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Developmental anomalies in ide (*Leuciscus idus* L.) larvae caused by copper and cadmium

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Ide embryos were incubated in 0.1 mg dm^3 of Cu or Cd or in clean tap water (control). Both metals significantly decreased swelling of eggs. They reduced the rate of embryonic development and the hatching rate. Six types of body malformations were found in newly hatched larvae: spine curvature, C-shaped body, head deformation, yolk sac deformation, heart oedema, and reduced body length. Only the first two types of deformations were observed in the control, while more severe malformations were found following Cu and Cd exposure. Copper exerted a detrimental effect mainly during embryogenesis (egg swelling and rate of development), while the toxic effects of cadmium were more significant in newly hatched larvae. Larval body deformities may be used as a bioindicator of water pollution with heavy metals.

KEYWORDS: fish, metal, toxicity, embryos, larvae

INTRODUCTION

The ide (*Leuciscus idus* L.) is a widespread fish in both Europe and Western Asia (Robins et al. 1991; Witkowski et al., 1997; Nico and Fuller, 2008). It is a large freshwater fish, with an average length of 30 cm, max. 85 cm, max. weight 4 kg, and max. lifespan 18 years (Wüstemann and Kammerad 1995; Kottelat and Freyhof 2007). It usually inhabits large lowland rivers and nutrient-rich lakes, at a temperature range of 4-20°C (Riehl and Baensch 1991). Adults are solitary, in contrast with juveniles. The ide feeds on various aquatic and terrestrial animals and on plant material, but larger individuals feed mainly on fish. Actively feeding larvae and juveniles live in shoreline habitats; only larger individuals migrate to deeper waters (Kottelat and Freyhof 2007). The ide is of significant economic value in many countries, including Poland, where it is farmed as a food fish, but also for recreation (Targonska et al. 2011; Froese and Pauly 2015). Production of summer fry for restocking and year-old fish accounted for 69% and 91% of total production of riverine cyprinids in Poland in 2000 and 2002 (Krejszeff et al. 2009). Unfortunately, the ecological status of the species is considered to be vulnerable (Lelek 1987; Schiemer and Spindler 1989; Lusk

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et al. 2004), and thus wild populations require protection and support.

Cu, and Cd are among the most important toxicants; they are difficult to eliminate and still detected at high concentrations in many aquatic environments (Iger et al. 1994; Meybeck et al. 2007), and thus continue to have a harmful effect on living organisms (Zheng et al. 2007).

Copper is an essential trace nutrient for all animals and a cofactor in cellular enzymes, e.g. oxidase, tyrosinase, laccase and ceruloplasmin (Fleming and Trevors 1989; Bieniarz and Epler 1994). It is a component of about 30 enzymes and glycoproteins, has important role in gastrointestinal tract and nervous system function, and is essential to haemoglobin synthesis (Sorensen 1991). Copper is naturally present in fresh water at concentrations ranging from 0.0002 to 0.03 mg/dm^3 (US EPA 2007). Water pollution with copper is mainly associated with mining, fertilizer production, municipal and industrial wastewater, and the use of copper salts as aquatic herbicides, algaecides, fungicides and bactericides (Michael 1986; Boyd 1990; Newman and Unger 2003). In excess, copper is toxic to living organisms (Moore and Ramamoorthy 1984), regarded as the second most toxic metal to fish after mercury, with a typical 96h LC_{50} value ranging from 0.017 to 1.0 mg/dm^3 for most freshwater fish species (Moore and Ramamoorthy 1984). At high concentrations Cu is an inhibitor of Na^+/K^+ ATPase in the basolateral membrane of the gills (Lauren and McDonald 1986; Morgan et al. 1995), with its main toxic effect consisting in disturbance of sodium homeostasis (Lauren and McDonald 1986). The gills are the organ most susceptible to copper toxicity: copper precipitates in gill mucus, which ultimately may result in asphyxiation (Karan et al. 1998; Stokes 1979). However, long-term exposure damages other organs as well, such as the liver, kidneys, or sensory organs (Baker 1969; Gardner and LaRoche 1973).

