Effects of ZnO nanoparticles on the Giant freshwater prawn (*Macrobrachium rosenbergii*, de Man, 1879): Reproductive performance, larvae development, CHH concentrations and anti-oxidative enzymes activity

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\textbf{ABSTRACT}

The Giant freshwater prawn (*Macrobrachium rosenbergii*) breeds when in captive conditions. The eggs of a clutch are attached to the abdomen of berried females. Zinc oxide (ZnO) is one of the most important metal oxide-nanoparticles (NPs) that is widely used in various industries and is released into aquatic environments from wastewater management facilities. The present study was conducted to evaluate effects of ZnO on values for the reproductive variables: larvae development, crustacean hyperglycemic hormone (CHH) release from the X-organ into the hemolymph and anti-oxidative enzymes activity of *M. rosenbergii*. There were five groups including a group not treated (control), and groups treated with 10, 20, 50, 100 mg/L ZnO in triplicate during a 90-day period. Results indicated that ZnO-NPs have marked effects on reproductive performance, offspring development, CHH release from the X-organ into the hemolymph and anti-oxidant enzymes activities with there being no spawning of brood-stock in the 100 mg/L ZnO group and in the prawns treated with 50 mg/L there was spawning but there was larvae mortality immediately subsequent to hatching. Also, values for viability rate of eggs, dry weight of eggs, brood-stock inter-spawn period and egg clutch somatic index (ESI) reproductive variables were affected by the NP. This NP was found to have a dose-dependent effect on CHH release from the X-organ into the hemolymph and also superoxide dismutase (SOD) and catalase activities in *M. rosenbergii*. The results indicate that *M. rosenbergii*, a freshwater decapod crustacean, is an appropriate species to study nano-material effects on reproduction in freshwater ecosystems.

1. Introduction

Nanoparticles (NPs) are materials that at least, one of the dimensions is in range of 1–100 nm and are widely used for various purposes in different industries (Qu \textit{et al.}, 2013). Zinc oxide (ZnO) NPs are one of these materials which have a white to yellowish-white color and is a crystalline powder that is soluble in water (Dimapilis \textit{et al.}, 2018). This NP is one of the most important metal oxide NPs and due to the large surface ratio to size and marked catalytic activities is widely used in various industries such as for rubber, paint, coating, cosmetic (Jiang \textit{et al.}, 2018), biomedical ointment, and anti-microbial (Siddiqi \textit{et al.}, 2018; Raghunath and

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Physicochemical properties, oxidizing agent production, duration in time cells are in contact and concentration are the main factors that pose a threat to the health of aquatic species when there is use of the metal oxide nanoparticles (MeO-NPs) for different applications (Raghunath and Perumal, 2017; Vali et al., 2020). The NPs can affect different organs of aquatic species, and signals may be transmitted between these organ systems, affecting body functions (Brohi et al., 2017). There can be effects of these agents directly on primary ovarian follicles and/or subsequent disruption of vitellogenesis of aquatic organisms (Wang et al., 2011).

