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High Resistance of Resting Eggs of Cladoceran *Moina macrocopa* to the Effect of Heavy Metals

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Abstract

The research aimed to determine critical concentrations of heavy metals at which survival of resting eggs of the cladoceran *Moina macrocopa* is negatively affected. Resting eggs' viability was not affected over a 30-days exposure towards copper, cadmium, zinc or nickel at concentrations up to 60–70 g/L. When resting eggs were exposed to sediment contaminated with heavy metals for 8 months, the hatching success was affected at 30 g copper/kg. Thus, resting eggs of Cladocera can tolerate heavy metals at concentrations that far exceed lethal concentrations of heavy metals to active life stage and exceed low or moderate levels of environmental pollution. Follow up investigation of life table parameters of hatchlings from resting eggs exposed to heavy metals demonstrated that neither lifespan nor fecundity of hatchlings differ from control animals. These results demonstrate that zooplankton may rapidly recover from resting egg bank once aquatic habitat becomes unpolluted.

Keywords Resting eggs · Heavy metals · Resistance · Hatching success · Life table parameters

Cladocerans belongs to filter-feeding zooplankton that play a key role in aquatic food webs. Such taxa as *Daphnia* or *Moina* are widely used as test organisms to determine the toxicity of chemicals in aquatic systems (e.g. Farre and Barcelo 2003). Under unfavorable conditions, many species of Cladocera produce resting eggs (Alekseev et al. 2007). Resting eggs can survive harsh environmental conditions as such drying or freezing (Radzikowski 2013). Resting eggs produced in different years are accumulated at the bottom sediments, where they form a resting egg bank (Hairston et al. 2000). Natural populations can recover from these banks with eggs being triggered by stimuli such as photoperiod, light intensity, resuspension of sediments, which ultimately supports the stability of zooplankton (Brendonck and De Meester 2003).

Despite the apparent importance of resting eggs for the ecology and evolution of zooplankton, few studies have estimated the effects of toxicants on them (e.g. Kerfoot et al. 1999). It was demonstrated that resting eggs are more resistant to toxic substances relative to active animals (e.g. Jiang et al. 2007; Raikow et al. 2007; Alekseev et al. 2010). Some studies demonstrated modifications in the life history of animals hatched from resting eggs exposed to toxicants (e.g. Navis et al. 2013). In this context, heavy metals (copper, cadmium, zinc, nickel and other) often pose a threat to freshwater zooplankton communities through exposure via the water phase (e.g. Donnachie et al. 2014). Moreover, due to adsorption, concentrations of metals in the sediments may exceed those in the water column and pose an additional threat to benthic biota (e.g. Avila-Perez et al. 1999). However, studies focusing on both the resistance of resting eggs to heavy metals and consequences on the life table parameters of animals hatched from resting eggs exposed to these toxicants are scarce.

The aim of this research was to determine and compare critical concentrations of heavy metals at which adverse effects on active females of Cladocera, resting eggs and females hatched from the eggs exposed to heavy metals are observed. We exposed resting eggs to a wide range of concentrations of selected heavy metals and perform life table experiments with hatchlings from these eggs. Prior we performed acute toxicity tests to have reference values needed to compare the toxicity of the heavy metals we assessed to

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active animals and resting eggs and to estimate environmental importance of observed responses.

Materials and Methods

We used a laboratory culture of cladoceran *Moina macrocopa* that had been maintained in the Institute of Biophysics SB RAS (Krasnoyarsk) for the last 10 years. Animals were cultivated in a climate chamber at a temperature (25 ± 1°C) and photoperiod (16 h light:8 h dark) optimal for their growth and reproduction (Zadereev and Gubanov 1996). Tap water (pH – 7.3; total permanent hardness – 62.9 mg·equivalent of $CaCO_3$/L; total content of cations (macro- and trace elements) – 26.4 mg/L) aged for at least 72 h was used as culture medium. Animals were fed with the non-axenic green algae *Chlorella vulgaris*, which was batch cultured under constant light and aeration in 500 mL flasks in Tamiya medium (Tamiya et al. 1953). Before being used as food, the algae were concentrated by centrifugation (1200×*g*). The concentration of the algae in the medium was adjusted to the desired level by dilution and determined with a CASY TTC particle counter (SCHÄRFE SYSTEM GmbH, Germany).

