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EFFECTS OF WATER pH ON COPPER TOXICITY TO EARLY LIFE STAGES OF THE COMMON CARP (*CYPRINUS CARPIO*)XANDER J.H.X. STOUTHART,* JEROEN L.M. HAANS, ROBERT A.C. LOCK and SJOERD E. WENDELAAR BONGA
University of Nijmegen, Department of Animal Physiology, Faculty of Science, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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Abstract—Carp eggs were exposed immediately after fertilization to Cu concentrations of 0.3 and 0.8 $\mu\text{mol/L}$ at water pH 7.6 or pH 6.3. Mortality, the incidence of spinal cord deformation, heart rate, tail movements, hatching success, and whole-body content of K, Na, Mg, Ca, and Cu were determined over time. Light microscopical preparations of eggs (48 h after fertilization) and larvae (168 h after fertilization) were studied. At pH 7.6, Cu did not affect egg mortality, heart rate, tail movements, and whole-body K and Mg content. Hatching success increased only in the 0.3 $\mu\text{mol/L}$ Cu group. Exposure to 0.8 $\mu\text{mol/L}$ Cu increased larval mortality and larval deformation and decreased whole-body Na and Ca content. At pH 6.3, exposure to 0.8 $\mu\text{mol/L}$ Cu increased egg mortality and decreased heart rate and tail movements. Furthermore, premature hatching, a concentration-dependent increase of larval mortality, and larval deformation was observed. Exposure to 0.3 and 0.8 $\mu\text{mol/L}$ Cu decreased the whole-body content of K, Na, Mg, and Ca. Uptake of Cu after hatching increased two-fold at pH 6.3 compared to the pH 7.6 groups. At pH 6.3, all Cu-exposed larvae were unable to fill their swim bladder. Also, after 168 h the yolk sac remained largely unabsorbed in the 0.3 and 0.8 $\mu\text{mol/L}$ Cu group. Exposure to 0.8 $\mu\text{mol/L}$ Cu resulted in coagulation of proteins in eggs and yolk sacs. No significant changes in any of the assessed parameters were observed in control groups of pH 6.3 and pH 7.6.

Keywords—Acid stress Copper Embryonic development Carp *Cyprinus carpio*

INTRODUCTION

Copper (Cu) is an essential metal for nearly all organisms, including fish [1,2], but becomes toxic when exceeding natural concentrations ($<0.05 \mu\text{mol/L}$) [3,4]. Indeed in polluted rivers such as the Rhine River concentrations exceeding $1 \mu\text{mol/L}$ Cu have been reported, which together with a decreasing water pH could seriously threaten aquatic life. In fish, Cu seriously interferes with branchial ion transport [5–8], plasma ion concentrations [9,10], hematologic parameters [11], and enzyme activities [6,12,13]. In addition, Cu may cause immunosuppression [14], vertebral deformities [15,16], and neurological disorders [17].

It is not known whether these effects of Cu will already manifest themselves during the early developmental stages of fish, particularly at a decreasing water pH. In this context it is important to consider that chemical speciation of Cu strongly depends on water pH. At pH 7 and higher, carbonate and hydroxide species are the dominant forms. Below water pH 7 the amount of free Cu ions rapidly increases, thereby enhancing the toxicity of this metal [18]. In addition, a lower water pH may also promote branchial uptake of Cu by protonating the binding sites for Cu on the epithelial cell surface [19].

During early life, the yolk sac stage is considered the most sensitive one, followed by the embryonic stage prior to completion of gastrulation [20]. This study describes the influence of water pH on Cu toxicity during the early developmental stages of the carp (*Cyprinus carpio*) immediately after fertilization. Attention was paid to deformation, malfunctioning, heart rate, tail movements, total body content of K, Na, Mg, Ca, Cu, hatching success, and histologic changes at the light microscopic level.

MATERIALS AND METHODS

In vitro fertilization and incubation of eggs

Fertilization and incubation of eggs from carp (*Cyprinus carpio*) were carried out in the experimental setup as described previously [21]. In four separate experiments, individual batches of eggs from seven different females were each fertilized by sperm from a different male carp resulting in seven genetically different batches of eggs ($n = 7$). For each batch, one Petri dish of eggs was used to examine mineral content, heart rate, and tail movements of the developing embryos and another Petri dish was used to determine mortality of eggs and larvae, deformation of larvae, and hatching success. Thus, for each batch of eggs 12 Petri dishes were used: two sets of parameters (two Petri dishes) \times six groups (two controls and four experimentals) = 12 Petri dishes. Immediately after fertilization, the Petri dishes (300–400 eggs per dish) with the eggs attached to the bottom, were placed in 4-L aquaria. To avoid low concentrations of Cu (0.03 – $0.24 \mu\text{mol/L}$) normally present in Nijmegen tap water, chemically defined water was used, which was demineralized water with the following concentrations (mmol/L) of salts added: KCl (0.06); NaHCO_3 (0.40); MgSO_4 (0.20); CaCl_2 (0.80).

The eggs were placed in water of 23°C (8.7 mg/L oxygenated, 12-h photoperiod, water hardness level of 32 mg/L Ca^{2+} and $24 \text{ mg/L CO}_3^{2-}$) at pH 7.6 or pH 6.3 (controls) and exposed to environmentally realistic Cu^{2+} concentrations (as $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$) of 0.3 or 0.8 $\mu\text{mol/L}$ (experimental groups). Water pH of the experimental groups was adjusted to pH 7.6 or pH 6.3 via gradual addition of 0.01 M sodium hydroxide or 0.01 M sulfuric acid, using pH-stat equipment (variation $< \text{pH } 0.1$). Constant Cu concentrations were maintained via a flow-through system with partial replacement of the water, resulting in a complete turnover of the water in 24 h. Actual total Cu concentrations were within 5% of the calculated values as verified regularly using atomic absorption spectrophotometric analysis.