The MeO-NPs are relatively new materials and there are concerns about the possible hazards on the reproduction of organisms because animal cells can easily uptake these NPs through the cytoplasmic membrane. One of these synthetic MeO-NPs is ZnO can have surface defects such as edges and corners that results in abrasive actions in disrupting cell walls (Stoimenov et al., 2002). Even though there are reports of zinc oxide NPs in the *M. rosenbergii* diet when feeding occurs as a supplement in aquaculture enterprises (Thirunavukkarasu et al., 2014, 2019), there continues to be incorporation of ZnO in diets in the micro-sized, but not nano-sized forms. The MeO-NPs in micro-size forms aren’t toxic and can be safely used in various animal feed and food industries. Even though this is the situation, there are many reports that ZnO-NPs due to the small size, structure and water solubility properties are toxic to aquatic organisms (Franklin et al., 2007; Aruoja et al., 2009; Bai et al., 2010; Hao and Chen, 2012; Tomilina et al., 2014; Chupani et al., 2018). The ZnO-NPs are released into the aquatic ecosystems through domestic and industrial wastewaters and can cause adverse effects on aquatic organisms and result in toxicity as a result of effects on reproductive organs (Rajput et al., 2018; Wu et al., 2019). Reproductive toxicity occurs when there are adverse effects on reproductive performance in adults, as well as toxicity during developmental stages, therefore, affecting production of offspring (Kumar et al., 2018; Rezaei Tavabe et al., 2020). There is greater toxicity of NPs in females because of effects on the reproduction system, and germ cells as well as during embryogenesis (Brohi et al., 2017). There are some reports that ZnO in nano-size forms has acute and chronic effects on the reproductive capacity of *Daphnia magna* (Wiench et al., 2009), earthworms *Eisenia fetida* (Cañas et al., 2011) and Zebrafish *Danio rerio* (Choi et al., 2016). Nevertheless, the reproductive toxicity of NPs and ZnO NPs on aquatic organisms and especially reproductive capacity of decapod crustaceans has not been studied in depth.

Different aquatic organisms have different tolerances to stress; therefore, there may be differences in the physiological response in different species and reproductive consequences to stressors. Furthermore, in aquatic organisms the physiology associated with maturation and spawning appears to be tightly coupled with stress physiology responses (Schreck et al., 2001; Rezaei Tavabe et al., 2020). Depending on the stage of animal development, stress factors have different reproductive consequences and the stress response is a process that requires energy. Aquatic organisms also mobilize considerable energy resources for reproduction (Contreras-Sánchez et al., 1998). Like most invertebrates, crustaceans when in stressful conditions switch to an alternative anaerobic energy metabolism via glycolysis and hyperglycemia with the process being regulated by crustacean hyperglycemic hormone (CHH) which has been evaluated as a biochemical stress biomarker (Chung et al., 2010). In decapod crustaceans, CHH is primarily involved as a metabolizing factor and has important functions in reproduction and molting regulation (Tavabe et al., 2013). Also, these stress conditions can result in induction of production of reactive oxygen species (ROS) and oxidative stress in aquatic animals (Chang et al., 2012). Decapod crustaceans because of the ecological and physiological characteristics including wide habitat distribution, minimum mobility capacity, well-known ecological characteristics, numerical abundance, suitability for laboratory experiments and sensitivity to different stressors are excellent bio-indicators and bio-monitors for different contaminants in freshwater ecosystems (Rezaei Tavabe et al., 2010, 2019; Chen et al., 2020; Zhang et al., 2020). The giant freshwater prawn, *Macrobrachium rosenbergii*, is an important decapod

![Fig. 1. Morphological structure and Scanning Electron Microscope (SEM) images of ZnO-NPs at two different scales A: 50 K magnification, scale bar =1 μm, B: 200 K magnification, scale bar =200 nm (Tescan mira 3, CZE).](image-url)
crustacean aquaculture species; originally from the southeastern region of Asia, however, during the past decades these animals have been transplanted throughout many other countries globally, for aquaculture purposes (Tidwell et al., 2005; New and Nair, 2012). Because this species breeds readily in captivity and has marked reproductive capacities and the eggs are produced in clutches with egg attachment being to the abdomen section of the ovigerous females (Tavabe et al., 2013; Rafiee et al., 2015; Rezaei Tavabe et al., 2015a, b; Rezaei Tavabe et al., 2017), it is an appropriate benthic species for toxicological studies in laboratory conditions. The objective of the present study, therefore, was assessment of the possible effects of ZnO-NPs on reproductive performance, larvae development, CHH release from the X-organ into the hemolymph and anti-oxidative enzyme activity of *M. rosenbergii*.