We used copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), cadmium sulfate octohydrate ($3CdSO_4 \cdot 8H_2O$), zinc chloride ($ZnCl_2$) and nickel hexahydrate ($NiCl_2 \cdot 6H_2O$) as model toxicants. These heavy metals are also widely used as model toxicants in toxicological research with cladocerans (Wong and Wong 1990; Wong 1992).

Concentrations of heavy metals in water samples were quantified using an iCAP 6300 Duo ICP-OES spectrometer (Thermo Scientific, England, 2010) according to EPA 200.7 (2001). ICP-OES spectrometer operating conditions were: RF power – 1150 W, flash pump rate – 50 rpm, analysis pump rate – 50 rpm, nebulizer gas flow – 0.70 L/min, auxiliary gas flow – 0.5 L/min. A nebulizer type was SeaSpray (ARG-07-USS2, Glass Expansion). Scandium solution (5 mg/L, dissolved from 92504 Scandium Standard for ICP, Fluka, Switzerland) was used as an internal standard, which was aspirated via Y piece and mixing loop. Merck (Darmstadt, Germany) "ICP multielements solution IV" was used for the method calibration. Accuracy of the calibration was checked by analyzing "Multielement standard solution 6 for ICP" (Fluka, Switzerland). 18 MΩ water was used for the standard solutions. Tap water was used as a blank for the experimental water samples measurements.

Concentrations of heavy metals in experimental sediment were determined by flame atomic absorption spectrometry (AAS) in oxygen–acetylene flame using a "Kvant 2A" spectrometer (Kortek, Russia). High purity single element water solutions of Cd, Cu, Ni and Zn (Ural Plant of Chemical Reagents, Russia), were used for a calibration. A solution of metal ions "KS-1" (Ekros, Russia) was used for working solutions control. A blank solution was prepared similarly to the sediment samples. To perform analysis sediment samples were dried at 105°C until a constant weight and 1 g of each sample was digested in 10 mL of HNO_3:$HClO_4$ (1:1, analytical grade) on a laboratory hotplate (Gladyshev et al. 2001).

Method detection limits (MDL) and wavelengths (λ) for the elements detection were for ICP-OES (Cu – λ = 324.7 nm, MDL = 0.0002 mg/L; Cd – λ = 214.4 nm, MDL = 0.0001 mg/L; Zn – λ = 213.8 nm, MDL = 0.0001 mg/L; Ni – λ = 231.6 nm, MDL = 0.0008 mg/L) and for AAS (Cu – λ = 324.8 nm, MDL = 0.075 mg/kg; Cd – λ = 228.8 nm, MDL = 0.011 mg/kg; Zn – λ = 213.9 nm, MDL = 0.023 mg/kg; Ni – λ = 232.0 nm, MDL = 0.105 mg/kg).

Solutions of heavy metals for acute toxicity tests were prepared by successive dilution of the stock solution of each salt with predetermined concentration of the toxicant. After we measured concentrations of heavy metals in each solution using the method described above.

For acute toxicity test, 1-day-old juvenile females (size 0.5–0.6 mm) hatched by mothers kept individually in the climate chamber in 20 mL of the daily renewed medium with food concentration of 200,000 cells/mL were used. These are standardized, favorable for parthenogenesis conditions to produce animals for individual tests or life table experiments (Zadereev and Gubanov 1996).

We placed females individually into beakers with 20 mL of culture medium without food, with a toxicant (measured concentrations: Cu – 0.004, 0.005, 0.006, 0.007, 0.009, 0.010, 0.012, 0.013, 0.015, 0.019, 0.021; Cd – 0.013, 0.036, 0.070, 0.153, 0.376, 0.784; Zn – 0.09, 0.14, 0.22, 0.42, 0.53, 0.83, 1.06, 1.70; Ni – 0.47, 0.90, 1.85, 3.37, 8.71, 17.27 mg/L); tap water with no toxicant was used as control. For each concentration of every toxicant and for the control, we tested 30 individually kept animals. The number of dead individuals after 24 h and 48 h was counted.

