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Cadmium and Zinc Reversibly Arrest Development of *Artemia* Larvae

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Despite the widespread distribution of heavy metals such as cadmium and zinc in the environment and their well-known cytotoxicity and embryotoxicity in mammals, comparatively little is known about their effect on aquatic organisms, particularly invertebrates. Acute toxicity tests have been performed with a few aquatic species, but the effects of sub-acute concentrations have been studied less frequently. This may be due in part to the relative difficulty of detecting and quantifying parameters indicative of sub-lethal toxicity. Early embryonic and larval stages of development, provided they can be conveniently observed in the laboratory, have the advantage that they can be clearly defined and progression from stage to stage can be used as a parameter of normal biological function potentially disrupted by toxic substances. Cadmium at 10 uM causes skeletal abnormalities in sea urchin pluteus larvae (Pagano et al. 1982). The acute cadmium LC50 for Daphnia magna has been reported to be 30 ug/L, or about 0.27 uM (Schuytema et al. 1984), and Hatakeyama and Yasuno (1981) have observed reproductive impairement in another cladoceran, Moina macrocopa, fed cadmium-loaded Chlorella.

Post-gastrula and early larval development of the brine shrimp, Artemia, present some useful advantages for studies of developmental aspects of environmental toxicology. Dormant encysted gastrulae, erroneously called "brine shrimp eggs", can be obtained commercially and raised in the laboratory under completely defined conditions. Following a period of post-gastrula development within the cyst, pre-nauplius larvae emerge through a crack in the cyst shell. A few hours later, free-swimming nauplius larvae hatch. There is already extensive knowledge of the physiology, biochemistry and molecular biology of this period of Artemia development (Hentschel and Tata 1976; Bagshaw and Warner 1979; Persoone et al. 1980), presenting the possibility that toxic effects can be analyzed in terms of specific molecular events. Cadmium is acutely toxic to both adults and nauplius larvae of Artemia, but the reported LC50s are as high as 10 mM, depending on larval age (Trieff, 1980; Sleet and Brendel, 1985). In this paper

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we show that pre-nauplius larvae prior to hatching are much more sensitive to cadmium than are hatched nauplius larvae. At 0.1 μM, cadmium retards development and hatching of larvae; higher concentrations block hatching almost completely and thus are lethal. However, the larvae arrested at the emergence stage survive for 24 hours or more before succumbing to the effects of cadmium, and during this period the potentially lethal effect is reversible if the larvae are placed in cadmium-free medium. The effects of zinc parallel those of cadmium, although zinc is somewhat less toxic than cadmium at equal concentrations.

MATERIALS AND METHODS

Artificial sea water was prepared by dissolving the following in deionized (Milli-Q) water: NaCl, 24.70 g/L; KCl, 0.54 g/L; $MgCl_2$-$6H_2O$, 4.59 g/L; $MgSO_4$-$7H_2O$, 6.29 g/L $CaCl_2$, 0.15 g/L; $NaHCO_3$, 0.04 g/L. *Artemia* embryos were purchased from San Francisco Bay Brand, Newark, CA, and were sterilized prior to use by the antiformin treatment of Nakanishi et al. (1962). Prior to toxicity tests, embryos were fully hydrated by soaking overnight at 4° in sterile artificial sea water containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.2% (w/v) sodium tetraborate. Cadmium and zinc were added where indicated from concentrated stock solutions of $CdCl_2$ or $ZnCl_2$ in deionized water. For toxicity tests, 6-10 replicate samples of 20-30 encysted embryos each were added to 3 ml of sterile artificial sea water in plastic 30 mm petri dishes. Samples were incubated at 28° with constant agitation (100 rpm) and illumination. The number of emerged and hatched larvae was scored with the aid of a dissecting microscope. We define E_1 as the stage at which the cyst shell has cracked and the pre-nauplius larva has begun to emerge, and E_2 as the stage at which the larva is completely outside the shell but still enclosed in the hatching membrane. Unless otherwise specified, the term "emerged" refers to the sum of E_1 and E_2 forms. Significance levels were determined by the two-tailed "Student" t test.

To determine whether pre-nauplius larvae could hatch and continue development after exposure to cadmium, embryos were incubated in sea water containing 10 μM Cd Cl_2 until the emergence stage. Larvae collected either shortly after emergence (16 hr) or after an additional 24 hr exposure to cadmium were washed with and returned to sea water without cadmium, and incubation under standard conditions was continued.

