.1. Survivor and Hatching Rate

Exposure to Pb did not affect survival in all exposed groups, and no significant differences were observed in the mortality rate compared to the control (Figure 1a). The hatching in zebrafish reared at 28 °C occurs from 48 to 72 hpf but can also take more time depending on the water temperature. Thus, time to hatch is not a staging index in this species, and development progress is independent of whether the embryo remains in the chorion or not. In our experiment, the control group hatching rate was 90% within 102 hpf (96 h from the start of the experiments), and all embryos had hatched after 150 hpf. No significant change in the hatching rate was observed in low concentration group. Animals exposed to the high Pb concentration showed a significant hatching delay compared to the control (*** $p = 0.0002$), and after 102 hpf (96 h of exposure), only 55.17% of individuals were hatched; however, they were swimming free out of their chorion within 150 hpf (144 h of exposure) (96.15%; Figure 1b).

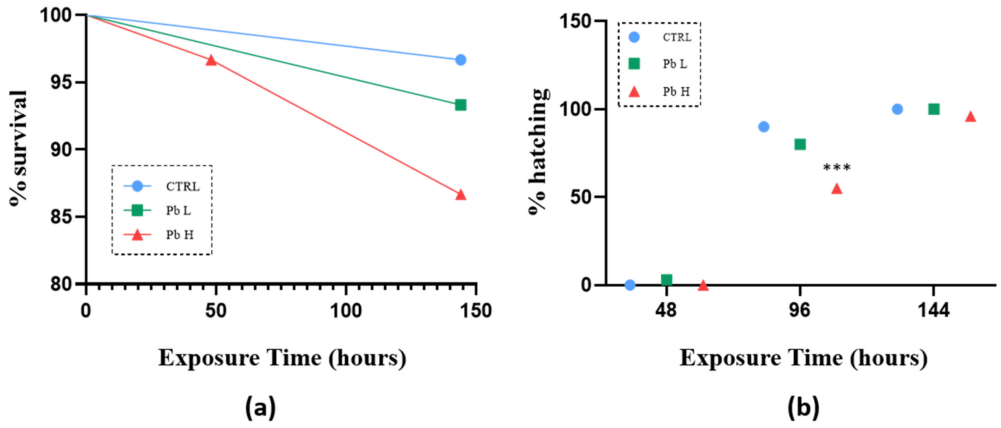

Figure 1. Survival and hatching percentage (%) of zebrafish embryos and larvae exposed to 2.5 and 5 µg/L of Pb. (**a**) Kaplan–Meier survival graph. Different symbols represent the treatment groups; Log-rank (Mantel–Cox) Test was used to compare the survivals during the whole exposure period; (**b**) hatching rate graph of zebrafish embryos. Asterisks indicate significant differences between the treated groups and the control group using Chi-square and Fisher's exact tests (*** $p < 0.0002$).

3.2. Developmental Toxicity

3.2.1. Control Group

To determine the developmental stage of zebrafish embryos and larvae in the present experiment, we used the data available in the literature as a reference [63,66], and no

differences or discrepancies in the development of control specimens were detected. Furthermore, no individuals from the control group showed morphological abnormalities over the whole period considered.

At 54 hpf, the embryos, still enveloped within the chorion, showed high pigmentated eyes, a prominent yolk sac, and a well recognizable caudal fin. (Figure 2a). At 102 hpf, the yolk sac was still prominent, the caudal fin was further developed, and the pectoral fin was forming. Otolith-containing chambers in the otic vesicle, heart, and muscle were clearly visible. The swim bladder started to inflate and was recognizable by the accumulation of melanin, giving it a dark appearance (Figure 2b,c). At 150 hpf, the facial structure was completed, and the mouth was visible. Larvae had completely resorbed the yolk, and both the spinal cord and the notochord were well distinguishable and the swim bladder was completed and inflated (Figure 2d).