2. Materials and methods

2.1. Preparation and characterizations of ZnO-NPs

The required Zinc oxide NPs was obtained from Sigma-Aldrich Co. (USA) and the scanning electron microscope (SEM) (Tescan mira 3, CZE) images were prepared. The SEM images indicated the mean dimension of the ZnO-NPs was about 50 nm (Fig. 1). Also, to assess quality of the purchased ZnO NPs, X-Ray diffraction (XRD) was conducted using a Philips PW-1730, NED in the range of 10–80° (2θ). In Fig. 2, there is a depiction of the ZnO-NPs XRD spectra with the recorded peak points being precisely consistent with the standard patterns.

2.2. Experimental animals

Prawns (n = 400) used in this study were procured from the Ghasreshirin prawn hatchery center in the western region of Iran. The experiment was conducted at the Aquatic Animal Laboratory of the Aquaculture and Environmental Sciences Department of Tehran University. The average weight of the prawns was 14 ± 2 g and when transferred into the laboratory prawns were placed in a 5000 L tank for adaptation about 10 days prior to conducting the study. In the adaptation tank, water quality factors, light period and feedings were recorded and were consistent with the recommendations of Tavabe et al. (2013) and Rafiee et al. (2015).

2.3. Experimental conditions and design

Based on results from ZnO monitoring in aquatic environments and different wastewaters, the concentration of these NPs are variable in the environments with concentrations being as great as 100 mg/L reported (Nezhadheydari et al., 2019). The experiments, therefore, were conducted with assignments of *M. rosenbergii* to five groups including a group not treated (control), or groups treated with 10, 20, 50, 100 mg/L of ZnO-NPs. Treatments were tested in triplicate and each of aquaculture units (500-L recirculating systems) was stocked with, 12 females and four males for a 90 day research period. During the study, in accordance with recommendations of Tavabe et al. (2013) for the same species, total ammonia-nitrogen (TAN: NH₄-N) and total nitrite-nitrogen (NO₂-N) were maintained at less than 0.2 and 0.1 mg/L, respectively. Gentle aeration was applied in the water in all aquaculture tanks. Average water temperature and dissolved oxygen in the water were 28 ± 2 °C and 7 ± 1 mg/L. A lighting system was installed, providing for 900–1000 lx in a 12 h day photoperiodic pattern. The prawns were fed *ad libitum* a commercial formulated extruded feed for marine shrimp (Faradaneh Co. Shahrekord, Iran) twice a day (at 0700 and 1900). Also, larvae collection, larvae-culture, water quality and larvae feeding were conducted using the procedures described by Rafiee et al. (2015). The larvae were fed *Artemia franciscana* (Great Salt Lake strain)

![Fig. 2. X-Ray Diffraction (XRD) spectra of the ZnO-NPs (Philips PW1730, NED) and the peak points of 31.8, 34.5, 36.6, 47.6, 52.9, 62.9, 67.9, and 69.1 were recorded to the crystalline plates.](image-url)
nauplii twice daily at 0700 and 1900.

2.4. Evaluation variables

2.4.1. Prawns brood-stock reproductive variables

Inter-molt period, inter-spawn period, viable egg percentages, egg weights, fecundity, egg clutch somatic index (ESI), weight gain (WG) and survival rates of the prawns were recorded during the study. Giant freshwater prawn females have eggs and the egg clutches attached to their swimming legs. Egg clutches were removed from ovigerous females to estimate fecundity, viable egg percentages, egg weights and ESI. The WG, ESI, viable egg percentages and fecundity were calculated using standard equations described by Rezaei Tavabe et al. (2015a, b) and Javanmardi et al. (2020). Egg and egg clutch quality were evaluated using a stereo microscope to determine normal and abnormal eggs, non-viable eggs and eggs with small amounts of yolk using the characteristics for structure and color described previously (Tavabe et al., 2013). Weight gain (WG) was determined using the formula where %WG = [(Final mean weight (g) - Initial mean weight (g))/Initial mean weight (g)] × 100; ESI was determined using the formula, ESI = Egg-clutches weight/Body weight × 100; and there was determination of Viable Egg (%) using the formula = Viable Eggs (%) (Normal eggs/Total fecundity) × 100.

