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Toxicity of mercury on *in vitro* development of parthenogenetic eggs of a freshwater cladoceran *Daphnia carinata*

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ABSTRACT

Chronic toxicity test duration of 21 days for daphnid is time consuming and expensive. Therefore, the developmental stages of *Daphnia carinata* eggs that could be used as potential endpoints for sublethal and chronic toxicity tests have been investigated and defined. Daphnid egg test is simple, easy to conduct and handle in the laboratory, and cost-effective. The 72 h 'egg arrest' bioassay system could be an alternative to the classic 21-day chronic test with neonates of daphnid. The main aims of the study were to establish easy to identify stages of *D. carinata* egg that could be used as potential endpoints for toxicity tests with *in vitro* cultures of daphnid parthenogenetic eggs. Commonly available Indian freshwater cladoceran *Daphnia carinata* parthenogenetic eggs *in vitro* were exposed to water borne mercury concentrations, ranging from 0.1 to 32 $\mu g\,l^{-1}$. Adult female cladoceran *D. carinata* have eight main developmental stages of parthenogenetic reproduction based on the release of external and internal membranes, formation of cephalic and body regions, appearance of secondary antennae, presence of two pink eyes, than a single black eye, and finally caudal or shell spine separation and finally free-swimming neonate within 65–72 h. At 1, 3.2 and 10 $\mu g\,l^{-1}$ of Hg concentrations; the 25, 50 and 70% embryonic developmental arrests were observed. The lower concentrations of Hg (0.32, 1, and 3.2 $\mu g\,l^{-1}$) tested in the present study are not generally harmful to the neonates and adults daphnid species, but the same are highly toxic to the embryos of *D. carinata*. The 48 h and 72 h EC_{50}s and their 95% confidence limits for survival and hatchability were lower than previously reported 48 h EC_{50}s for *Daphnia magna* immobilization assay. The egg of *D. carinata* turned out to be a suitable alternative model for ecotoxicological and water quality assessment studies.

1. Introduction

Freshwater cladocerans with particular reference to *Daphnia magna* have been of recent interest as a test model organism due to need for developing a non-mammalian test system for ecotoxicological risk assessment of hazardous pollutants for improving the water quality criteria for aquaculture, drinking purposes and human health. Water fleas (*Daphnia* sp.) have a relatively short life cycle, require little space, are easily adaptable to laboratory conditions and are sensitive to a broad range of aquatic contaminants. In acute and chronic toxicity tests with daphnids, young female *Daphnia*, aged less than 24 h are generally used. Although, it is possible to obtain information on lethal and sublethal toxic effects but such test design could not provide information about the effects on embryonic survivorship and morphological abnormalities during development [1]. Until now, only a few investigations have been undertaken to determine the effects of environmental pollutants on the daphnid developmental stages whose neonates are commonly used in ecotoxicological research. Treatment with ethylenethiourea (ETU), a teratogenic and mutagenic compound to mammals, induced morphological abnormalities in the carapace of *D. magna* [2]. However, embryonic abnormalities at later stages (stages 5 and 6) of development were not observed with chlorophenols [3], aniline derivatives [4] and TCDD [5] with egg bioassay tests of *D. magna*. Thus, these compounds may not have a specific toxic mode of action like ETU towards embryonic developmental processes. Researchers have defined various stages of abnormal embryonic development based largely on development of the daphnid eye, carapace and secondary antennae [6,7]. LeBlanc et al. [8] and Mu and LeBlanc [9] studied the developmental toxicity in *D. magna* of endocrine disruptors chemicals. The details of *D. magna* normal developmental stages are well studied [10,11]. Sobral et al. [12] defined the developmental stages of *D. magna* eggs and suggested that egg maturity, duration of egg stage, egg diameter, and egg abnormalities could be potential endpoints for sublethal toxicity tests. Embryo-larval tests with fish have been successfully used as an accurate tool to predict sublethal effects [13].