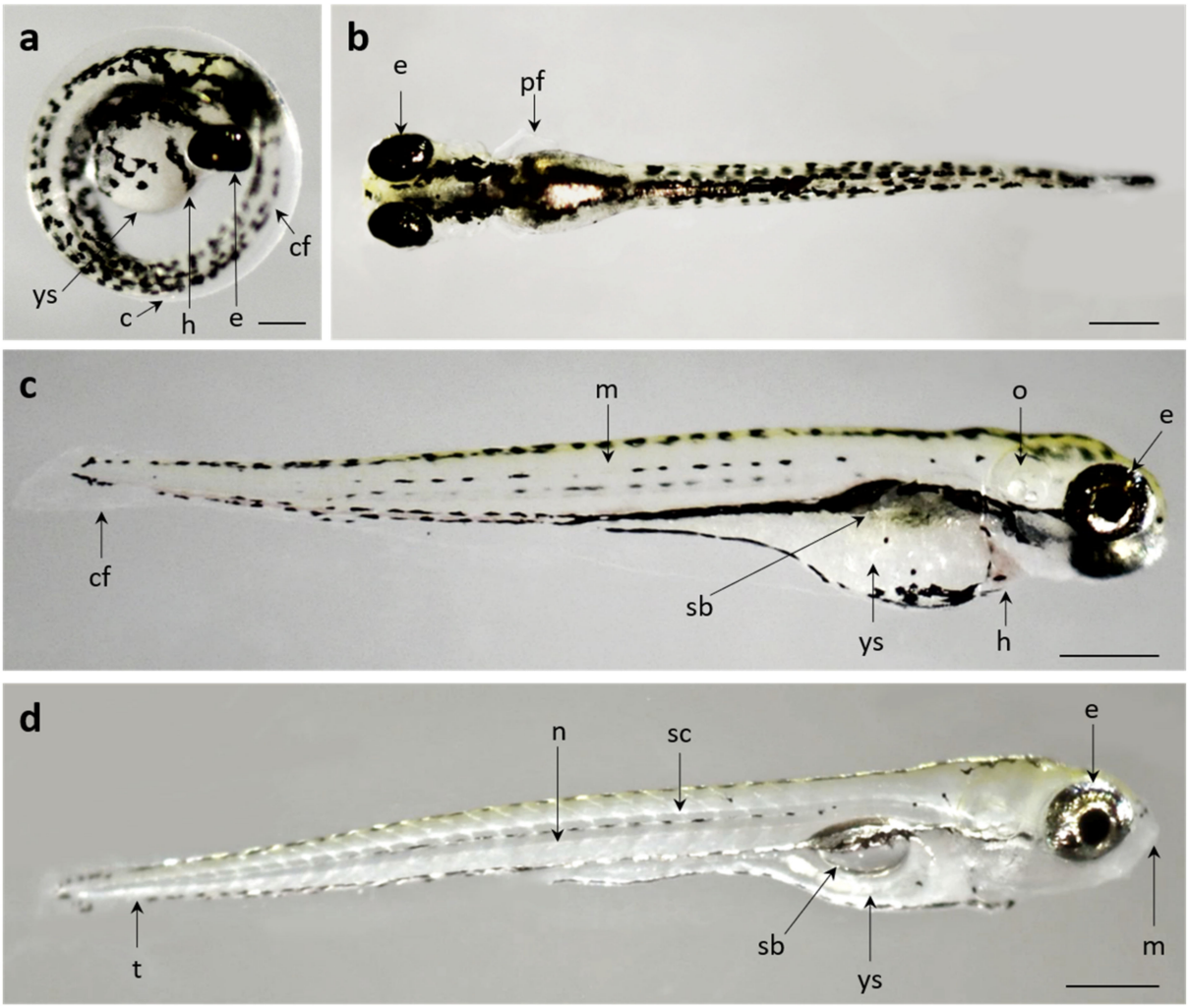

Figure 2. Gross morphology of zebrafish embryos and larvae in the basal conditions: (**a**) at 54 hpf, the embryo within the chorion has a prominent yolk sac and a well recognizable caudal fin. Note the high pigmentated eyes and the heart; (**b**) dorsal view of zebrafish larvae at 102 hpf; (**c**) at 102 hpf the yolk sac of the larvae is still prominent, and the swim bladder started to inflate; (**d**) at 150 hpf the larvae have resorbed the yolk sac, and the swim bladder is completed and inflated. C = chorion, ys = yolk sac, cf = caudal fin, e = eyes, h = heart, pf = pectoral fin, sb = swim bladder, o = otolith, m = muscle, sp = spinal cord, n = notochord, m = mouth, t = tail. All scale bars = 200 μm.