2.4.2. Larvae development indices

Larvae weight, larvae condition index (LCI), larvae stage index (LSI), survival rate and time of the first post-larvae (PL) appearance indices were calculated and recorded. Larvae weight and survival rate (%) were determined at 5, 15, and 25 days post hatching (dph) for all groups. The LCI and LSI indices were determined at 5, 10, 15, and 20 dph as described by Tayamen and Brown (1999) using the formula LCI = Σ P/10 N where N was the number of larvae examined; and P was the score recorded for each larve.

2.4.3. CHH concentrations

Crustacean’s hyper-glycemic hormone (CHH) concentrations in hemolymph were quantified using procedures described by Levenson et al. (1999). The hemolymph sample in each well was mixed 1:1 (v/v) with sodium bicarbonate buffer. After washing with buffer solution (10 mM/l PBS, pH 7.4 and 0.1% Tween 20) the plate was blocked with 100 μl of a specific blocking buffer (10 mM/l PBS, 0.1% Tween 20, 2% BSA) for 2 h and then incubated with anti-CHH solution (dilution 1:10,000 in blocking buffer) for 2 h at the laboratory temperature. The plate was washed and incubated with the secondary antibody, anti-rabbit IgG peroxidase (Sigma A4914), for 2 h. The plate was washed again and 100 μl of Tetramethylbenzidine (TMB) ELISA substrates were added into each well to initiate the enzymatic reaction for 10–30 min at 37 °C. There was subsequently adding of 2 M/l H2SO4 so that the enzymatic reaction was stopped and there were detections for CHH quantitation at 450 nm on an ELISA-reader (Cyberlab Inc., USA).

2.4.4. Anti-oxidant enzyme activity assay

At the end of research period, there was collection of tissues from three prawns from each treatment group. Prawns were anaesthetized and hepatopancreas tissue of an individual prawn was removed then catalase and superoxide dismutase (SOD) enzyme activities were assayed. Catalase enzyme activity was quantified using the methods described by Takahara et al. (1960). Furthermore, the SOD enzyme activity in the hepatopancreas was quantified using the methods described by Du et al. (2019), based on the oxidation of epinephrine to adreno-chrome by the enzyme.

2.5. Ethics statement

The authors confirm that the trial protocol was approved by the Ethics Committee for the Animal Research, University of Tehran (IR.UT.REC); none of the prawns suffered from starvation, trauma or electrical shock and all animals were completely anesthetized before tissue sampling.

2.6. Data analysis

The data were evaluated using the Shapiro-Wilk test and all collected data distributions were normal. So, the data were analyzed using a one way ANOVA and there were considered to be mean differences when there was a P < 0.05 using the Duncan test in SPSS version 24 (IBM, USA).

3. Results

3.1. Brood-stock reproductive variables

When there were larger ZnO-NP concentrations imposed on the prawns in the treatment groups, values for reproductive variables were markedly affected. In the 100 mg/L ZnO treatment group, the brood-stock did not spawn and in the 50 mg/L treatment group, the prawns spawned but all larvae were detected to be non-viable immediately after hatching in the experimental tanks. Similar concentrations of ZnO-NPs that had these detrimental effects had no effect on the total fecundity of the prawn brood-stock, but had significant effects on viable egg rate, egg weight, brood-stock inter-spawning period and values for ESI variables. For the control treatment, viable egg rates and egg weights were 97 ± 3% and 26 ± 2 μg while in the 50 mg/L treatment group the values for these
variables were $23 \pm 5\%$ and $19 \pm 1 \mu g$, respectively (Table 1). There were differences in egg weight and viable egg rates among the treatment groups and there were effects on ESI values and quality. Depictions in Fig. 3 are indicative of differences in egg clutch quality among treatment groups in comparison to the control group.

