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Bioaccumulation of copper and toxic effects on feeding, growth, fecundity and development of pond snail *Lymnaea luteola* L.

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ABSTRACT

We studied the bioaccumulation and the toxic effects of Cu on survival, number of eggs and eggmasses laying, embryo development, growth, and food consumption in an Indian pond snail, *Lymnaea luteola* L. exposed for 7 weeks. Copper caused loss of chemoreception, locomotion and inhibited food consumption significantly during 7 weeks of exposure. Food consumption in Cu exposed snails significantly decreased and at 56 and 100 $\mu g\,L^{-1}$, snail stopped feeding activity. Mean number of eggmasses or eggs significantly decreased in Cu concentrations during the 7 week study. The percentage hatching decreased in Cu concentrations but there was more than 95% hatched in control in 10–11 days after spawning. Egg development was completely inhibited at 100 $\mu g\,L^{-1}$, while abnormal embryonic development observed at 32 and 56 $\mu g\,L^{-1}$ of Cu. The Cu concentration in tissues increased in Cu treated snails and bioaccumulation factor ranged from 2.3 to 18.7. Snail growth at 5.6 and 10 $\mu g\,L^{-1}$ was reduced by 6.2% and 16.9%, respectively. The study revealed that snail embryos and adults could be used as *in vivo* test models for ecotoxicological studies. Findings of present study are helpful for advancing water quality guidelines for protecting aquatic biota.

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1. Introduction

Copper (Cu) like zinc is an essential trace metal to all aquatic biota and human beings, but even a small excess amount of it is extremely toxic to aquatic organisms [1]. Copper has been reported to be frequently occurred in freshwater habitats at concentrations of approximately 2–3 $\mu g\,L^{-1}$ [2]. It is usually discharged by metal finishing industries, mining process, copper-based products in agriculture as fertilizers, fungicides, herbicides, algaecides and molluscicides into aquatic environment and its deleterious effects on freshwater fauna and flora has been widely studied and well documented [3]. Among several invertebrates, pulmonate gastropods are known for their ability to accumulate heavy metals from the aquatic environment [4]. In an investigation of the European freshwater snail *Lymnaea peregra* exposed to Cu concentrations for 28 days, it was found that in the digestive gland; Cu concentrations varied between 64 and 133 $\mu g\,kg^{-1}$ [5].

Chronic toxicity data on heavy metals are only available for a few European pulmonate snails *Lymnaea stagnalis* and *L. palustris* [6,7]. There is also a clear need for chronic toxicity data for Cu and other toxic heavy metals to freshwater snails which are of ecological relevance for the Asian freshwater environments. Snails satisfy all the conditions of good biological indicators [8]. Their biological and ecological characteristics are well known; they strongly accumulate heavy metals and can be easily reared both in the laboratory and commercially. The dose-dependent inhibition of their growth following exposure to heavy metals has been demonstrated [9]. The sublethal effects of heavy metals induce changes in feeding and, reproductive behavior, growth, survival success, and ultimately the population growth of aquatic invertebrates [10–13]. However, effects of copper on reproduction, embryo development, growth, bioaccumulation, and feeding activity are still unknown. Therefore, the goal of the present study was to investigate these toxicological endpoints in an Indian ubiquitous species *L. luteola* exposed to Cu in chronic bioassay. This species was chosen for ecological reasons, as it is commonly found in freshwater systems of India, Pakistan, Nepal and Bangladesh and other South-East Asian countries and form an important link in aquatic food chain(s).

2. Materials and methods

2.1. Snail collection and culture

Individuals of adult *L. luteola* L. of similar size and weight were carefully collected from non-contaminated artificial fish culture ponds situated at Indian Institute of Toxicology Research (IITR),

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Gheru Campus, Lucknow, and transferred to the laboratory. They were maintained in glass aquaria. Snails were acclimatized to laboratory conditions for 2 weeks before experimentation, at temperature 21 ± 1 °C and fed daily *ad libitum* with thoroughly washed freshwater green aquatic plant (*Marsilia* sp.) leaves. They had an average wet weight of 450 mg (range, 350–550 mg) and shell length 21 mm (range 19–25 mm).

2.2. Test compound and exposure conditions

A stock solution of copper (1 mg mL^{-1} of Cu) (as $CuSO_4{\cdot}5H_2O$; Sigma Chemical Co, St. Louis, MO, USA) in double distilled water was prepared and from this solution; a series of log-based Cu concentrations were prepared. Groups of 20 young snails were placed in transparent polystyrene beakers of 2000 mL test water (Tarsons Pvt. Ltd., Lucknow, India). There was a control group and rest 6 groups were exposed to a series of Cu concentrations for 7 weeks (49 days). Photoperiod was controlled to simulate the natural day:light cycle (12 h:12 h). Fluorescent light with two 48w lamps was used as light source. Nominal test concentrations of 0, 5.6, 10, 32, 56, 100 and 180 μg L^{-1}of Cu were used in the study. Six replicates were employed in each Cu concentration and test water was carefully renewed every alternate day. The criterion for determining snail death was the failure of adult snails to respond to prodding of their 'foot' with a blunt needle.

2.3. Embryo toxicity tests

The eggmasses lay on the test container walls and plant leaves were collected when test water was changed. Eggmasses were incubated in transparent polystyrene Petri plates (size: 90 mm diameter × 15 mm height; make-Axygen® Scientific, Inc., Bath BA2 9AP, UK) containing same Cu concentration in which their parents were exposed under the similar environmental conditions up to hatching or maximum for 25 days. The number of eggs per eggmass was counted. The number of eggmasses and eggs per individual snail were also determined. After egg laying, eggmasses under incubation were observed for development with an inverted microscope (Olympus IX 170, Japan) until hatching. Embryogenesis inhibition was defined as embryo development blocked at one stage for more than 3–4 days and when no movement of the embryo in the egg capsule was observed it was considered as dead. The normal and abnormal development of the embryos was counted and the main abnormalities and malformations detected at the various stages of embryogenesis were recorded. The duration of incubation, survival and the number of hatchings were noted for each tested Cu concentration.

2.4. Physico-chemical analysis of test water

At the beginning of the experiments, physico-chemical properties of test water such as pH, total dissolved solids (TDS), dissolved oxygen, chloride, hardness and alkalinity were determined using the routine standard methods [14]. The mean and range of test water physico-chemical characteristics were as follows: pH 7.5 (7.35–7.65); dissolved oxygen 6.5 (5.8–6.9) mg L^{-1}; total dissolved solids 940 (910–1023) mg L^{-1}; chloride 13 (10–17) mg L^{-1}; total hardness 230 (218–240) mg L^{-1} as $CaCO_3$ and alkalinity 180 (170–210) mg L^{-1} as $CaCO_3$. Heavy metals were determined using inductively coupled plasma-mass spectrometry (ICP-MS Interpid II XDL Duo; Thermo Electron Corporation, USA). Mean and ranges of selected heavy metals (μg L^{-1}) in control test water were: Zn, 5.4 (4.1–6.5); Cu, 4.3 (3.1–5.6); Ni, 4 (3.2–6.8); Fe, 40 (25–59); Cd, 3 (1–6); and Cr, 4 (1–6.1).