3.2.2. Pb Exposed Groups

Starting from 48 h of exposure to Pb, several types of deformities were observed in all exposed groups. Phenotypic malformations included: spinal and tail deformity, pericardial edema, and yolk swelling. As development proceeded, uninflated swim bladder was also recognizable in samples exposed to Pb.

After exposure to both low and high Pb concentrations, the severity of morphological abnormalities and the frequency of malformations (i.e., cumulative deformity) significantly increased with the increase of exposure time (**** $p < 0.0001$; Table 1).

Table 1. Frequency (%), cumulative deformity (%) and severity of morphological abnormalities in zebrafish embryos and larvae (n = 90) exposed to 2.5 and 5 µg/L of Pb for 48, 96 and 144 h.

		CTRL			2.5 µg/L			5 µg/L		
		48 h	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**
Deformity rate (%)	Spinal deformity	0	0	0	30 (a **)	50 (a ****)	60 (a ****)	90 (ab ****)	90 (a ****)(b **)	100 (a ****)(b **)
	Tail deformity	0	0	0	20 (a *)	50 (a ****)	50 (a ****)	90 (ab ****)	90 (a ****)(b **)	100 (ab ****)
	Yolk sac swellig	0	0	0	0	30 (a **)	60 (a ****)	0	40 (a ***)	70 (a ****)
	Pericardial edema	0	0	0	0	0	0	20 (ab *)	50 (ab ****)	60 (ab ****)
	Uninflated swim bladder	0	0	0	0	0	60(a ****)	0	0	80 (a ****)
	Cumulative deformity	0	0	0	60 (a ****)	90 (a ****)	90 (a ****)	90 (a ****)(b *)	90 (a ****)	100 (a ****)
	Score value	0	0	0	12 (a *)	35 (a ****)	58 (a ****)	58 (ab ****)	74 (ab ****)	144 (ab ****)

a = significant difference between treated group and control group; b = significance difference between high concentration and low concentration group; * $p < 0.05$; ** $p < 0.002$; *** $p < 0.001$; **** $p < 0.0001$.

In detail, after 48 h of exposure to the low Pb concentration, the first recorded alterations were spinal and tail deformity, which significantly increased compared to control (Table 1) (Figure 3a,b). Incidence of such malformation increased after 96 h of exposure when also the swelling of the yolk sac was recorded (Figure 3d). After 144 h of exposure, larvae also exhibited uninflated swim bladder (60%) (Figure 3f).

The Pb-induced deformities were dose dependent, and the overall incidence of deformities (deformity rate and score value) was significantly higher in the high-Pb exposed group (**** $p < 0.0001$; Table 1). Indeed, in the high concentration group, all individuals exhibited deformities at the end of the exposure period (Table 1).

After 48 h of exposure to the high Pb concentration, spinal and tail deformity incidence was significantly higher than in the control and low concentration groups (Table 1). Moreover, pericardial edema was often observed (Figure 4a,b).

After 96 h, the swelling of the yolk sac and pericardial edema were frequently observed with an incidence increased compared to low concentration (Figure 4a,b) (Figure 5c). After 144 h of exposure, all individuals showed spinal and tail deformity (Figure 4e–j). In addition, the frequency of larvae with pericardial edema (Figure 4f) and yolk sac swelling (Figure 4h,i) increased. Moreover, compared to the low concentration group, larvae with uninflated swim bladder were frequently observed (Figure 4e–j).

3.3. Gene Expression

Quantitative RT-PCR showed that Pb exposure induced the modulation of all analysed genes.

Metallothionein, mtf—After exposure to the low concentration of Pb, the gene was significantly upregulated compared to the control at all exposure times (* $p < 0.05$; Figure 5a). A similar pattern was observed in the high concentration group when the expression further increased (**** $p < 0.0001$; Figure 1a), reaching a peak after 96 h of exposure (**** $p < 0.0001$; Figure 5a).