Cadmium is a xenobiotic present in water as a result of natural and anthropogenic processes (Asagba et al. 2008; Bouraoui et al. 2008; Czeczot and Skrzycki 2010). It is as a key component in the production of batteries, pigments, and electroplating (Smith et al. 1999; Scoullos et al. 2001). The main mechanism of Cd toxicity is the antagonistic interaction between uptake of Ca^{2+} and Cd^{2+}, which disrupts Ca^{2+} absorption and homeostasis (McGeer et al. 2011). Cadmium accumulates mainly in the liver, kidneys, and gills (McGeer et al. 2011). It can disturb the life of fish by affecting various biochemical and physiological processes (Drąg-Kozak et al. 2019). It causes anaemia, vertebral fractures (Larsoon, 1977), hypocalcaemia, hypokalaemia, and hyperglycaemia (Sorensen, 1991); reduces digestive capacity (Sastry and Gupta 1979); and affects olfactory sensitivity (Scott et al. 2003). Cadmium is also considered an endocrine disruptor, whose effect can take place via the hypothalamus-pituitary-interrenal (HPI) and hypothalamic-pituitary-gonadal (HPG) axes (Szczerbik et al. 2006; Lizardo-Daudt et al. 2007; Sandhu and Vijayan 2011; Drąg-Kozak et al. 2018).

In fish ontogeny, sensitivity to metals generally decreases with age, with larvae considered to be the most susceptible (von Westernhagen 1988; Hwang et al. 1995; Jezierska and Witeska 2001). Data obtained by various authors show the adverse effect of copper and cadmium, especially on hatchability (Calta 2001; Gonzales-Doncel et al. 2003), rate of development (Hodson et al. 1978; Jezierska and Słomińska 1997; Johnson et al. 2007), survival, and morphological abnormalities (Cheng et al. 2000; Chow and Cheng 2003; Hallare et al. 2005; Fraysse et al. 2006; Zhu et al. 2013; Sfakianakis et al. 2015). The effects of copper and cadmium on the early stages of many cyprinid fish species have been well documented (Jezierska et al. 2009), but data concerning Cu and Cd toxicity to ide embryos and larvae are still incomplete.

The aim of the present study was to investigate the effects of embryonic exposure to copper and cadmium present in the water on body deformities in newly hatched larvae of ide.

MATERIALS AND METHODS

The study was performed on embryos and newly hatched larvae of ide exposed to copper or cadmium during embryonic development. Eggs from five females and sperm from five males were obtained during artificially stimulated spawning in the Samokleski fish farm in Kamionka, Poland. The material was transported at 5°C to the laboratory of the Department of Animal Physiology, University of Natural Sciences and Humanities in Siedlce. The eggs were fertilized about 2 h after stripping. Fertilized eggs were placed in glass Petri dishes, and these in 2 L aquariums, and divided into three groups (four replications with 160 eggs in each): C (control) – clean tap water, Cu – 0.1 mg dm^3 Cu (from $CuSO_4$ solution) and Cd – 0.1 mg dm^3 Cd (from $CdCl_2$ solution). The water (16°C) was changed every day and continually aerated. The diameters of 25 eggs and their yolks were measured under a stereoscopic microscope (12 × 1.6 magnification), two hours after fertilization. Swelling was calculated as $S = (c - d) \times 100/d$, where S is the swelling (% increase in egg diameter), c is the egg diameter, and d is the yolk diameter. As the eggs were opaque during cleavage, the rate of embryonic development could not be evaluated until the body formation stage. Newly hatched larvae were counted and inspected. Each deformed larva was examined and classified according to deformation catalogues by Jezierska et al. (2000) and Lugowska and Kubik (2011). The percentage of each type of deformation among all deformed larvae in each group was calculated. Embryos and larvae were observed and photographed daily using the MultiScan computer image analysis system and a stereoscopic microscope connected to a camera. The photographs were used to create a catalogue of ide larval deformations.