2.5. Determination of Cu in test water, and snail tissues

After the specified time period, the snails were sacrificed and samples of the various tissues/organs were carefully dissected out, washed thoroughly in deionized water, and analyzed following the detail method described elsewhere [15]. Briefly, snail tissues and shells were processed by adding concentrated 5 mL HNO_3 plus 0.5 mL $HClO_4$ to 0.5 g sample of tissue in Teflon beakers. Each mixture was heated to near dryness on a hot plate and then cooled. The gelatinous precipitate was then dissolved in concentrated HCl diluted 1:1 (v/v) with deionized water, with gentle heating for 5 min and filtered through Whatman® No. 41 paper, collected in polystyrene test tubes and diluted to 10 mL with deionized water and stored at 4 °C until analysis. Prior to analysis, the test water sample was concentrated 10-fold by heating at 60 °C. One volume of concentrated HCl and HNO_3 were added to 10 volume of test water concentrate and heated on a sand bath for 1–2 h. The remaining solution was than made up to 10 mL and stored at −20 °C until analysis. The concentrations of Cu and other heavy metals were estimated using ICP-MS. Metal nitrate salt standards were used for calibration curves. Bioaccumulation factors (BCFs) corresponding to the ratio of the Cu in the foot, shell, digestive gland (μg kg^{-1}) and whole animals to the nominal Cu concentration in the water (μg L^{-1}).

2.6. Estimation of feeding rate

Unconsumed aquatic plant *Marsilia* sp. leaves were collected from test beakers when test water was changed on alternate day. Since the same mass of fresh food was given to all groups, the unconsumed leaves were used as an estimation of the food consumption rate. The results were expressed in grams of wet mass of consumed leaves in grams of snail per week.

The feeding rate of *L. luteola* was calculated for each Cu concentration using a simplified version of Gauld's equation [16].

$$F = \frac{V(C_i - C_f)}{nt}$$

where F = feeding rate in mg snail^{-1} week^{-1}, C_i = initial leaf weight (mg), C_f final leaf weight (mg), V = volume of test medium in the test beaker (mL), n = total number of snail survived in the experiment and t = exposure period.

2.7. Estimation of growth

To measure weights, snails (including shell) from each replicate were wrapped with filter paper to absorb condensed moisture on the snail shell surface and then weighed on an analytical digital balance (model 120 XB 120A, Precisa, Switzerland) to determine the wet weight. Growth was measured weekly by measuring the weight of the snail. The growth coefficients (mean weight after 1, 3, 5, and 7 weeks × 100/mean weight at the start of the experiment) were calculated.

2.8. Statistical calculation

At the end of the experiments, the calculated data of the treatments were compared with those of the controls. Comparison among treatments were made using analysis of variance (ANOVA), student's '*t*' test and followed by Dunnett's test ($p \leq 0.05$) to compare treatments against controls, and also with multiple comparisons (Tukey post hoc test, $\alpha = 0.05$) test to compare means among treatments [17]. The proportions of the embryo stages between different Cu concentrations were compared with Chi-square (X^2) test ($p \leq 0.05$) [18]. The number of eggmasses and the number of eggs per individual were also determined and related to the individual by considering the numbers of

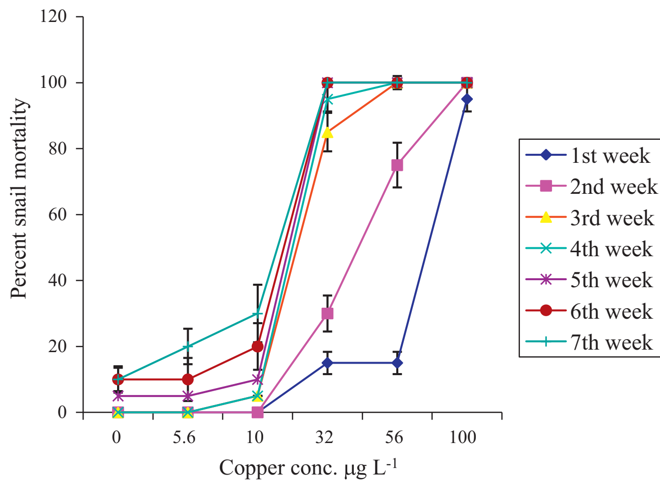

Fig. 1. Cumulative percent mortality of adult snail *L. luteola* exposed to Cu concentrations for 7 weeks.

individuals surviving at the beginning of the two day period before the eggmasses were harvested. The EC_{50} values and their 95% confidence limits were calculated by moving-average-angle method [19].

3. Results

3.1. Mortality of adult snail

Snails exposed to 100 and 56 $\mu g L^{-1}$ of Cu resulted in 100% and 75% mortality after 2 weeks whereas at 180 $\mu g L^{-1}$ of Cu, all snails died within 3 days of exposure. Over the same period, none of the snails died in control, 5.6 and 10 $\mu g L^{-1}$ of Cu (Fig. 1). Snail death in control and at 5.6 $\mu g L^{-1}$ of Cu occurred near the end of the experiment after intense reproductive and feeding activity. The EC_{50} values (50% mortality) and their 95% confidence limits from 1 to 7 week of Cu exposure were calculated (Table 1).

Table 1
The EC_{50} values and 95% confidence limits at different periods of Cu exposure for adult snail *L. luteola*.

Exposure period	EC_{50} values and 95% confidence limits ($\mu g L^{-1}$)
1st week	63.62 (56.89–70.15)
2nd week	40.88 (37.42–44.76)
3rd week	20.94 (17.05–25.83)
4th week	15.89 (13.19–18.69)
7th week	9.47 (8.42–10.73)

3.2. Behavioral responses

Copper exposed snails showed a well defined series of symptoms. The first symptom was the loss of chemoreception, so that the snails were no longer attracted to food and walls of the test container. At higher Cu concentrations (180, 100 $\mu g L^{-1}$), snails spent most of their time at the bottom of the beaker and died without showing any locomotion and feeding activities. At these concentrations, the foot becomes discolored; leading to paralysis and finally death appeared. However, in lower concentrations (5.6, 10 $\mu g L^{-1}$ of Cu) and in control test beakers, most of the animals remain at the surface of the water or attached to the wall of the test container and back surface of *Marsilia* sp. leaves and showed intense reproductive activity by laying several eggmasses during the experimental