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Mercury (Hg) contamination of the aquatic environment is a subject of widespread concern since this acutely metal is known to enter the food chain(s) and undergo bioaccumulation through tropic levels and endangering human health [14,15]. Acute and chronic toxicity of Hg to cladocerans with particular reference to *D. magna* is well documented [16,17]. However, information of Hg on cladocerans embryonic developmental stages is not clearly reported so far. Because of the paucity of information on this subject, the present study was undertaken to determine the toxic effects of Hg on a freshwater cladoceran *Daphnia carinata* developmental stages survival, morphological abnormalities, developmental arrests, and to establish easy to identify egg stages that could be potential endpoints for embryo-larval toxicity tests. The water flea *D. carinata* was chosen for ecological reasons, as it is ubiquitous species found in Indian lakes, ponds, and rivers and also a significant member of aquatic food chain(s). In the acute and chronic toxicity tests with heavy metals, cladoceran *D. carinata* has been previously used [18].

2. Materials and methods

Water fleas *D. carinata* were obtained from ponds situated at Gheru Campus of Industrial Toxicology Research Centre, Lucknow and have been maintained in hard water at $20 \pm 1\,^{\circ}C$ under a 16/8-h light/dark cycle [19]. Daphnid were fed daily with the living unicellular green alga *Chlorella vulgaris* (5×10^5 cell ml^{-1}). Water flea (*D. carinata*) embryos are capable of normal development outside of the brood pouch and are therefore suitable for *ex vivo* studies. Medium Elendt M4 was used for culture of isolated parthenogenetic eggs [20]. Glass beakers of 2 l were used as the culture vessels including 1.5 l medium and 50–70 adult *Daphnia*. Gravid females were selected from the cultures and examined microscopically for the level of development (i.e., stages 1–2, as described below) were removed by applying gentle pressure to the posterior region of the brood chamber with a dissecting needle. Extruded embryos were collected and pooled. Eggs were washed several times, successfully adding and removing medium with a fine glass dropper. At the start of Hg exposure, 2–6 h old eggs (between stages 1 and 2) were used. Eggs were dark brown and round in shape (diameter approximately 400 μm). Tests were performed in 24-well tissue culture plates and eggs were exposed individually in 2 ml test solution for each Hg concentration. Embryos were incubated at $20 \pm 1\,^{\circ}C$ with 16 h photoperiod and were examined microscopically every 24 h during the test period. We scored embryos for stage of development and recorded mortality and any abnormalities in development after exposure to a series of Hg concentrations. Dissolved oxygen, hardness, and pH in the test solutions during testing period were 5.8–6.5 mg l^{-1}, 230–245 mg l^{-1} as $CaCO_3$, and 7.3–7.6, respectively. The development time was recorded for every hatched young animal. In addition, gross morphological abnormalities (e.g., formation of the carapace, first and second antennae, eye, brood chamber, abdomen protuberance, shell spine, and pigmentation in body) of hatched, survival and dead embryos. Test organisms were inspected under a low magnification inverted microscope. In all experiments, at least five test concentrations were arranged in a geometric series. Five replicates for each Hg concentration were tested. Test water was not renewed during the test period. Final test concentrations selected were based on earlier tested broad range Hg concentrations. All biological results were expressed in terms of the nominal concentrations of Hg to facilitate a comparison with other available toxicity data. Embryonic development was monitored microscopically and discernible developmental stages were recorded with a JVC color video digital CCD camera affixed to the Olympus inverted microscope. The median effective concentrations (EC_{50}s) and their 95% confidence limits were calculated by moving-average-angle method [21].