Resting eggs for experiments were produced in the batch culture of *M. macrocopa*, which had been started from 20 females in 4 L of the medium renewed every 3 days with the concentration of *Chlorella* adjusted to 1 million cells/mL. Such growth conditions ensured rapid population growth, depletion of food and production of a large number of resting eggs. Resting eggs were collected from the batch culture within approximately 3 weeks and kept in the darkness at 4°C for 12 months before experiments. For experiments, we selected undamaged ephippia containing two fertilized embryos. Resting eggs were exposed either to solutes of heavy metals or to artificial sediment contaminated with heavy metals (see details below).

Solutes of heavy metals were prepared by successive dilution of the stock solution of each toxicant with aged tap water to reach the desired concentration which was

confirmed as detailed above. For each concentration of every toxicant (measured concentrations: Cu – 0.1, 0.8, 3.2, 10.1, 16.7, 49.6, 53.8; Cd – 0.7, 1.1, 6.3, 11.1, 18.6, 38.8, 66.3; Zn – 2.3, 4.8, 9.9, 20.0, 39.8, 82.9; Ni – 0.3, 0.7, 4.1, 8.8, 16.5, 39.5, 72.0 g/L) 60–70 resting eggs were tested. Resting eggs were stored for 30 days in the darkness at a temperature of 3°C at the bottom of an 8-mL vial with ground glass stopper containing 3 mL of the medium with the respective concentration of the target toxicant.

Artificial sediment was prepared following the standard protocol for the production of artificial sediments (OECD 1984, 2004). *Sphagnum* peat was air-dried, ground to fine powder (particles ≤ 1 mm) and moistened with deionized water. The suspension was conditioned for 2 days at 20 ± 2°C, to stabilize pH (6.0 ± 0.5). Then the peat suspension (5%–10%, dry weight) was blended with kaolin clay (20%, dry weight) and quartz sand (70%–75%, dry weight). The resulting sediment was mixed thoroughly and moistened with deionized water to obtain a homogeneous substance. The pH of the final mixture was 7.0 ± 0.5.

To enrich the artificial sediment with the model toxicants, stock solutions of heavy metals were prepared. The sediment was divided into 10 g portions. Deionized water with a dissolved toxicant at the desired concentration was added to each portion of the sediment. The amount of water was calculated to adjust sediment humidity to 35%–40% of its dry weight. The sediment was thoroughly mixed, kept under experimental conditions for 24 h. Samples were taken from each enriched artificial sediment to control the humidity and measure the concentration of heavy metals using the method described above.

Resting eggs were buried in the artificial sediment contaminated with heavy metals (measured concentrations: Cu – 5.6, 11.0, 33.6, 67.4; Cd – 5.3, 17.0, 50.7; Zn – 8.0, 12.6, 30.1, 59.5; Ni – 4.4, 8.0, 16.2, 37.2, 56.2 g/kg) and kept in the darkness at a temperature of 4°C for 8 months. For each concentration of each metal, 60–70 resting eggs were tested.

After exposure, eggs were washed in distilled water, kept for 30 min in a solution of ethylenediaminetetraacetic acid (20 mM/L) to remove metal cations from the egg surface (Vazquez et al. 1999), and, finally, washed once more with distilled water. This treatment was introduced after a preliminary experiment where we observed mortality of hatchlings from resting eggs that have been exposed to high concentrations of heavy metals. We assumed that mortality of hatchlings was associated with the diffusion of heavy metals initially bound to the surface of the resting eggs to the experimental medium. The treatment detailed above did not cause any mortality of hatchlings. The washed eggs from each treatment were placed for reactivation into 500 mL glass jars containing *Chlorella* as food at a concentration of 400,000 cells/mL. Eggs were hatched under a constant temperature (25 ± 1°C) and photoperiod (16 h light, 8 h dark). The medium and food were renewed every 3 days. The number of hatched neonates was recorded daily. We monitored hatching success for 2 weeks after the start of reactivation. The hatching success was calculated as the ratio of hatched eggs to the total number of eggs.

