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Effects of copper on the egg development and hatching of a freshwater pulmonate snail *Lymnaea luteola* L.

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ABSTRACT

A freshwater invertebrate egg development and hatching toxicity test with an Indian freshwater pulmonate snail, *Lymnaea luteola*, comprising the following developmental endpoints was described: mortality, development, formation of eyes and foot structure, heart rate, duration of different larval stages, and hatching time. Developmental stages were morula, and at third, fifth, and eighth days; the trochophore, veliger, and hippo larvae, respectively. At the age of about 9th to 11th days after egg laying; more than 95% young snail hatched in control laboratory conditions. To evaluate effects on embryonic development, the pulmonate snail eggs of 24-h old were exposed to a series of nominal copper concentrations. The percentage survival of embryos treated in 10–32 $\mu g\,l^{-1}$ of Cu after 240 h of exposure drops sharply at veliger and hippo stages. All embryos died at 100–320 $\mu g\,l^{-1}$ of Cu within 168 h of exposure at trochophore and early veliger stages. The detected abnormalities were malformation of foot, eyes, thinness and incomplete formation of shell, growth retardation, and slow rotation of embryo within the egg capsule as compared to control embryos. Lethal and sublethal effects in terms of mortality and significant delay in hatching could be found in the 3.2, 5.6 and 10 $\mu g\,l^{-1}$ of Cu concentrations. This species is widely distributed in the Indian subcontinent freshwater reservoirs and more sensitive to Cu than other tested aquatic test organisms; therefore, could be used as a test model of Cu and possibly other pollutants for rapid risk assessment of environmental pollutants. The snail egg embryo bioassay is simple, rapid, highly sensitive, cost-effective, and easy to test under standardized laboratory conditions.

1. Introduction

Embryo-larval toxicity bioassays have grown to be important tools for environmental risk and hazard assessment in recent years [1–3]. The common pond snail is a freshwater species widely used in embryological and ecotoxicological studies [4–7]. The pulmonate snail *Lymnaea luteola* (L.) is a subtropical grazing omnivore and abundantly found in Indian subcontinent freshwater rivers, ponds and lakes and has been earlier successfully used in aquatic environmental pollution studies [8,9]. Embryogenesis in pond snail may be used as an inexpensive and readily available model for embryo-larval lethal and sublethal toxicity experiments [10,11]. Tripathi and Singh [12] reported the effects of cypermethrin and alphamethrin insecticides on the reproduction, hatching, and embryogenesis of an Indian freshwater pulmonate snail *Lymnaea acuminata*. Furthermore, *L. luteola* egg assay can also serve as a model species for an invertebrate embryo test, as, e.g., the subtropical zebra fish, *Danio rerio* for fish and vertebrates [13,14]. There are only a few standardized test protocols like Dar'T or AMPHITOX for vertebrates [15], while such tests are lacking for invertebrates. Pond snail *L. luteola* eggs are transparent, which provides an opportunity to follow the development of embryo from zygote to hatching stage. Embryo toxicity tests have several advantages such as they required short duration in comparison of life-cycle tests, easy to perform under laboratory conditions, simple, rapid, cost-effective, and more importantly; they cover highly sensitive stages of development. So far, only a few studies have been reported on effects of heavy metals on freshwater snail embryos with particular reference to *L. stagnalis* [16–18]. The detailed information concerning the effects and mechanisms of action of heavy metals and industrial chemicals in invertebrates developing stages has been obtained only for a few freshwater invertebrate species, although invertebrates represent more than 95% of the known species in the animal kingdom [19].

Copper, like iron, is an essential transition metal ion for plants, animals and human health because it is useful in a number of catalytic and transport functions in living cells and their organelles, particularly the mitochondria [20]. Even the trace excess copper in the living cells causes toxicity due to formation of reactive oxygen species which can damage lipids, nucleic acids and

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proteins [21]. Copper enters into the aquatic environment through a wide variety of industrial effluents, mining wastes, fossil-fuel combustion, and municipal sludge sewage disposal sources [22]. Copper has been reported to often occurring freshwater habitat at concentrations of approximately 2–3 $\mu g\,l^{-1}$ [23]. Excess amount of Cu, like other toxic heavy metals was reported form Indian freshwater reservoirs [24,25] and its widespread uses generated much considerable research interests [26]. U.S. EPA [27] recommended 12–43 $\mu g\,l^{-1}$ of Cu for freshwater organisms. Although human can tolerate generally high levels of copper (1.5 $mg\,l^{-1}$ of Cu, WHO drinking water limit), a vast majority of organisms of aquatic ecosystem, unfortunately would not tolerate or survive at such high levels of copper [8,28]. Copper concentrations generally ranged from 1.3 to 17.5 $\mu g\,l^{-1}$ in streams and values of up to 100 $\mu g\,l^{-1}$ have been occasionally measured in extremely polluted streams [29]. Even a mining effluent discharge standard of 50 $\mu g\,l^{-1}$ of Cu [27] may endanger to sensitive aquatic organisms [22].

Lymnaeids are world widely distributed [30] and Indian freshwater pond snail *L. luteola* acts as an intermediate host of trematode parasites, the causative agent of helminth diseases [31]. The morphological and behavioral aspects of the development of *Lymnaea* species have been well described [32–34]. The acute toxicity of copper is higher than that of cadmium for *L. luteola* [8], but to our knowledge, no reports have appeared regarding toxic effects of Cu on the egg development and hatching. Therefore, the present study was designed to help to fill this knowledge gap. This paper serves to introduce Indian freshwater hermaphroditic pulmonate snail *L. luteola* (L.) as a test organism for toxicity bioassays using egg embryos since they reproduce easily under standard laboratory conditions. Our present study focused on effects of Cu on embryo mortality at various stages of development, growth retardation, developmental arrest, malformation of organs, and hatchability of an Indian pond snail *L. luteola*. Test concentrations were chosen in the experiment, which are reportedly found in the natural and industrial polluted waters [26].

2. Materials and methods

2.1. Snail collection and culture

Freshwater pulmonate snail *L. luteola* L. (Gastropoda: Lymnaeidae) is commonly found in stagnant and slowly running freshwater of India, Pakistan, and Bangladesh. Pond snails were collected from unpolluted fish ponds situated at Gheru Campus of Indian Institute of Toxicology Research (IITR), Lucknow. Adult snails (150 snails) were cultured in glass aquaria (size – 40 l) for 14 days before experimentation at temperature 21 ± 1 °C and fed daily *ad libitum* aquatic plant (*Marsilia* sp.) leaves during acclimation period. They had an average wet weight of 0.5 g (range, 0.45–0.75 g) and shell length 21 mm (range, 19–25 mm). Lymnaeid snails laid egg masses on the aquarium walls and to the back surface of *Marsilia* plant leaves. Snail eggs of 0–24 h old were collected and incubated in sterile Petri plates containing underground (well) water (pH 7.2).