3. Results

Eight readily discernible stages of daphnid embryonic development were easily distinguished. At stage 1, *D. carinata* egg is uncleaved, homogenous, dark brown and round in shape, and covered with an egg envelope (Fig. 1a and b). Later on embryo undergoes early cleavage. Blastomeres formed a symmetrical sphere during stage 1, with no evidence of cellular reorganization. Stage 1, typically persisted during the first 15 h following deposition of the embryos into the brood chamber and it is considered as zero time of development. At stage 2, the egg periphery becomes lighter, due to mass retraction and external and internal membrane becomes visible (Fig. 1c and d). Release of the external membrane is considered the end of the first egg stage. Stage 2 represents gastrulation. Blastomere reorganization, took place as evident by the formation of a cellular ring just below embryonic membrane. The area within this ring was typical dark, and it may represent the blastocoel. Symmetry of blastula was lost during stage 2 with the formation of blastopore and the loss of the first embryonic membrane and stage 2 typically persisted from 20 to 30 h after deposition of embryos into the brood chamber. Stages 1–2 are round in shape. Later the cephalic region can be distinguished from the body region and form the headless embryos. Stage 3 embryos had a discernible head capsule and second antennae (Fig. 1d). Release of external membrane, which is considered the end of second stage and the start of third stage, occurs after 30 h after zero. From stage 3 onwards, the symmetry of the egg changes from radial to bilateral (Fig. 1e). Lateral protrusions corresponding to the antennas become clearly visible. However, the antennae were underdeveloped, and dark eye pigments were not yet evident. Stage 4 embryos (35–45 h) after deposition into the brood chamber had well-developed second antennae and the eye was well defined, with dark pigment. Lateral protrusions corresponding to the antennae become visible and two separate pink eyes appear in the cephalic region (stage 5, Fig. 1f–h). Rupture of the second embryonic membrane represented the onset of stage 5 embryos (45–50 h). Second antennae of stage 5 embryos extended from the body. Setae associated with the second antennae were not yet fully developed. Later on the shell spine on the posterior carapace remained tightly folded onto the carapace (Fig. 1i). Stage 6 (>48 h) represents organisms as typically viewed following release from the brood chamber. At stage 6, two red eyes clearly visible and the secondary antennae becomes free (Fig. 1j); thereafter, double black eye appears at stage 7, Fig. 1k) and finally two black eyes fuse to form a single black eye The distal region of the antennae separates from the body, and permitting some movement. Setae were well developed on the second antennae, the shell spine was fully extended and formed a 90° angle with the posterior end of the carapace, and animals were freely swimming in the medium. Separation of caudal spine and releasing thin cuticle covering of the feeding apparatus occurs 65–70 h after zero. This event, which is considered the end of the stage 8, concludes egg development (Fig. 1l). Thereafter, organisms present free movement and feeding capacity. In control groups, embryonic development (from an egg to a free swimming animals proceeded completely within 65–72 h with more than 95% hatchability.

Examples of arrested development in embryos and neonates deformities recorded at various mercury concentrations with the increase of exposure time are presented in Fig. 2. Forty percent of the embryos died during the developmental progression from stages 5 to 6 at 3.2 μg l^{-1} of Hg. Effects of Hg concentrations on