3.2. Larvae development indices

In the $100 \text{ mg/L}$ ZnO treatment group, the brood-stock did not spawn and in the $50 \text{ mg/L}$ treatment group all larvae were non-viable immediately after hatching in the experimental tanks. Even though there were differences in the incremental effects of ZnO-NPs on the larvae and larvae quality variables in the other as compared with the $100 \text{ mg/L}$ treatment groups, these effects of ZnO-NPs on larvae variables were marked. With increasing ZnO concentrations among the other treatment groups, the LCI, LSI, larvae dry weight and larvae survival variables were markedly less than these values for the control group. Furthermore, for these groups synchrony in development to the PL stage and time of the first PL development were markedly prolonged (Tables 2 and 3, Figs. 4 and 5).

3.3. CHH release from the X-organ into the hemolymph

The results highlighted that CHH release into the $M. \text{ rosenbergii}$ hemolymph from the hepatopancreas for the $10 \text{ mg/L}$ treatment group was similar in comparison to the control group, while in the other groups this hormone release from the X-organ into the hemolymph was greater when there were the larger treatment concentrations of the ZnO-NPs (Fig. 6).

3.4. Enzyme activity

In the hepatopancreas tissue, there were associations of values for activities of both SOD and catalase with the concentrations of ZnO-NPs in the experimental treatment groups. The SOD enzyme activities were markedly different among the treatment groups with there being increasing activities as concentration of ZnO-NP treatments increased. Catalase enzyme activities were similar in the 50 and $100 \text{ mg/L}$ treatment groups, however, the values for the other treatment groups differed markedly among the different groups (Figs. 7 and 8).

4. Discussion

Reproductive tissues are very sensitive to the adverse effects of NPs in the bio-organisms (Brohi et al., 2017). The NPs can cross biological barriers and are potential factors having detrimental effects on reproductive organs in susceptible females (Wang et al., 2011). In aquatic animals and environments, timing of reproductive functions including puberty, follicular atresia, follicular maturation and ovulation are affected by physiological responsive to environmental factors and stressors (Schreck et al., 2001). Findings in the present study indicated that $M. \text{ rosenbergii}$ reproductive variables, offspring development, CHH release from the X-organ into the hemolymph and anti-oxidative enzyme activities were markedly affected by ZnO-NPs in laboratory conditions. The NPs may have actions on primary ovarian follicles directly and/or subsequently affect vitellogenesis of zebra-fish ($D. \text{ rerio}$; Wang et al., 2011). The ZnO-NPs actions are primarily through enhancing apoptosis in reproductive organs and in the endoplasmic reticulum signaling pathway (Tang et al., 2019). Exbrayat et al. (2015) confirmed that when the effects of NPs in crustaceans are chronic, the growth of these animals is less and reproductive functions are less. The NPs can adversely affect organisms such as the Wistar rat reproduction organ as well as mechanisms and functions during vitellogenesis and embryogenesis (Rollerova et al., 2015).

The results from the present study confirmed that by increasing the ZnO-NPs concentrations, the values for the reproductive variables of prawns were markedly affected. In crustaceans, the effects of NPs result from ingestion and adsorption through surface epithelia cells of these compounds (Walters et al., 2016). As soon as the NPs enter the body of organisms, these compounds induce

<table>
<thead>
<tr>
<th>Water contaminated by ZnO NPs (mg/L)</th>
<th>WG (%)</th>
<th>Survival (%)</th>
<th>Inter-molt period (days)</th>
<th>Inter-spawn period (days)</th>
<th>Egg dry weight (µg)</th>
<th>Fecundity (eggs/female)</th>
<th>Viable Eggs (%)</th>
<th>ESI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 100</td>
<td>25.76±2.75</td>
<td>36 %</td>
<td>24±3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>68.75±4.32</td>
<td>95 %</td>
<td>25±2</td>
<td>35±3</td>
<td>26±2</td>
<td>3855±432</td>
<td>97±3</td>
<td>9±1</td>
</tr>
<tr>
<td>20</td>
<td>68.24±2.00</td>
<td>87 %</td>
<td>24±4</td>
<td>38±3</td>
<td>26±1</td>
<td>4150±563</td>
<td>92±2</td>
<td>9±2</td>
</tr>
<tr>
<td>50</td>
<td>57.33±3.21</td>
<td>62 %</td>
<td>23±4</td>
<td>45±2</td>
<td>19±1</td>
<td>3750±461</td>
<td>23±5</td>
<td>6±1</td>
</tr>
<tr>
<td>100</td>
<td>25.76±2.75</td>
<td>36 %</td>
<td>24±3</td>
<td>At this treatment concentration the brood-stock did not spawn.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1