RESULTS AND DISCUSSION

Figure 1 illustrates the normal progression of emergence and hatching of *Artemia* larvae. After about 12-14 hr of incubation at 28° in uncontaminated sea water a crack appears in the cyst shell and the pre-nauplius larvae begin to force their way out, a stage we refer to as E_1 (Fig 1A). After emerging completely from the shell, the pre-nauplius larvae remain within the hatching membrane, which is normally anchored to the empty shell, giving

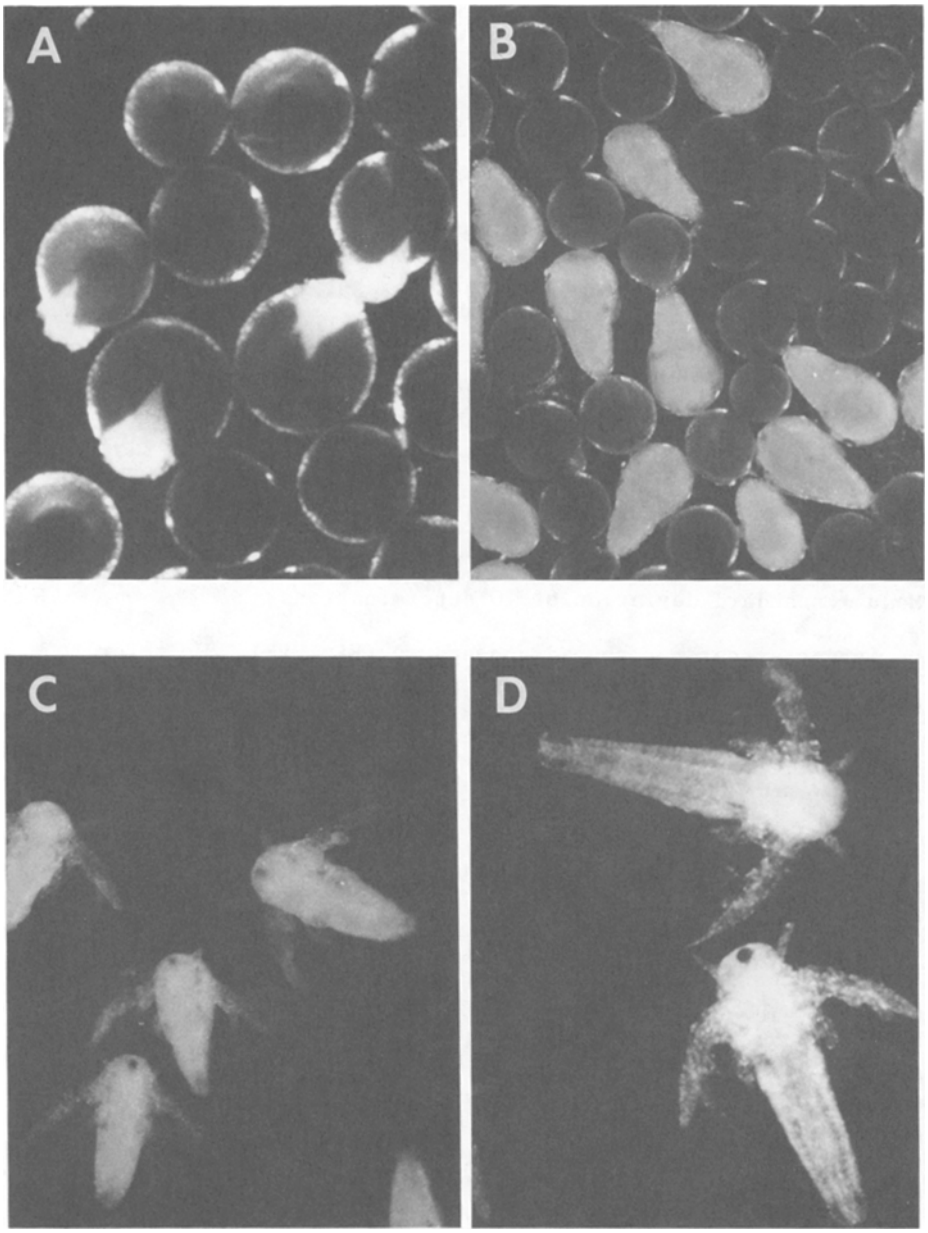

Figure 1. Normal development of Artemia. A) Emerging pre-nauplius larvae at 12 hr of incubation (x90); B) Fully emerged pre-nauplius larvae at 16 hr (x50); C) Hatched nauplius larvae at 24 hr (x50); D) Hatched nauplius larvae at 48 hr (x50).