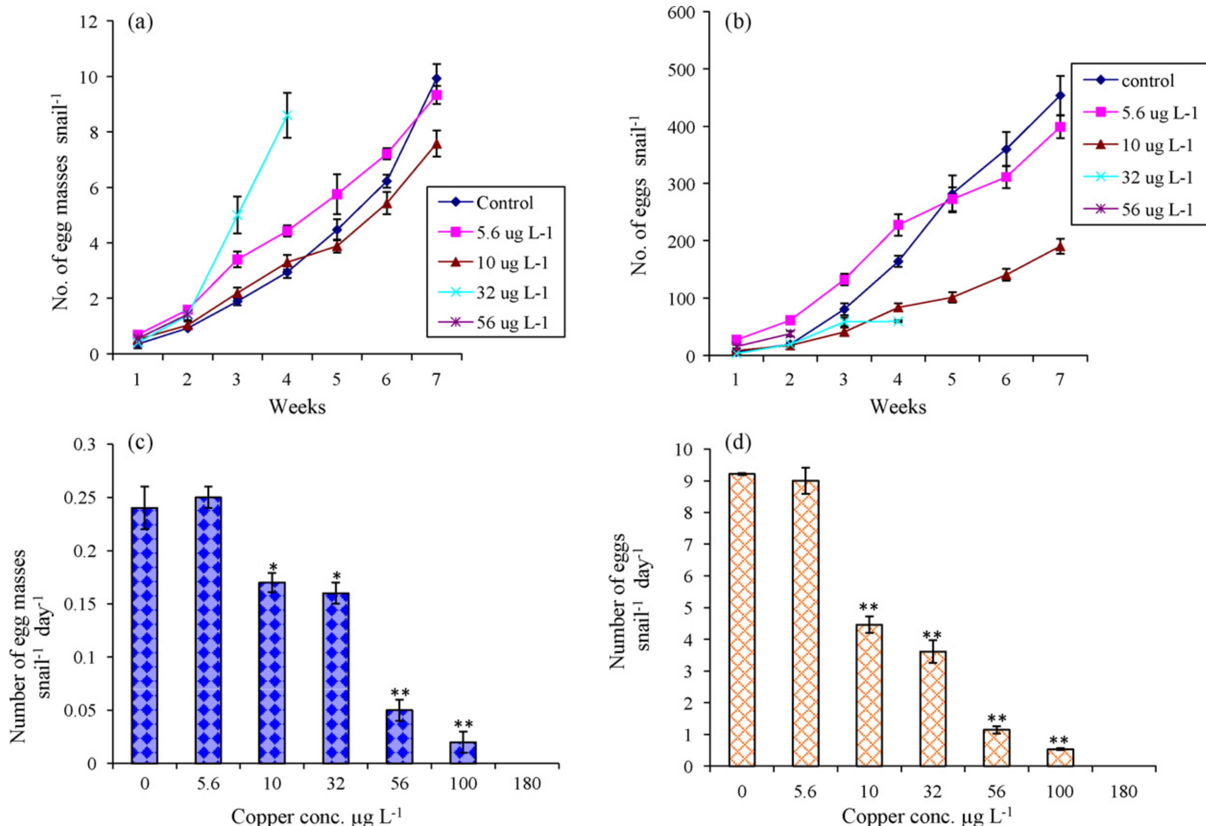

Fig. 2. (a)–(d) Effects of copper on the number of eggmasses (a) and eggs (b) per snail laid by mature *L. luteola* from 1 to 7 weeks. Data are shown as cumulative means of number of eggs and eggmasses laid by per snail during 7 weeks exposure. At 10, 32, and 56 $\mu g L^{-1}$ the mean differed ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$) ANOVA from that of unexposed controls (0 $\mu g L^{-1}$) at the 7 weeks. The number of eggmasses per snail per day (c) and number of eggs per snail per day determined (d).

Table 2
Effects of copper on eggs and eggmasses production of pond snail *Lymnaea luteola* exposed for 7 weeks.

Nominal Cu conc. ($\mu g\,L^{-1}$)	Measured Cu conc. ($\mu g\,L^{-1}$)	Mean number of eggmasses		Mean number of eggs		
		From weeks 1 to 3	From weeks 4 to 7	Total eggs from week 1 to 7	Mean per eggmass	Mean eggs per week
Control	4	37.8	141	8165 ± 291.3	45.64	1166.4
5.6	14	62	115.6	7178 ± 150.4*	40.42	1025.4
10	30	43.8	85	3235 ± 128.7**	25.22**	462.1
32	51	25	0.8	550 ± 49.3***	21.32***	78.6
56	75	11.4	0	305 ± 16.1***	26.75***	43.6
100	119	1	–	28	–	–

Mean ± standard deviation; significant difference compared with control:
* $p \le 0.05$.
** $p \le 0.01$.
*** $p \le 0.001$.

period. At 56 $\mu g\,L^{-1}$ of Cu test concentrations, shell became discolored and almost all the snails refused to consume *Marsilia* plant leaves offered as food after 2 weeks of exposure.

3.3. Ovipository activity

The cumulative production of eggmasses and eggs per snail decreased in a dose-dependent manner during 7 weeks of Cu exposure (Fig. 2a, b). At 5.6 $\mu g\,L^{-1}$ of Cu and in control experiments, no significance difference was observed in the total number of eggs and the number of eggmasses produced. Eggmasses were significantly less numerous during the first 1–3 weeks of 56 $\mu g\,L^{-1}$ of Cu exposure (mean 11.4, SD = 1.50) than the control (mean = 37.8, SD = 2.51). Effect of Cu was significant for the reduction of eggmass size (ANOVA, F = 79.4, d.f. 5,30; $p < 0.001$). At 10 $\mu g\,L^{-1}$ of Cu concentration, distinctly a reduced number of eggs (190.3) and the number of eggmasses per snail (7.58) were noticed as compared to those numbers recorded for unexposed controls (453.7, 10.93). At intermediate concentration tested (32 $\mu g\,L^{-1}$ of Cu), egg production of exposed snails, was also significantly different from that of controls either in terms of eggmasses or in terms of egg produced after 4 weeks.

The nominal and measured Cu concentrations, total number of eggs and eggmasses from 1 to 3 week and 4 to 7 week, mean eggs per eggmass and mean eggmass per week are given in Table 2. There was a significant difference between the nominal concentration of Cu and the measured because of the water hardness, pH and alkalinity of test water. As hardness and pH of test water increases the solubility of Cu decreases in the test water. Copper exposure caused a striking reduction in the number of egg and eggmasses at 10, 32 and 56 $\mu g\,L^{-1}$ of Cu after 3 and 7 weeks exposure. The number of eggmasses and eggs per snail per day significantly decreased at 10 $\mu g\,L^{-1}$ and above Cu concentrations (Fig. 2c, d). Normal eggmasses of 20 to 40 eggs were noticed but there were smaller eggmasses with only a few eggs, so the mean number of eggs per eggmass was lower in Cu concentrations. Egg laying frequency increased during third and fourth weeks of exposure. At 100 $\mu g\,L^{-1}$ of Cu, only one eggmass produced with 28 eggs in the first week of Cu exposure.

