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Heavy metal effects on P, Ca, Mg, and total protein contents in embryonic pleopodal eggs and stage-1 juveniles of freshwater crayfish *Astacus leptodactylus* (Eschscholtz, 1823)

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Abstract: To determine the potential biochemical effects of heavy metal pollution on aquatic organisms, pleopodal eggs, and stage-1 juveniles of the freshwater crayfish, *Astacus leptodactylus* (Eschscholtz, 1823), was exposed to a non-essential (Cd) and an essential (Cu) metal, either singly or in combination. Three different sublethal doses (0.1, 0.5, and 1 ppm) and 1 interaction dose (0.1 ppm Cu + 0.1 ppm Cd) were used to evaluate the accumulation and toxicity. The effects of 2 doses of metals on P, Ca, total protein, and Mg contents in the embryonic pleopodal eggs and in stage-1 juveniles were examined by an autoanalyzer. The same analyses were also performed on recently spawned eggs through the transition to the first juvenile stage (3 weeks). Ten eggs or stage-1 juveniles were sampled during the 3-week period to measure the bioaccumulation of metals. Cd and Cu levels in the eggs and recently spawned eggs were determined by flame atomic absorption spectrophotometer (FAAS). The results showed that both Cd and Cu accumulated in eggs and in stage-1 juveniles. The results also indicated that metal interactions also occurred, which in this case were probably antagonistic. A comparison of the effects of Cd alone with those of Cd+Cu in combination showed that the mixture was more toxic than Cd alone, but not more toxic than Cu alone. Cd also affected the Ca level in the eggs. The level of Mg increased, while the levels of P, Ca, and total protein decreased in pleopodal eggs exposed to different doses of Cu and Cd. All biochemical parameters and protein levels measured were affected by both metals.

Key words: Crayfish, Cd, Cu, egg, stage-1 juveniles, P, Mg, Ca

Kerevit *Astacus leptodactylus* (Eschscholtz, 1823) embryonik pleopodal yumurta ve stage–1 juvenillerindeki P, Ca, Mg ve toplam protein miktarı üzerine ağır metallerin etkisi

Özet: Araştırmada, iki ağır metale (gerekli olan (Cu) ve biyolojik yapıda bulunmayan (Cd)) maruz bırakılan kerevit (*Astacus leptodactylus* (Eschscholtz, 1823) plepodal yumurta ve stage–1 junevillerin biyokimyasal değişimlerinin belirlenmesi amaçlanmıştır. Çalışma, farklı üç sublethal doz (0,1, 0,5, 1 ppm) ve bir etkileşim dozunda gerçekleştirmiştir. Farklı ağır metal dozlarına maruz bırakılan plepodal yumurta ve stage–1 junevillerin P, Ca, Mg ve toplam protein miktarı otoanalizatörde ölçülmüştür. Analizler hem embriyonik plepodal yumurta hem de stage–1 junevillerde (üçüncü haftada) yapılmıştır. On adet plepodal yumurta ya da stage–1 junevil 3 hafta boyunca, haftalık olarak ağır metal miktarları belirlenmiştir. Yumurta ve yeni çıkmış stage–1 junevilde Cd ve Cu miktarları alev atomik absorpsiyon spektrofotometresinde (FAAS) ile belirlenmiştir. Sonuçlar, her iki ağır metalin de (Cd, Cu) yumurta ve yeni çıkmış stage–1 junevilde biriktiğini göstermektedir. Aynı zamanda Cu ile Cd arasında antagonistik bir ilişki olduğu düşülmektedir. Cd+Cu etkisi, tek başına Cd ile karşılaştırıldığında, karışım halinde (Cd+Cu) daha toksik olduğu buna karşın Cu ile kıyasla daha toksik olmadığı belirlenmiştir. Farklı Cd dozlarında, kadmiyumun yumurtadaki Ca seviyesini etkilediği saptanmıştır. Mg miktarının plepodal yumurta ya da stage–1 junevil süreye bağlı olarak artış gösterirken, P, Ca, ve toplam protein miktarında azalma belirlenmiştir. Tüm biyokimyasal parametreler ve toplam protein miktarı her iki ağır metal miktarına göre değişmektedir.