* To whom correspondence may be addressed.

Table 1. Mortality of eggs and larvae, larval deformation, heart rate, and tail movements during exposure to Cu at pH 7.6

[Cu] ($\mu\text{mol/L}$)	Egg mortality ^a	Larval mortality ^a	Larval deformation ^a	Heart rate ^b	Tail movements ^b
n.d. ^c	11 \pm 5	1 \pm 1	2 \pm 2	121 \pm 9	21 \pm 7
0.3	11 \pm 4	1 \pm 1	2 \pm 2	123 \pm 14	20 \pm 6
0.8	13 \pm 3	15 \pm 4 ^d	43 \pm 6 ^d	124 \pm 10	21 \pm 4

^a Mortality and deformation are expressed as percentage of total number of embryos and larvae, means \pm SE ($n = 7$) are given.

^b Heart rate and tail movements are given in beats/min, means \pm SE are given ($n = 7$).

^c n.d. = Nondetectable; less than 1.10^{-6} $\mu\text{mol/L}$.

^d Significant at the level of $p < 0.001$ compared to control values.

Table 2. Mortality of eggs and larvae, larval deformation, heart rate, and tail movements during exposure to Cu at pH 6.3

[Cu] ($\mu\text{mol/L}$)	Egg mortality ^a	Larval mortality ^a	Larval deformation ^a	Heart rate ^b	Tail movements ^b
n.d. ^c	11 \pm 4	1 \pm 1	2 \pm 1	120 \pm 11	15 \pm 6
0.3	12 \pm 5	48 \pm 8 ^d	41 \pm 6 ^d	115 \pm 5	17 \pm 7
0.8	37 \pm 7 ^d	82 \pm 5 ^d	77 \pm 13 ^d	101 \pm 12 ^c	4 \pm 4 ^e

^a Mortality and deformation are expressed as percentage of total number of embryos and larvae, means \pm SE ($n = 7$) are given.

^b Heart rate and tail movements are given in beats/min, means \pm SE are given ($n = 7$).

^c n.d. = Nondetectable; less than 1.10^{-6} $\mu\text{mol/L}$.

^d Significant at the level of $p < 0.001$ compared to control values.

^e Significant at the level of $p \leq 0.01$ compared to control values.

Mortality and deformation

Dead eggs and larvae were counted at 6, 24, 48, 76, 96, 120, 144, and 168 h after fertilization and immediately removed to prevent fungal growth. Eggs were considered dead when parts of the content turned opaque and white, or when heart beat had stopped. For the 0.8 $\mu\text{mol/L}$ Cu group at pH 6.3 tail movements had to be used to determine egg mortality. The percentage of deformation of the larval spinal cord (including dead ones) was determined after microscopic examination. Deformed larvae were unable to swim.

Heart rate and tail movements

Fifty hours after fertilization, heart rate and rate of tail movements (both in beats/min) were examined for 20 embryos per genetic batch.

Mineral content and copper accumulation

To determine their mineral content, 10 eggs or larvae from each group were collected at 6, 24, 48, 76, 96, 120, 144, and 168 h after fertilization. The eggs and larvae were freeze-dried