3.4. Effects on food consumption

The cumulative food consumption of *L. luteola* in control and Cu exposed snails was determined from 1 to 7 weeks of experimental period (Fig. 3a). The feeding rate of snails was also expressed as food consumed per snail per week (wet wt mg snail^{-1} week^{-1}) (Fig. 3b). It is clear that as Cu concentrations increased; the feeding rate significantly decreased. In treatments 180 and 100 $\mu g\,L^{-1}$ of Cu, snail stopped feeding and no food was consumed by snails; but in lowest Cu concentration (5.6 $\mu g\,L^{-1}$) snails consumed near about 12.25 g aquatic plant (*Marsilia*) leaves in 7 week. At 56, 32 and 10 $\mu g\,L^{-1}$ of Cu, the food consumption was 1.63, 3.13, 5.97 g, respectively after 7 weeks of exposure. Feeding rates in Cu treated snails considerably decreased per snail per week in a dose-dependent manner. Analysis of variance (ANOVA) indicated that food consumption was significantly ($p \le 0.01$) reduced in 10 $\mu g\,L^{-1}$ of Cu exposed groups after 1, 2, 3 and 4 weeks exposure. Copper at 10 $\mu g\,L^{-1}$, produced a partial inhibition of feeding and such feeding inhibition appeared because some snails consumed the food while others ceased feeding. It has been observed that time spent on feeding of *Marsilia* leaves in Cu exposed snails was reduced as compared with those of control. Snail *L. luteola* showed significant reduction in their feeding rates when exposed to sub-lethal concentrations of Cu (F = 12.67, d.f. 5,36, $p \le 0.01$). Pair wise comparisons also indicated that snails feed lesser amount of food in Cu test containers than those in the control experiments.

3.5. Effects of Cu on adult snail growth

Concentrations of 10 and 32 $\mu g\,L^{-1}$ inhibit the growth of *L. luteola* comparing the mean wet weights of the control snails during experiment (Table 3). Copper induced a dose-dependent inhibition of individual growth. Control snails increased their weight substantially during the 7 weeks, but snails exposed to 5.6, 10 and 32 $\mu g\,L^{-1}$ of Cu, showed small increase in weight. Growth of *L. luteola* was inhibited after 3 weeks of exposure at 32 $\mu g\,L^{-1}$ of Cu (ANOVA, $p \le 0.05$). The growth coefficient of snails was higher in lower Cu concentrations indicating a higher growth rate, than other higher Cu concentration groups.

3.6. Snail embryo development

3.6.1. Normal development of L. luteola

The general description of normal developmental stages of freshwater pulmonate snails (*Lymnaea* spp.) has been previously published elsewhere [20,21]. Briefly, we are able to identify four common stages of development (Fig. 4a–h) and use them to investigate the toxic effects of copper. The following normal embryonic stages were recorded: (1) morula: embryo leaves the vitelline membrane and moves freely within the egg capsule by means of cilia (Fig. 4a); (2) trochophore: in 3-day old trochophore larva showing the shell gland and prototroch. Embryo was more transparent, and rotates within the egg capsule (Fig. 4b, c); (3) veliger: when larva was 5 day old then regular heartbeat observed. At this stage foot, reddish eyes in color, tentacles, appearance of helical shell structure noticed (Fig. 4d, e); (4) hippo or veliconcha: the foot and viscera were well separated; embryo fully occupied the whole egg capsule (Fig. 4f, g). The larval structures degenerate gradually

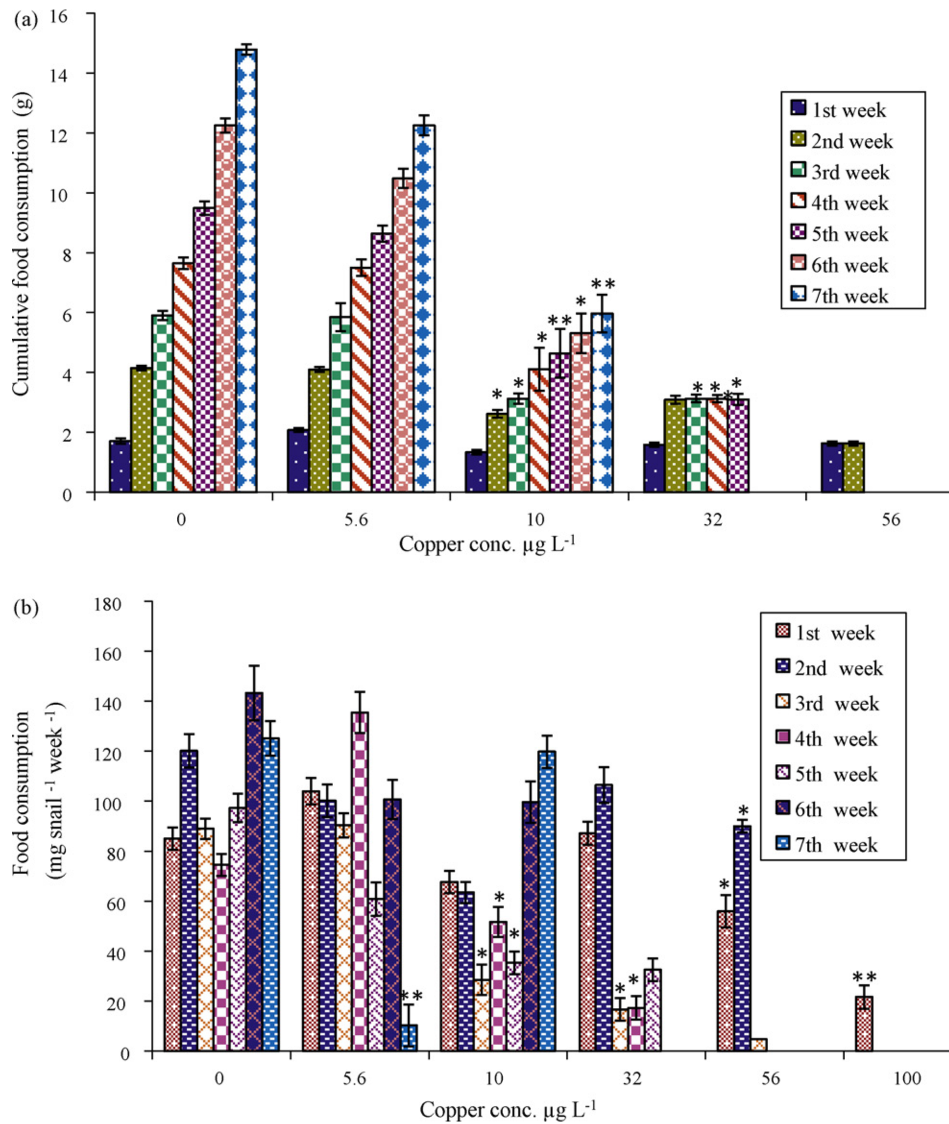

Fig. 3. Effects of 7 week of Cu exposure on cumulative food consumption parameters of *L. luteola*. (a) Cumulative food consumption in 7 weeks, (b) food consumption in mg per snail per week. At 32, 56, and 100 $\mu g\,L^{-1}$ mean values differ ($^{*}p \le 0.05$; $^{**}p \le 0.01$) ANOVA from that of unexposed controls (0 $\mu g\,L^{-1}$) at the 7 weeks.

with following 2 days and at 10–11 days, the young snail hatched (Fig. 4h).

3.6.2. Abnormal egg development

An overview of the toxic effects of the copper concentrations on the development of snail eggs at 32, and 56 $\mu g\,L^{-1}$ of Cu is given in Fig. 5A–C. Normal development of *L. luteola* was observed in control groups and at the 5.6 $\mu g\,L^{-1}$ of Cu and most of eggs hatched between day 10 and 14 after spawning (Fig. 5A_1–A_3). Aberrant snail egg embryo development was observed after 5, 7 and 9 days at 32 and 56 $\mu g\,L^{-1}$ of Cu treated groups. Inhibition of shell growth, eyes and tentacle formation, and shell gland was significantly affected at 32 $\mu g\,L^{-1}$ of Cu at different periods of exposure (Fig. 5B_1–B_3). The development of *L. luteola* eggs was completely

Table 3
Effects of copper on average wet weight of Indian pond snail *Lymnaea luteola* L.