Anahtar sözcükler: Kerevit, Cd, Cu, yumurta, stage–1 juvenil, P, Mg, Ca

Introduction

Heavy metals, such as copper (Cu) and cadmium (Cd), enter aquatic ecosystems from a number of different sources including municipal wastewater, liquid industrial waste discharges, mining operations, and river runoff. These metals can cause adverse effects on aquatic organisms if significant concentrations of these chemicals come into contact with the biological membranes of the organisms. At higher levels of biological organization (tissue, organ, and whole organism), heavy metals induce changes in metabolism, biochemistry, and physiology, which are often expressed through inhibition of protein synthesis (1,2).

Non-ferrous metal mines represent a major source of release of Cd, a non-essential, extremely toxic trace metal (3), to aquatic environments. Normally found at low (i.e. parts per billion) concentrations in rivers, lakes, and ponds, Cd is readily accumulated by many aquatic organisms, including crayfish (*Procambarus clarkii*) (4), *Palaemon elegans* (Decapoda), *Echinogammarus pirloti* (Malacostraca), and *Eliminius modestus* (Cirripedia) (5). Cd interferes with the calcium metabolism of animals, causing hypocalcaemia in fishes (6). Cd is among the most toxic of all metals in the aquatic environment, possesses no known biological role and exhibits high toxicity if allowed to accumulate in metabolically-active tissues (7).

Cu, in contrast, is essential for several aquatic species, including *Astacus leptodactylus* and *Oncorhynchus mykiss* (8). Crustaceans accumulate Cu in their bodies in direct proportion to the increase in its bioavailability from water and food-chains (9). Decapod crustaceans are able to metabolically regulate tissue levels of Cu below a threshold level (5-10).

It is well known that Cd and Cu have negative effects on aquatic organisms, not only on adults but also on eggs and juveniles (11,12). Studies by various authors have shown adverse effects of Cd, especially on hatchability (13-15). Cd is one of the metals most commonly occurring in polluted natural waters and has been implicated in disruption of early stages of fish development (16,17) and quality of larvae (11). Unfortunately, in crayfish biology, we know less about the comparable effects of metals, e.g. Cd and Cu, on egg and juvenile development, particularly in terms of concentrations and exposure times.

The narrow-clawed crayfish, *Astacus leptodactylus* (Eschscholtz, 1823), an economically important species, has been extensively studied as a bioindicator of effects of pollutants in aquatic environments. It is an important crayfish species in Europe due to its aquaculture potential and its wide consumer demand. In the past, Turkey was the main supplier of *A. leptodactylus* for export to Western Europe, with exports between 1971 and 1985 ranging from 1300 to 2500 t. However, after 1985, crayfish production was dramatically reduced in most Turkish lakes; the presence of the crayfish plaque fungus (*Aphanomyces astaci*) was reported as the causative agent (18-20).

Astacid crayfish reproduce only once a year, have low fecundity, and a long embryonic development (6–9 months) in natural conditions (13-15). Much literature exists concerning the effects of heavy metals on this crayfish species, including studies of toxicity and accumulation (3,17,21-24). These metals have been reported to cause deleterious effects including impairment of reproduction, disruption of molting, failures in limb regeneration, alteration of blood glucose levels, and color changes (11). The potential for embryonic and juvenile stage toxicity also exists, but this has been poorly studied. Therefore, due to the common and widespread distribution of *A. leptodactylus* in Turkey, and also a similar pattern for potential heavy metal pollution, this study has focused on the effects of sublethal doses of Cd and Cu on *A. leptodactylus* eggs and stage-1 juveniles.

Materials and methods

Approximately 100 adult female *A. leptodactylus* specimens with pleopodal eggs were collected from DSI (The General Directorate of State Hydraulic Works) Dam Lake (N41°44′E27°22′) near Üsküp, Kırklareli, Turkey. They were transferred to 4 glass aquaria (50 × 50 × 100 cm, 100 L) in the aquarium room and maintained at 23 °C, 12:12 light: dark regime (21). This arrangement was left undisturbed for 2 weeks, during which time the animals became accustomed to the room conditions. They were fed once, on alternate days, throughout the experimental period with a diet of trout pellets, carrot, and potato (24).