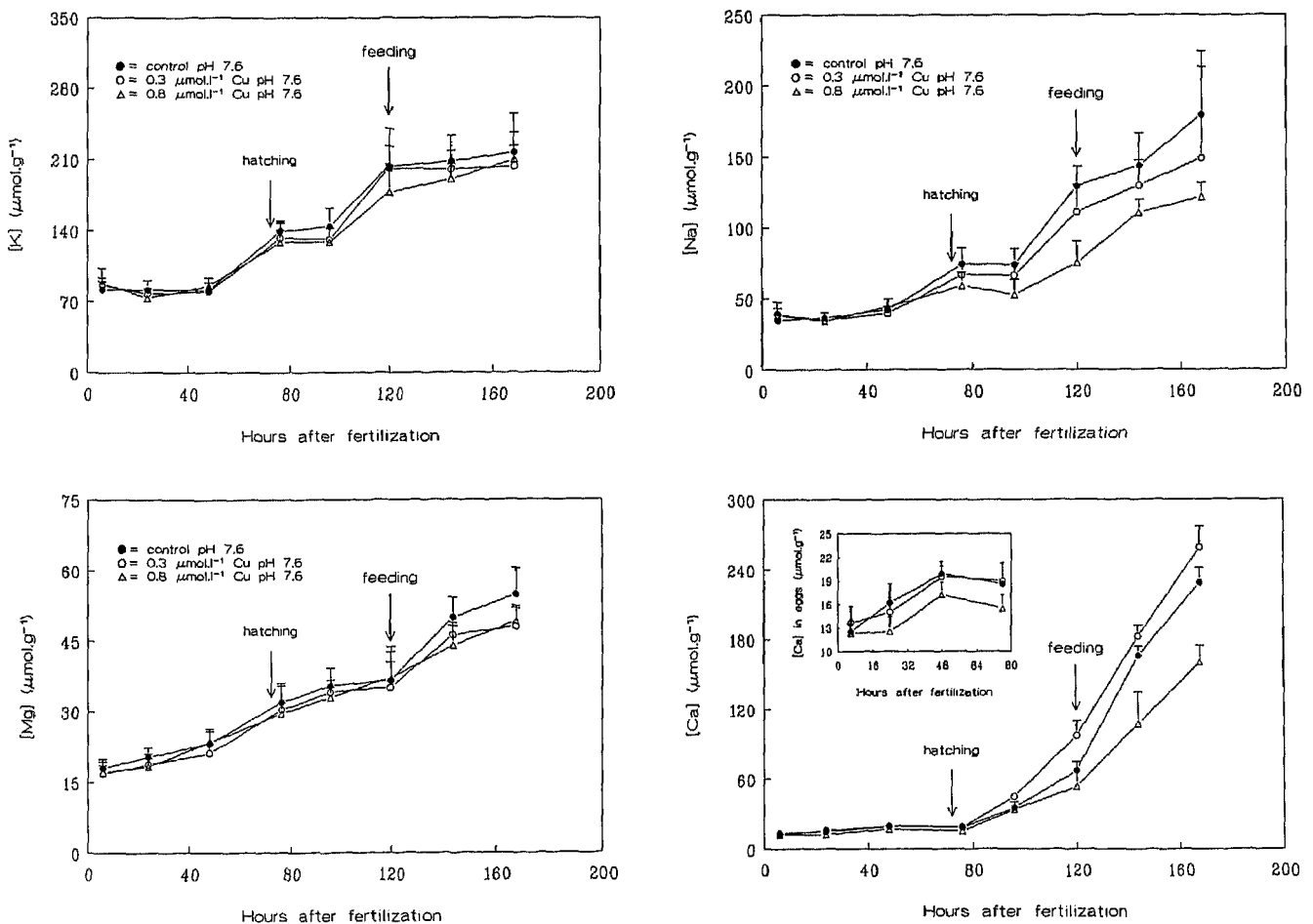


Fig. 1. Whole-body K, Na, Mg, and Ca concentrations ($\mu\text{mol/g}$ dry weight) of carp eggs and larvae exposed to Cu at pH 7.6. Data are expressed as means \pm SE ($n = 7$). Closed circle (\bullet) represents control, open circle (\circ) exposure to 0.3 $\mu\text{mol/L}$ Cu, and triangle (Δ) exposure to 0.8 $\mu\text{mol/L}$ Cu. Inset on the graph for Ca shows the first 80 h, plotted at a larger scale.