2.2. Snail egg embryos

The eggs were deposited in egg masses (egg jelly) and each containing about 30–100 egg capsules which were randomly mixed before they were selected for toxicity testing. Egg masses containing between 30 and 40 embryos were placed in Petri plates. Each copper concentration was tested in six replications. Thus, the effect of Cu on embryonic development was evaluated using approximately 180–250 eggs per Cu concentration. Egg masses were gently dislodged and suctioned into a 10-ml pipette, which had its tip removed. In each Cu concentration, one egg mass was used. The egg capsules were not freed from the egg mass. Disposable sterile polycarbonate Petri plates (size – 90 mm diameter × 15 mm height; make – Axygen® Scientific, Inc., Bath, BA2 9AP, UK) containing one egg mass was gently filled with test water (50 ml) and allowed to sink to the bottom. There might be some adsorption of Cu on the wall of the Petri plates, therefore it is suggested that it could be better to use glass plates or any other suitable material to reduce adsorption of pollutants during the exposure period.

2.3. Test compound and exposure conditions

A stock solution of copper (as $CuSO_4{\cdot}5H_2O$, E. Merck, India) in double distilled water was prepared and from this solution; a series of log-based Cu concentrations were prepared in underground water. The nominal Cu concentrations of 0, 1, 3.2, 5.6, 10, 18, 32, 56, 100, 180 and 320 $\mu g\,l^{-1}$ were used in the semi-static embryo toxicity tests. At the start of copper exposure, 24-h-old embryos were used in the semi-renewal static bioassay tests as outlined in standard methods [35] were carried out in 50 ml Petri plates for 14 days in an environmental growth chamber at 21 ± 1 °C. Control experiments were run in the underground water without addition of nominal Cu concentration. The test medium was replaced at interval of twice a week. Test water pH was measured just before and after the renewal of test solution. During the study, dissolved oxygen concentrations were 5.9 $mg\,l^{-1}$ or greater, pH ranged from 7.1 to 7.4, alkalinity ranged from 180 to 210 $mg\,l^{-1}$ and total hardness ranged from 220 to 240 $mg\,l^{-1}$ as $CaCO_3$. Mean and range of selected heavy metals ($mg\,l^{-1}$) in control test water (underground water) were Zn, 0.054 (0.041–0.065); Cu, 0.043 (0.031–0.056); Ni, 0.040 (0.032–0.068); Fe, 0.040 (0.025–0.059); Cd, 0.006 (0.004–0.008); and Cr, 0.004 (0.001–0.031). Heavy metals were analyzed using a simultaneous multi-element Atomic Absorption Spectrophotometer (PerkinElmer analyst 300, USA). The added low concentrations of Cu had no effect on the pH of test water. Glass aquaria water also contains the same underground water in which snails were cultured. The measurement of temperature, dissolved oxygen, total hardness and alkalinity parameters were determined by routine procedure [35]. Photoperiod was controlled to simulate the natural day:light (12:12 h) cycle. Fluorescent light with two 48 W lamps was used. Food was not offered to snail egg embryos during the test period.

2.4. Assessment of toxic effects on snail embryos

The following parameters are most feasible and recorded daily or at least at the suggested days of development as described in Tables 1 and 2. From day 3 onwards, formation of foot; from day 4 onwards, formation of the eyes; and at day 8 heart rate measured. From day 8 onwards, the Petri plates were checked for hatching. Furthermore, the mortality recorded daily from day 3 onwards, when eggs start to clear up and become transparent. The mortality was defined by coagulation of the embryo or cessation of the heartbeat and no movement or rotation of embryo within the egg capsule. Developing embryo within the egg capsule was observed from the third day old stage until hatching of the young snail or till the end of experiment. Inverted microscope (make – Olympus Japan; model – IX 170) was used to determine the following endpoints: mortality (%), malformation (%), formation of eyes, foot structure and tentacles (%), heartbeat rate (min^{-1}), shell length (mm) and hatching success (%). Maximum shell length was measured with an ocular micrometer with a precision of ±0.005 mm. Furthermore, the wet weight was recorded after hatching of newly young snail. The weights of the snails were measured after transfer of the snails from the Petri plate to the surface of the filter paper. After 1 min the adhesive water was removed completely

Table 1
Embryonic growth, stages of development and characteristics feature in *L. luteola*.

Embryonic stage	Duration	Characteristics features
1. Morula	60–72 h (2.5–3 days)	Embryo is yellow, very slow motions
2. Trochophore	Starts after 3 days last between 48 and 60 h (2–2.5 days)	Clear to whitish, "grainy" embryo, uninterrupted rotation, prototroch rises over body surface, embryo transparent, embryo is still rounded
3. Veliger stage	Starts after 5 days lasts for 48 h (2 days)	Eyes reddish dark in color can be seen head, foot region can be demarcated, rotation irregular, embryo loss its round shape, soft body and shell can be seen
4. Hippo stage	Starts after 7 days last between 48 and 72 h (2–3 days)	Complete metamorphosis to young snail embryo fully developed foot is used for locomotion, sporadic rotation, shell cover the whole visceral complex, eyes and heartbeat can be seen, shell and foot clearly separated. Foot reaches the shell at posterior end. The eyes are dark. Shell is coiled

Note: Based on information extracted from Lalah et al. [7], Morrill [11], Sarkar et al. [31], and Meshcheryakov [34].

and respectively 10 individuals were pooled and weighted at a time on an analytical balance having readability of 0.1 mg (make – Sartorius; model – Precisa XB-120A). The normal and abnormal development of the embryo was noted and photographed by digital color CCD camera (make – JVC Thailand; model – TK C241 EG) and stored in computer hard disk. Embryogenesis inhibition was defined as embryo development blocked at one stage for more than 2–3 days and very slow or no movement of the embryo in the egg capsule was observed. The duration of the incubation and the number of hatchlings were also noted for each Cu-treated and control groups.

2.5. Statistical analysis

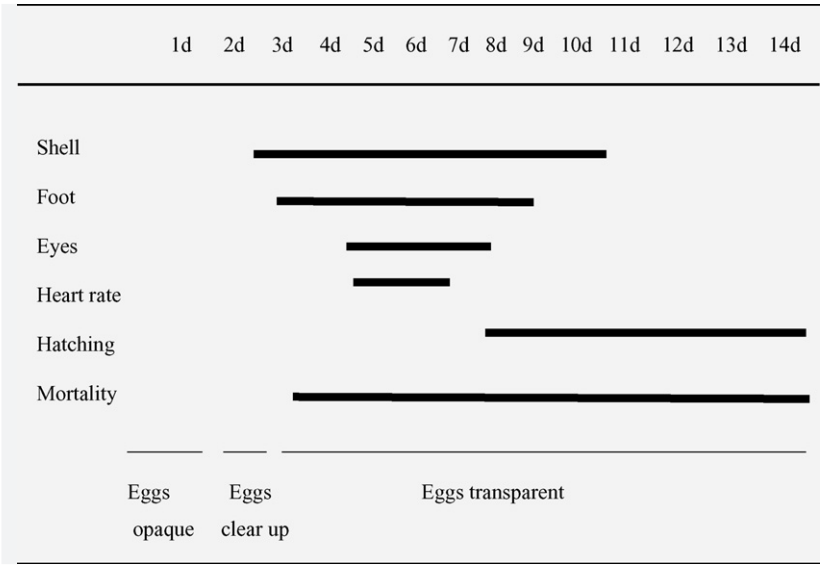
For each Cu treatment and controls, at least six replicates ($n = 6$) of egg embryo tests were investigated. For control, mortality less than 10% was accepted. Data were given as means ± standard deviation. At the end of the experiment, the calculated percentage of hatchings in Cu-exposed groups was lower compared with that of the controls. The EC_{50} values (effective concentration at which 50% embryo immobilization or hatching responses were recorded) and their 95% confidence limits were calculated by the moving-average-angle method [36]. Student's *t*-test was used to determine the significance of differences between Cu-exposed and Cu-unexposed snail egg embryos [37]. The α-level for significant differences was set at $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