Copper conc. ($\mu g\,L^{-1}$)	Start of experiment	1st week		3rd week		5th week		7th week	
	Mean weight (mg) SD	Mean weight (mg) SD	Growth coefficient	Mean weight (mg) SD	Growth coefficient	Mean weight (mg) SD	Growth coefficient	Mean weight (mg) SD	Growth coefficient
Control	438.83 ± 8.78	465.5 ± 3.86	106.08	490.5 ± 2.99	111.99	625.30 ± 2.67	142.59	650.17 ± 2.67	148.16
5.6	439.5 ± 8.22	462.67 ± 2.13	105.27	485.33 ± 2.73	110.43	579.17 ± 2.41	131.78	610.33 ± 1.97	138.87
10	452.33 ± 1.97	463.17 ± 2.54	102.40	482.67 ± 1.60	106.71	520.67 ± 1.79*	115.11	540.17 ± 2.19*	119.42
32	451.67 ± 6.69	454.33 ± 1.97	100.59	463.5 ± 2.63	102.62	–		–	
56	444.0 ± 3.51	458 ± 4.65	103.15	–		–		–	
100	442.5 ± 3.82	–		–		–		–	

Mean ± standard deviation; significant difference compared with control:
* $p \le 0.05$.

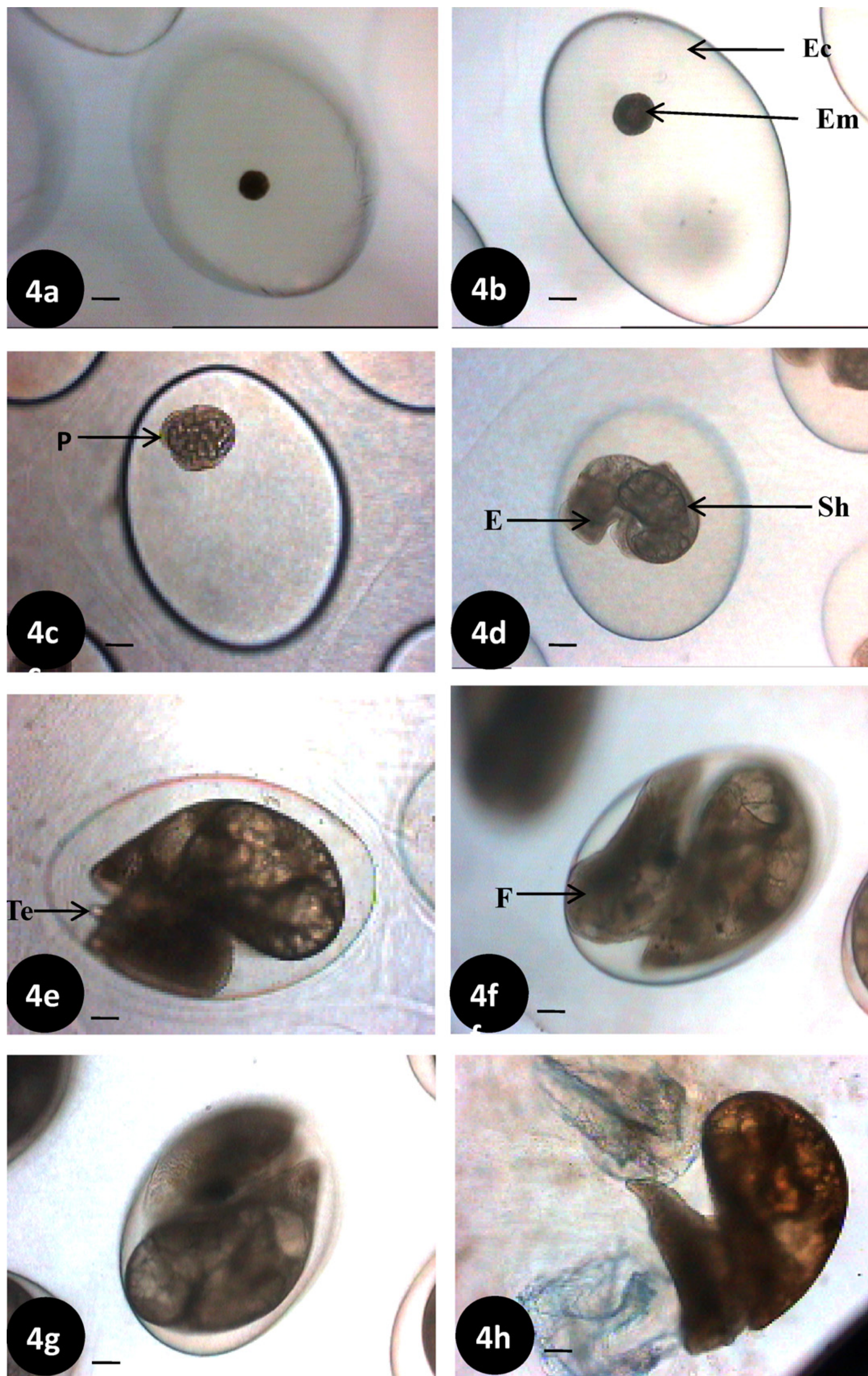

Fig. 4. Normal developmental stages of *L. luteola* at different days after egg laying until hatching. (a) Morula stage at days 2–3 days (b, c) early trochophore and mid trochophore stages 3–4 days, (d) early and mid veliger stages, 5–7 days. (e, f) late veliger stage 7–8 days, (g) hippo stage 9 days, (h) newly hatched snail at 10 days. E, eye; F, foot; Ec, egg capsule; Em, embryo; Sh, shell; Te, tentacle; P, prototroch. Scale bar = 0.22 mm (magnification 10×10).