Weight gain (WG), survival and values for reproductive variables (mean ± SD) of the $M. \text{ rosenbergii}$ female brood-stock treated with different concentrations of ZnO-NPs ($n=3$ brood-stock).

Values with different letters are different ($P<0.05$).
Fig. 3. Giant freshwater prawn brood-stock eggs and egg clutch quality when there were different ZnO-NP treatments at 2 days after spawning, a: control treatment with normal and spherical eggs; b: 10 mg/L ZnO treatment with normal and abnormal eggs and some eggs with a small amount of yolk contents; c: 20 mg/L ZnO treatment resulting in non-viable and abnormally developed eggs; d: 50 mg/L ZnO treatment resulting in non-viable and development of small eggs with a small amount of yolk content; (The brood-stock treated with the 100 mg/L ZnO concentration didn’t spawn); Scale bar =0.5 mm.

Table 2
Larvae Condition Index (LCI) at different days post hatching (dph) and synchrony in the development to the Post-Larvae (PL) stage (mean ± SD) of the larvae from the *M. rosenbergii* female brood-stock when there were treatments with different ZnO-NP concentrations (*n* = 10 larvae).

<table>
<thead>
<tr>
<th>Water contaminated by ZnO NPs (mg/L)</th>
<th>Larvae Condition Index (LCI) at different dph</th>
<th>Synchrony for PL stage (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>1.83 ± 0.08 ‚</td>
<td>1.88 ± 0.04 ‚</td>
</tr>
<tr>
<td>10</td>
<td>1.62 ± 0.05 b</td>
<td>1.55 ± 0.03 b</td>
</tr>
<tr>
<td>20</td>
<td>1.33 ± 0.04 c</td>
<td>1.25 ± 0.05 c</td>
</tr>
<tr>
<td>50</td>
<td>At this treatment concentration, all larvae were detected to be non-viable immediately after hatching.</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>At this treatment concentration, the brood-stock did not spawn.</td>
<td></td>
</tr>
</tbody>
</table>

Values for treatment groups with different letters in same columns are different (*P < 0.05*).

Table 3
Larval stage index (LSI) at different days post hatching (dph) and time of the Post-Larval (PL) development (mean ± SD) of the larvae from the *M. rosenbergii* female brood-stock when there were different concentrations of ZnO-NP treatments (*n* = 10 larvae).

<table>
<thead>
<tr>
<th>Water contaminated by ZnO NPs (mg/L)</th>
<th>Larvae Stage Index (LSI) at different dph</th>
<th>Time of the first PL appearance (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>3.36 ± 0.23 a</td>
<td>6.00 ± 0.52 a</td>
</tr>
<tr>
<td>10</td>
<td>3.15 ± 0.26 a</td>
<td>6.14 ± 0.56 a</td>
</tr>
<tr>
<td>20</td>
<td>2.10 ± 0.33 b</td>
<td>4.47 ± 0.40 b</td>
</tr>
<tr>
<td>50</td>
<td>At this treatment concentration, all larvae were detected to be non-viable immediately after hatching.</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>At this treatment concentration, the brood-stock did not spawn.</td>
<td></td>
</tr>
</tbody>
</table>