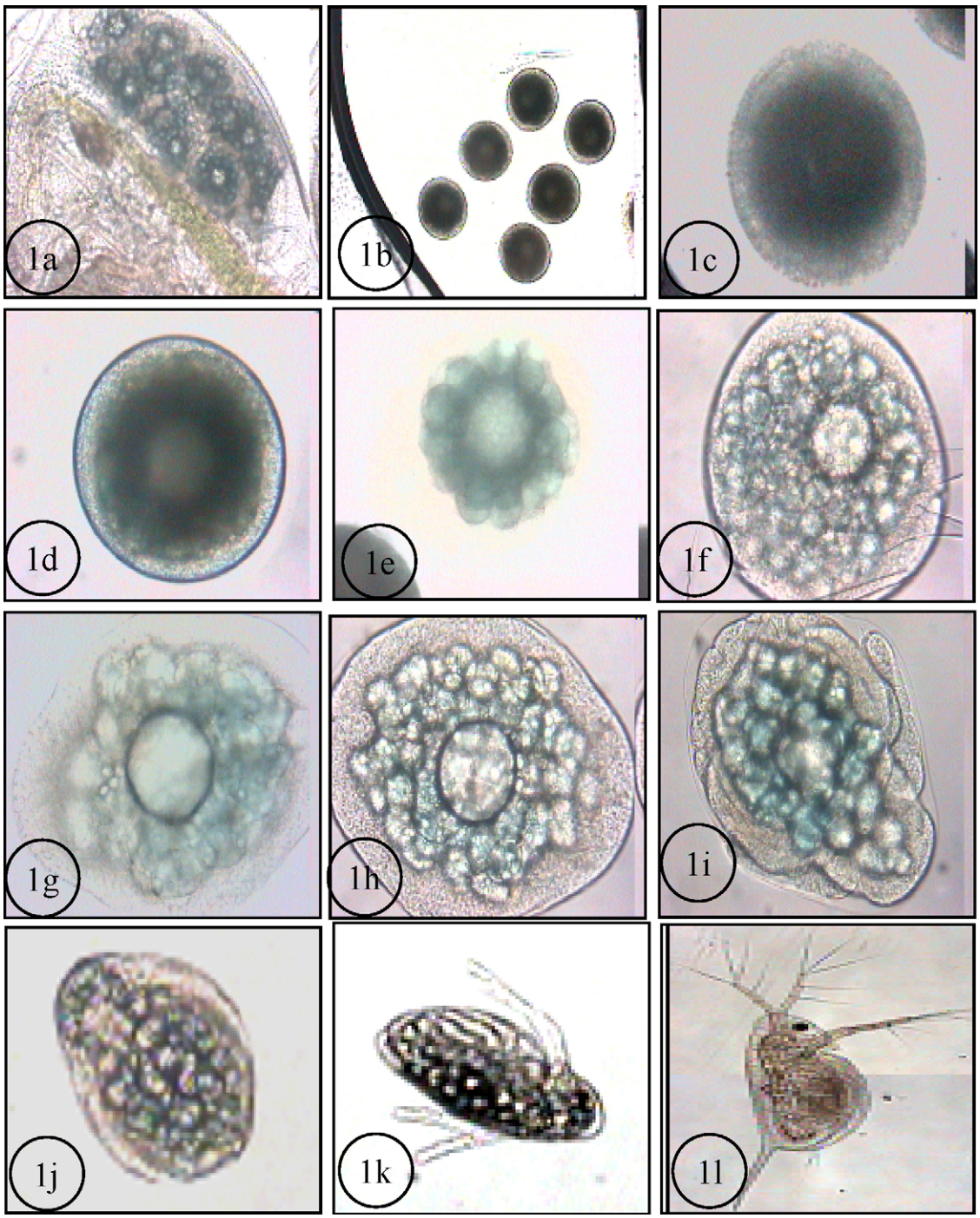

Fig. 1. Developmental stages of control cladoceran *Daphnia carinata* at different time in control experiments. (a and b) Daphnid carapace chamber showing several eggs in stage 1 egg at higher magnification (×100); (c) stage 1 uncleaved eggs, round in shape and covered with egg envelope (×100); (d) at stage 2 egg periphery becomes lighter and internal external membranes clearly visible (×100); (e) stage 3 showing head and body regions (×40); (f–h) embryo at stage 4 showing undeveloped second antennae (×40); (h) stage 5 embryo showing well-developed second antennae and dark pigmented eye, symmetry change from radial to bilateral. Also note the two separate pink eyes in the cephalic region (×40); (i) stage 6 embryo (×40); (j) stage 6 note the second antennae becomes free from carapace (×40); (k) stage 7 note that shell spine still unfolded in the posterior carapace region (×40); (l) stage 8 freely swimming neonate of *D. carinata* with shell spine, and single black eye (×40).