One-day-old neonates (size group 0.5–0.6 mm) hatched from heavy metal exposed resting eggs were placed individually into beakers containing 20 mL of the medium and *Chlorella* at a concentration of 200,000 cells/mL. For each tested concentration of each heavy metal we tested 15 hatchlings from resting eggs exposed to solutes of heavy metals and 20 hatchlings from resting eggs exposed to contaminated sediment (overall about 900 animals were tested). The medium and food in experimental vessels were renewed daily. Experiments were run until death of all animals. For each female, we counted the number of offspring and recorded the time until death. The average lifespan (days) and fecundity (hatchlings per female) were calculated for each concentration of each metal.

LC_{50} values in acute toxicity tests were determined in drc package for R (Ritz et al. 2015). In life table experiments for all treatments (water with solutes or contaminated sediment, four heavy metals) we tested the effect of each heavy metal individually among concentrations on each life table parameter (lifespan and fecundity) using ANOVA (STATISTICA 8.0). For each heavy metal to calculate relative to control values of lifespan and fecundity we (1) divided each treatment value of a parameter to the average value of respective control and (2) averaged these relative to control values.

Results and Discussion

Obtained LC_{50} values allowed ranking of heavy metals based on theirs acute toxicity towards female *M. macrocopa* (Cu–Cd–Zn–Ni, from more toxic to less toxic ones) (Table 1). Even though there is a difference of various orders of magnitude between the present and earlier studies, we can see that our ranking is consistent with literature.

Hatching success of resting eggs was not affected by the 30 days exposure to the wide range of heavy metal concentrations (from the background level to 60–70 g/L) dissolved in water (Fig. 1a). When the exposure to heavy metals was prolonged, we observed some adverse effects: in the experiment with contaminated artificial sediment, hatching of resting eggs was completely suppressed after 8-month of exposure to high concentrations of copper (34 and 67 g/kg). None of other treatments, however, affected hatching success of resting eggs (Fig. 1b).

We observed resistance of resting eggs to much higher concentrations (up to 60–70 g/L) of heavy metals than it had been demonstrated before for resting eggs of zooplankton in general (e.g. Alekseev et al. 2010; Jiang et al.

Table 1 Acute toxicity ($LC_{50} \pm SE$, mg/L) of copper (Cu), cadmium (Cd), zinc (Zn) and nickel (Ni) to females of *M. macrocopa*

Heavy metal	LC_{50} (24 h) (mg/L)	LC_{50} (48 h) (mg/L)	References
Cu	0.0094 ± 0.0002	0.0094 ± 0.0002	Our data
	0.710	–	Nandini et al. (2007)
	0.090	0.080	Wong et al. (1991)
Cd	0.284 ± 0.029	0.169 ± 0.020	Our data
	–	0.053	Xu et al. (2011)
	0.218	0.013	Pokethitiyook et al. (1987)
	0.418	–	Garcıa et al. (2004)
Zn	1.19 ± 0.18	0.34 ± 0.02	Our data
	1.01	–	Nandini et al. (2007)
	2.04	1.17	Wong et al. (1991)
Ni	10.41 ± 0.52	5.01 ± 0.58	Our data
	–	7.00	Wong et al. (1991)
	2.20	0.46	Pokethitiyook et al. (1987)
	–	6.84	Tabche et al. (2000)
	23.59	6.48	Wong (1992)