3. Results

3.1. Normal egg embryo development in control groups

Pond snail *L. luteola* laid eggs in elongated egg masses, which at the beginning of the reproductive period contain several eggs. Each egg is located in an egg capsule. An extensive description of normal developmental stages of Indian and European freshwater pulmonate snails (*Lymnaea* spp.) has been previously published elsewhere [10,11,34]. Briefly, the different stages of growth and development in *L. luteola* and some of the characteristic features are summarized in Table 1. In the present study, we were able to identify four common stages of development and use them to investigate the toxic effects exposing egg masses to copper. The normal control groups, four developmental stages of *L. luteola* were illustrated in Fig. 1. As proposed for *L. stagnalis* [4] and in accordance with our findings, the following embryonic stages were recorded: (1) morula: embryo leaves the vitelline membrane and moves freely within the egg capsule by means of cilia (Fig. 1a); (2) trochophore: the shell gland and prototroch developed and embryo rotate within the egg capsule (Fig. 1b); (3) veliger: stage at

Table 2
Schedule to investigate different parameters of embryonic development of Indian pond snail *Lymnaea luteola*.



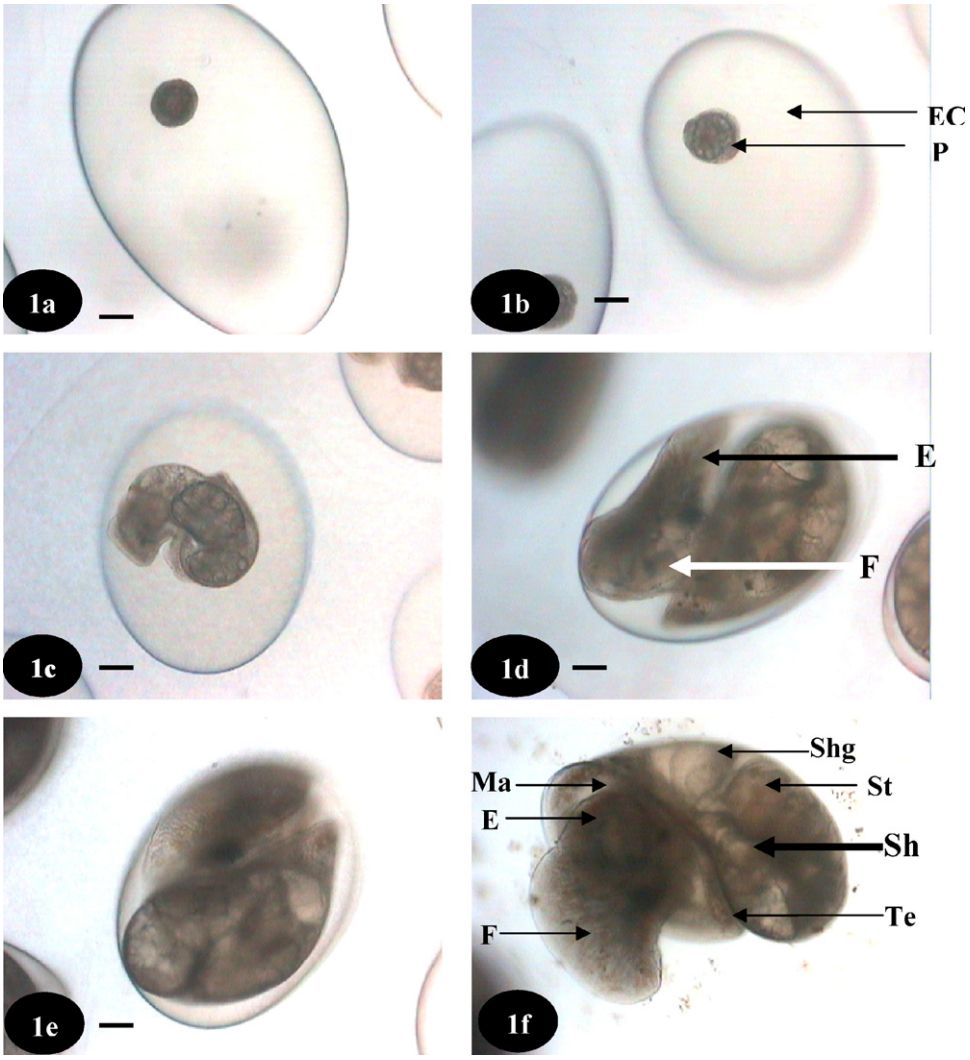


Fig. 1. Normal developmental stages of the pond snail *Lymnaea luteola*. (a) 2-day-old morula stage appeared in round shape, (b) at 3-day old trochophore larval stage foot and shell formation begins. Shell gland rudiment looks like a small depression, (c) early veliger larva (4–5 days old) showing shell and foot formation, (d) 6-day-old late veliger, showing well developed foot, shell, eyes, tentacles and digestive gland, (e) 7-day-old hippo stage occupying the whole space in the egg capsule, and (f) newly hatched snail (magnification 10 × 10). E, eye; F, foot; Ma, mantle; Ec, egg capsule; Sh, shell; Shg, shell gland; St, stomach; P, Prototroch; Te, tentacle. Scale for a–f, 0.2 mm.

which foot, eyes, and tentacles and regular beating of heart could be observed (Fig. 1c–d); (4) Hippo or veliconcha: the foot and viscera were well separated (Fig. 1e). The eyes of the embryo could be distinguished; and embryo fully occupied the whole egg capsule and ready for hatching. The Petri plates were checked for hatching from day 8 onwards (Fig. 1f). The developmental endpoints studied at different periods of Cu exposure are described in Table 2.

3.2. Egg embryo mortality

Egg masses were examined to determine the percentage of dead embryos (embryo lethality), the incidence of malformed snail embryos (teratogenicity %) and the percentage of hatching delay until 14 days after Cu exposure. All embryos, which developed further after copper exposure, normally or abnormally, were regarded as survivors. Cumulative percent mortality and EC_{50} values (50% embryo mortality) and their 95% confidence limits are presented in Table 3. The 96 h and 240 h EC_{50} values and 95% confidence limits calculated were 28.31 (21.86–36.64) and 18.19 (16.72–19.26) $\mu g\,l^{-1}$ of Cu, respectively. Survival of eggs drastically decreased and incidence of malformation and abnormality increased with the increase in Cu concentrations from 10 to 100 $\mu g\,l^{-1}$ of Cu. Less than 1% mortality appeared in control and 1 $\mu g\,l^{-1}$ of Cu-treated groups in 14th days of the experimental period, whereas nearly 85%, 67.4%, and 18.5% mortalities were observed at 32, 18, and 10 $\mu g\,l^{-1}$ of Cu, respectively. Thus, a dose-dependant relationship was observed for egg embryo mortality exposed in a series of increasing concentrations of Cu. Mean survival at 1, 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu after 14 days of exposure was significantly higher than 18, 32, and 100 $\mu g\,l^{-1}$ of Cu concentration.