daphnid embryo mortality and percent hatching at different periods of exposure were recorded (Table 1). Exposure of mercury at 10 $\mu g\,l^{-1}$ for 24–72 h induced retraction of egg mass (Fig. 2a) abnormal development in the cephalic and body regions and the undeveloped formation of second antennae (Fig. 2b). In the cephalic and body regions, cell arrangements completely disrupted. In the 3.2 $\mu g\,l^{-1}$ of Hg treated groups some embryos showed abnormal development without well differentiation of eye, second antennae and carapace (Fig. 2d) as compared to control daphnids (Fig. 2c). In few eggs, exposed for 24 h at 32 $\mu g\,l^{-1}$ of Hg showed dark egg capsule without organogenesis differentiation (Fig. 2e). Several abnormal embryos died at various developmental stages with the increasing concentrations from 10 to 32 $\mu g\,l^{-1}$ of Hg after 48 and 72 h of exposure (Fig. 2f and g). In control experiments, after 65–72 h, a free-swimming neonate daphnid appeared. Few numbers of developing daphnid neonates did not have well-developed

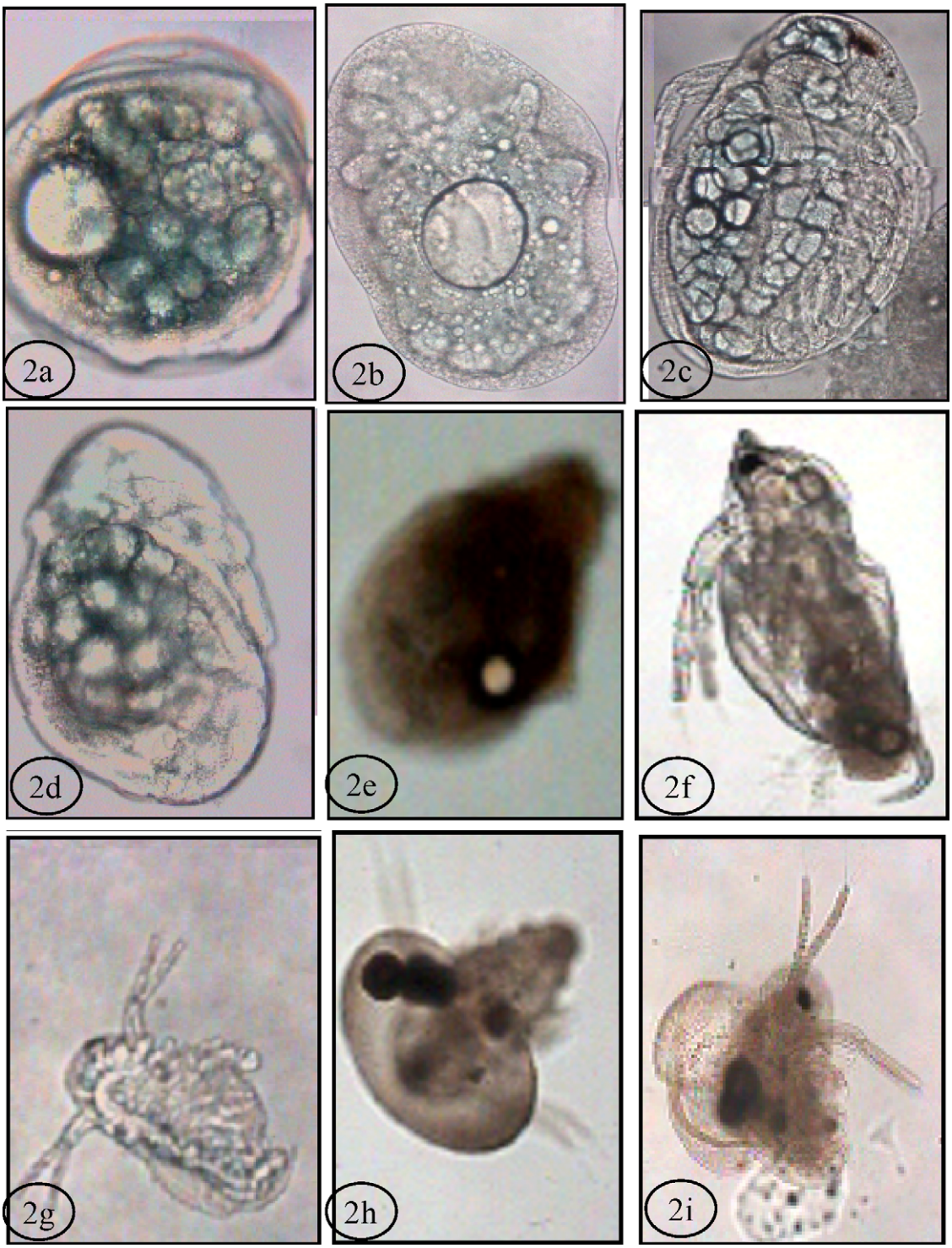

Fig. 2. Developmental stages of *D. carinata* following after mercury exposure. (a) Embryo (×100) exposed to Hg (10 μg l^{-1}) for 24 h. Note the retraction of egg mass. (b) Stage 3 embryos (×100) showing abnormal morphology of cell arrangement with poor development of cephalic and body regions. (c) Control well-developed embryo at stage 5 (×40). Note the developing second antennae, two black-pigmented eyes. (d) Stage 5 embryo after 10 μg l^{-1} of Hg exposure for 24–48 h caused developmental retardation delay and abnormalities (×40). (e) Egg embryo (×40) exposed to 32 μg l^{-1} of Hg for 24 h not showing organogenesis differentiation. (f) Abnormal embryo (×40) at 72 h exposed to 32 μg l^{-1} of Hg. (e–g) Deformed neonates (×40) exposed to Hg at 10 and 32 μg l^{-1}.