Table 1. Effect of cadmium and zinc on hatching of Artemia larvae

Condition	Incubation time 24 hr % emerged*	24 hr % hatched*	48 hr % emerged*	48 hr % hatched*
Control	8.7±4.3	56.1±8.1	5.1±3.2	64.7±7.7
10 μM $CdCl_2$	51.4±4.0	3.3±2.2	56.8±7.2	3.7±2.5
1 μM $CdCl_2$	52.3±4.4	4.4±3.9	59.3±5.4	5.1±3.3
0.1 μM $CdCl_2$	36.7±8.2	26.5±6.3	23.7±4.0	31.9±16.2
10 μM $ZnCl_2$	65.1±4.9	4.0±2.8	59.8±7.8	6.3±5.9
1 μM $ZnCl_2$	51.7±7.4	12.9±5.8	40.2±19.0	20.6±7.8
0.1 μM $ZnCl_2$	31.8±15.1	24.1±10.2	22.3±7.7	49.7±6.1

*Mean ± standard deviation of 10 determinations

the larvae a teardrop shaped appearance (Fig. 1B). We refer to this stage as E_2. Within 4 hr the hatching membrane ruptures and the nauplius larvae swim away (Fig. 1C). Larval development is rapid, and larvae 24 hr after hatching have passed through the first molt, are longer than newly hatched larvae and have a complete gut tube (Fig. 1D).

The presence of cadmium or zinc profoundly affects the hatching of Artemia larvae as shown in Table 1. Whereas hatching of control samples is near the maximal value after 24 hr of incubation, $CdCl_2$ at 1 μM or 10 μM blocks hatching almost completely, even after 48 hr. At 0.1 μM $CdCl_2$, the number of hatched nauplius larvae is about half the control value at both 24 hr and 48 hr. All test values were significantly different from controls ($p<0.01$). Zinc has a qualitatively similar but quantitatively less dramatic effect, with the number of nauplii in 1 μM or 0.1 μM being higher but still below controls. In all cases the total number of embryos that developed at least as far as the emergence stage was similar to controls, i.e., cadmium or zinc did not begin to affect development before emergence. For example, after 48 hr of incubation the percent of embryos that had reached or passed the emergence stage (E_1 + E_2 + nauplii) was 59.9 ± 7.5 in 10 μM $CdCl_2$ and 65.4 ± 6.3 in 10 μM $ZnCl_2$ compared with 69.4 ± 8.3 for controls. It is apparent that cadmium or zinc have little or no detectable effect on pre-emergence development, presumably owing to the impermeability of the cyst shell. (DeChaffoy et al. 1978).

An additional aspect of the heavy metal effect is illustrated in Fig. 2. In the presence of 10 μM $CdCl_2$ the pre-nauplius larvae are blocked at the E_1 stage of emergence (Fig. 2A). After

