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Sensitivity of isolated eggs of pond snails: a new method for toxicity assays and risk assessment

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Abstract The concentration of heavy metals in the environment is normally low. We here address whether using the development of isolated pond snail *Radix auricularia* eggs would provide a more sensitive endpoint and whether the gelatinous matrix of the egg mass surrounding the eggs indeed protects the snail embryos. In the present study, artificial removal of the gelatinous matrix of egg masses greatly increased the sensitivity of developing eggs to a heavy metal (cadmium). The sensitivity of isolated eggs to cadmium was determined using several convenient endpoints, including mortality, hatching rate, and heart rate, with an acute toxicity test and a subchronic test. In the acute toxicity test, a 96-h LC_{50} value of 58.26 μg/L cadmium was determined. In the subchronic toxicity test, sublethal effects in terms of a significant reduction in hatching rate could be found in the 25-μg/L treatment, and a significant decrease of heart rate was observed in both treatments (5 and 25 μg/L). The high sensitivity of isolated eggs indicates that such tests can be efficient for toxicity assays and risk assessment, although one needs to keep in mind that the ecologically relevant measure of toxicity will be how eggs are affected when they are still inside the egg mass.

Keywords Lymnaeidae · Embryo · Hatching · Heart rate

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Introduction

Cadmium is one of the few nonessential elements and is toxic to all forms of life tested so far, including aquatic organisms (Chandra 2004; Kim 2004; Thompson and Bannigan 2008). The main sources of Cd are Zn industry, mining, and the application of sewage sludge and fertilizers to agroecosystems (Adriano 2001; Oporto and Carlo Smolders 2007). When river water and drinking water have elevated cadmium concentrations, this is normally attributed to one of the abovementioned anthropogenic sources (Jain et al. 2005; Wang et al. 2011). For example, water bodies nearby (former) mining areas are often polluted by cadmium and other heavy metals, far above the recommended levels (Florea et al. 2005; Oporto and Carlo Smolders 2007).

A considerable number of investigations reflect the embryotoxicity of cadmium on fish and snails (Witeska et al. 1995; Coeurdassier et al. 2003; Schirling et al. 2006; Cao et al. 2009). In snails, cadmium decreases the total number of laid eggs, delays the hatching time, and

causes reduction of growth rate and food consumption (Schirling et al. 2006; Ansaldo et al. 2009; Das and Khangarot 2010). Besides, exposed snails can synthesize Cd-binding metallothionein which plays a crucial role in cadmium detoxification and can act as a molecular biomarker in invertebrates given its dose–response relationship with cadmium (Chabicovsky et al. 2003; Dallinger et al. 2004; Timmermans et al. 2005).

Snails have been widely used in toxicity assays, especially for metals, but many of these studies were conducted using juveniles or/and adults (Khangarot and Ray 1988; Gomot 1997; Pyatt and Pentreath 2002; Das and Khangarot 2010). Considering that one aim of toxicological tests with animals should be to reduce, refine, and replace (known as the three R's; Mehlman et al. 1989; Gad 1990), it is worthwhile to test whether different endpoints are more suitable and/or efficient. In this context, egg masses of snails have been used in toxicity assays (Gomot 1998; Schirling et al. 2006). Several embryological endpoints have been developed for such embryo test model. Hatching is one important event, given that the newly hatched animals start to interact with and be exposed to their environment, both via their food and surrounding medium. Hatching has turned out to be one of the most sensitive parameters in the investigation of the embryotoxicity of chemicals (Hallare et al. 2006). Khangarot and Das (2010) reported that heart rate can be affected by heavy metal (copper), and many a study has taken heart rate as a developmental endpoint (Schirling et al. 2006; Filla et al. 2009; Sawasdee and Köhler 2009; Sawasdee and Köhler 2010). In the present study, we also use hatching and heart rate as endpoints.