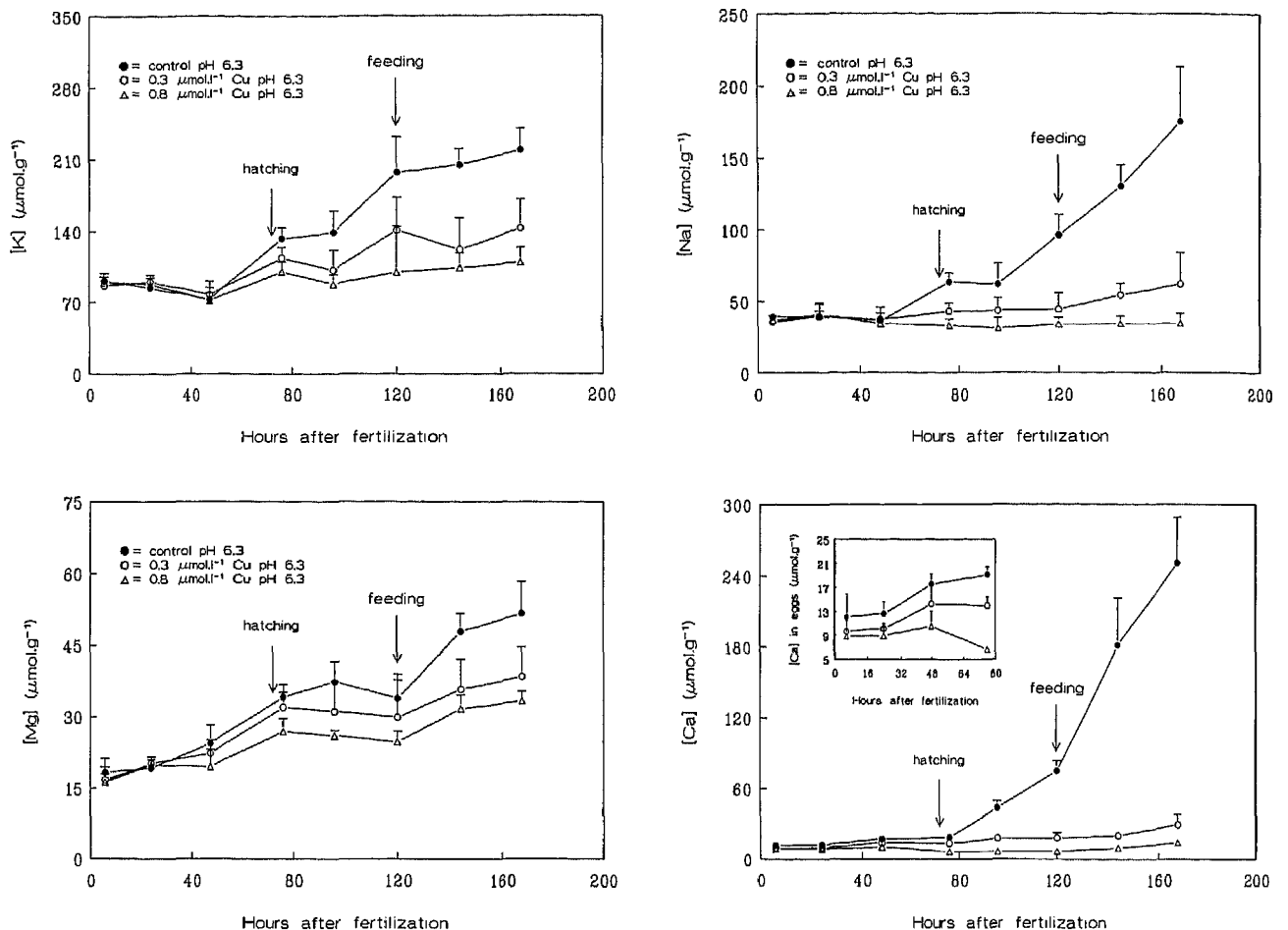


Fig. 2. Whole-body K, Na, Mg, and Ca concentrations ($\mu\text{mol/g}$ dry weight) of carp eggs and larvae exposed to Cu at pH 6.3. Data are expressed as means \pm SE ($n = 7$). Closed circle (\bullet) represents control, open circle (O) exposure to 0.3 $\mu\text{mol/L}$ Cu, and triangle (Δ) exposure to 0.8 $\mu\text{mol/L}$ Cu. Inset on the graph for Ca shows the first 80 h, plotted at a larger scale.

to constant (dry) weight and total K, Na, Mg, Ca, and Cu content were measured. Tissues were dissolved for 24 h at 70°C in 65% HNO_3 . The K and Na concentrations were analyzed in a flame photometric auto analyzer (Technicon); Mg and Ca were determined with inductively coupled plasma atomic emission spectrometry (Plasma IL200, Thermo Jarrell Ash); Cu was measured with atomic absorption spectrophotometry (AAS). All concentrations are expressed as $\mu\text{mol/g}$ dry weight. Feeding with brine shrimp larvae (*Artemia franciscana*) was started at 120 h.

Hatching success

Hatching success was defined as the percentage of larvae that had hatched every hour, during 54 h until 80 h after fertilization. Hatching was defined as rupture of the chorion by the tail. Fully, as well as partially hatched larvae were included.

Swim bladder experiment

Pilot experiments showed that carp larvae exposed to 0.3 and 0.8 $\mu\text{mol/L}$ Cu at water pH 6.3 had no functional swim bladder. A parallel experiment therefore was designed to determine whether this organ was indeed missing or merely devoid of air. To this end, a batch of 20 fertilized eggs of each group was put on a coverslip and placed in a plastic tube submerged in the different aquaria. The top end of each tube was covered with a nylon mesh to prevent the larvae from reaching the

surface. Tubes without mesh were used as controls. The bottom end was closed off. After the control larvae had filled their swim bladders, larvae in the tubes were taken out for examination, and were then allowed to reach the surface.

Histology

At 48 h and at 168 h after fertilization, eggs and larvae were collected for examination under a light microscope. After fixation in Bouin's solution, dehydration, and embedding in paraffin, sections were cut at 5- μm thickness, stained with a trichrome solution (using Alcian Blue, Nuclear Red, phosphotungstic acid, aniline blue, and Orange G), and sealed with Entellan.

Statistical analysis

Data are expressed as means \pm SE ($n = 7$). Significant differences were tested using the unpaired Student's *t*-test. Significance was accepted for $p \leq 0.01$. Significance is expressed at the level of $p \leq 0.01$ or $p < 0.001$ compared to control values.

RESULTS

Mortality and deformation

No significant difference in egg mortality was found between the groups exposed to Cu at pH 7.6 and the controls (Table 1).

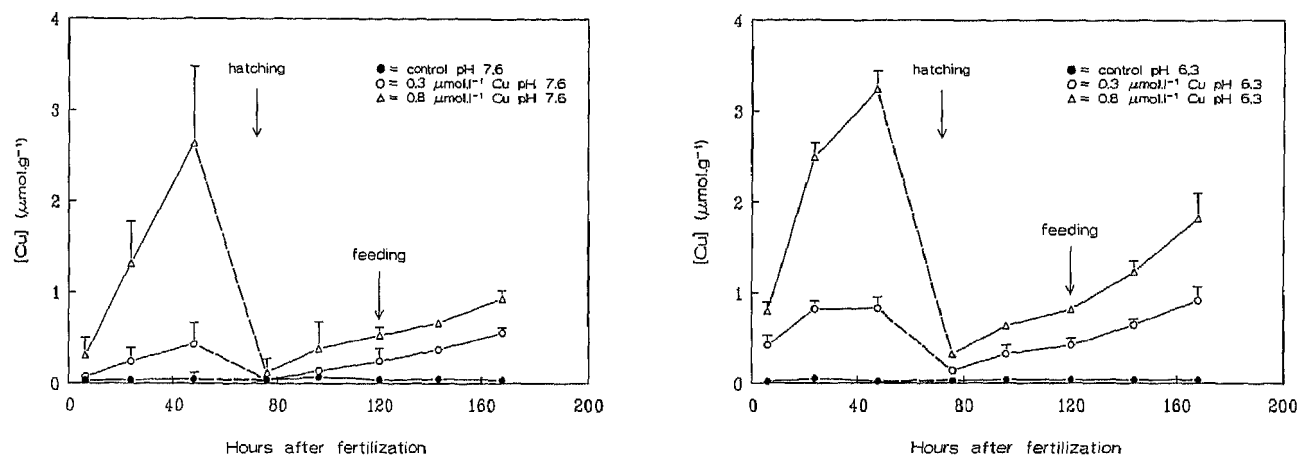


Fig. 3. Accumulation of Cu by carp eggs and larvae exposed to Cu at pH 7.6 and pH 6.3. Data are expressed as means \pm SE ($n = 7$).