The results were analysed using STATISTICA 10 software. Normality of distribution was tested using Shapiro-Wilk's test, and homogeneity of variance using Levene's test. Egg swelling and rate of embryonic development, which showed normal distribution, were analysed by ANOVA, followed by Tukey's post-hoc test. For the frequency of types of larval deformations (data that did not meet the assumptions of ANOVA), the non-parametric Kruskal-Wallis test for multiple comparisons test was performed. The level of significance was set at $p < 0.05$. Data are presented as means ± SD.

RESULTS

At 120 minutes after fertilization the highest swelling percentage was obtained in the control (81%), while in the groups exposed to metals it was significantly reduced, particularly in the case of copper (49% in group Cu and 77% in group Cd; Fig.1).

Both metals reduced the rate of embryonic development, especially at the stage when the first body movements appeared, which took place about 44 hours later than in the control, resulting in delayed hatching (Table 1). The effects of the two metals on hatching were different: Cu slightly prolonged hatching to 22 h, while Cd reduced it to 16 h, compared to 21 h in the control.

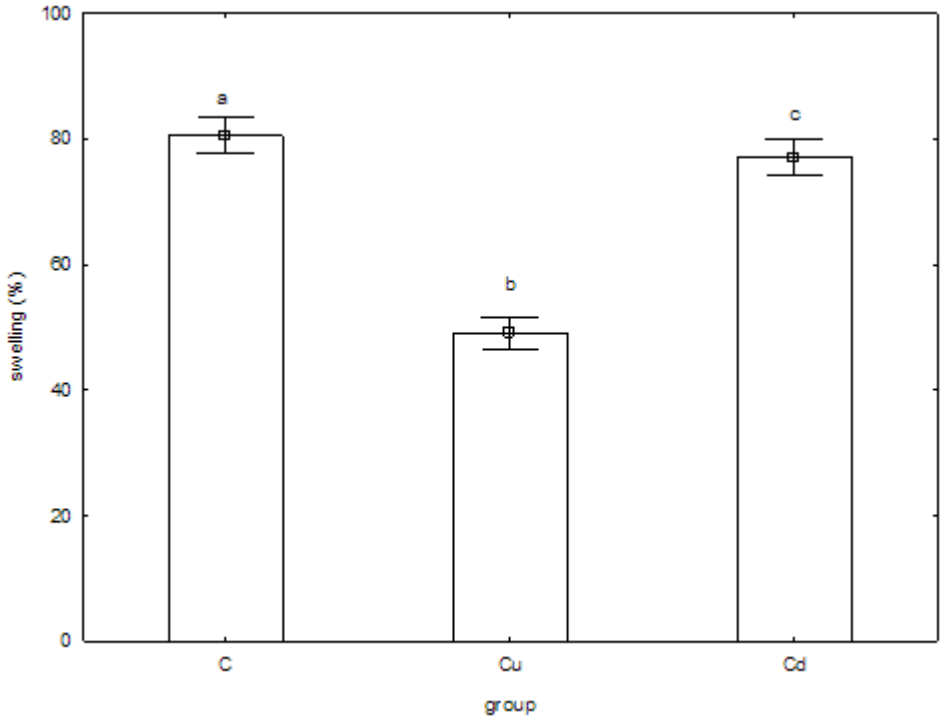


Fig. 1. The effect of copper and cadmium on swelling of ide eggs (Tukey's post-hoc test; n = 25, different superscript letters a, b, c indicate significant differences between means for experimental groups at $P \leq 0.05$).

Table 1.