single eye, secondary antennae, setae on antennae, and abnormal shaped carapace. Highly abnormal living daphnids with different shapes and sizes with poorly developed carapace, caudal spine, second antennae and head region were observed at the completion of test period at 10–32 μg l^{-1} of Hg concentrations (Fig. 2g and i). Lateral protrusions corresponding to the antennas were not clearly visible. Two separate pink eyes not appear in the cephalic region. In all the tested mercury exposures, from 3.2 to 32 μg l^{-1}, a significant developmental delay in stages from 3 to 8 was observed. Most of the egg embryos exposed to 32 μg l^{-1} of Hg for 24–48 h, died without showing further development beyond stage 4. Eggs remain in homogenous and dark brown appearances until the end of the

Table 1
Effect of mercury exposure on percentage mortality and hatching

Hg concentrations ($\mu g\,l^{-1}$)	24 h	48 h	72 h	Percent hatching at 72 h
32	90	100	100	No hatching
10	35	50	90	No hatching
3.2	25	40	60	25
1.0	10	25	40	50
0.32	–	–	20	70
0.1	–	–	–	85
Control	–	–	–	95

test. Eggs mortality increased with the increase of Hg concentration and exposure time. At Hg concentrations of 3.2–10 $\mu g\,l^{-1}$, developmental deformities such as curved or unexpended shell spine and unexpended second antennae were observed after 48 and 72 h of Hg exposure (Table 2). These deformities are characteristics of late-stage toxicity.