3.3. Abnormal egg development in Cu concentrations

At higher Cu concentrations (180 and 320 $\mu g\,l^{-1}$ of Cu) none of the snail eggs survived after 48 h of exposure and all the snail eggs died before reaching to trochophore stage; while at 100 $\mu g\,l^{-1}$ of Cu all snail embryos died at veliger stage and no hatching occurred. From 18 to 180 $\mu g\,l^{-1}$ of Cu, development is often blocked at the trochophore stage with only a very few number of the embryos reached the late veliger and hippo stages and again no hatching occurred. At 100 $\mu g\,l^{-1}$ of Cu, most of the embryos died within 96 h of exposure, they never develop normally, and all of them were with abnormal shell and digestive gland and arrested development (Fig. 2a). Normal trochophore and veliger stages were never been observed at 100 $\mu g\,l^{-1}$. Some of the snail eggs abnormally devel-

Table 3
Effects of copper on percent mortality of snail embryos at different periods of exposure.

Nominal Cu conc. ($\mu g\,l^{-1}$)	Measured mean Cu conc. ($\mu g\,l^{-1}$)	Percent mortality at					
		24 h	48 h	96 h	168 h	240 h	336 h
320	197	100	100	100	100	100	100
180	164	20	60	100	100	100	100
100	119	–	6.7	73.6	100	100	100
32	51	–	–	12.5	25	72	85
18	35	–	–	10.7	17.3	53.3	67.4
10	30	–	–	–	–	50	67
5.6	14	–	–	–	–	15.7	18.5
3.2	8	–	–	–	–	–	1.8
1.0	4	–	–	–	–	–	0.47
Control	2.5	–	–	–	–	–	0.56
EC_{50} and 95%				28.31		18.19	15.92
C.L. ($\mu g\,l^{-1}$ of Cu)				21.86–36.64[a]		16.72–19.26	14.97–17.16

[a] C.L.: confidence limits.

oped with malformation of shell and foot structure during 7 days of exposure up to late trochophore and veliger stages in 32 $\mu g\,l^{-1}$ of Cu before death occurred (Fig. 2b). All embryos which developed further after Cu treatment, normally or abnormally were regarded as survivors. At 10 $\mu g\,l^{-1}$ of Cu after 7 and 10 days; numerous egg embryos become highly abnormal in shape, size, and morphological features, the formation of foot, shell, shell gland were not clear at veliger stage and embryos were smaller in size as compared to control embryos (Fig. 2c–d). At lower Cu concentrations, snail embryos show a higher percentage of survival, but many of them become abnormal. At day 10, embryos in 3.2 and 5.6 $mg\,l^{-1}$ of Cu experiments showed a trend to a delayed development in comparison to the controls as symbolized by a reduced mean percentage of visible eyes, and shell formation, shell thinness, digestive gland, slow rotation of embryo in egg capsule, and undeveloped foot and tentacles structures (Fig. 2e and f). Dead cells are extruded from the small-celled foot and head structures, and from the mantle ridge observed at 3.2 $\mu g\,l^{-1}$ of Cu of after 10 days of exposure. The close contact of foot and mantle ridge and shell edge disappears in many snail embryos. At 1 $\mu g\,l^{-1}$ of Cu, growth retardation and delay in hatching was observed at 14 days exposure as compared to control groups (Fig. 2g and h).

3.4. Developmental delay and malformation

Effects on developmental stages were made every day from the start of experiment. On the seventh day of Cu exposure, 90% of the embryos in control tests were in hippo and 10% were in veliger stages, whereas in 5.6 $\mu g\,l^{-1}$ of Cu-exposed egg masses, 20% of the surviving embryos were in hippo, 35% in veliger, 35% in trochophore, and 10% in morula stages (Fig. 3a). At lower Cu concentrations (1 and 3.2 $\mu g\,l^{-1}$ of Cu) after 7 days of Cu exposure, most of the embryos were in hippo and veliger stage and all survived, appeared healthy and show normal rotation in the egg capsule. These observations suggested that over the seventh day growth period, development was significantly delayed for 2–4 days at 5.6 and above Cu concentrations. On the 10th day, all snail embryos hatched in control groups. However, at 10 $\mu g\,l^{-1}$ of Cu, only 10% embryo reached at hatching stage and remaining 90% were at hippo, veliger, trochophore and morula stages (Fig. 3b). On the 9th day, all most all the unhatched embryos appeared in the hippo stage in control experiments compared to about 17% in copper (from 1 to 18 $\mu g\,l^{-1}$ of Cu) treated embryos.

The percentage of individuals reaching to veliger and hippo stages in 6–8 days was higher in control. At 10 $\mu g\,l^{-1}$ of Cu, most of the snail eggs remained in morula, trochophore and veliger stages after 7 and 10 days of exposure (Fig. 3a and b). The maximum percentage number of snail eggs remained in trochophore, veliger and hippo stages for 2–7 days at 3.2–10 $\mu g\,l^{-1}$ of Cu without showing further development. As Cu concentrations increases; the percentage of veliger and hippo stages drastically decreases. In control, eggs development reached to next higher stage in shorter duration (2–4 days) than in higher Cu concentrations (5.6 and 10 $\mu g\,l^{-1}$) treated groups. At 10 and 18 $mg\,l^{-1}$ of Cu exposure, delay in formation of eyes, tentacles, foot structure, shell gland, and shell was observed at veliger stages.

In 90% of the embryos in control groups, the trochophore stage lasted for less than 3 days, while trochophore stage in 80% of the 5.6, 10 and 32 $\mu g\,l^{-1}$ of Cu-treated groups lasted for more than 3.5–5 days. Similarly, the duration of the veliger stage was less than 3 days in control. At 3.2, 5.6 and 10 $\mu g\,l^{-1}$ of Cu more than 75% embryos remained in veliger stage for 3–6 days and further development to next hippo stage considerably delayed. Inhibition of embryogenesis has been observed at different stages of development after 10 days of copper exposure. At 14 days, 99% embryos hatched in control; while in 3.2 $\mu g\,l^{-1}$ of Cu, nearly 40% were still in hippo stage, respectively. The duration of the hippo stage was less than 2 days in control groups. At 5.6 and 10 $\mu g\,l^{-1}$ of Cu several egg embryos remained living at hippo stage for longer periods but most of the snail embryos failed to hatch in 14 days of experimental period.

The percentage of malformed snail embryo increases in a dose-dependant manner. As Cu concentrations increases from 3.2 to 10 $\mu g\,l^{-1}$ of Cu, the number of malformed embryos increases significantly. At 1 $\mu g\,l^{-1}$ of Cu and in control no malformation was noted during 14 days of study. However, at 3.2, 5.6 and 10 $\mu g\,l^{-1}$ of Cu there were 10%, 37% and 50% malformation, respectively in surviving embryos at 7 days. At 14 days of copper exposure malformation significantly increases with Cu exposure. These results were significantly differed from control groups.