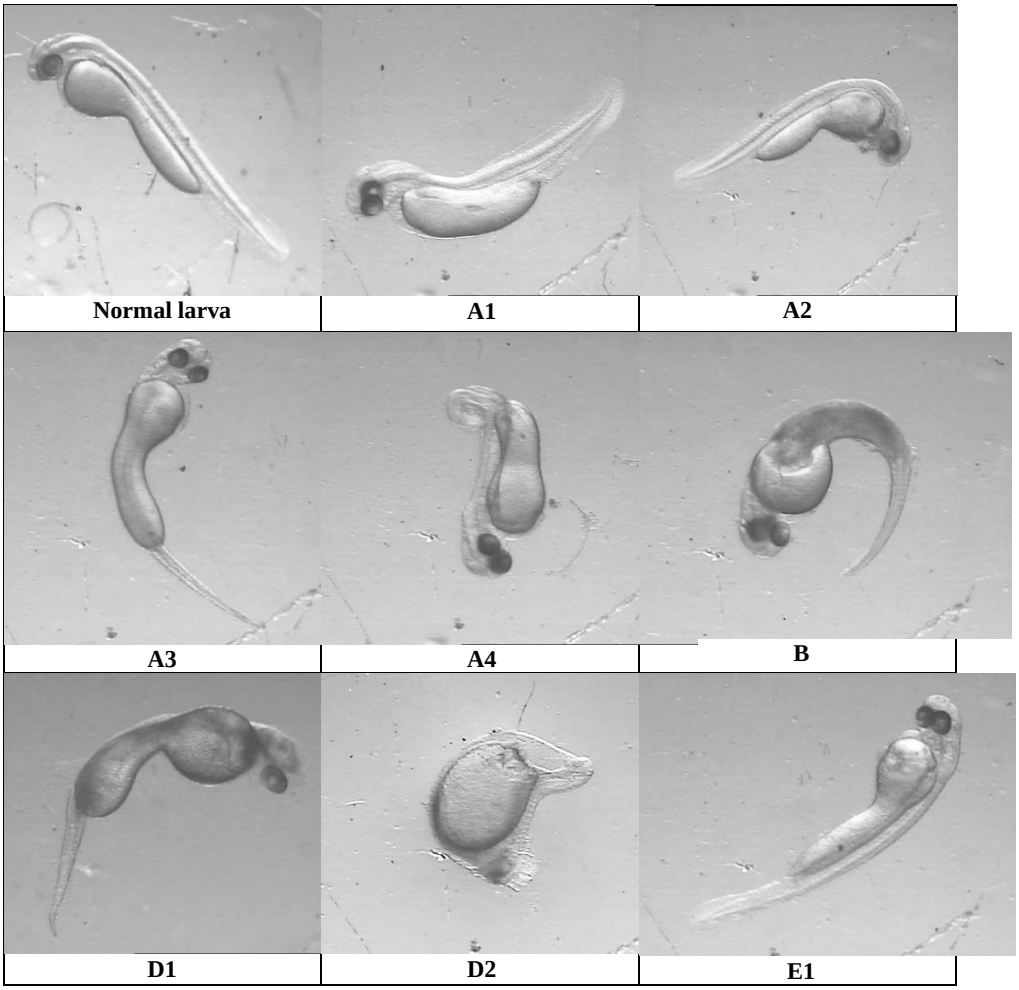
Effect of copper and cadmium on rate of ide embryonic development (time in hours after fertilization, Tukey's post-hoc test; n=25).

Stage	Control	Cu	Cd
Small-cell blastula	2.0 ±0.07^a	5.0 ±0.25^b	2.5 ±0.35^c
Embryonic body formation	19 ±0.07^a	24 ±0.28^b	21 ±0.35^c
Eye pigmentation	65 ±1.37^a	72 ±2.12^b	68 ±2.12^c
Body movements	71 ±1.41^a	115 ±1.78^b	115 ±1.86^b
Start of hatching	114 ±0.71^a	128 ±2.12^b	125 ±1.79^c
End of hatching	135 ±0.56^a	150 ±1.99^b	141 ±1.71^c

Different superscript letters a, b, c indicate significant differences between means for experimental groups at $P \leq 0.05$.

Table 2.