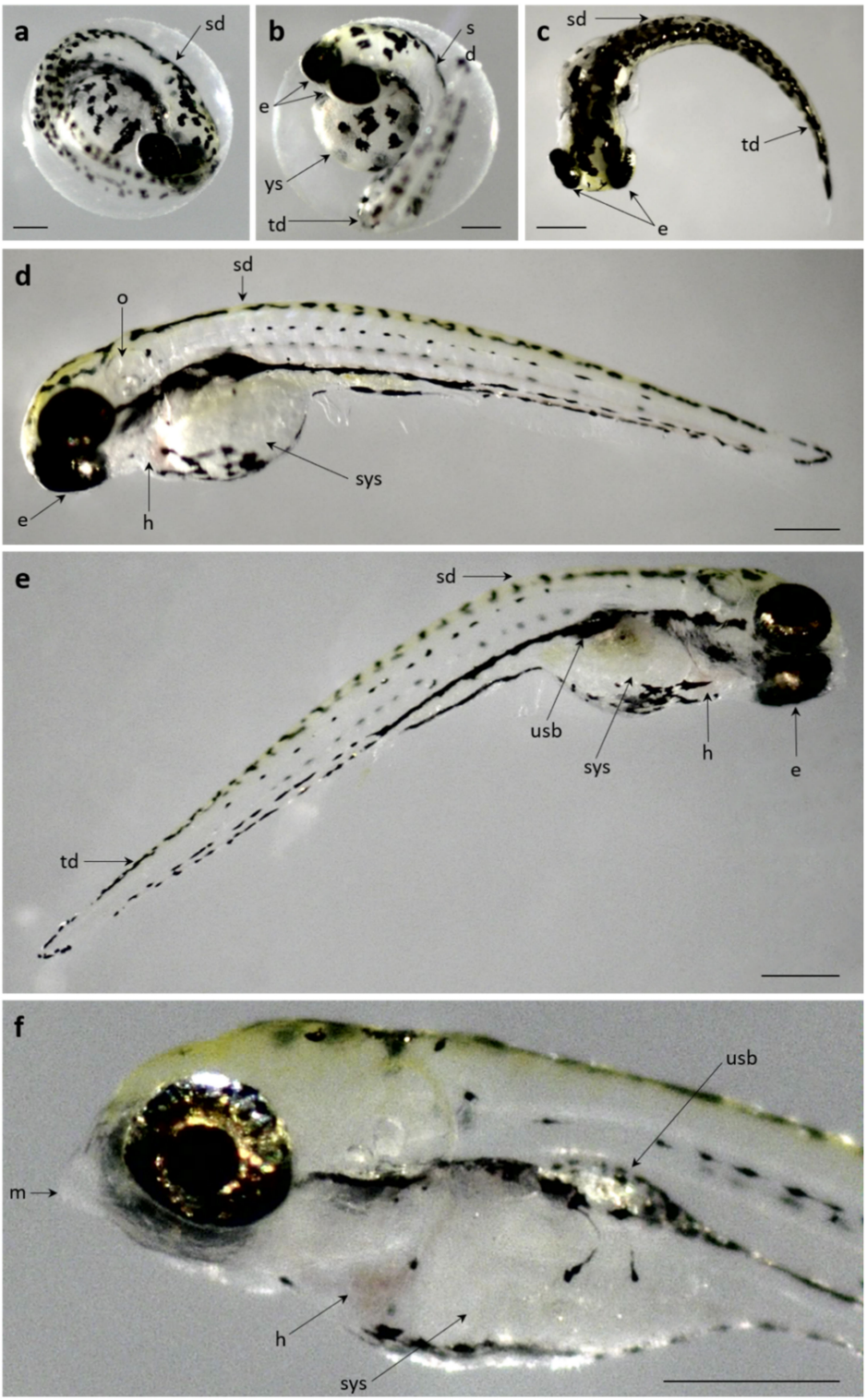

Figure 3. Gross morphology of zebrafish embryos and larvae after exposure to 2.5 µg/L of Pb: (**a**,**b**) note the appearance of spinal and tail deformity after 48 h of exposure to Pb; (**c**) dorsal and (**d**) frontal view of spinal deformity after 96 h of exposure to Pb. Note the swelling of the yolk sac; (**e**,**f**) after 144 h of exposure to Pb, the severity of spinal and tail deformity increases. Also, the swelling of the yolk sac is evident along with the uninflated swim bladder. Sd = spinal deformity, td = tail deformity, sys = swelling of the yolk sac, usb = uninflated swim bladder, e = eye, m = mouth, o = otolith, h = heart, ys = yolk sac. All scale bars = 200 µm.