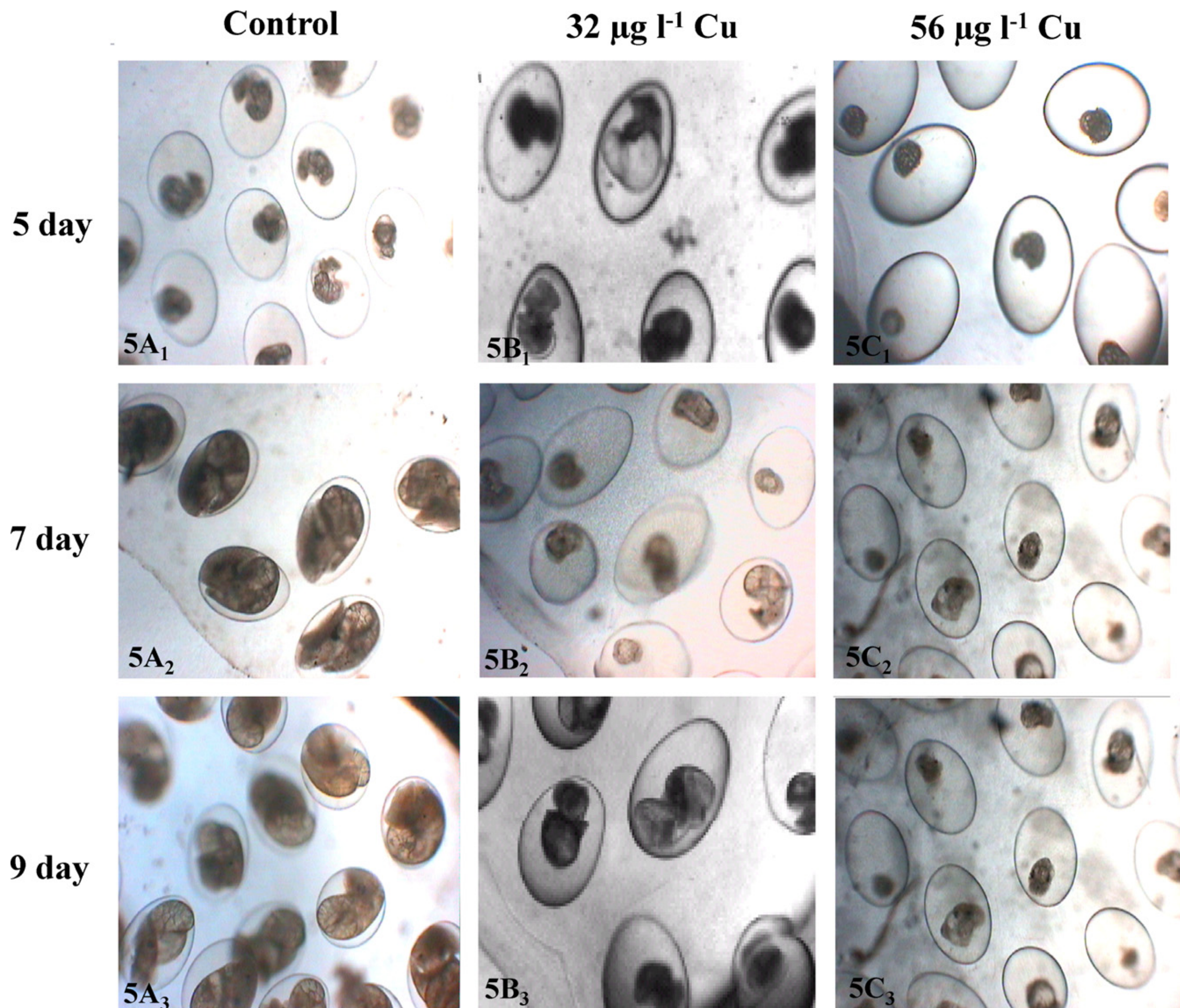

Fig. 5. Microphotographs showing the delayed and incomplete development of *L. luteola* embryos over time day 5, 7, 9 exposed to control, 32 and 56 $\mu g L^{-1}$ of Cu. Note the normal development of snail embryo in control (A_1–A_3) abnormal and malformed embryos noticed in exposed to 32 $\mu g L^{-1}$ of Cu after 5, 7 and 9 days (B_1–B_3). Retardation of developmental stages occurred after exposure to 56 $\mu g L^{-1}$ after 5, 7, and 9 days (C_1–C_3). Scale bar = 0.66 mm (magnification 10 × 4).

inhibited at 56 $\mu g L^{-1}$ of Cu, while abnormal embryonic development, i.e., without shell formation, highly abnormal shell gland and foot was observed after 5, 7 and 9 days (Fig. 5C_1–C_3). Egg development was blocked at trochophore and veliger stages for several days at 32 and 56 $\mu g L^{-1}$ of Cu. Survival of eggs considerably decreased, when the Cu concentrations increased from 10 to 56 $\mu g L^{-1}$ Effects of Cu on percent mortality of egg embryos were noted from 3 to 25 days in control and Cu treated groups (Table 4). Individuals were considered dead when, developmental stage, cells were coagulated (i.e., clumped together and whitish in color), or when no movement

Table 4
Effects of copper on the percent mortality of egg embryos of *L. luteola*.

Cu conc. ($\mu g L^{-1}$)	Percent egg mortality at					
	3 day	7 day	10 day	15 day	20 day	25 day
Control	–	–	–	–	0.1 ± 0.14	0.1 ± 0.1
5.6	–	–	–	0.13 ± 0.03	0.26 ± 0.03	0.26 ± 0.02
10	4.03 ± 0.03[a]	3.97 ± 0.19	4.03 ± 0.06	3.84 ± 0.41	9.82 ± 0.78	14.24 ± 1.34*
32	4.14 ± 0.16	8.23 ± 0.33	10.90 ± 0.97	16.54 ± 1.50	18.26 ± 0.85**	21.94 ± 1.25***
56	16.33 ± 2.21	16.5 ± 2.22	20.5 ± 1.17	26.29 ± 0.99	26.86 ± 2.95***	27.16 ± 2.23***
100	28.85 ± 1.08***	21.38 ± 0.51	46.33 ± 2.69	94.54 ± 1.35	95.62 ± 3.55***	99 ± 1.15***

[a] Mean ± standard deviation; significant difference compared with control:
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

Table 5
Effects of copper on percentage of hatching success of *L. luteola* at different concentrations over a period of 9–21 days.

Copper conc. ($\mu g L^{-1}$)	Percent hatching at (day)				
	9	10	11	15	21
Control	34.88 ± 0.95	82.57 ± 1.36	95.87 ± 1.55	97.04 ± 1.53	99.9 ± 0.01
5.6	11.92 ± 0.01	54.92 ± 1.50*	92.04 ± 1.007	95.11 ± 1.52	99.80 ± 1.49
10	1.24 ± 0.02	52.14 ± 0.80*	80.93 ± 0.68	84.44 ± 2.38	83.21 ± 1.87**
32	–	17.46 ± 0.65***	54.83 ± 0.74	72.98 ± 1.26	74.43 ± 0.94***
56	–	12.03 ± 0.56***	17.35 ± 0.41	53.26 ± 1.96	71.29 ± 1.02***
100	–	–	–	–	–

Mean ± standard deviation; significant difference compared with control:
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

of developing embryo could be observed or cessation of the heart beat.