Experimental design

Crayfish were separated into 8 groups of 6 female individuals with eggs. Each group was exposed to a different concentration of Cd and/or Cu in replicate in 100 L experimental glass aquaria. As a control, another group was kept in an aquarium filled with only filtered tap water. In order to avoid cannibalism, a number of short-length polyvinylchloride pipes (5 cm diameter) were placed at the bottom of the aquaria. Prior to the experiments, aquaria were filled with the respective solutions of Cd and/or Cu for 48 h for initial adsorption of Cd and Cu onto inner tank surfaces. After 48 h, this solution was discarded and experiments were initiated with fresh Cd and/or Cu solutions (24).

Metal salts used for preparation of stock solution were $CdSO_4.7H_2O$ (Merck) for Cd and $CuSO_4.7H_2O$ (Merck) for Cu. Stock solutions of 1000 mg/L were prepared by dissolving the Cd and Cu salt in tap water. The required volume of stock solution was added to the respective experimental aquaria to achieve the desired concentrations of 0.1, 0.5, and 1.0 mg/L. All aquaria were constantly aerated. Water parameters during the experiment remained constant as follows: dissolved oxygen 9.49 ± 3.73 mg/L, temperature 23 ± 1 °C, pH 8.06 ± 0.76, and conductivity 637.69 ± 33.45 μhos.

At the end of the accumulation exposure period (after 1, 2, and 3 weeks), 2 female crayfish with eggs were collected. By the end of the second week, all eggs were at stage 1.

Chemical analysis

For biochemical analysis (P, Ca, Mg, and total protein), 5 eggs were homogenized in 1.2% NaCl saline solution and centrifuged at 4000 rpm for 5 min. P, Ca, Mg, and total protein analyses were performed by a Cobas Integra 700 autoanalyzer (Roche Diagnostic, GmbH, Mannheim, Germany).

At the same time, for flame atomic absorption spectroscopy (AAS) analysis, 5 eggs were weighed and digested in a mixture of equal volumes (1 mL:1 mL) of concentrated nitric/perchloric acids (Merck) and heated at 80-100 °C until a clear yellow liquid was produced (approximately 3 h) (24,25). After digestion of the eggs, the residues were analyzed by AAS (Unicom 929 AA) using 4 standards. The standards used to make a calibration curve were 1, 3, 4, and 5 mg\L.

Statistical analysis

The Student-Newman-Keuls (SNK) test was used to compare Cd and Cu concentrations among replicates, among Cd and Cu treatments, and among eggs. As metal accumulation in replicate aquaria was not significantly different, samples were pooled and a two-way ANOVA analysis was performed, followed by a SNK test as a post hoc test. Groups were considered to be significantly different from each other if $P < 0.05$. All analyses were performed using SPSS (version 11) and MS Excel.

Results and discussion

Water parameters during the experiment remained constant: dissolved oxygen 9.49 ± 3.73 mg/L, temperature 23 ± 1 °C, pH 8.06 ± 0.76 and conductivity 637.69 ± 33.45 μhos. There was no mortality during the experimental period. Both Cd, a non-essential, extremely toxic trace element, and Cu, a trace element, were accumulated by *A. leptodactylus* eggs during the 3-week exposure period. Cd accumulations in the *A. leptodactylus* eggs were greater than Cu accumulations. During the 3 weeks of exposure, the maximum accumulation occurred at the third week at the maximum Cd concentration (1 ppm). Cd concentrations in the control group were below the detection limit. The maximum Cu accumulation was found in the second week at 0.5 ppm concentration, which means that, for both metals, the levels of metals in eggs of all exposure groups were significantly different from those of the control group ($P < 0.01$, Figure 1).