3.5. Effects on hatchling

At 1 $\mu g\,l^{-1}$ of Cu concentration; the normal egg development was noticed and there was no significant difference in percent hatching success when compared to control groups. There was a complete hatching failure at 32 $\mu g\,l^{-1}$ of Cu and 72–85% embryo mortalities, noticed at 10th and 14th days of Cu exposure. The snail embryo in the control group hatched earlier than those exposed to 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu. On the other hand, a significant effect of copper was noticed in the time of hatching. In the copper treated groups (from 1 to 10 $\mu g\,l^{-1}$ of Cu), the cumulative hatching was significantly reduced ($p \leq 0.01$) with an average of 30.66% and at day 10, only 45.4% had hatched at ($p \leq 0.01$) at 14 days. In case of control group average hatching at 10 days was 98% and at 14 days it was 99%. The exposure to 5.6 and 10 $\mu g\,l^{-1}$ of Cu induced a significantly ($p \leq 0.05$, Student's t-test) reduced hatchability after 10 and 14 days Cu exposure, when compared to 1.0 $\mu g\,l^{-1}$ of Cu and control. Furthermore, in control groups, 78.3% hatched successfully

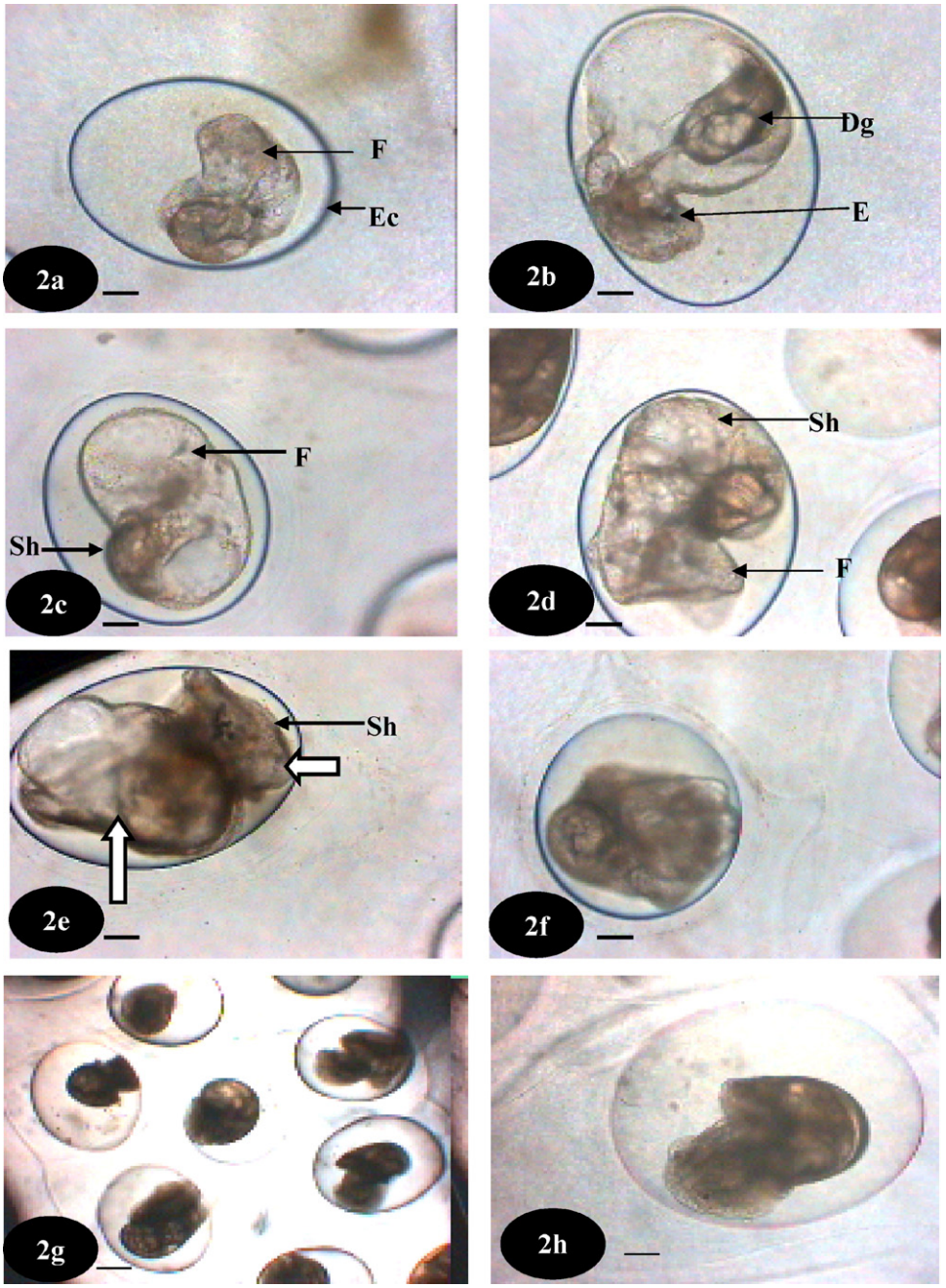


Fig. 2. Microphotographs of snail (*L. luteola*) embryonic stages at different periods of Cu concentrations. (a) At 100 μg l^{-1} of Cu and above after 5 days embryo remains at trochophore stage and the formation of shell, foot and eyes not observed; (b) at 32 μg l^{-1} of Cu after 7 days of exposure most of the embryos were abnormal and remained for longer periods in trochophore and veliger stages; (c and d) at 10 μg l^{-1} of Cu after 7 and 10 days exposure the formation of eyes, shell, shell gland and tentacles were not observed (⟸) shell and digestive gland formation not clear and are abnormal shaped in several egg embryos and majority of the embryos showed the abnormal shape and smaller size as compared to control, embryo is still in early veliger stage; (e and f) abnormal embryos (⟸) observed at 3.2 and 5.6 μg l^{-1} of Cu after 10 days exposure. Note the growth retardation; (g) at 1 μg l^{-1} of Cu after 7 days normal development observed with the growth retardation and delay in hatching; (h) 10-day-old larva after 1 μg l^{-1} of Cu for 10 days of experimental period. Note the abnormal shape, size, malformation and growth retardation. Scale for a–h, 0.2 mm and g, 0.66 mm. Dg, digestive gland; Ec, egg capsule; F, foot; Sh, shell.

(>50% hatching) at 9 days, while at 1.0 and 3.2 μg l^{-1} of Cu, only 45.7% and 25.7% hatching observed, respectively (Table 4). The 10-day EC_{50} value and their 95% confidence limits for snail egg hatching calculated were 2.18 (1.95–2.59) μg l^{-1} of Cu, respectively.

3.6. Effects on newly hatched snail weight

The average weight of newly hatched snails raised in the control group was 155 μg per snail while from the 3.2 and 5.6 μg l^{-1} of Cu had a mean weight of 90 μg and 80 μg per snail (Fig. 4). The highest weight was measured in the control and 1 μg l^{-1} of Cu after recently hatched juvenile snail. A comparison of the mean wet weight of newly hatched juvenile snail after 14 days showed that weight decreased significantly relative to that of control groups. However, a significantly ($p \leq 0.05$, Student's *t*-test) lower weight as compared to the average weight of the controls was recorded at 3.2 and 5.6 μg l^{-1} of Cu.