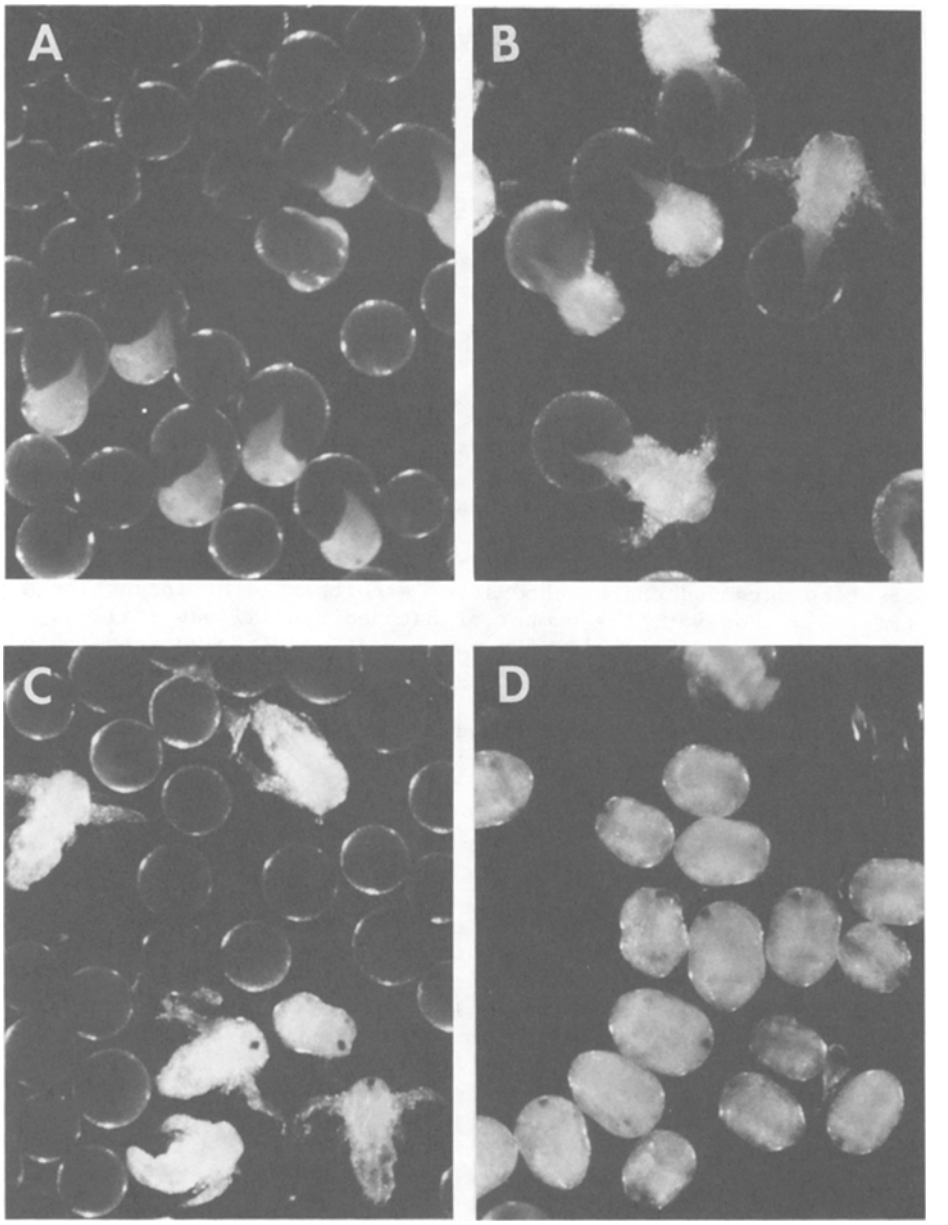

Figure 2. Abnormal development of Artemia larvae exposed to cadmium. Larvae incubated in artificial sea water containing cadmium as follows: A) 10 µM $CdCl_2$, 24 hr (x50); B,C) 10 µM $CdCl_2$, 40 hr (x50). C) 1 µM $CdCl_2$, 24 hr (x50).

prolonged incubation (72 hr or more) a small percentage of these larvae hatch without ever completing emergence (Fig. 2B) and will swim about with the shell still attached. Those hatched larvae that were completely released from the shell tended to have shortened and distorted bodies (compare Fig. 2C with Fig. 1D). At 1.0 μM $CdCl_2$ the larvae proceeded to the E_2 stage and, unlike controls, detached from the cyst shell but remained enclosed in the hatching membrane (Fig. 2D). Similarly, pre-nauplius larvae in 0.1 μM $CdCl_2$ reached the E_2 stage and detached from the shell. However, this concentration of cadmium delayed hatching but did not prevent it entirely. After prolonged incubation (96 hr) in 0.1 μM $CdCl_2$, 55.0 + 2.4% of the larvae had hatched, approaching the control value (Table 1) which did not change after 48 hr.

In order to test the ability of cadmium-exposed larvae to recover and continue development E_1-stage larvae emerged in 10 μM $CdCl_2$ were transferred to uncontaminated sterile sea water, and the results are shown in Table 2 and Fig. 3. The ability of pre-nauplii to survive and hatch depended upon the length of time they were exposed to cadmium. When newly emerged pre-nauplii were rescued after incubation in 10 μM $CdCl_2$ for 16 hr, a substantial fraction survived and hatched after a further 24 hr incubation (Fig. 3). However, the number of hatched nauplii was still significantly ($p < 0.01$) below the number derived from uncontaminated emerged larvae (Table 2). When arrested pre-nauplii were washed after 40 hr of exposure to 10 μM $CdCl_2$ and further incubated in clean sea water, none had hatched after 24 hr and only a few hatched after 72 hr (Table 2). It was clear that the arrest of development induced by cadmium was largely reversible if the emerged pre-nauplius larvae were exposed for only a few hours, but prolonged exposure drastically reduced their probability of survival.