Rainbow and White (1993) previously investigated the accumulation strategies of the decapod for Cu and Cd (9). Decapods appeared to have concentrated Cu, but verification of regulation awaits separate identification of incoming and already accumulated metal. The decapods were net accumulators of the nonessential metal Cd. In fact, regulation as a metal accumulation strategy seems to be restricted to essential metals.

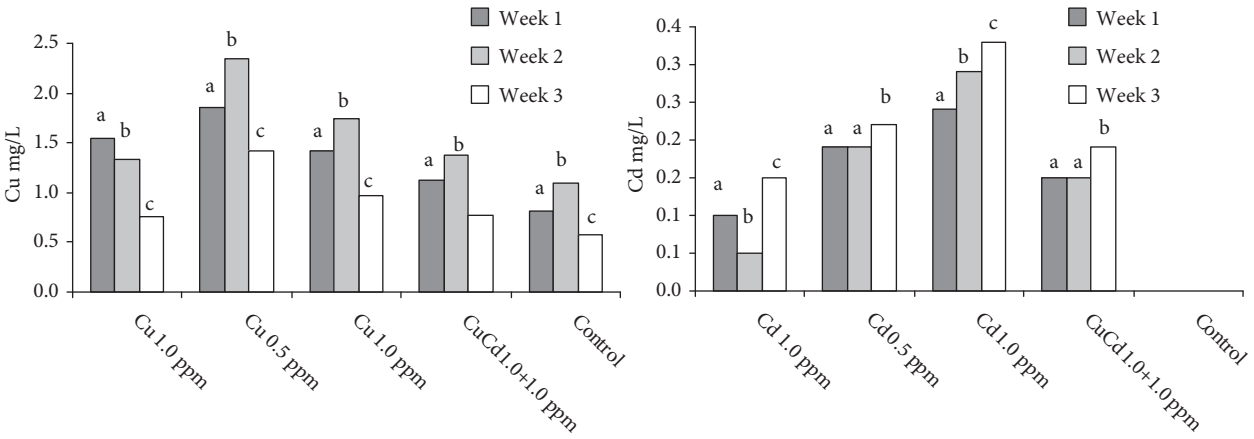

Figure 1. Accumulation of Cu and Cd at *A. leptodactylus* eggs over a 3 week exposure. Letters a, b, c letters indicate significant differences between weeks ($P < 0.01$).

Figure 2 and 3 and the Table present the results from all test groups of crayfish eggs, where different Cu and Cd concentrations were used for 3 weeks. During this time, the phosphorus concentration in the egg content decreased substantially, with increasing Cu and Cd concentrations in the water. The calcium concentration in the egg was increased at the third week in both Cu and Cd treated conditions; this was most likely because of spawning.

In the Cd test groups, Cd accumulation caused calcium hypocalcaemia (abnormally low Ca levels), probably by inhibiting calcium uptake from the water (see Ca results in Figure 2). On the other hand, the same concentration of Cu did not change the Ca content of eggs in the Cu test groups.