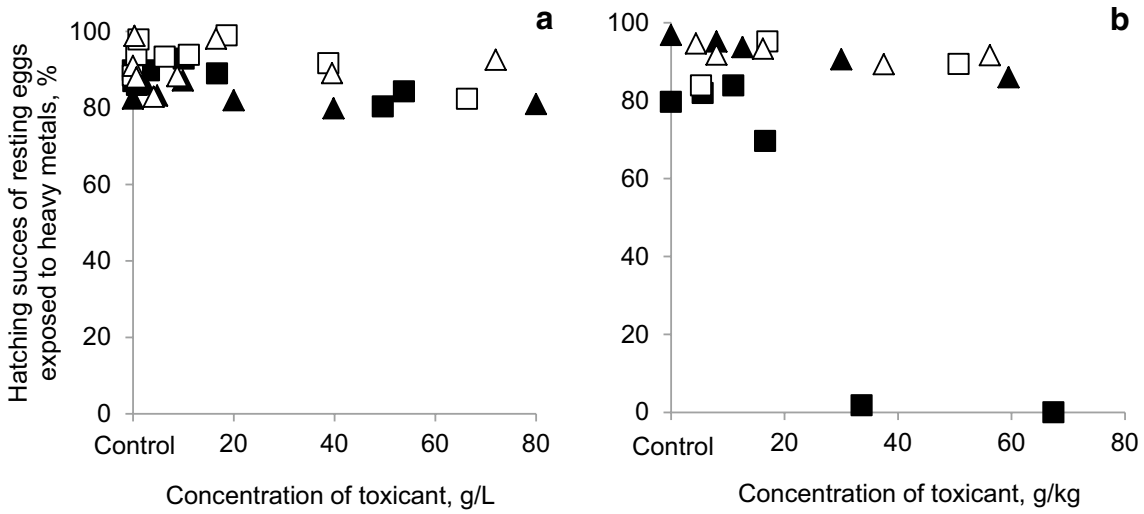

Fig. 1 The hatching success of resting eggs of *M. macrocopa* after exposure to heavy metals (black squares – copper, white squares – cadmium, black triangles – zinc, white triangles – nickel). **a** 30 days exposure to water with dissolved heavy metals; **b** 8-month exposure to artificial sediment contaminated with heavy metals

2007). Recently it was shown that resting eggs of cladoceran *Daphnia* are good biosorbent of heavy metals (Sacmaci et al. 2014). Thus, most probably protective structures of the ephippium are able to absorb heavy metal ions and protect enveloped embryos from toxic exposure.

The effect of toxicant concentration on life table parameters of hatchlings from metal exposed eggs was insignificant for all the heavy metals and all treatments (Fig. 2). We observed only two type of responses in our experiments. Either the eggs were killed (high concentrations and long contact of eggs with the most toxic heavy metal (Cu)) or eggs were hatched and the offspring did not differ from the control group.

Other toxic compounds could induce different effects on resting eggs and the life table parameters of their hatchlings. For example, the exposure to pesticides (carbaryl and fenoxycarb) affected not only the reactivation of exposed resting eggs of *Daphnia magna* but also the survival and life table parameters of their hatchlings (Navis et al. 2013). Later, studying the sensitivity of resting eggs of *D. magna* to fenoxycarb, the authors showed that ephippium did not protect embryos against the pesticide (Navis et al. 2015).

Most probably, the effect of toxic compounds on resting eggs depends on the interactions of toxicants with protective structures of resting eggs. Alekseev et al. (2010) compared the toxicity of heavy metals, organic substances and