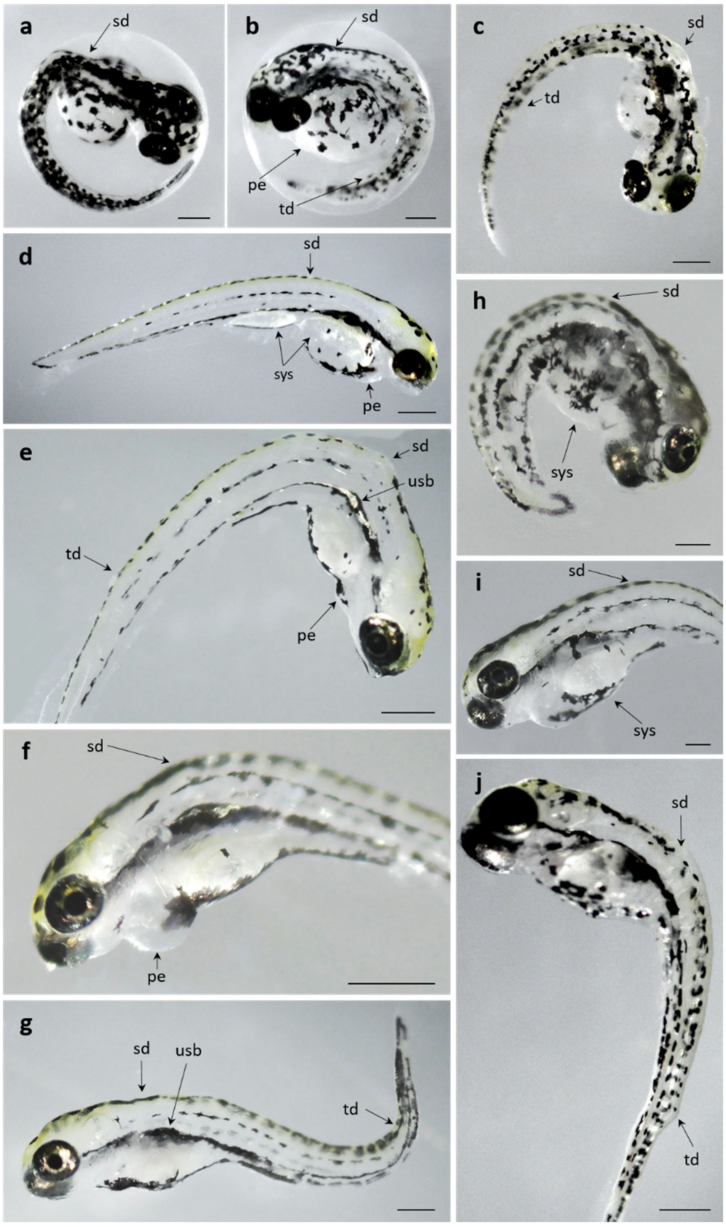

Figure 4. Gross morphology of zebrafish embryos and larvae after exposure to 5 µg/L of Pb: (**a**,**b**) spinal and tail deformity are frequently detected after 48 h of exposure. Note the appearance of pericardial edema; (**c**,**d**) after 96 h of exposure, both yolk sac swelling and pericardial edema are commonly observed; (**e**–**j**) all larvae show spinal and tail deformity after 144 h of exposure. Note pericardial edema, yolk sac swelling, and uninflated swim bladder. Sd = spinal deformity, td = tail deformity, pe = pericardial edema, sys = swelling of the yolk sac, usb = uninflated swim bladder. All scale bars = 200 µm.

Superoxide dismutase 1, sod1—Pb exposure resulted in a significant increase of *sod1* expression in both concentration groups at all exposure time, compared to the control, also showing a dose-dependent trend (** $p < 0.01$; Figure 5b).

Lactate dehydrogenase, ldh—A significant upregulation of *ldh* expression was recorded in both concentration groups compared to the control (**** $p < 0.0001$; Figure 5c). The increase in gene expression was higher in the high concentration group, showing a statistically significant difference even compared to the low concentration group (#### $p < 0.0001$; Figure 5c).