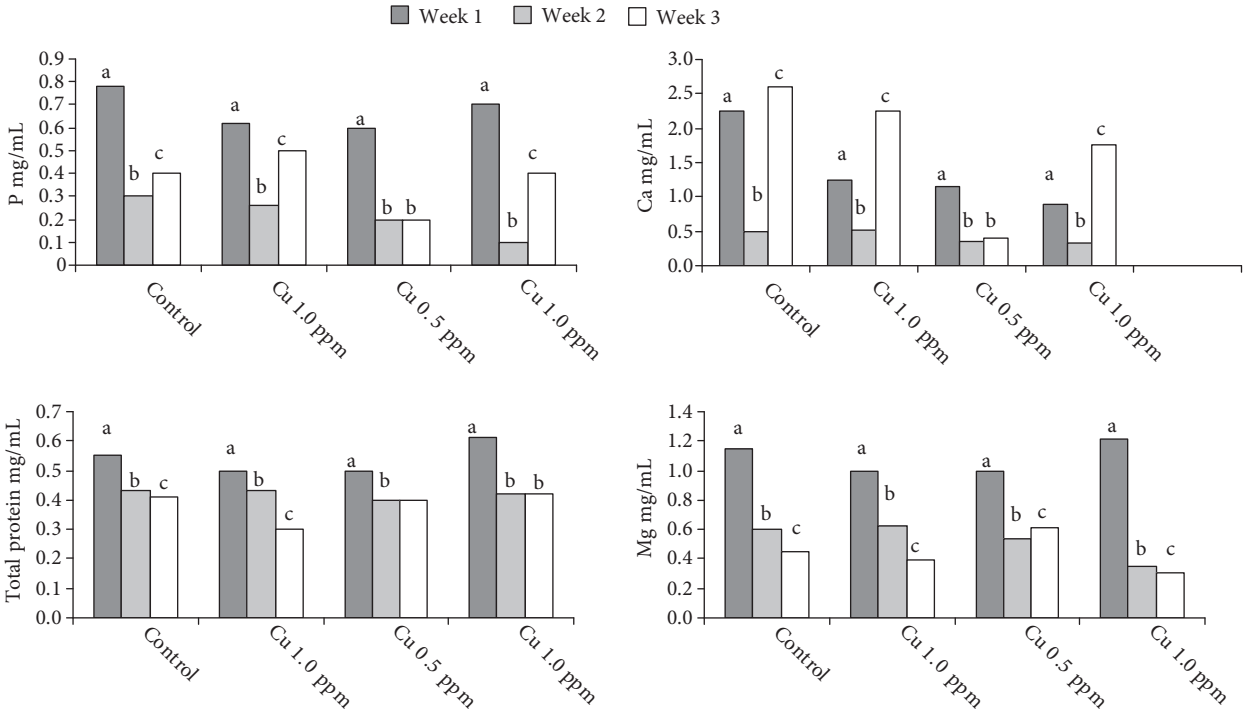

Figure 2. Effect of copper on the content of phosphorus, calcium, protein, and magnesium concentration in *A. leptodactylus* eggs. Letters a, b, c indicate differences between weeks ($P < 0.01$).