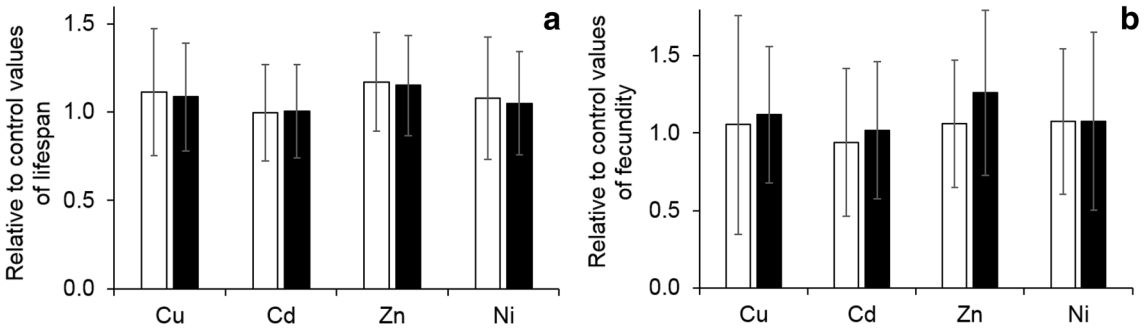

Fig. 2 Lifespan (**a**) and fecundity (**b**) of *M. macrocopa* females hatched from resting eggs exposed to heavy metals (averaged for all tested concentrations expressed as relative alteration compared to the respective control). White bars – eggs exposed to solutes of heavy metals in water. Black bars – eggs exposed to metal contaminated artificial sediment. Whiskers – standard deviation. Control values: Lifespan, days ± SD: LS_{water} = 9.42 ± 3.17, $LS_{sediment}$ = 9.38 ± 2.72; Fecundity, number of neonates per female ± SD: F_{water} = 43.69 ± 22.56, $F_{sediment}$ = 39.08 ± 15.54

low-molecular-weight compounds to resting eggs of eight species from the Porifera, Bryozoa and Crustacea including *M. macrocopa* and suggested that toxic effect depends on the ability of toxicants to penetrate the protective structures of the ephippium. We mentioned before that resting eggs of cladocerans sorbs heavy metals that can explain theirs high resistance.

We observed resistance of resting eggs to high concentrations of heavy metals over a relatively long time (up to 8 months). Concentrations of heavy metals in natural habitats are usually lower, even in the case of severe pollution. For example, copper concentrations in sediments reached only 5 g/kg in contaminated swampy areas around mines in China (Deng et al. 2004), 0.5 g/kg in the places where the city run-off enters the water body (Vesk and Allaway 1997), and 5 g/kg in a mining region in Mexico (Razo et al. 2004). In our study, mortality of resting eggs was observed after 8-month exposure to 30 g/kg of copper while for cadmium, zinc or nickel, concentrations of up to 50 g/kg had no effect on eggs' survival. These insights suggest that for many habitats negatively impacted by heavy metals zooplankton may recover from resting egg bank once aquatic habitat are restored.

Concentrations of toxicants that pose threat to resting eggs will be lower if we consider long-term storage of resting eggs in sediments (e.g., for several years). For example, Rogalski (2015) observed that hatching rate of *Daphnia* hatched from diapausing eggs isolated from sediments from four lakes that experienced varying levels of metal contamination (Cu, Cd, Zn, Hg, Pb) was negatively influenced by metal contamination and sediment age. However, as long-term storage reduces the viability of resting eggs and the contribution of resting eggs to the zooplankton community in lakes varies between 10 and 20 years (Hairston et al. 2000), the ecological significance of long-term heavy metal exposure may be rather limited but can still not be excluded.

Also, with high concentrations of heavy metals in sediments, elevated concentrations of toxicants will be observed in water. Studying the effect of zinc on resting eggs of *Artemia*, Sarabia et al. (2008) suggested that in natural habitats, chronic toxicity of heavy metals dissolved in water to active animals would be more important than the effect on resting eggs. However, we should not underestimate evolutionary implications of the effects of heavy metals on active zooplankton community and the resting egg bank. For example, metals in freshwater environments can modulate diapause adaptive efficacy and the selection process in egg banks (e.g., Aránguiz-Acuña and Pérez-Portilla 2017).

We can summarize that resting eggs are quite resistant to heavy metals. Our research and several other studies have demonstrated that the survival of eggs is affected by both the duration of contact with heavy metals and their concentrations (Jiang et al. 2007; Rogalski 2015). Hence, we consider it highly important to investigate mechanisms that are responsible for the viability of resting eggs under various concentrations of toxicants and durations of direct contact of eggs with a toxicant, and to understand the interactions of toxicants with protective structures of the ephippium.

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Data Availability Data, associated metadata, and calculation tools are available from the corresponding author (egor@ibp.ru).

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