Types of body malformations of newly hatched ide larvae: A – spine curvature (A1 – lordosis, A2 – kyphosis, A3 – scoliosis in caudal region, A4 – scoliosis in caudal and abdominal regions); B – C-shaped larva with shortened body, spine curvature and yolk sac deformation; D – head deformation accompanied by spine curvature and yolk sac deformation (D1 – partial lack of eye pigmentation, D2 – lack of eyes); E – yolk sac malformation accompanied by scoliosis in abdominal region (E1 – oedema in caudal part of yolk sac E2 – enlarged caudal region of yolk sac – pea-shaped); F – heart oedema (F1 – heart oedema and spine curvature in caudal and abdominal regions, accompanied by yolk sac malformation and blood clots in yolk sac vessels); G – shortened body (G1 – shortened body accompanied by complex spine curvature, oedema and yolk sac malformation).



E2	F1	G1

The normal, correctly developed ide larva has a straight spine, with the yolk sac wider in the caudal region and gradually narrowing in the abdominal region (Table 2). Newly hatched ide larvae showed six main types of body malformations, classified according to the deformation catalogues by Jezierska et al. (2000) and Lugowska and Kubik (2011).

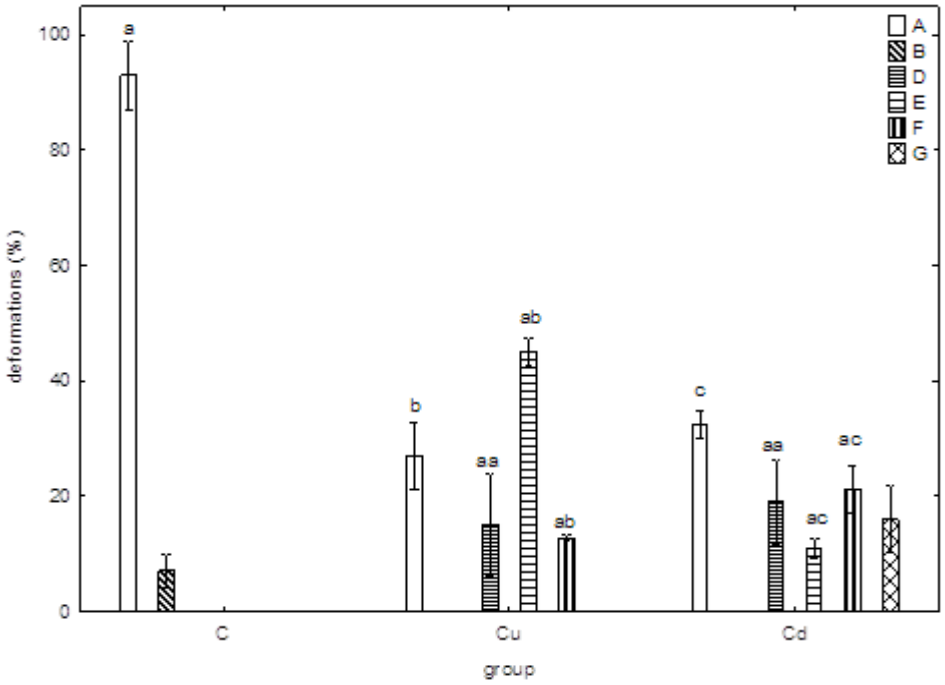


Fig. 2. Effect of copper and cadmium on incidence of various types of body malformations in ide larvae (Kruskal-Wallis test, $p<0,05$; different superscript letters indicate significant differences between experimental groups; A – spine curvature; B – C-shaped larva with shortened body, spine curvature and yolk sac deformation; D – head deformation accompanied by spine curvature and yolk sac deformation; E – yolk sac malformation accompanied by scoliosis in abdominal region; F – heart oedema; G – shortened body).

In the control group, most deformed larvae exhibited spine curvature, while only 7% of them had a C-shaped body (Fig. 2). In the Cu and Cd groups, spine deformations were observed in about 30% of larvae, whereas the C-shape was not detected. More complex malformations (types D-G) were observed only in metal-exposed groups. Head deformations (type D) in both the Cu and Cd groups were noted in about 17% of deformed individuals. Yolk sac malformations were significantly more frequent in the Cu group, while heart oedema was more common in the Cd group. Larvae with a shortened body were observed only in the Cd group.