However, at pH 6.3 a significantly ($p < 0.001$) increased egg mortality was observed during exposure to 0.8 $\mu\text{mol/L}$ Cu (Table 2). These embryos already showed deformation of the head and spinal cord. In this group almost all of the eggs turned opaque, but most of them remained alive as indicated by tail movements of the embryos. Many of them also had opaque yolk sacs. This effect was already seen, although much less severe, in the 0.3- $\mu\text{mol/L}$ -Cu group at pH 6.3. In controls, and Cu-exposed groups at pH 7.6, the perivitelline fluid (pvf; between embryo and chorion) was clear. At pH 7.6, exposure to 0.8 $\mu\text{mol/L}$ Cu significantly (both $p < 0.001$) increased larval mortality and larval deformation, whereas at pH 6.3 these parameters increased rapidly in a concentration-related manner (Table 2).

At pH 6.3, a large number of the 0.8- $\mu\text{mol/L}$ -Cu larvae displayed swollen, opaque yolk sacs, and other larvae turned opaque white in the head region. Apart from the obvious spinal cord deformations, these larvae also had deformed heads. Compared to controls, the upper jaw was grossly underdeveloped. The swimming pattern of the 0.8- $\mu\text{mol/L}$ -Cu group at pH 7.6 and of the 0.3- $\mu\text{mol/L}$ -Cu group at pH 6.3 deviated from the controls. The larvae of the 0.8- $\mu\text{mol/L}$ -Cu group at pH 6.3 did not swim at all, but lay on the bottom, displaying uncoordinated spurts or frantically turning circles.

Heart rate and tail movements

At pH 7.6, Cu exposure did not increase heart rate and tail movements compared to the control (Table 1) whereas at pH 6.3 exposure to 0.8 $\mu\text{mol/L}$ Cu significantly ($p < 0.01$) decreased heart rate and tail movements (Table 2). These embryos often showed a tetanuslike movement, which continued after hatching.

Mineral content

At pH 7.6 (Fig. 1) no effect of Cu was observed on whole-body K content of either eggs or larvae. Whole-body Na content of larvae exposed to 0.8 $\mu\text{mol/L}$ Cu decreased significantly ($p < 0.01$) after 96 h compared to controls. Whole-body Mg content was not affected in eggs and larvae exposed to 0.3 and 0.8 $\mu\text{mol/L}$ Cu. In the egg stage, whole-body Ca content of the 0.8- $\mu\text{mol/L}$ -Cu group decreased significantly ($p < 0.01$) after 24 to 48 h compared to controls. The larval stage showed a significant ($p < 0.01$) increase after 72 h in whole-body Ca for the 0.3- $\mu\text{mol/L}$ -Cu group as compared to controls, whereas the

0.8- $\mu\text{mol/L}$ -Cu group demonstrated a significant ($p < 0.01$) decrease after 144 h compared to controls.

At pH 6.3 (Fig. 2) after hatching, whole-body K and Na content of larvae exposed to 0.3 and 0.8 $\mu\text{mol/L}$ Cu decreased significantly ($p < 0.01$) after 72 h compared to controls. After 144 h, whole-body Mg content of the 0.3- $\mu\text{mol/L}$ -Cu-exposed larvae was significantly ($p < 0.01$) decreased compared to controls. Exposure to 0.8 $\mu\text{mol/L}$ Cu, however, decreased whole-body Mg content of larvae significantly ($p < 0.01$) after 72 h. In both the 0.3 and 0.8- $\mu\text{mol/L}$ -Cu-exposed groups whole-body Ca contents of eggs and larvae significantly ($p < 0.01$) decreased after 24 h compared to controls.

Copper accumulation

At pH 7.6, 98% of the Cu was adsorbed to the chorion. The remaining amount was found in the embryo, whereas at pH 6.3 more than 8% was found in the embryo. Immediately after hatching, larvae started to accumulate Cu in a concentration-related manner. At pH 6.3, Cu accumulation during the larval stage reached a level twice the observed at pH 7.6 (Fig. 3).

Hatching success

For eggs incubated at pH 7.6 and 0.3 $\mu\text{mol/L}$ Cu hatching success increased significantly ($p < 0.01$) after 64 h compared to controls (Fig. 4). At pH 6.3 and 0.8 $\mu\text{mol/L}$ Cu, significant ($p < 0.01$) premature hatching was also observed after 54 h (Fig. 4). In addition, the 0.3- $\mu\text{mol/L}$ - and 0.8- $\mu\text{mol/L}$ -Cu-exposed groups at pH 6.3 showed a significant ($p < 0.01$) decrease in hatching success after 75 h, compared to controls (Fig. 4).

Swim bladder

Through macroscopic observation of the larvae during the experiment, no swim bladder could be detected in larvae of both Cu groups at pH 6.3. In controls, the swim bladder was seen as a silvery organ, whereas in both Cu groups only a yellow patch was observed. Larvae of the 0.3- $\mu\text{mol/L}$ -Cu group were still able to float and swim. At pH 7.6, the 0.8- $\mu\text{mol/L}$ -Cu group appeared to be a mixture of fish with normal swim bladders and other with small swim bladders. When the larvae were prevented from reaching the surface, none of the larvae displayed a filled swim bladder. During the experiment all larvae were constantly swimming up, followed by passive sinking. Fifteen minutes after being allowed to reach the surface, all of