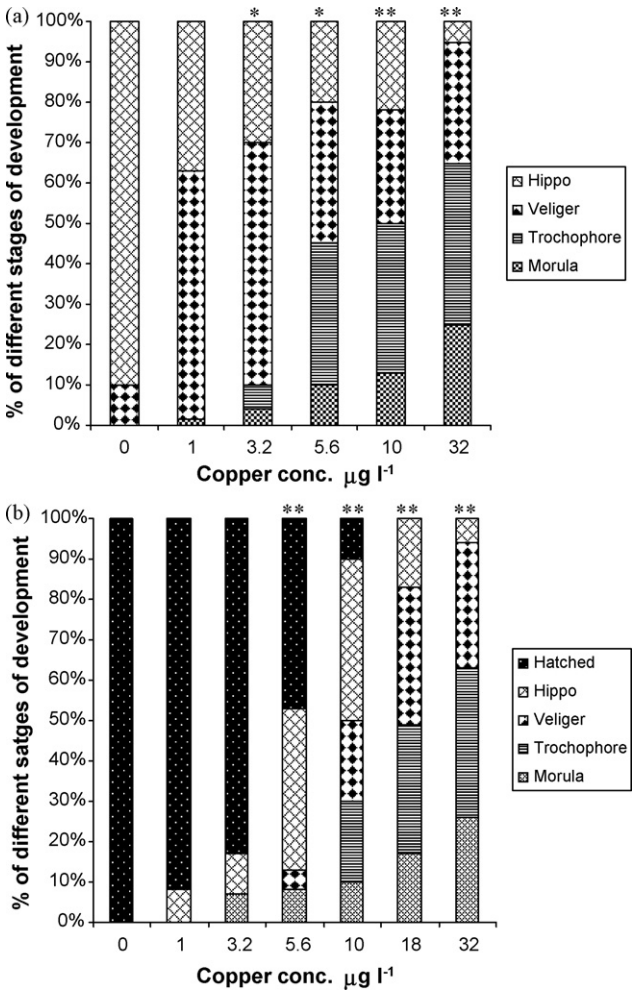


Fig. 3. (a and b) Inhibition of embryogenesis of *L. luteola* of living embryos at various stages of development exposed to increasing concentrations of Cu from 0 to 32 $\mu g\,l^{-1}$ for 7 (a) and 10 days (b) (* and ** indicate the proportion of the stages are significantly different from the control proportions Student's *t*-test, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$).

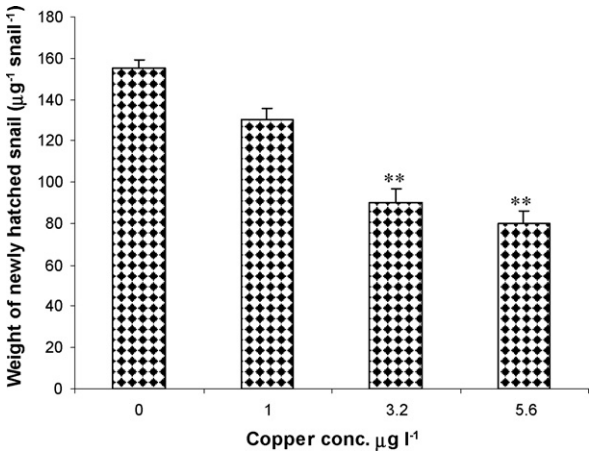


Fig. 4. Effects of control, 1, 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu exposure for 14 days on individual wet weight of newly hatched *L. luteola* (in mg per snail). $^{**}p \leq 0.01$.

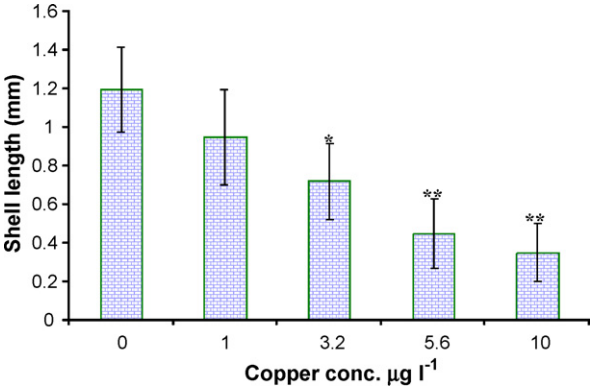


Fig. 5. Effects of increasing concentrations of Cu on shell length of *L. luteola* after 7 days exposure at 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu and control (* and ** indicate the proportion of the stages which are significantly different from the control proportions Student's *t*-test, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$).

3.7. Effects on shell formation

In control group, mean shell length at 7 days was 1.2 mm while in 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu; the shell length was 0.93 mm and 0.45 mm, respectively. Copper treatment had adverse effects on shell length, when determined at 7 days of Cu exposure (Fig. 5). There was a marked decline in shell length due to copper exposure. At 5.6 and 10 $\mu g\,l^{-1}$ of Cu, highly abnormal shell formation and there was delay in shell formation at veliger stage were noticed after 10 days. Copper exposed eggs also showed thin formation of shell, but again this only happens in 3.2 $\mu g\,l^{-1}$ of Cu and above copper exposed groups as compared with the control groups ($p \leq 0.05$).

3.8. Effects on eyes and tentacles formation

In control groups, eyes and tentacles formation starts at veliger stage after 7 days of experimental period (Fig. 6). The formation of eyes and tentacles was delayed for individual snail eggs exposed to 5.6 and 10 $\mu g\,l^{-1}$ of Cu. As copper concentrations increases, a very small brownish spot of eye bud formation was noticed at veliger and hippo stages. In control and 1 $\mu g\,l^{-1}$ of Cu, there was an 80% and 77% tentacle formation was very clear, while at 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu, only 40% and 30% embryos showed tentacles formation.

Table 4
Effects of different copper concentrations on percent hatching of larvae from egg capsule.

Cu conc. ($\mu g\,l^{-1}$)	Percent hatching at			
	8-day	9-day	10-day	14-day
18	–	–	–	–
10	–	–	2.7[a] ± 0.46	5.45[a] ± 1.08
5.6	–	2.88 ± 0.54	14.76[a] ± 1.23	32.43[a] ± 2.86
3.2	5.67[a] ± 4.63[b]	25.67[a] ± 3.88	32.67[a] ± 2.81	55[a] ± 5.76[a]
1.0	8.67[a] ± 5.75	45.67[a] ± 5.99	72.5[a] ± 2.42	88.67 ± 4.13
Control	47 ± 11.91	78.3[a] ± 9.24	98 ± 1.09	99 ± 0.83

[a] Significant difference compared with respect to control ($p \leq 0.01$).
[b] Mean ± standard deviation of six observations.