Clearly, the egg mass has evolved to protect snail eggs during development from environmental factors that may negatively affect development (Marois and Croll 1991; Kuang et al. 2002). Of course, in toxicity assays, the egg mass may also protect eggs from the test substance. Therefore, an artificial removal of the egg mass matrix surrounding snail eggs may improve the sensitivity and reliability of toxicity assays and risk assessment. So far, little information has been available on the application of isolated pond snail eggs to toxicity assays and risk assessment. We here focus on a pond snail species, *Radix auricularia*, that is very common in China (among other places) but for which little toxicological data from metal exposure are currently available. Using isolated eggs of this species, we aim to develop a new method to improve the sensitivity of toxicity assays and risk assessment in freshwater animals. Therefore, we here address whether the gelatinous matrix of egg masses surrounding the eggs indeed protects the snail embryos and whether development of eggs would provide more sensitive endpoints.

Material and methods

Animal collection and acclimation

The pond snail *R. auricularia* is a pulmonate gastropod belonging to the suborder Basommatophora and the family Lymnaeidae. This hermaphrodite is distributed commonly in lakes and ponds of China (where it is native) and can be collected easily in the field. The pond snail egg masses were collected from uncontaminated waters from March to May in the Xiaoqing River flowing through the city of Jinan, China, and were acclimatized in glass Petri dishes (90 mm in diameter) under experimental conditions: 21±1 °C, day/night cycle (14:10 h).

Separation and egg size

We adopt the convention of Plesch et al. (1971) that the egg refers to the ovum surrounded by an amount of perivitelline fluid, which is bounded by two thin membranes, the membrana interna and the membrana externa. Five days post laying, egg masses (at the late trochophore stage, Pande et al. 2010) were selected and opened by cutting through the limiting membrane (called the tunica interna, Plesch et al. 1971) at one end of the egg mass. Eggs were carefully pushed out through the opening using a pair of forceps. Further separation was carried out with a sharp Pasteur pipette to get rid of the remaining gelatinous materials. Normal embryos were randomly selected. These isolated pond snail eggs will be referred to as *isolated eggs* in the following. Isolated eggs were measured by determining the length of the long axis and short axis of the membrana externa using an ocular micrometer (n=50). In order to get accurate data, the measurements were done at ×400 magnification.

Chemical test

A stock solution of cadmium (as $CdCl_2 \cdot 2.5\ H_2O$, Sinopharm, Shanghai, China) was prepared in deionized water, and from this solution, a series of cadmium concentrations were also prepared in deionized water with pH7.3±0.1. Freshwater quality criteria (USEPA, 2006) for chloride (chronic) is 230 mg/L, which is far above the concentration of chloride that got dissolved in our experiments (see each test for actual concentrations); therefore, we chose not to test the effects of chloride ion separately.

The protective role of egg masses

Firstly, we evaluated the effect of the isolation procedure on isolated eggs and confirmed the protective role of egg masses. Isolated eggs as well as intact egg masses were exposed to nominal cadmium concentrations of 0, 75, and 150 μg/L (chloride concentrations were 0, 47.4, and 94.78 μg/L, respectively), which were established according to previous preliminary tests. Either 10 isolated eggs or one egg mass containing 35–85 eggs were exposed to each concentration. Three replications were used in each treatment. Tests were conducted in 12-well plates to supply enough oxygen for egg masses in the 96-h exposure. Mortality was defined as the coagulation of embryos or the cessation of heartbeat (Das and Khangarot 2011) and was recorded at every 24-h interval. Dead embryos were discarded.

Acute toxicity

A series of nominal cadmium concentrations of 0, 25, 40, 55, 70, and 85 μg/L (chloride nominal concentrations—0, 15.8, 25.3, 34.8, 44.2, and 53.7 μg/L, respectively) were used to determine the 96-h LC_{50}. Groups of 10 isolated eggs were exposed to each cadmium concentration. The number of dead embryos was documented for every 24 h. The definitions of mortality and test system were according to the test of the protective role of egg masses (see above).