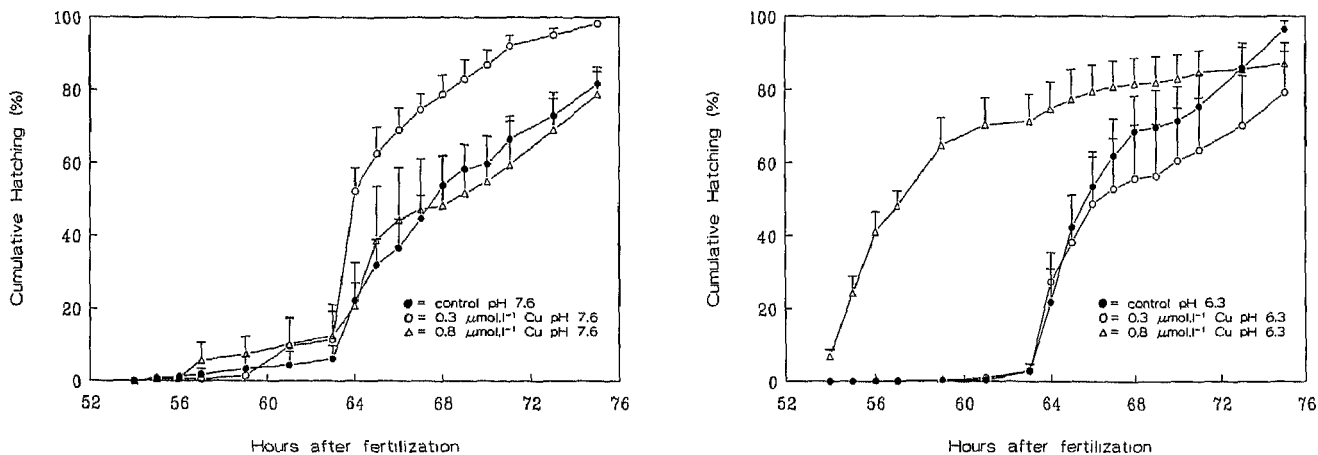


Fig. 4. Cumulative hatching success of carp eggs exposed to Cu at pH 7.6 and pH 6.3. Data are expressed as means \pm SE ($n = 7$).

the larvae showed a filled swim bladder, except for the Cu-exposed groups at pH 6.3.

Histology

Histological examination showed that the eggs exposed to 0.8 $\mu\text{mol/L}$ Cu at pH 6.3 had coagulated particles inside the chorion. Also, the yolk sac of most of these embryos displayed coagulation. In the other groups the pvf was clear (Fig. 5A and B). In the 0.8- $\mu\text{mol/L}$ -Cu group at pH 7.6 and the 0.3- and 0.8- $\mu\text{mol/L}$ -Cu group at pH 6.3 the yolk sac of the larvae had not been absorbed. Furthermore, other organs (e.g., intestine and liver) looked atrophied and the swim bladder lumen in these three groups was much smaller than in controls. The epithelium of the swim bladder lumen was present, but appeared as a compact mass showing large cells, possibly macrophages (Fig. 6A to D).

pH controls

In the controls of pH 6.3 no significant changes of any of the studied parameters were observed when compared to the controls of pH 7.6.

DISCUSSION

Our results clearly show that a decreased water pH leads to increased Cu toxicity during the early life stages of the carp. This enhanced toxicity could be explained by the increased

concentration of Cu^{2+} at lower water pH. Indeed, at pH 7.6, 32% of the total Cu is present as Cu^{2+} , and this amount increases to 92% at pH 6.3 [18]. This implies that the total amount of bioavailable Cu^{2+} is virtually the same to both the larvae of the 0.8- $\mu\text{mol/L}$ -Cu group at pH 7.6 and to the larvae of the 0.3- $\mu\text{mol/L}$ -Cu group at pH 6.3, that is, 0.26 $\mu\text{mol/L}$ Cu^{2+} (32% of 0.8 $\mu\text{mol/L}$ at pH 7.6) and 0.28 $\mu\text{mol/L}$ (92% of 0.3 $\mu\text{mol/L}$ at pH 6.3). Indeed, the total Cu concentration in larvae exposed to 0.8 $\mu\text{mol/L}$ at pH 7.6 and larvae exposed to 0.3 $\mu\text{mol/L}$ at pH 6.3 are approximately equal. This result supports the general view that Cu forms such as Cu-carbonates and Cu-hydroxides (pH > 7), although potentially toxic, are hardly available for uptake by fish [18,19,22].

At pH 6.3, coagulation of the perivitelline proteins occurred only at 0.8 $\mu\text{mol/L}$ Cu, leading to high mortality of the eggs. This coagulation may be explained by the interaction of Cu with SH groups, denaturing the perivitelline proteins. Embryonic deformation was already observed at 0.8 $\mu\text{mol/L}$ Cu (pH 7.6) and at 0.3 and 0.8 $\mu\text{mol/L}$ Cu (pH 6.3). Cu significantly decreased Ca contents of Cu-exposed eggs at both pH 7.6 and 6.3 and, to a lesser extent, Mg contents (only in eggs exposed to 0.8 $\mu\text{mol/L}$ Cu at pH 6.3). It should be stressed that Cu exposure started immediately after fertilization, thus before hardening of the chorion, enabling Cu^{2+} to pass easily through this membrane. After hardening, the chorion acts as a cation exchanger, preventing metal ions with high binding affinities such as Cu^{2+} , Hg^{2+} , and