The results presented here show that the hatching stage of Artemia development is highly sensitive to the presence of heavy metals. The developing post-gastrula embryos are protected by an impermeable chitinous shell and suffer little or no apparent damage. The emerging pre-nauplius larvae, however, encounter a toxic environment that can have catastrophic effects on their further development. A concentration of cadmium as low as 0.1 μM (11.1 ppb) significantly retards normal development and hatching, and higher concentrations block hatching almost completely. After hatching, however, the nauplius larvae are far less sensitive to heavy metal toxicity. In agreement with the results of Sleet and Brendel (1985) we observed no mortality of normally hatched nauplii transfered to 100 μM $CdCl_2$. Similarly, 10 μM $ZnCl_2$ produced no significant mortality in hatched nauplii; we were unable to test higher concentrations because of the limited solubility of zinc in the artificial sea water formula we use.

The mechanisms by which heavy metals interfere with normal development of Artemia larvae remain to be determined. It may be that cadmium and zinc interfere directly with the hatching process, for example by inhibiting rupture of the hatching membrane. Alterna-

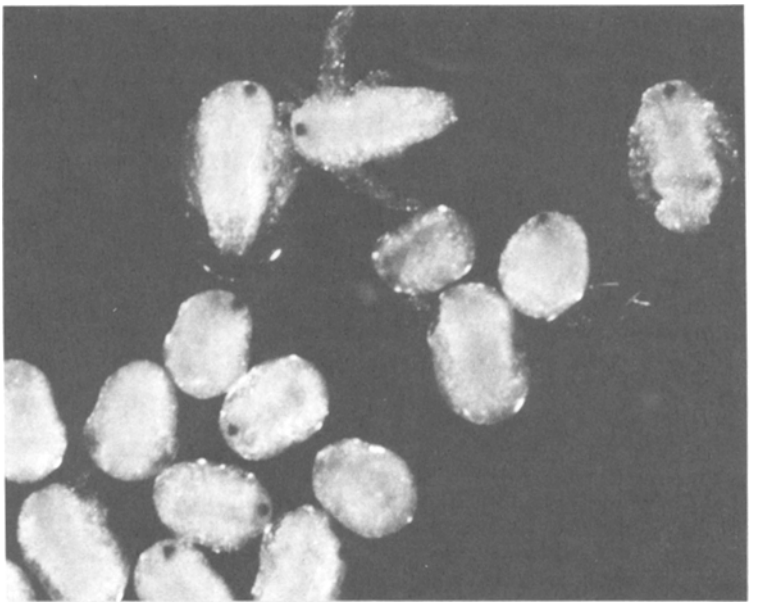

Figure 3. Recovery of cadmium-exposed Artemia larvae, Pre-nauplius E_1 larvae emerged in 10 μM $CdCl_2$ at 16 hr were washed and incubated 24 hr in uncontaminated sea water, resulting in detached E_2 pre-nauplii and hatched nauplii (x50).

Table 2. Hatching of pre-nauplius larvae exposed to cadmium.

Conditions	% hatched*
Control: emerged (16 hr) in sea water, incubated additional 24 hr (n=8)	87.0±8.2
Emerged in 10 μM $CdCl_2$, washed at 16 hr, incubated additional 24 hr (n=7)	61.1±11.5
Emerged in 10 μM $CdCl_2$, washed at 40 hr, incubated additional 24 hr (n=10)	0
Emerged in 10 μM $CdCl_2$, washed at 40 hr, incubated additional 72 hr (n=10)	16.0±8.5

*Mean ± standard deviation

tively these metals may directly block development of the pre-nauplius larvae, preventing them from reaching a stage capable of hatching. In any case we have identified a developmental process that is highly sensitive to heavy metal contamination. The extensive knowledge of biochemical aspects of Artemia development will facilitate further elucidation of this phenomenon, providing valuable information concerning the cellular and molecular targets of these common environmental contaminants.

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