Subchronic toxicity

The isolated eggs were exposed for 7 days to cadmium concentrations of 0, 5, and 25 μg/L (chloride concentrations were 0, 3.2, and 15.8 μg/L, respectively). Hatching and heartbeat per 20 s were recorded every 24 h. Hatching was defined as a complete separation of the juvenile from the capsule. Eggs were examined under a dissecting microscope to count heartbeat. Because of the rotational behavior of embryos, heartbeat could usually be kept in view for 20 s. Heartbeats per 20 s were recorded and therefore lasted 20 s from the moment that the heartbeat was visible. Those recordings that lasted for less than 20 s were excluded.

Statistical analysis

SPSS 13.0 was used to test the significance of treatments using ANOVA, followed by Tukey post hoc testing in the case of multiple comparisons. We used a logistic regression to test for the effects of the factors cadmium treatment and egg type (i.e., isolated eggs or intact egg mass), and their interaction, on the dependent nominal variable mortality (with replicate nested within treatment).

Results

Protective role of egg masses

Eggs measured on average 0.93±0.02 by 0.69±0.02 mm (mean±SD, n=50). There was a clear effect of the presence of cadmium on mortality (cadmium treatment: χ^2=120.75, df=2, P<0.0001). As can be seen in Fig. 1, no isolated eggs survived in all cadmium treatments, while the mortality of embryos in the egg masses was less than 30 % in both treatments of 75 and 150 μg/L (4.5 and 25.6 %, respectively). On the other hand, the logistic regression revealed no significant difference of the factor egg type (χ^2=2.43, df=1, P=0.119); there was a significant interaction between the two factors (cadmium treatment × egg type: χ^2=50.39, df=2, P<0.0001). This indicates, as can be seen in Fig. 1, that cadmium has a detrimental effect on both isolated eggs and intact egg masses, but that the effect is much stronger and becomes evident at a lower concentration in the former eggs. Finally, the replicates differed significantly from each other (replicate nested in cadmium treatment: χ^2=17.49, df=6, P=0.008), which was due to differences in egg masses, not isolated eggs. A predictable phenomenon was observed that most of the dead embryos existed at the edge of the egg masses where the

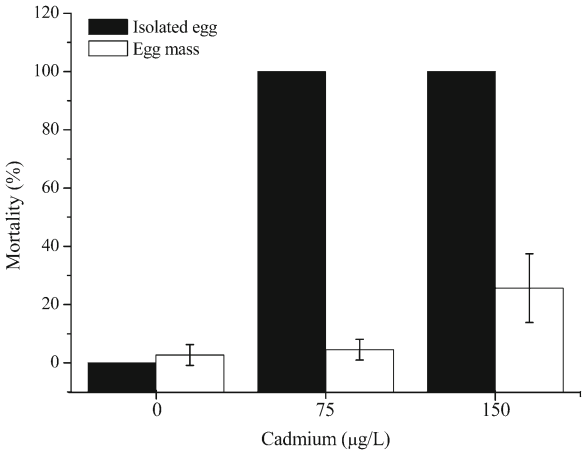

Fig. 1 Different responses of isolated eggs and intact egg masses to cadmium. Data are shown as mean±SD; see text for statistics

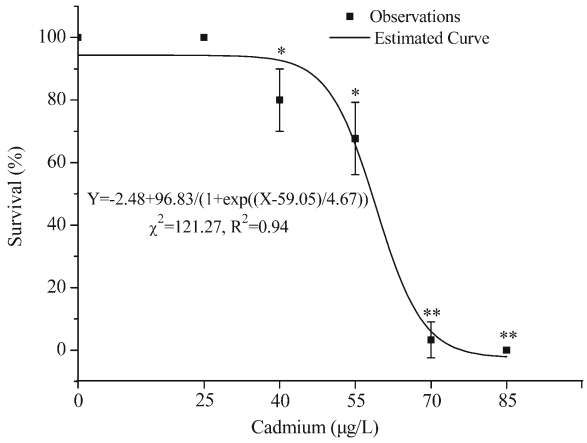

Fig. 2 Changes in survival of isolated eggs after the 96 h exposure. Data are shown as mean±SD and significant difference between treatments are indicated with asterisks (* $P<0.05$; ** $P<0.01$)