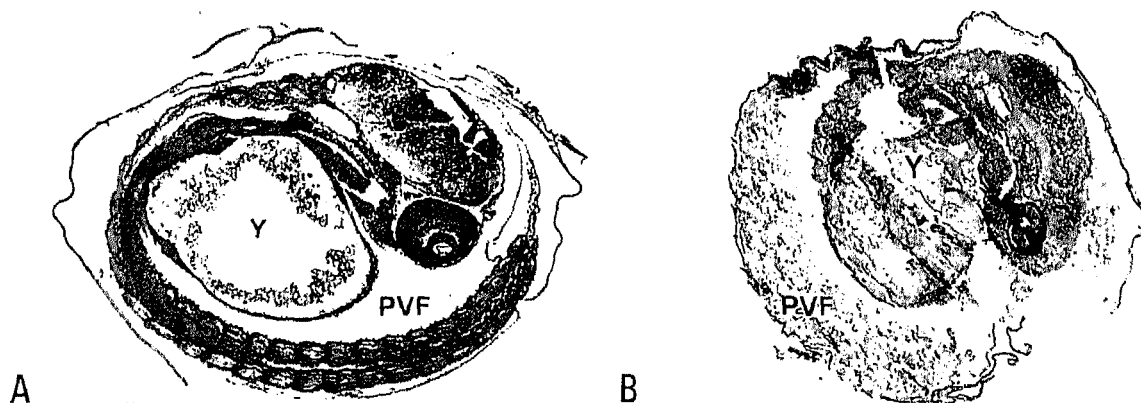


Fig. 5. (A) Cross section through control embryo (48 h after fertilization) raised at water pH 6.3 showing a translucent perivitelline fluid ($\times 40$). (B) Cross section through embryo (48 h after fertilization) exposed to 0.8 $\mu\text{mol/g}$ Cu at water pH 6.3 showing an opaque perivitelline fluid ($\times 40$). PVF = perivitelline fluid, Y = yolk sac.

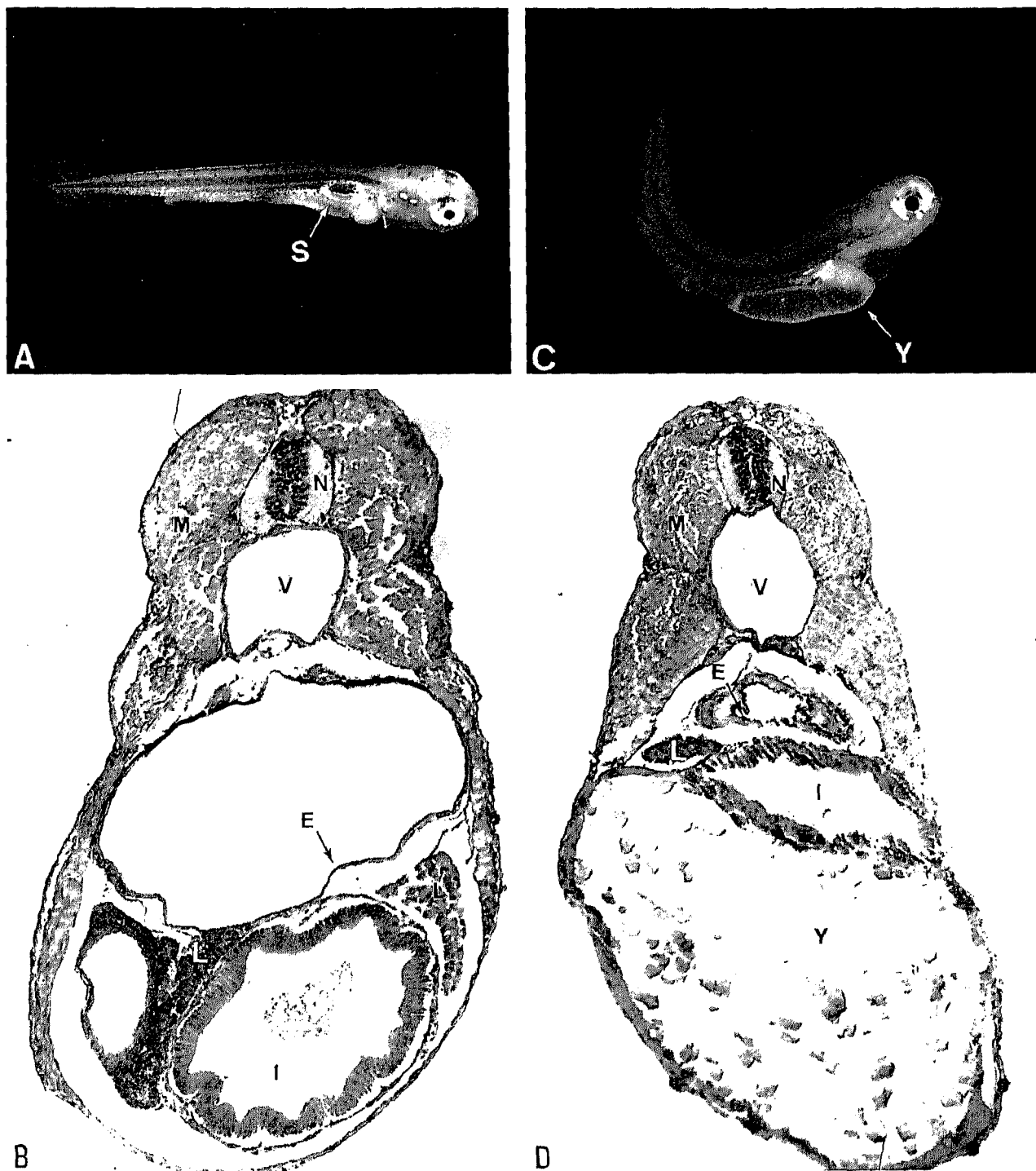


Fig. 6. (A) Lateral view ($\times 8$) and (B) cross section ($\times 100$) of a control larva (168 h after fertilization) raised at water pH 6.3 showing a prominent swim bladder and completely absorbed yolk sac. (C) Lateral view ($\times 8$) and (D) cross section ($\times 100$) of a larva (168 h after fertilization) exposed to $0.3 \mu\text{mol/g}$ Cu at water pH 6.3 showing a grossly reduced swim bladder and a nonabsorbed yolk sac. E = swim bladder epithelium; I = intestine; L = liver; M = muscles; N = neural tube; S = swim bladder; V = vertebra, and Y = yolk sac.