3.7. Effects of Cu on embryogenesis and hatching

Embryos of the control group developed normally and hatching started from the 9–10th day and more than 95% hatching occurred in 11 days after egg laying (Table 5). The time required for snail eggs to hatch was significantly different among the five copper concentrations (F = 11.73, d.f. 5,18, $p \leq 0.01$). Significant effects of copper were visible in the time of hatching. At day 10, 82.57% of the snails had hatched in the control and at day 11 more than 95.87% had hatched. In the 56 $\mu g L^{-1}$ of Cu concentration, the hatching at day 10 was only 12.03% and at day 15 it was 53.26%. A concentration dependent augment of hatching delays was noticed at the 32, 56 and 100 $\mu g L^{-1}$ of Cu. There was 74% hatching success in the 32 $\mu g L^{-1}$ of Cu after 21 days. The proportions of each stage in Cu exposed eggmasses were significantly different to those of the control. Time required for snail egg embryos to hatch in 10 and 32 $\mu g L^{-1}$ was significantly different from laboratory control tests. At 100 $\mu g L^{-1}$ of Cu, more than 75% of the egg embryos remained at trochophore and early veliger stages which had abnormal shaped (foot, shell, eyes and tentacles not properly developed) and 21.43% were blocked at morula stage at 7 days of exposure (Fig. 6a). In this group, no egg embryo hatched and all the individuals died by the 21 days. In 5.6 and 10 $\mu g L^{-1}$ of Cu, 99%, 83.21% embryos hatched and 0.29, 14.09% were in veliger stage after 21 days, respectively (Fig. 6b). At 21 days of 56 $\mu g L^{-1}$ Cu exposure, nearly 71.29% embryo hatched, 17% remained in morula and 12.59% in veliger stages. The minimum development time of the embryos was significantly affected by the Cu exposure and was longer for the eggmasses laid by adults exposed to 32 and 56 $\mu g L^{-1}$ of Cu (ANOVA). Hatching rates of eggmasses laid by these snails were also significantly lower than the eggmasses laid by controls.

3.8. Effects of Cu on embryo length

The average length of all embryos in control, and Cu solutions on day 7 and 12 was measured (Table 6). The effects of Cu exposure on length of snail embryo differed significantly relative to controls (ANOVA, F = 9.61; d.f. 5,30; $p \leq 0.005$). Control embryos measured an average of 0.92 ± 0.032 mm compared to embryos exposed to 10, 32, 56 and 100 $\mu g L^{-1}$ of Cu, which measured 0.52 ± 0.06 mm, 0.47 ± 0.12 mm, 0.35 ± 0.09 mm, and 0.30 ± 0.09 mm, respectively. Growth and development of the snail embryos was on day 12 for control (1.06 mm), 10 $\mu g L^{-1}$ (0.87 mm), and 56 $\mu g L^{-1}$ (0.65 mm), respectively. Embryos exposed to 56 $\mu g L^{-1}$ of Cu grew significantly less than the embryos submitted to all other Cu concentrations, including the controls (Tukey's *post hoc* test). Embryo length significantly decreased as Cu concentrations increased.

3.9. Accumulation of Cu in snail

The Cu concentrations in foot, shell, digestive gland and whole soft body of snail *L. luteola* exposed to Cu for 7 weeks were observed (Table 7). A dose–response was noticed in foot and digestive gland. Copper accumulated in snail tissues in the following decreasing order: foot > digestive gland > whole body > shell, with a maximum in foot (144.33 ± 6.05 $\mu g g^{-1}$ wet wt), and a minimum in the shell (74.57 ± 1.25 $\mu g g^{-1}$ wet wt) at 32 $\mu g L^{-1}$ of Cu exposure. In general, Cu accumulation was linear throughout the exposure time in foot and digestive gland. Copper accumulated principally in foot and in the digestive gland. Bioconcentration factors (BCFs) ranged between 4.5 and 18.7 in foot and 3.8 and 12.6 in digestive gland. For all soft tissues (foot and viscera) BCFs were 3.3–11.1 for 5.6, 10 and 32 $\mu g L^{-1}$ of Cu.

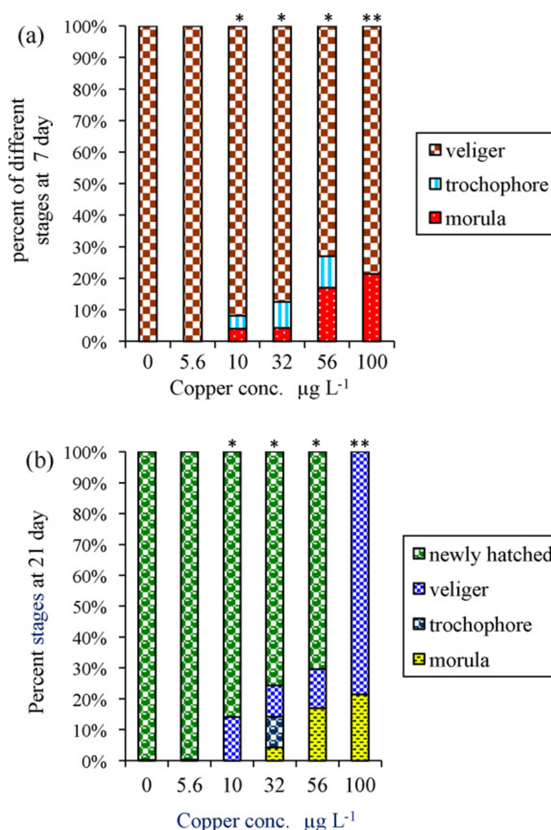

Fig. 6. Inhibition of embryogenesis of *L. luteola* at different stages of development versus the Cu concentrations in test water. At 7 day (a), and 21 day (b). (* and ** indicate that the proportions of the stages are significantly different from the control proportions, X^2 test * $p \leq 0.05$; ** $p \leq 0.01$).

Table 6
Effect of copper on the average size of shell length snail egg embryos after 7 and 12 day of exposure.

Copper conc. (μg L^{-1})	Snail embryo size (mm) at	
	7 day	12 day
Control	0.92 ± 0.032	1.06 ± 0.08
5.6	0.82 ± 0.04*	0.95 ± 0.08**
10	0.52 ± 0.06**	0.87 ± 0.06**
32	0.47 ± 0.12**	0.84 ± 0.03**
56	0.35 ± 0.09***	0.65 ± 0.16***
100	0.30 ± 0.09***	All died

Mean ± standard deviation; significant difference compared with control:
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

4. Discussion

The results suggested that as Cu concentrations and exposure time increases; the embryo mortality, malformation and developmental time increased, thus indicating concentration-dependent responses. In the present study, the 7 and 14 days EC_{50} values and their 95% confidence limits were 63.62 (56.89–70.15) and 40.88 (37.42–44.16) μg L^{-1} of Cu, respectively. Our results agree with studies carried out on the adult *L. luteola* Cu acute toxicity tests. For example, 96 h acute toxicity tests of Cu to adult snail *L. luteola* were conducted and the EC_{50} values and 95% confidence limits calculated were 27 (23–35) μg L^{-1} of Cu [13]. In another experiment, Mathur et al. [22] determined the 96 h EC_{50} value of 172 μg L^{-1} of Cu for *L. luteola* in hard water (hardness: 315 mg L^{-1} as $CaCO_3$). Water hardness is an important physico-chemical characteristic of test water which could change the acute and chronic toxicity of copper and other heavy metals [23].

A brief description of Cu effects on the reproduction, growth, and feeding of molluscs with particular reference to snails are presented in Table 8. Recently, Khangarot and Das [21] reported delay in embryonic development and hatching failure in *L. luteola* after exposure to low concentrations of Cu (3.2, 5.6 and 10 μg L^{-1}). Pulmonate snail veliger stage was considered the most sensitive stage for toxicity studies [6]. Cadmium caused aberrant embryonic development and reduced hatching success in two European freshwater pulmonate snails, *L. stagnalis* and *L. palustris* [6,7].