gelatinous matter was thinner. No mortality was observed in the control of isolated eggs, while a mortality of 2.7 % was seen in the control of intact egg masses; this difference was not significant (χ^2 test: $\chi^2=0.434$, $df=1$, $P=0.510$) but is possibly due to organisms (annelids) that were observed in a few egg masses.

Acute toxicity

Isolated eggs were used in the acute toxicity test. After the 96-h period of exposure, there was a significant dose–response decrease in the survival of isolated eggs exposed to 40–85 μg/L cadmium (ANOVA, $F_{5,12}=140.925$, $P<0.001$; Tukey post hoc test, $P<0.05$ at 40–55 μg/L and $P<0.01$ at 70–85 μg/L) compared to the control (Fig. 2). The 96-h LC_{50} derived from the estimated equation (Fig. 2) in our study was 58.26 μg/L. No mortality was observed in the control.

Subchronic toxicity

The normal hatching period is 12–14 days at the experimental temperature for this species. The embryos used in the present study had developed for 5 days, including the days when they were collected and acclimatized. Hence, the hatching rate reached its peak (93.7 %) in the control at day 7 after exposure, exhibiting a good synchrony with the expected hatching period. The synchrony could be attributed to the isolation procedure. Within the 5-μg/L cadmium treatment, hatching rate decreased to 90.0 %, which showed no significant difference from the control; however, administration of cadmium at 25 μg/L significantly reduced the hatching rate of the isolated eggs compared to the control (ANOVA: $F_{2,6}=16.964$, $P=0.003$; Tukey post hoc test, $P=0.850$ at 5 μg/L and $P=0.004$ at 25 μg/L; Fig. 3). No developmental inhibition was observed in all treatments, but delayed hatching occurred in all treatments.

In this test, we also measured the heartbeat per 20 s. After 5 days of exposure, regular heartbeat can be observed in all groups. The results indicated that the heartbeat of embryos was 27.77±2.69 times per 20 s in the control. The 5 and 25 μg/L cadmium reduced heartbeats at 24.28±2.37 and 21.89±3.74 times,

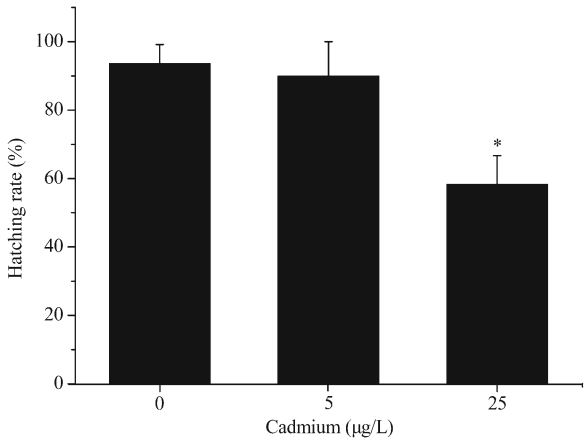

Fig. 3 Effect of cadmium on hatching rate of isolated pond snail eggs. Data are shown as mean±SD, and significant difference between treatments are indicated with asterisks (*$P<0.01$)

respectively. All treatments showed significant differences compared with the control (ANOVA: $F_{2, 55}$=19.928, P<0.001; Tukey post hoc test, P<0.001 at 5 μg/L and P<0.001 at 25 μg/L; Fig. 4).