4. Discussion

The toxic effects of Hg on freshwater cladocerans have been largely documented, but most of the studies were devoted to acute and chronic toxicity to juvenile or adult *D. magna* in 48 h and 21 days toxicity experiments [16,17]. The test duration of 48 h and 21 days are recommended for acute and chronic toxicity tests for cladocerans [22,23]. The results of the present study suggest that daphnid egg test could be an attractive cost-effective alternative bioassay to the classic 21-day reproductive test. The effects of heavy metals on life-history traits of different species of cladocerans have also been evaluated, particularly on reproduction [24,25]. Some preliminary investigations about viability of eggs (number of hatching/number of eggs) exposed to different heavy metals have shown that embryonic development is a highly sensitive stage in daphnid species. For example, embryos of *D. magna* were blocked at early stages when Cd and Cu concentrations were increased [24]. In *D. magna*, early embryonic stages are often decreased as being more sensitive than juveniles to heavy metals and other toxicants based on EC_{50} values [7]. Developmental stages of *D. magna* parthenogenetic eggs have been successfully used as potential endpoints for toxicity tests with heavy metals and other toxicants [12]. More *in vitro* parthenogenetic egg culture tests are urgently required to standardize toxicity endpoints and protocols with various types of environmental pollutants.

Results from the present study demonstrate that embryonic development of *D. carinata* proceeds from initial cleavage to complete organogenesis during a period of 65–72 h at $21 \pm 1\,^{\circ}C$. Embryonic development of the water flea is a continuous uninterrupted process in brood chamber under suitable natural conditions. Embryonic structures at various developmental stages are an easy way to explain the continuity of the process with the help of an optical microscope. Under controlled conditions, at $20 \pm 2\,^{\circ}C$ female water flea release a new brood every 3 days. During this period, eggs inside the brood chamber pass through various developmental stages, which can be easily distinguished by morphological alterations. Our studies demonstrate that embryos develop normally when they are removed from the brood chambers of parental organisms and could be incubated in culture media [20]. The 72 h EC_{50} values and their 95% confidence limits calculated for *D. carinata* survival were 1.8 (1.4–2.3) $\mu g\,l^{-1}$ and 0.9 (0.6–1.4) $\mu g\,l^{-1}$ of Hg for hatchability. The survival response of *D. carinata* to mercury concentrations observed in the present study is significantly lower than the reported literature values of (48 h EC_{50}; 5.2 $\mu g\,l^{-1}$) for acute tests with *D. magna* neonates [26]. Thus, *D. carinata* embryo egg arrest assay system is more sensitive than generally used 24 h old neonate *D. magna* bioassays. Similar observations were recorded with 4-nonylphenol at 281 $\mu g\,l^{-1}$ after 96 h of exposure in *D. magna* [27]. An increase in neonate deformities and embryo lethality (arrested egg development) was noticed at 50 $\mu g\,l^{-1}$ of 4-nonylphenol in 96 h study. The sensitivity of *in vitro* development of *D. carinata* eggs to Hg exposure (100% mortality at 32 $\mu g\,l^{-1}$) was higher than the sensitivity of juveniles of *D. magna* [17]. However, the present findings do not corroborate the conclusion of Boadar et al. [28] about the sensitivity of heavy metals of early life stages of *D. magna* compared with neonate or adult stages. In their study, the lesser toxicity of Zn, Cu, Cd, and Pb was assessed using *in vitro* cultures of parthenogenetic eggs, which was explained by the protective function of egg external and internal membranes.

Effects of environmental contaminants like 4-nonylphenol (an endocrine disrupting chemical), pesticides, chlorophenols, anilines, heavy metals, fungicides, and testosterone on *D. magna* developing stages, hatching success, embryo mortality, and morphological abnormalities have been reported recently (see-Table 3). Now, there is an urgent need to develop a standardized egg assay system for the rapid screening of heavy metals, pesticides, teratogens and endocrine disruptors using cladoceran egg. Furthermore, daphnid egg assay could be used for detecting mammalian teratogens with high predictive accuracy because daphnid life cycle is short, and their developmental stages are easy to identify with a simple binocular microscope. Parthenogenetic adult female daphnids are available round the year, which produce large number of genotypically isogenic eggs after every 3 days under favorable laboratory conditions. The developmental stages are transparent, which allows for relatively easy detection of internal and external morphological malformations. Large number of eggs of particular stage can be collected and counted. It is essentially a low cost whole embryo-teratogen screening test, which can be completed in 72–96 h. It has been observed that young *Daphnia* sp. fully developed *in vivo* is not different from those hatched in the brood pouch with regard to their morphology and reproductive ability [29,30]. The technique used in the present study and by others for *D. magna* could be applied as a detective system for developmental toxicity of chemicals to biologically important organisms. Eggs are large enough, therefore; collection and distribution of eggs to 96-well titer plates or small petridish can be conducted easily. Further studies with other toxicants, including defined specific toxicants (i.e., teratogens, mutagens, carcinogens or endocrine-disrupting chemicals, heavy metals, and pesticides), will be needed to verify the detection of various toxic actions of chemicals and to develop quantitative structural activity relationships (QSARs) for prediction of toxicity responses. Such types of experiments are in progress in our laboratory at ITRC, Lucknow.