Analysis of variance indicated that growth of *L. luteola* significantly ($p \leq 0.01$) reduced after 7 weeks at 32 and 10 μg L^{-1} of Cu. Snails exposed to concentrations of 5.6 μg L^{-1} of Cu, continued to grow at slower rates than the control but did not reveal any significant inhibitory effect during the 7 weeks of exposure. No inhibitory effect of copper was clear during the first 2 weeks of the experiment.

Copper serves as a catalytic and structural cofactor for several enzymes that drives an ample array of vital biochemical processes which are crucial for energy generation, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting, signal transduction, and a host of other process [24]. Physiological and biochemical studies have suggested the presence of two independent activities responsible for Cu uptake in eukaryotic cells [25]. Ceruloplasmin, a serum glycoprotein that harbors over 95% of the plasma Cu which is speculated as an important part of the copper-uptake machinery. Copper mainly accumulated in soft tissues and respiratory organs [26]. Snails are excellent bioindicator species for biological monitoring of toxic heavy metals [27]. Concentration of Cu in tissues is due to the combination of the process of sorption–absorption, excretion and storage. Short-term exposure of Cu induced changes in survival, and uptake of Cu in Florida apple snail, *Pomacea paludosa* [28]. The actual processes involved in Cu uptake in pulmonate snails are not yet well understood. Copper toxicity in snails occurs through a variety of mechanisms. Heavy metals including Cu bind to free thiol groups, disrupting proteins structure or function. Copper generate reactive oxygen species through autooxidation reactions. Copper-induced oxidative stress can damage the cell membrane through lipid peroxidation, leading to membrane permeability and cell death [24,29]. The metal detoxification from the digestive gland cells of *L. stagnalis* may occur via faeces or via basal exocytosis towards hemocytes dispersed by the connective tissues in the visceral mass [30]. We employed rela-

Table 7
Copper concentration (μg kg^{-1}) in tissues/organ of *L. luteola* after exposure to copper concentrations for 7 weeks.

Copper conc. (μg L^{-1})	Cu accumulation in (μg kg^{-1} wet wt)							
	Foot	BCFs	Shell	BCFs	Digestive gland	BCFs	Whole body	BCFs
Control	76.18 ± 1.93[a]		60.79 ± 1.16		76.28 ± 2.38	54.9 ± 2.24		
5.6	104.9 ± 3.59	18.7	65.38 ± 1.27	11.7	70.68 ± 1.71	12.6	62.33 ± 1.36	11.1
10	118.17 ± 4.22*	11.8	70.68 ± 1.71	7.1	103.67 ± 2.05*	10.4	94.75 ± 1.04*	9.5
32	144.33 ± 6.05**	4.5	74.57 ± 1.25	2.3	123.0 ± 1.63**	3.8	106.42 ± 3.57**	3.3

[a] Mean ± standard deviation.
* Significant difference compared with control, $p \leq 0.05$.
** Significant difference compared with control, $p \leq 0.01$.

Table 8
Effects of copper on growth, feeding activity and reproduction of molluscs.

Test organism	Concentration	Observation	Reference
Lymnaea luteola	10–56 μg L^{-1}	At 10 to 32 μg L^{-1} of Cu decreased number of eggmasses and number of eggs, growth retardation, and feeding inhibition in 49 days	Present study
Pomacea canaliculata	20–67.5 μg L^{-1}	Exposure to 20 μg L^{-1} for 36 days increased, feeding rate and reduced the snail feeding feeding rate and growth rate up to 28%	[39]
Potamopyrgus jenkinsi	10–30 μg L^{-1}	At 10 μg L^{-1} inhibition of growth At 30 μg L^{-1} inhibition of reproduction in 20–36 days	[40]
Physa integra	28 μg L^{-1}	Stopped growth	[41]
H. pomatia		Acute toxicity	[42]
H. aspersa	1180 μg g^{-1}	Inhibiting effect on growth	[43]
H. engaddensis	4–2500 μg g^{-1}	Inhibited feeding and growth	[37]
L. luteola	27 μg L^{-1}	Acute toxicity	[13]
Stagnicola vulnerata	44 μg L^{-1}	Affected growth rate, reproduction and embryo hatching	[44]
S. vulnerata	2700 μg L^{-1}	Effect on hatching	[44]
H. aspersa	1000 and 2000 μg g^{-1}	Inhibited growth	[45]

tively low concentrations of Cu and found tissue concentrations after exposure to be in the similar range as reported by some other workers in a European freshwater pulmonate snail *L. peregra* [5]. It has been reported that digestive gland may operate as a storage area of heavy metals [31]. The digestive gland showed bioaccumulation of Cu and there was a rise in copper concentration of this organ from 76.28 $\mu g\,g^{-1}$ (control) to 123 $\mu g\,g^{-1}$ (with exposure at 32 $\mu g\,L^{-1}$ of Cu) in *L. luteola*. At higher concentrations, copper regulated physiological process may be impaired [32], due to cytotoxic and/or genotoxic properties of Cu and hence destroyed the Cu regulatory mechanism [33]. Furthermore, the soft tissues of gastropods contain various sub cellular metal containing granules [34], and these may function as a route for the accumulation, storage and subsequent detoxification/excretion of heavy metals [35]. Soft tissue bioaccumulation is likely to result from the relative ease of transport and subsequent binding in these tissues [36].

The effect of Cu treatment on food consumption rate was negligible at 5.6 $\mu g\,L^{-1}$ of Cu; however, at higher concentrations; a rapid drop in food consumption was observed. Both Cu and Cd in the diet were found to have inhibitory effect on feeding and growth of terrestrial snail *Helix engaddensis* in a dose-dependent manner [37]. Snails assimilate and accumulate Cu efficiently from food because in natural environmental Cu always occurs in concentrations near the minimum nutritional requirements of these animals [8]. Therefore, it appears that snails spent the first 2 weeks of the experiment, accumulating Cu from the test solution. As a result of that, Cu reached high concentrations inside the *L. luteola*, causing them to reduce or stop feeding. Our findings indicate that *L. luteola* were able to detect high concentrations of Cu in test water therefore, they reduced food consumption before toxicity takes place. Other test organisms, such as the isopod *Porcellia scaber*, indicated significant reduction in feeding rate when fed zinc-contaminated plant leaves [38]. The exact mechanism involved in growth inhibition of organisms particularly freshwater snails exposed to elevated metals in the diet or in test water is still unknown.

5. Summary and conclusion

The present adult snail and embryo toxicity procedure described is simple, cost-effective, and easy to rear and conduct in the laboratory. Our results showed that reproductive and feeding activities and abnormal embryo development were sensitive indicators of Cu toxicity. The present results support *L. luteola* L. as a suitable natural bioindicator for tropical and subtropical freshwater environments. The environmentally realistic concentrations of Cu in freshwater, especially in areas with copper mines, copper producing industries could cause sublethal effects in the pulmonate snail *L. luteola* and these effects may eventually lead to significant population level effects in the longer term. The number of eggs per eggmass, the number of eggmass per snail drastically decreased in dose-dependent manner. The fecundity, growth, feeding inhibition and the stages of embryo development were useful parameters for the toxicity of Cu and deserve complete investigation with other heavy metals, organic and industrial pollutants.

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