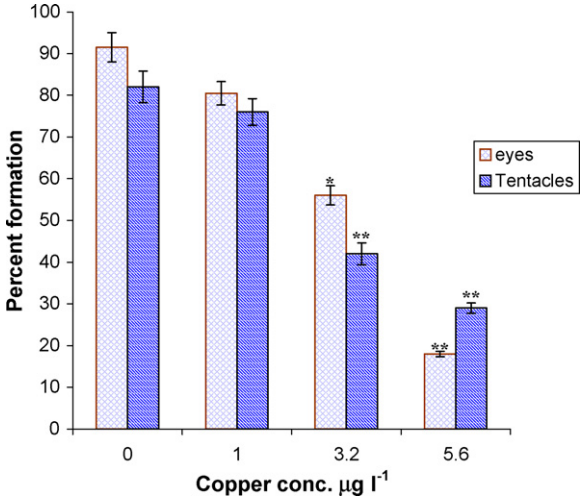


Fig. 6. Effects of increasing concentrations of Cu on the formation of eyes and tentacles after 7 days exposure at 3.2 and 5.6 $\mu g\,l^{-1}$ of Cu and control (* and ** indicate the proportion of the stages which are significantly different from the control proportions Student's *t*-test, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$).

3.9. Effects on heart rate

The direct effects of 1–5.6 $mg\,l^{-1}$ of Cu exposure were cessation of heartbeat. As the test concentrations increases from 1 to 5.6 $mg\,l^{-1}$ of Cu, the heart rate (heartbeat per minute) decreases at 7 days. The measurement of heart rate revealed a significant decline in the Cu-treated groups. There were 58 ± 4.8 beats per minute (min^{-1}) in 5.6 $mg\,l^{-1}$ of Cu-exposed embryos as compared to the 75 ± 5.4 beats per minute in control embryos. For the Cu treatment, the lowest heartbeat rate was counted with 10 $mg\,l^{-1}$ of Cu. There was a slow rotation of egg embryo in egg capsule at trochophore and veliger stages at 3.2, 5.6 and 10 $\mu g\,l^{-1}$ of Cu. Several egg embryos reached up to veliger stages, but rotation rate per minute drastically decreased in Cu-exposed groups.

4. Discussion

The results of present study clearly show its range of sensitivity that covers both acute and subacute toxicity. In the Cu-exposed test containers, embryo death started after 24 h and remained higher than in the control groups until the end of the experiment. The effects of tested concentrations of Cu demonstrated the possibility to evaluate effects on different developmental endpoints in pond snail. The formation of eyes, foot structure, tentacles, shell as well as heart beat rate and hatching parameters were easily accessible and these endpoints observed in the present study reflect the developmental stress of the animal. The hazardous potential of Cu and its adverse effects on a large number of aquatic organisms are well known [28], but the cellular and molecular mechanisms of Cu toxicity on the developing snail embryo is still unknown. Copper is an essential part of hemocyanin, the oxygen-carrying molecule of snails and arthropods. The excess of the copper could cause of dysfunction of estrogen receptor resulting in reproductive disorders [38]. It is possible that Cu causes a dysfunction of egg embryos by influencing the neuroendocrine regulation of developing snails. The physiology of Cu metabolism in snail and other invertebrates has been well studied [39]. Mechanisms of biotoxicity of copper to aquatic organisms and its distribution in the environment were reviewed earlier [40]. The results suggested that as Cu concentrations and exposure time increases; the embryo mortality, malformation and developmental delay in snail embryo also increase, thus indicating a dose-dependant response of Cu exposure. Our results agree with studies carried out on the adult *L. luteola* acute toxicity tests. For example, acute toxicity of copper to adult snail was conducted and the 96 h EC_{50} values and 95% confidence limits were 27 (23–35) $\mu g\,l^{-1}$ of Cu [8]. In another experiment, Mathur et al. [9] calculated the 96 h EC_{50} values of 172 $\mu g\,l^{-1}$ of Cu for *L. luteola* in hard water (hardness - 315 $mg\,l^{-1}$ as $CaCO_3$). Copper toxicity in aquatic invertebrates including freshwater pulmonate snails varies considerably according to the species and environmental factors [41]. The 96 h EC_{50} values varied from 6 to 390 $\mu g\,l^{-1}$ of Cu to a commonly distributed Indian pond snail *Viviparus bengalensis* [42]. Hardness of test water is an important physicochemical characteristic, which could change the acute and chronic toxicity of copper [43]. The present snail egg embryo tests were carried out in hard water (mean hardness – 230 $mg\,l^{-1}$). There was a significant difference between nominal and actual Cu concentration at the highest concentrations studied (e.g. 320/197 $\mu g\,l^{-1}$ of Cu). The difference might be due to higher values of total hardness (220–240 $mg\,l^{-1}$ as $CaCO_3$) and pH (7.1–7.4) of the underground test water used in the present study. It is known that at higher pH and hardness, less amount of Cu dissolved in the water [43]. Copper is highly toxic heavy metal and high toxicity of copper to molluscs could be possibly explained by a greater accumulation rate of Cu, with gills and respiratory organs being the main target organ [44]. Aquatic molluscs particularly snails accumulate persistent heavy metals to a great extent than other organisms, therefore these organisms could serve as excellent bioindicator species for biological monitoring of toxic heavy metal pollutants for improving the water quality standards for diverse uses [45].

The embryo and larvae of the aquatic organisms are generally more sensitive to toxic substances than adults [46–48] and embryos of pulmonate snails including *Lymnaea* species have been recognized as valuable tools for water quality monitoring [48]. In other bioassays, the avoidance behavior of the pulmonate snails to copper and other heavy metals were successfully used [49]. Calabrese and Nelson [50] measured the growth of a bivalve mollusc *Mercenaria mercenaria* exposed to 48 h EC_{50} values of copper concentration and found the retarded shell growth to 52% of control growth. The adverse effects of low concentrations of cadmium were studied on reproduction, development, and hatching in a European freshwater pulmonate snail *L. stagnalis* [4]. Cadmium like copper also caused the incomplete development of foot, eyes, tentacles, shell, digestive gland, shell thinness, developmental arrests and delay in hatching. Other ecotoxicologists working with embryos of *L. stagnalis* with herbicides have noticed similar effects [51]. These findings suggested that pond snail and the study of their reproduction; development and hatching success could be a good model for estimating the ecological significance of various types of population. First, pulmonate snail are sensitive to Cu and to other toxic heavy metals such as Cd, Cr, Hg, Ni, Ag, and Zn [8], and second they have also been used to test the toxicity of endocrine disrupters chemicals and other pollutants [6,48,52]. The comparative sensitivities of heavy metals, pesticides and endocrine disrupters on molluscan embryo development with particular reference to developing pulmonate snails are given in Table 5.

The most surprising finding of this study was that hatching was delayed for 2–5 days at 1–3.2 $\mu g\,g^{-1}$ of Cu. In particular, the exposure of copper and its potent effects on hatching were astonishing. A good number of freshwater quality guidelines for Cu are around 1.3–2.0 $mg\,l^{-1}$ of Cu [27,53]. Hatching is often the result of a joint effort of chemical, osmotic and mechanical mechanisms [54]. Therefore, the effect of Cu on hatching may result from effects not only one mechanism but from a combination of them. The results show that the embryo can react to Cu at concentrations, which are sometimes below those that affect egg embryo survival. We suggest that the time for hatching may serve as sublethal response variable for freshwater snail egg embryonic developmental toxicity tests.

Table 5
Effects of environmental pollutants on mollusks embryo development.