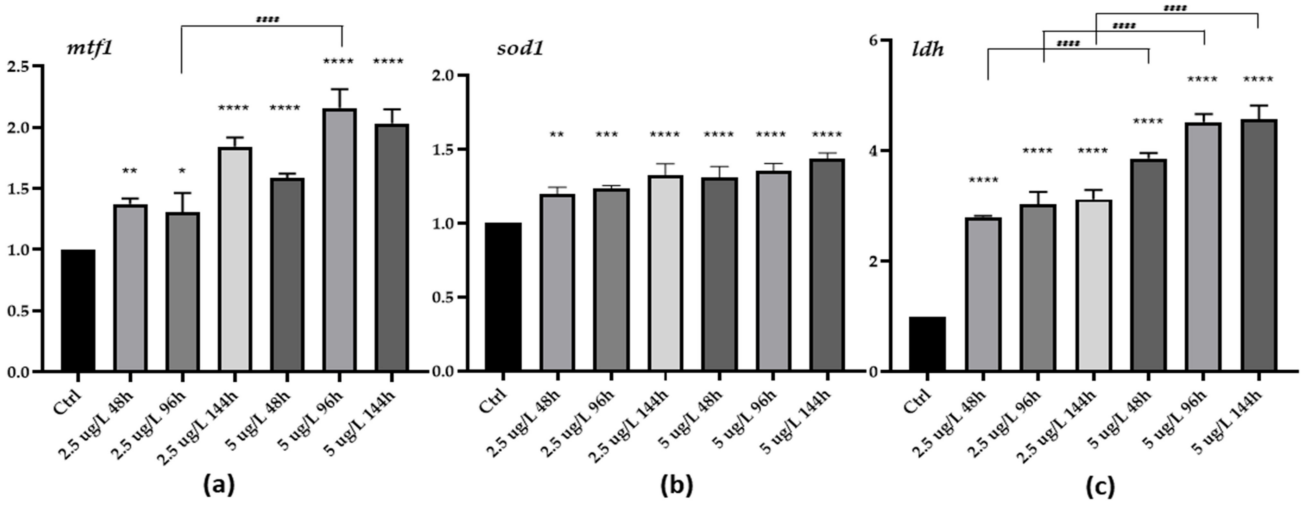

Figure 5. Gene expression in zebrafish embryos and larvae exposed to 2.5 and 5 µg/L of Pb for 48, 96, and 144 h: relative mRNA expression of metallothionein (**a**), superoxide dismutase 1 (**b**), lactate dehydrogenase (**c**). The bars represent the mean ± S.D. Asterisks indicate significant differences between treated groups and control using one-way ANOVA followed by Tukey's multiple comparisons test. Hashtags indicate significant different between high concentration and low concentration groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; #### $p < 0.0001$.

β-catenin, *ctnnb1*- The low Pb concentration resulted in a significant increase in *ctnnb1* expression after 96 and 144 h of exposure compared to the control group (**** $p < 0.0001$; Figure 6a). In the high concentration group, *ctnnb1* expression significantly increased, compared to the basal condition, starting from 48 h of exposure (* $p < 0.05$; Figure 6a). The expression further rose after 96 and 144 h of exposure, becoming significantly relevant compared to both control and low concentration group (**** $p < 0.0001$; Figure 6a).