Discussion

Separation and egg size

To the best of our knowledge, no quantitative measurement has been done on the egg size of *R. auricularia*. Our results indicated that the egg of *R. auricularia* is similar in size to that of *Lymnaea acuminata* (i.e., 1.18×0.64 mm: Pande et al. 2010). Like in most other pond snail species, the egg is transparent, making embryogenesis observation easy (as in embryos of medaka and zebra fish observed by Kimmel et al. 1995; Iwamatsu 2004, respectively). Furthermore, efforts in the isolation procedure get rid of the gelatinous materials, allowing us to determine the heartbeat conveniently in the subchronic test. No eggs were damaged in our procedure indicating that eggs were able to easily survive this isolation procedure.

Protective role of egg masses

Pond snail embryos have been the subject of many studies, but in most cases, toxic effects, such as hatching, were determined for intact egg masses (Gomot 1998; Khangarot and Das 2010; Das and Khangarot 2011). The protective design of egg masses limits the sensitivity of snail embryos to some extent, so we could get a more reliable method for toxicity assay and risk assessment adopting the isolation procedure. The differences between isolated eggs and intact egg masses in their responses to cadmium convincingly confirm the protective role of the gelatinous matrix of egg masses. Clearly, the gelatin matrix around eggs has evolved to protect the eggs against threats from the environment during their development. In terms of mortality, isolated eggs are much more sensitive to cadmium than intact egg masses. A previous study developed an embryo test system using divided apple snail egg masses, and the sensitivity of the test turned out to be equal to or even higher than other test species and systems/endpoints (Schirling et al. 2006). However, the study did not focus on the protective role of the egg mass. Gomot (1998) reported that the inhibition by cadmium was much stronger at the edges of the egg mass than at the center, which implied the occurrence of a diffusion gradient of cadmium in the material surrounding the eggs. This is consistent with our observations.

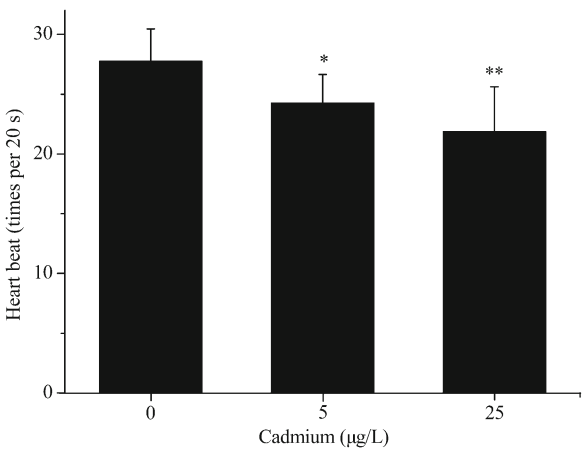

Fig. 4 Effect of cadmium on heart rate of isolated pond snail eggs. Data are shown as mean±SD, and significant difference between treatments are indicated with asterisks (*P<0.01; **P<0.001)

Egg masses of this species are frequently infected by parasites in the field. These infected eggs could be discarded by the isolation procedure. Hence, the isolated eggs seem better suitable for toxic assays and risk assessment since they lower the individual differences between developing eggs. Nevertheless, eggs are deposited in egg masses in the natural environment, so one needs to keep in mind that the ecologically relevant measure of toxicity will be how eggs are affected when they are still inside the egg mass. No significant difference was exhibited between the isolated eggs and intact egg masses in the control, which indicates that the isolation procedure had little adverse effect on the mortality of embryos. Knowing this, the acute and subchronic toxicity tests were applied in order to quantify the sensitivity of the isolated pond snail eggs to cadmium.