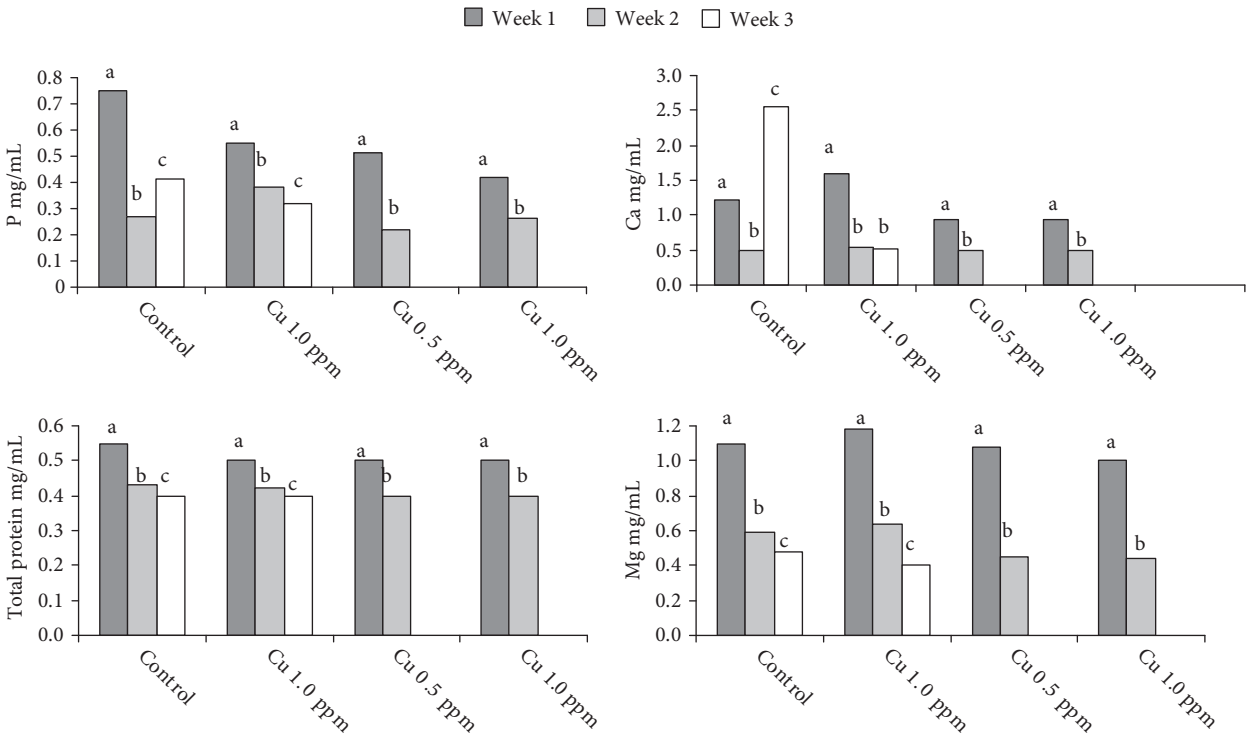

Figure 3. Effect of cadmium on the phosphorus, calcium, protein, and magnesium concentration in eggs of *A. leptodactylus*. Letters a, b, c letters indicate significant differences between weeks ($P < 0.01$).

Metallothioneins (MTs) can be induced by the essential metals Cu and Zn and by the non-essential metals, Cd, Ag, and Hg, in both vertebrates and invertebrates (25), but we noted no consistent increase in total protein content during the 2-week exposure.

There were no statistically significant treatment-dependent differences between Cu and Cd treatments in terms of Mg levels. There were no time effects within treatments (ANOVAs, $P > 0.01$), except some variability in the mean Mg values for egg data. Figures 2 and 3 also show no relationship between egg Mg content and either Cu or Cd concentrations in *A. leptodactylus*.

Differences in toxicity to Cd and Cu in embryonic development can be a result of differential uptake and accumulation of each metal into the egg. No data are available on Cu accumulation, which is an essential metal probably taken up into the embryo. According to many studies, most cadmium is accumulated into the egg outer shell (the chorion), such that only a small amount appears in the embryo (26,27). It is likely that Cd was accumulated by the chorion for the first 2 weeks of the experiment, but by the end of the second week all eggs were stage-1 juvenile and Cd accumulation was much higher.

No differences in the effect of Cd exposure (0.1 ppm) and co-exposure with less toxic Cu (0.1ppm Cd + 0.1 ppm Cu) on egg metal content were observed (ANOVAs, $P > 0.01$). These results indicate metal interactions, which in this case were probably antagonistic (Figures 1, 2, and 3).

Heavy metals can have effects on different aspects of the physiology of aquatic animals; for example, in oxygen consumption, water permeability, and osmoregulation. Considering the toxicity of these metals, and Cd in particular, it is important to be able to monitor their levels in the environment in an accurate and representative way (29,30). In *A. leptodactylus*, the embryos are unable to osmoregulate during most of their development. They are osmotically protected from freshwater by the egg envelopes, according to a mechanism that still has to be fully explained (31,32), and become able to osmoregulate in fresh water shortly before hatching. Accumulation of both Cu and Cd affected osmoregulation of the eggs.

Table. P, Ca, Mg, and total protein level of crayfish eggs and stage-1 juveniles exposed to 0.1, 0.5, and 1.0 ppm copper and cadmium for 3 weeks (mean ± standard deviation, ↓↑ indicate a decrease or increase, respectively, with respect to the control, * shows statistical significance $P<0.05$).