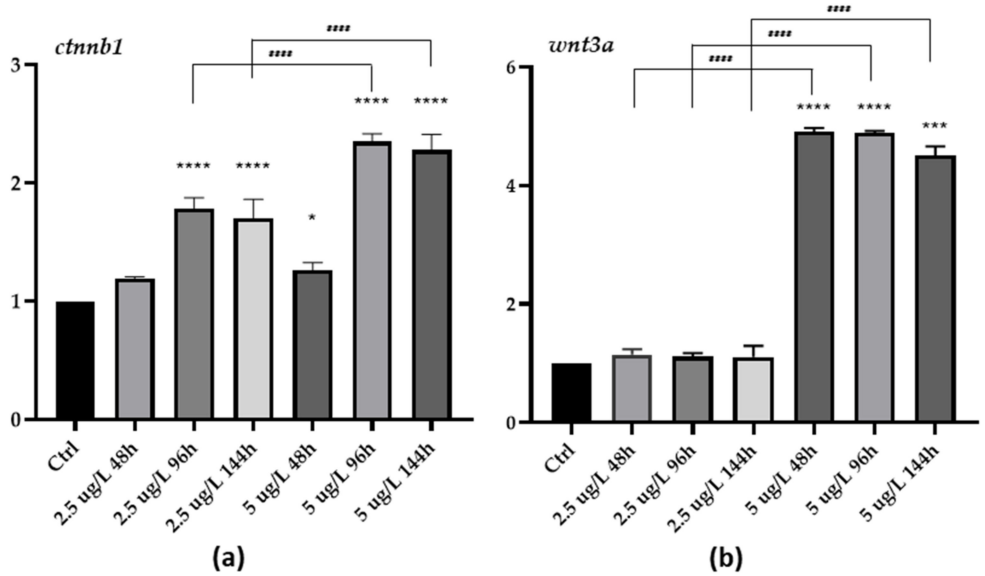

Figure 6. Gene expression in zebrafish embryos and larvae exposed to 2.5 and 5 µg/L of Pb for 48, 96, and 144 h: relative mRNA expression of *β-catenin* (**a**) and wingless-type MMTV integration site family (**b**). The bars represent the mean ± S.D. Asterisks indicate significant differences between treated groups and control using one-way ANOVA followed by Tukey's multiple comparisons test. Hashtags indicate significant different between high concentration and low concentration groups; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, #### $p < 0.0001$.

Wingless-type MMTV integration site family, wnt3a- In the low concentration group, no significant modification in *wnt* expression was detected. However, the expression drastically increased in the high concentration group, becoming significantly relevant compared to both the control (*** $p < 0.001$; Figure 6b) and the low concentration group at all exposure times (### $p < 0.001$; Figure 6b).

4. Discussion

Over time, several species have been used as surrogate indicators of exposure to pollutants in order to estimate the risk to human health. Still, the accuracy with which vertebrate studies predict the effects of putative toxic substances in the environment has long been a topic of interest and concern [40]. Given its well-demonstrated translational value for human toxicity research, in recent years, the use of zebrafish has undergone rapid development, becoming a prominent model in a variety of scientific disciplines, including toxicology [41]. In particular, in all vertebrates, both gene programming and development are highly conserved during the early stages of life, also allowing the maintenance of numerous morphological similarities through the evolutionary scale [40,42]. Several endpoints have been evaluated to disclose signals of lead toxicity in fish sentinel species, including zebrafish. Neurotoxicity, oxidative stress induction, and hematological alterations are the mainly reported outcomes of lead exposure in both adults and embryos [8,22–24,29,30,34,44–47]. However, all these literature data relate to high lead concentrations, thus leaving a gap of knowledge regarding the adverse effects of lead at environmentally relevant concentrations [9,53].

Here, we investigated the effects of lead exposure during development in zebrafish, providing evidence of the high toxicity of this heavy metal even at very low doses. To the best of our knowledge, the present study demonstrated for the first time that short-term exposure to environmentally relevant concentrations of Pb induces severe morphological modifications in the early stages of zebrafish and modulates the expression of several genes involved in the toxicological responses of fish to heavy metals. The results from the current study also confirm that embryos and larvae can act as an early warning indicator of pollution and may play a major role in environmental monitoring to protect ecosystems and human health.

4.1. Morphological Modifications

The sublethal doses of lead tested in our experiments were very low but proved to be high enough to induce severe body deformities when administered during the early life stages. We showed that morphological modifications arose from 48 h in both exposed groups, and their incidence and intensity were time dependent. The severity of injuries was significantly greater in the high concentration group at all exposure times, thus proving a strong correlation between lead dose and the incidence of morphological abnormalities; interestingly, all individuals in the high concentration group exhibited some body deformities at the end of the experimental period. Spinal and tail deformities were the most frequently detected alterations under both experimental conditions. However, animals exposed to the high Pb dose also displayed pericardial edema, which became more severe with increasing exposure time. There are few data for comparison with results presented herein; still, our observations are consistent with the few available reports on the lead-induced alterations in embryos and larvae [49–52].

4.2. Gene-Expression

Exposure to heavy metals triggers several adaptive responses, among which the induction of metal-binding proteins (MTs) involved in both transport and detoxification of metals. Stage-specific differences in the implementation of compensatory responses have been discussed several times in fish exposed to heavy metals [59]. Osman and colleagues [67] showed no significant differences in MTs' expression after exposure to high lead concentrations in embryonic tissues of African catfish (*Clarias gariepinus*), suggesting

that high sensitivity during early stages may be due to the failure of homeostatic mechanisms. On the contrary, the significant increase in *mtf* observed here clearly indicates zebrafish embryos and larvae's ability to mount an adaptive response to lead injuries. According to Tang et al. [59], the differential activation of homeostatic mechanisms, including MT induction, may be responsible for species-specific and stage-specific differences in metal sensitivity observed in fishes. Besides, several studies reported that MTs significantly contribute to protecting the organism from oxidative stress acting as scavenging against free oxygen radicals [60–62].