DISCUSSION

Developmental disturbances may result from impaired cellular functions caused by metal-induced oxidative stress, including lipid peroxidation, DNA damage, protein carbonylation, or impaired gene expression (Cavas, 2008; Cavas et al., 2005; Grygoryev et al., 2008; Jia et al., 2011).

The chorion is a thick, multi-layered coat entirely surrounding the mature fish oocyte (Cotelli et al., 1988). It has two fundamental roles: first, in the fertilization process it prevents polyspermy and probably provides recognition signals from gamete interactions, and secondly, it forms a protective barrier between the embryo and the external environment after egg swelling (Mizell et al., 1996; von Westernhagen, 1988; Weis and Weis, 1991; Gellert and Heinrichsdorff, 2001). Swelling begins when the eggs first come into contact with water. The permeable chorion allows water and ions to penetrate the egg, which takes place due to the high osmotic pressure produced by protein colloids. They are released into the perivitelline space through the yolk membrane and retained by the chorion (Hoar and Randall, 1969; Peterson and Martin-Robichaud, 1982). Swelling is possible only while the chorion is permeable, i.e. before it hardens. According to Hoar and Randall (1969), hardening of the chorion is caused by its internal glycoprotein layer and by calcium ions, phospholipids and hardening enzymes present in the perivitelline fluid. The fully hardened chorion protects the embryo against both mechanical injury and toxic substances from the environment.

In the present study, both metals significantly reduced swelling of ide eggs. Data on the effects of environmental factors on fish egg swelling are very scarce. Decreased swelling has been reported only for common carp, at various concentrations of copper, i.e. 0.05 mg/dm^3 and 0.2 mg/dm^3 (Jezierska and Slominska, 1997), and cadmium: 0.001-0.01 mg/dm^3 (Witeska et al., 1995), 5-50 mg/dm^3 (Calta, 2001), and 0.2-0.7 mg/dm^3 (Sikorska and Lugowska, 2005).

During swelling, waterborne metal ions can easily penetrate the egg, but this becomes difficult after the chorion hardens (Nguyen et al., 1998, Williams and Holdway, 2000; Kong et al., 2013). According to Beattie and Pascoe (1978), metals are accumulated mainly in the chorion; after 22-hour exposure of *Salmo salar* eggs to Cd at 10 mg/dm^3, 98% of the metal was bound to the chorion, 1.8% had accumulated in the embryo, and 0.2% in the yolk sac. Very similar results were obtained by Michibata (1981) for eggs of *Oryzias latipes* exposed to the same concentration of cadmium. Water contamination with metals during swelling may affect further embryonic development in two ways: through ineffective hardening of the chorion, which enables further penetration of metals into the egg (Gonzales-Doncel et al., 2003), or through inhibition of swelling.

A likely cause of swelling inhibition by waterborne metals is their accumulation in the chorion and penetration into the egg. Stouthart et al. (1994) suggested that lead binds to membrane mucopolysaccharides, thereby altering chorion permeability, which results in disturbances of ion exchange between the perivitelline fluid and the external environment. Metals may also affect the

physical properties of the egg surface. Benoit and Holcombe (1978) reported that zinc-treated eggs of *Pimephales promelas* became sticky and breakable soon after spawning. According to Korwin-Kossakowski (1996), an embryo in an insufficiently swollen egg cannot change its position (this takes place every 5-10 seconds during the final embryonic stage), which is essential for normal development and hatching.

In the present study, the metals delayed embryonic development, particularly the onset of body movements, which resulted in a delay in the start of hatching. Hatching in the Cu-exposed group was prolonged by one hour, while in group Cd it was reduced by six hours. Retardation of embryonic development due to incubation in water contaminated with various metals has also been observed by Rask (1983), Cleveland et al. (1986), Jezierska and Słomińska (1997), and Ługowska and Jezierska (2000).