Table 2
Percentage incidence of specific developmental abnormalities associates with exposure to mercury

Hg concentrations ($\mu g\,l^{-1}$)	Curved or extended shell spine	Underdeveloped antennae	Early developmental arrest
32	No formation	No formation	100
10	89	75	70
3.2	32	16	45
1.0	16	8.2	25
Control	1.8	1.1	3.2

Table 3
Effects of environmental pollutants on a freshwater cladoceran *Daphnia* sp. embryo development

Test compound	Concent-ration	Abnormalities observed	Reference
4-Nonyphenol	738 $\mu g\,l^{-1}$	EC_{50} embryo lethality	[27]
4-Nonyphenol	263 $\mu g\,l^{-1}$	EC_{50} curved or unextended shell spine	[27]
4-Nonyphenol	~44 $\mu g\,l^{-1}$	Developmental abnormalities	[8]
Chlorophenols		Reduce hatchability	[3]
Carbaryl (Insecticide)	1–5 $\mu g\,l^{-1}$	Developmental abnormalities, and delayed maturation	[6]
Aniline derivatives		Embryo hatching inhibited	[5]
Ethylenethiourea	50 $mg\,l^{-1}$	Egg development arrested	[2]
Ethylenethiourea	20 $mg\,l^{-1}$	Prolonged egg developmental time, bent carapace, and premature death	[2]
2,3,7,8-Tetrachloro dibenzo-*p*-dioxin (TCDD)	0.1–1000 $\mu g\,l^{-1}$	Reproduction and development affected	[4]
3,4-Dichloroaniline (3,4-DCA)	20–40 $\mu g\,l^{-1}$	Developmental delay and abnormal neonates	[12]
Dodecyl benzyl sulfonate (DBS)	1.25–10 $mg\,l^{-1}$	Egg died without development, egg diameter decreased	[12]
Cadmium	0.062–0.248 $mg\,l^{-1}$	Developmental delay	[12]
Copper	24 $\mu g\,l^{-1}$	Embryo death	[12]
Propiconazole (Fungicide)	0.015–0.25 $mg\,l^{-1}$	Underdeveloped second antennae and carapace abnormalities	[6]
Testosterone	8 μM	Arrest embryonic Development and Abnormalities in carapace	[9]

5. Conclusion

The present study clearly demonstrated that water flea *D. carinata* parthenogenetic egg developmental stages can become an important research tool for the evaluation of lethal and sublethal toxicity and to test teratogenecity of environmental contaminants. This bioassay provides useful information to evaluate the lethal and sublethal toxicity of environmental chemicals and the differences in sensitivity in the developmental stages. Various experimental scenarios can be used to facilitate elucidation of the mechanism of toxicity as it relates to direct exposure of the embryos or maternal intervention. Developmental endpoints that were affected by mercury included developmental arrest at various stages of organogenesis and release of live neonates with abnormally developed second antennae, carapace, eye and shell spines. Further studies are warranted to define the specific mechanism responsible for the embryo toxicity of mercurial compounds and other toxic heavy metals. Such studies may provide insight regarding targets of developmental toxicity that are unique to crustaceans.

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