Acute toxicity

Extremely high sensitivity of isolated pond snail eggs to cadmium was determined from the results of the acute toxicity test. The value of the 96-h LC_{50} was

58.26 μg/L cadmium. Due to inadequate literature, we cannot compare our results to previous studies of isolated pond snail eggs. But previous research with apple snails revealed that the lethal concentration for embryos was up to 500 μg/L cadmium (Schirling et al. 2006), which indicated that isolated pond snail eggs were more sensitive to cadmium than apple snails. In comparison to the embryo test with medaka, *Oryzias latipes*, the sensitivity of pond snails is high, as Chen et al. (2001) reported a 96-h LC_{50} value of 0.3 mg/L cadmium. Previous work on the embryo of *Daphnia magna* with a 96-h LC_{50} value of >1 mg/L cadmium (Bodar et al. 1989) also supports the higher sensitivity of the isolated pond snail eggs than other species. Some polluted waters nearby mining areas have cadmium concentrations that range from 65 to 240 μg/L and are thus the same or higher than the LC_{50} value that we find here (Florea et al. 2005; Oporto and Carlo Smolders 2007). Therefore, the endpoint 96-h LC_{50} could be proposed as a valuable tool to assess cadmium contamination nearby mining areas.

Subchronic toxicity

Sublethal responses are commonly more sensitive parameters than survival (Dave and Xiu 1991; Hollert et al. 2003), which is consistent with our results. Hatchability is one of the most frequently used response variables in early life stage studies (Dave and Xiu 1991). In the present study, 25 μg/L cadmium significantly inhibited the hatchability of isolated pond snail eggs. A delayed hatching in isolated apple snail embryos was reported in up to 250 μg/L cadmium (Schirling et al. 2006), and the delay was observed in *Lymnaea stagnalis* embryos embedded in intact egg masses for cadmium concentrations between 25 and 100 μg/L (Gomot 1998). Other studies also reported higher effective concentrations in hatching delay of pond snails than the concentrations used in our experiment (Coeurdassier et al. 2003). Besides, the procedure of isolation resulted in a more synchronous hatching, as hatching time correlates with the position of eggs within egg masses (Marois and Croll 1991). Therefore, using isolated eggs can reduce the individual differences when hatchability is taken as an endpoint.

Heart rate can be negatively affected by cadmium in aquatic organisms, especially in their embryos. In this study, the exposure to 5 μg/L cadmium induced a significant decrease in heart rate during the development of isolated eggs. A similar effect was also observed in zebra fish embryos, where the cadmium concentration was up to 250 μg/L (Hallare et al. 2005). To the best of our knowledge, no lower effective concentrations than 5 μg/L were documented in previous studies on embryo test models. The molluscan heart is myogenic, but its beating is regulated by the central nervous system (Yasuo 1987; Buckett et al. 1990). Cadmium could replace Ca^{2+} in Ca^{2+}-binding proteins which are essential to the normal functioning of the nervous system (Kerschbaum et al. 1997; Thompson and Bannigan 2008). Such replacement may disrupt the normal function of the central nervous system and thus affect the heart rate. The maximum permissible concentration of cadmium in drinking water is 5 μg/L (0.005 mg/L) in China (Water Quality Standard for Drinking Water, GB5749-2006) and Europe (Drinking Water Directive) and 3 μg/L according to the World Health Organization guidelines. So, the response of heart rate in isolated eggs to cadmium is probably suitable for the assessment of drinking water in China and/or in Europe. In less polluted waters (compared to waters nearby mining areas), cadmium was observed in concentrations of 4 μg/L in freshwater (Jain et al. 2005) up to 12 μg/L in seawater (Kobayashi and Okamura 2004; Filosto et al. 2008). The response of heart rate could be capable of detecting cadmium pollution in these areas.

Overall, we regard our results as elevating the sensitivity of the embryo test model to cadmium and the embryo test with *R. auricularia* to be efficient in toxicity assays and risk assessment. Similar to *R. auricularia*, other species in family Lymnaeidae spawn egg masses. Therefore, the application of the present model would probably expand to the Lymnaeidae. Future studies should focus on validation of the model by examining its ability to predict toxicity of cadmium in a wide range of natural waters and expansion of the model within family Lymnaeidae.

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