Heavy metals are known to induce oxidative stress by stimulating the production of reactive oxygen species (ROS), considered a common toxicity mechanism driven by different environmental stressors in fish. Through their scavenging activity, antioxidant enzymes represent a protection mechanism against elevated ROS levels and play a crucial role in preventing pathological processes [68]. In zebrafish embryos, it has been previously reported that short-term exposure to high Pb concentrations induces increased ROS production and upregulation of the antioxidant enzymes [51]. We showed that two environmentally relevant concentrations of Pb induce a significant and time-dependent upregulation of *sod1*, a key antioxidant enzyme implicated in the first-line defense against oxidative stress [35,47,51,53,69,70]. Since Pb concentrations tested here are very low, our results reinforce the role of sod as sensitive and valuable biomarkers of lead-induced oxidative stress with important consequences thereof for the protection of aquatic life and human health. Furthermore, the increase in SOD activity observed in both embryo and larvae indicates an attempt to counterbalance increased ROS generation. However, in the early stages of development, when morphological, biochemical, and physiological development is incomplete, the overall antioxidant capacity may not fully counteract ROS production [71,72]. The imbalance between ROS formation and the antioxidant defence system can alter the normal developmental process and therefore be involved in the appearance of the morphological abnormalities observed here.

The LDH has been recognized in both toxicology and clinical chemistry as an indicator of energetic changes and a biomarker of cellular, tissue, and organ damage following exposure to xenobiotics [71,73,74]. In juveniles and adult fishes, both an increase and decrease of LDH have been observed after metal exposure [75–77]. Literature data on the effects of lead in the early developmental stages are scarce, and the only available study reports a decrease in LDH activity in *Clarias gariepinus* after exposure to three high concentrations of lead nitrate (100, 300, 500 µg/L) [74]. We do not have clear evidence regarding the mechanisms responsible for these different outcomes; nevertheless, we found in zebrafish a significant increase of *ldh* expression at all concentrations and exposure times. Similar results have been reported in the early life stages of several species, including zebrafish, after exposure to different pollutants, and confirmed the high energy requirement originating from chemical stress is involved in the upregulation of LDH in zebrafish [71,78–80].
β-catenin is a multifunctional molecule highly conserved through evolution and plays a pivotal role in animals' developmental and homeostatic processes [81]. Depending on its binding partners and cell localization, *β-catenin* functions as a mediator of cell adhesion through interactions with cadherins [82] or as a downstream effector of the canonical (*β-catenin* dependent) wnt signaling pathway. In zebrafish, as in other vertebrates, this pathway is activated at different stages, and it is required for axial growth, neural crest cell migration, and early development of the swim bladder [83,84].

The results presented in this study show that *β-catenin* is significantly upregulated after exposure to lead, and the modulation is dose dependent. Instead, exposure to low doses of Pb induces a slight increase in *wnt3* expression, which becomes significant in the groups exposed to high Pb concentration. The complex interactions of *β-catenin* with different transcription factors and the implications in many processes during animal development and tissue homeostasis have been reviewed by Valenta et al., [81], who outlined the intricate functions of this pleiotropic molecule that are only partially understood. In pathological conditions, the intracellular accumulation of free *β-catenin* may be caused by several molec-

ular events which inactivate scaffold molecules [85,86]. The increased concentration of free cytoplasmic *β-catenin* allows for its nuclear translocation and activation of *wnt/β-catenin* target genes, but the mechanism through which *β-catenin* is translocated is not fully understood [87,88]. Although many studies have focused on the *β-catenin* dependent wnt pathway during embryo development, much is unknown about the hierarchical dialogues between wnt and *β-catenin* and the cross-talking between the canonical and the canonical noncanonical wnt pathways. Taken together, our results provide the basis for a model in which disturbance of *β-catenin* may represent another pathway of lead toxicity.

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