Ag^{2+} from entering the surrounding pvf, whereas metals with low binding affinities (Zn^{2+} , Pb^{2+} , and Cd^{2+}) easily pass through the chorion and accumulate in the pvf according to the Donnan equilibrium [23,24]. In our experiment, Cu was located mainly in the chorion (98% at pH 7.6 and 92% at pH 6.3), and only a small amount of Cu was able to pass this barrier. This accumulation of Cu could change the selective permeability of the chorion, leading to an impaired cation exchange capacity between the pvf and the ambient water. The pvf contains a neg-

atively charged colloid [25], which attracts cations from the ambient water and maintains a potential difference between pvf and the ambient water, the so-called perivitelline potential (pvp). The exchange of cations such as Mg^{2+} and Ca^{2+} between the water and the pvf is crucial for normal embryonic development [26]. For example, when the pvp is affected by low pH or by a metal such as Cd^{2+} , the ability of the pvf to concentrate Ca^{2+} is reduced as predicted by changes in the magnitude of the pvp [27]. As a consequence, the passive diffusion increases. Simi-

larly, the presence of Cu in the chorion increases the probability of Cu to enter the pvf by either passive diffusion or as a result of exchange with other cations.

Copper exposure induced premature hatching at both pH 7.6 and pH 6.3, despite the decreased heart rate and tail movements of embryos exposed to 0.8 $\mu\text{mol/L}$ at pH 6.3. Heavy metals such as Cd and Zn are also known to induce premature hatching and this has been ascribed to softening of the chorion [28,29]. To our knowledge nothing is known about the effect of Cu on chorionic strength.

After hatching, Na and Ca are mainly taken up from the ambient water by the chloride cells, located on the yolk sac epithelium, skin and in the developing gill filaments [30]. In contrast, K and Mg are mainly taken up from food [31]. Our observation that only the whole-body Na and Ca concentrations are decreased in Cu-exposed larvae supports the notion that Cu interferes with the branchial uptake of these ions. The branchial chloride cells have been reported to be the main exchange site for Na and Ca [32]. The intimate contact of fish gills with water makes them very vulnerable to heavy metals, including Cu [5,6]. Indeed, Cu exposure has been shown to reduce plasma Na^+ and Ca^{2+} in rainbow trout (*Oncorhynchus mykiss*) [7,8,10] and decreases the whole body content of these ions in developing brown trout (*Salmo trutta* L.) [33]. During Cu exposure, Na^+ uptake in developing rainbow trout was strongly inhibited, probably because of a decreasing Na^+/K^+ -ATPase activity [6]. Freshwater fish gills have a tight epithelium, and transcellular influx of ions dominates over paracellular efflux. Movement of Ca^{2+} and Na^+ into the chloride cell is passive, down their electrochemical gradients over the apical membrane, and appears to be regulated through channel or carrier proteins [34]. After inhibition of Na^+/K^+ -ATPase in the basolateral membrane, Na^+ transfer to the blood will be decreased [35]. Na^+/K^+ -ATPase may also indirectly support Ca^{2+} uptake via maintenance of a transmembrane Na^+ electrochemical gradient. This in turn appears to be the driving force for the basolateral $\text{Na}^+/\text{Ca}^{2+}$ -exchanger [36,37], which may be affected by the drop in plasma Na^+ level. This means that this route of Ca^{2+} transport to the blood will also be inhibited by Cu. However, Cu exposure at pH 6.3 decreased whole-body K, Na, Mg, and Ca. Because whole-body K and Mg levels were already lowered before feeding, this observed decrease can not be explained by insufficient uptake of food. Therefore, it is more likely that this decrease is the result of enhanced branchial efflux of K, Na, Mg, and Ca. The lower water pH is considered to be the main cause of this leakage, by affecting the tight junctions between the branchial epithelial cells (paracellular route) resulting in increasing permeability [37].

Yolk sac absorption was also inhibited by Cu, particularly at pH 6.3. The rate of yolk absorption may have been affected by the metal via reduction of the metabolic activity of the yolk syncytium.

The failure of the larvae to fill their swim bladder during Cu exposure at pH 6.3 made them unable to maintain their position in the water. The larvae of many species fill the swim bladder soon after hatching, or at the end of yolk resorption, probably by swallowing air at the surface [38]. However, our results show that when Cu-exposed larvae were allowed to reach the surface, they were still unable to fill their swim bladder. One possibility to explain this phenomenon is that due to the underdeveloped upper jaw syndrome, larvae are unable to swallow air. Another possibility is that the gas exchange systems for fine regulation of buoyancy is affected by Cu. This could

explain the partial filling of several swim bladders in the 0.8- $\mu\text{mol/L}$ -Cu group at pH 7.6. Resorption and secretion of O_2 occur in specialized regions of the swim bladder as a result of differences in the partial pressures of O_2 between this organ and the blood. How Cu interferes with this process is still unknown and remains to be investigated.

The Dutch water quality standard for cyprinid species is ≤ 0.5 $\mu\text{mol/L}$ Cu. From the observed sublethal effects in the 0.3- $\mu\text{mol/L}$ -Cu group at pH 6.3 in our laboratory, it is feasible that the carp population under these conditions could also be affected in natural waters. Our results indicate that early life stages of fish prove to be a very sensitive bioassay for aquatic pollutants such as copper.

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