A reduction in the speed of hatching may be caused by a variety of mechanisms, such as reduced embryo motility, abnormal activity or distribution of the hatching enzyme chorionase (Rosenthal and Alderdice, 1976), the toxic effect of metals (Hagenmaier, 1974), or difficulty in breaking an insufficiently digested chorion (Sinha and Kanamadi, 2000). In the present study, the increased hatching speed due to metal exposure may have been linked to damage to the hatching glands (Miś et al., 1995, 1996; Miś and Bigaj, 1997), which could have led to earlier release of chorionase or to a stress-mediated increase in chorionase secretion.

In our study, six types of body deformities of ide larvae caused by incubation in water containing metals were distinguished: spine curvature (A), C-shaped larva (B), head deformation (D), yolk sac malformation (E), heart oedema (F) and shortened body (G). Except for type B, all were detected in the metal-exposed fish. These anomalies were similar to those described for other fish species following exposure to various metals, e.g. *Cyprinus carpio* (Jezierska et al., 2000; Ługowska and Witeska, 2004; Lugowska, 2007), *Ctenopharyngodon idella* (Ługowska et al., 2002; Ługowska and Kubik, 2011) and *Danio rerio* (Cheng et al., 2000).

The aetiology of body malformations is, however, not entirely clear. Korwin-Kossakowski (1996) considered retardation of egg swelling to be a possible cause of body deformations in developing embryos. The space inside inadequately swollen eggs was not large enough for the embryo to develop normally, which resulted in malformations in the hatched larva. Disturbances of ion exchange are regarded as another possible cause of larval body malformation. Competition between Cd^{2+} or Cu^{2+} and Ca^{2+} (as well as displacement of Ca^{2+} from paracellular junctions and Ca^{2+}-binding proteins) is one of the mechanisms of the toxic action of these metals on cell membranes, which are stabilized by Ca^{2+} (Nieboer and Richardson, 1980). Niyogi & Wood (2004) reported inhibition of Ca^{2+} uptake as a result of competition between Cd^{2+} and Ca^{2+}. According to Verbost et al. (1988), Cd^{2+} caused the inhibition of Ca^{2+} transport by occupying the Ca^{2+} transport site in Ca^{2+}-ATPase. Schoenmakers et al. (1992) confirm that inhibition of Ca^{2+} – is one of mechanisms by which Cd^{2+} suppresses intestinal calcium uptake and disturbs calcium homeostasis in fish. These disturbances of Ca^{2+} transport and homeostasis may result in skeletal abnormalities. Muramoto (1981) reported a reduction in calcium and phosphorus levels as a cause of Cd-induced damage in the vertebral column of common carp. Some authors have proposed possible causes of specific types of metal-induced malformations. For example, oedema is said to be caused by leakiness of the endothelial vessels supplying the yolk sac due to cardiovascular dysfunction (Guiney et al., 1990). Alternatively, it could be interpreted as an indicator of metabolic or osmotic

disruption, likely due to mitochondrial malfunction (Sinha and Kanamadi, 2000). Cheng et al. (2000) attributed spinal deformities to a reduction in the formation of both myosin and myotome, which are essential for the normal development of a healthy musculoskeletal system. Developmental defects in the tail, such as deformation and curvature, can be explained genetically as resulting from the inability of Cd-treated embryos to express the EVX1 gene, which is important during tail extension (Cheng et al., 2000), or developmentally, through altered migration of precursor cells of the somatic mesoderm (Ho and Kane 1990). Muscle contracture during the hatching process may also be considered a possible cause of skeletal deformations (Holcombe et al., 1976).

CONCLUSIONS

Exposure of ide embryos to Cu and Cd resulted in significant changes in their development.

1. Both metals significantly reduced egg swelling, slowed the rate of development (especially body movements), and delayed hatching, while the hatching duration itself was slightly prolonged by Cu and reduced by Cd relative to the control. Cu and Cd induced similar types of larval body malformations, but the presence of cadmium caused more complex abnormalities.
2. Comparison of the toxicity of the two metals during embryonic development of ide shows that copper exerts a greater effect during embryogenesis (swelling and rate of development), while the toxic effects of cadmium were more evident in newly hatched larvae.
3. Larval body deformities can be used as a bioindicator of water pollution with heavy metals.

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