Test compound	Concentration	Abnormalities observed	Organisms	Reference
Copper	1–10 $\mu g\,l^{-1}$	Malformation of shell, foot, eyes, and growth retardation, delay in development and embryo blocked at trochophore and veliger stages. The 96 h EC_{50} value 28.3 $\mu g\,l^{-1}$ of Cu (for embryo mortality) and 14 days 3.58 $\mu g\,l^{-1}$ of Cu (for hatching)	*Lymnaea luteola*	Present study
Copper	27 (22–35) $\mu g\,l^{-1}$	96 h EC_{50} values and 95% C.L.	*L. luteola*	[8]
Cooper	18.2 (16.7–19.3) $\mu g\,l^{-1}$	96 h EC_{50} values for adult	*L. luteola*	[9]
Copper	44 $\mu g\,l^{-1}$	No. of egg masses and number of eggs per egg mass here significantly lower in Cu treated. Hatching decreased	*Stagnicola vulnerata*	[29]
Copper	28 $\mu g\,l^{-1}$	Growth inhibited	*Physa integra*	[55]
Copper	10–30 $\mu g\,l^{-1}$	Inhibition of growth rate, inhibition of reproduction	*Potamopyrgus jenkinsi*	[56]
Cadmium	500 $\mu g\,l^{-1}$	One week delay in sexual maturity	*Biomphalaria glabrata*	[57]
Cadmium	80–320 $\mu g\,l^{-1}$	Embryonic development blocked at morula stage	*L. palustris*	[18]
Cadmium	160 $\mu g\,l^{-1}$	The number of egg masses per Individual and number of eggs per individual decreases significantly	*L. palustris*	[18]
Cadmium	25–400 $\mu g\,l^{-1}$	Egg production caused at 400 $\mu g\,l^{-1}$ hatching reduced to 0.4% with 200 $\mu g\,l^{-1}$, at 400 $\mu g\,l^{-1}$ eggs blocked in cleavage stage. At 100–200 $\mu g\,l^{-1}$ development of eggs was hilled at various stages of such as cleavage, gastrula, veliger and hippo. Conc. of 25–100 $\mu g\,l^{-1}$ slowed down hatching	*L. stagnalis*	[4]
Cadmium	200–500 $\mu g\,l^{-1}$ (12 days)	Significant delay in hatching at 250 $\mu g\,l^{-1}$ and at 500 $\mu g\,l^{-1}$ lethal for the snail embryo	*Marisa cornuarietis*	[2]
Cadmium	–	Significant delay in development and in hatching at low concentrations	*P. acuta*	[58]
Cobalt	1–10 $mg\,l^{-1}$	Number of eggs laid, number of egg Masses and egg at all tested conc.	*L. acuminata*	[12]
Heavy metals (Pb, Cd, Hg, Ni)		Reproduction and embryonic development affected	*B. glabrata*	[16]
Baygon	–	Development arrest, high embryo mortality, low hatchability	*L. stagnalis*	[59]
Cyperemethrin	4–12 $\mu g\,l^{-1}$	Increased number of egg masses and egg at all test concentration	*L. accuminata*	[12]
Alphamethrin	4–12 $\mu g\,l^{-1}$	Increased number of egg masses	*L. accuminata*	[12]
17α-Ethnylestradiol	10 $\mu g\,l^{-1}$	Significant decline in heart rate of embryos and weight increased	*M. cornuarietis*	[2]
17(-Ethnylestradiol (EE2)	1–100 $ng\,l^{-1}$	Embryo production increased relative to control at 1–25 $ng\,l^{-1}$, egg production decreased at 100 $ng\,l^{-1}$ of EE2	*Potamoeyrgus antitodarum*	[60]
Bisphenol A	52–100 $\mu g\,l^{-1}$	Significant decline in heart rate of embryos and weight increased	*M. cornuarietis*	[2]
Bisphenol A	1–100 $\mu g\,l^{-1}$	Embryo production increased relative to control at 1–25 $ng\,l^{-1}$, egg production decreased at 100 $ng\,l^{-1}$ of bisphenol A	*P. antitodarum*	[60]
Sewage effluent containing (EDCs)	–	Sewage effluent effect egg production both in dose and time related manner	*P. antitodarum*	[60]
p-Nonylphenol isomer (4 (3',6-dimethyl-3' heptyl)-phenol)	105 $\mu g\,l^{-1}$	Significant delay embryonic development, growth: specially in morula and veliger stages, embryo mortality, hatching success reduced in 20 days exposure	*L. stagnalis*	[7]
Trybutylin (TBT)	1.0–10 $\mu g\,l^{-1}$	Abnormal embryonic development hatching failed to survive, fecundity and survival significantly reduced	*Physa fontinalis*	[48]
Trybutylin (TBT)	125 $ng\,l^{-1}$	Egg production and cellular structure	*L. stagnalis*	[4]
Nonylphenol ethoxylate	0.1–1 .$mg\,l^{-1}$	Reduced fecundity, reduction in the Number of egg produced per snail, Egg masses, decreased, incidence of malformed snail eggs and hatching delay	*B. tenagophila*	[61]

The average concentrations of copper in river sediment of the Gomati River (Lucknow, India) a tributary of the Ganges could reach up to 37 $\mu g\,l^{-1}$ [25] and at such high ambient Cu concentrations many gastropods including *L. luteola* and their developing stages would be expected suffer adversely. The endpoints suggested in the study can be used to establish biomarkers that are specific and sensitive indicators for the presence of Cu and possibly of other environmental pollutants in the aquatic environment. In the present study, pond snail *L. luteola* was susceptible to the toxic effects of Cu at environmentally realistic concentrations found in the polluted areas as indicated by decrease in their embryonic development and hatching success. Our results also indicate that snail populations might eventually become extirpated in near future; if exposure continued at slightly higher Cu levels in metal mining and industrial effluent discharges sites. Ecotoxicological monitoring requires simple, inexpensive, and rapid methods that

can be used as a matter of routine by research laboratories without specialized equipment. There are several standard bioassays today for assessing water quality for diverse usages with embryos, larvae, and adult of aquatic test species. The present snail egg development and hatching study could serve as a simple, rapid, sensitive and cost-effective embryonic bioassay for water quality assessment.

5. Summary and conclusions

In summary, copper is deleterious to snail eggs at very low concentrations, causing acute effects at 32 $\mu g l^{-1}$ and above Cu levels and sublethal effects at lower concentrations (1–10 $\mu g l^{-1}$ of Cu). Among sublethal effects were decreased embryo survivals, delay or failure of hatching, malformation of foot, eyes, digestive gland, shell, developmental arrest, and thinness of shell and growth retardation at various developmental stages. As shown here, the sensitivity of embryo test is higher than adult snails, therefore; embryo-larval test can be regarded as an alternative or supplement for ecotoxicological studies. There is an urgent need to develop water quality guidelines or standards for Indian subcontinent freshwater habitats for the protection of aquatic fauna and flora. There is also a need to revaluate the current rate of Cu discharges levels to prevent further damage to affected ecosystems. Our findings imply that environmentally realistic Cu concentrations in freshwater, especially with high mining and heavy metals industrial areas can cause lethal and sublethal effects in the pulmonate snail *L. luteola* developmental stages; which form an important link in the aquatic food chain(s) and these effects may eventually lead to significant population-level effect in longer term. A further understanding of ecotoxicity of heavy metals to aquatic organisms would not be possible without considering the effects on their developing stages as these stages are highly sensitive to environmental pollutants.

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