		Dose ppm	P mg/dL	Ca g/dL	Total Protein Mg	Mg mg/dL
week 1	**Control**		**0.750 ± 0.071**	**1.200 ± 0.070**	**0.550 ± 0.071**	**1.065 ± 0.092**
	Cu	0.1	0.650 ± 0.354↓*	1.200 ± 0.141↓	0.500 ± 0.050↓	0.985 ± 0.021↓*
		0.5	0.600 ± 0.060↓*	1.100 ± 0.040↓	0.500 ± 0.045↓	0.980 ± 0.030↓*
		1.0	0.500 ± 0.050↓*	1.000 ± 0.080↓*	0.600 ± 0.040↑	1.180 ± 0.000↑
	Cd	0.1	0.550 ± 0.071↓*	1.500 ± 0.283↑*	0.500 ± 0.050↓	1.145 ± 0.007↓
		0.5	0.500 ± 0.030↓*	0.900 ± 0.080↓*	0.500 ± 0.060↓	1.030 ± 0.080↓
		1.0	0.400 ± 0.040↓*	0.900 ± 0.070↓*	0.500 ± 0.055↓	0.980 ± 0.080↓*
	CuCd	0.1+0.1	0.550 ± 0.071↓*	1.100 ± 0.141↓*	0.500 ± 0.040↓	0.990 ± 0.042↓*
week 2	**Control**		**0.275 ± 0.050**	**0.525 ± 0.189**	**0.425 ± 0.050**	**0.585 ± 0.256**
	Cu	0.1	0.300 ± 0.141↑	0.575 ± 0.287↑*	0.425 ± 0.050↑	0.648 ± 0.244↑*
		0.5	0.200 ± 0.060↓*	0.367 ± 0.058↓*	0.400 ± 0.047↓	0.453 ± 0.040↓*
		1.0	0.100 ± 0.030↓*	0.333 ± 0.058↓*	0.400 ± 0.029↓	0.367 ± 0.091↓*
	Cd	0.1	0.375 ± 0.206↑*	0.575 ± 0.222↑*	0.425 ± 0.050↑	0.640 ± 0.308↑*
		0.5	0.200 ± 0.141↓*	0.450 ± 0.212↓*	0.400 ± 0.030↓	0.465 ± 0.163↓*
		1.0	0.220 ± 0.045↓*	0.440 ± 0.134↓*	0.400 ± 0.029↓	0.470 ± 0.069↓*
	CuCd	0.1+0.1	0.350 ± 0.212↑*	0.750 ± 0.495↑*	0.450 ± 0.071↑	0.740 ± 0.368↓*
week 3	**Control**		**0.400 ± 0.030**	**2.600 ± 0.070**	**0.400 ± 0.040**	**0.470 ± 0.020**
	Cu	0.1	0.500 ± 0.090↑*	2.400 ± 0.080↓*	0.300 ± 0.010↓*	0.450 ± 0.030↓
		0.5	0.200 ± 0.010↓*	0.500 ± 0.010↓*	0.400 ± 0.034↓	0.500 ± 0.090↑
		1.0	0.300 ± 0.200↓*	1.700 ± 0.050↓*	0.400 ± 0.060↓	0.340 ± 0.070↓*
	Cd	0.1	0.300 ± 0.340↓*	0.500 ± 0.060↓*	0.400 ± 0.060↓	0.420 ± 0.010↓*
	CuCd	0.1+0.1	0.300 ± 0.060↓*	2.000 ± 0.060↓*	0.500 ± 0.030↑*	0.480 ± 0.020↑

Niyogi and Wood (2003) reported that metals penetrate aquatic species in a chemical form, not as free ions. In the water, the actual bioavailability and toxicity depends on the form and interactions with biological receptor sites on sensitive aquatic species, for example, juvenile crustaceans (33). Competition with natural substances in the water may minimize or eliminate the bioavailability, and therefore the toxicity, of the metals. The acute toxicity LC_{50} to metals for crustaceans over a 96-h exposure period exhibited an increased sensitivity. The rate of metal uptake into crustaceans was related to concentration and increased mortality was seen with higher metal concentrations (34).

In conclusion, our observations strongly suggest that increased concentrations of Cd, together with a decrease of protein, Mg, and P, are associated with disruption of osmoregulation in *A. leptodactylus* eggs. Exposure to Cd alone or a Cd-Cu mixture during embryonic development also reduced some parameters of egg Ca and P. Both Cd and the Cd-Cu mixture caused similar effects on egg contents, but the

Cd-Cu mixture was more toxic to eggs than cadmium alone. Future studies should focus on analysis of other biochemical parameters that will show the effect of heavy metal accumulations during the embryonic development of the crayfish.

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