Values with different letters in same columns are different (*P < 0.05*).
tissue stress and subsequently actions lead to cell and tissue damage (Huang et al., 2017). The NPs can distribute to different tissues in organisms especially reproductive tissues and organs, and signals may be transmitted between these organ systems that result in exacerbation of the effects of these compounds (Brohi et al., 2017). Armenti et al. (2008) reported that reproductive hormonal control systems in rats have dynamic functions that are susceptible to stress factors. Although, increases of ZnO-NP concentrations in the treatment groups of the present study had no effect on the brood-stock fecundity, there were marked effects on viable egg percentages, egg dry weight, brood-stock inter-spawning period and values for ESI variables. Sun et al. (2013) reported that the effects of toxic materials on the animal reproduction system may impair normal embryogenesis, hormone release and the structure or function of the accessory reproductive organ structures. The NPs can have detrimental effects on fertility and development of the offspring (Hougaard and Campagnolo, 2012). These previous findings were similar to the results in the present study with there being effects of NPs in aquatic animals after spawning as a result of there being fewer viable eggs produced. Also, Sun et al. (2013) confirmed that NPs adversely affect the female reproduction system and fertility of aquatic animals. In aquatic organisms, and especially macro-benthos decapod bio-indicators, there needs to be further research to evaluate effects of the NPs on the gametes and gametogenesis.

Fig. 4. Larvae dry weight (mean ± SD) at different days post hatching (dph) for different treatment groups (n = 10 larvae); The comparison is inter-group and different letters indicate differences among the treatment groups (P < 0.05); At the 100 mg/L ZnO treatment, the brood-stock did not spawn and in the 50 mg/L group, larvae were all detected to be non-viable immediately after hatching.

Fig. 5. Larvae survival rate (mean ± SD) at different days post hatching (dph) for different ZnO-NP treatment groups (n = 10 larva); The comparison is inter-group and different letters indicate there were differences among the treatment groups (P < 0.05); In the 100 mg/L ZnO treatment group, the brood-stock did not spawn and in the 50 mg/L group all larvae were non-viable immediately after hatching.
Sreenivasula Reddy et al. (2011) reported that stress factors induce glucose release from the hepatopancreas tissue into the hemolymph in decapod crustaceans via secretion of CHH from the eyestalk nervous organ into the hemolymph to decrease the effects of stress factors in the fresh water crab, Oziotelphusa senex senex. The increment of the release of CHH from the X-organ into the hemolymph in crustaceans is directly associated with the extent of effects of the stress factor. Hyperglycemia and increased hemolymph CHH from the hepatopancreas into the hemolymph in response to different stressors in decapod crustaceans have been reported (Stentiford et al., 2001; Lorenzon et al., 2002; Nezhadheydari et al., 2019). Reddy and Sainath (2009) reported that the variations in the CHH concentration values and hemolymph glucose concentrations in response to stress factors could be used as an effective technique to monitor a variety of stress responses in decapod crustaceans. The sinus gland in the X-organ modulates reproduction processes in crustaceans via secretions of various gonadal inhibiting hormone (GIH), molting inhibiting hormone (MIH), mouth organ inhibiting hormone (MOIH) and CHH. Not only is CHH a stress-metabolizing hormone it also regulates reproductive functions (Chung et al., 2010).

Results from the present study indicated that in M. rosenbergii, SOD and catalase enzymes activities in the hepatopancreas tissue are directly associated with concentrations of ZnO-NPs in the treatment groups. Catalase is an important anti-oxidative agent and is present in all oxygen respiring tissues (Klotz et al., 1997), and also SOD is an enzyme that alternately catalyzes the ROS radicals that are

Fig. 6. Concentrations of hyper-glycemic hormone (CHH) of M. rosenbergii (mean ± SD) in hemolymph when there were different concentrations of ZnO-NP treatments (mg/L) (n = 3 brood-stock); Different letters indicate there are differences among the treatment groups (P < 0.05).

Fig. 7. Superoxide dismutase (SOD) enzyme activity in hepatopancreas tissue of M. rosenbergii (mean ± SD) for different ZnO-NP treatment groups (mg/L) (n = 3 brood-stock); Different letters indicate there are differences among the treatment groups (P < 0.05).
produced in response to oxygen and other molecules in various tissues (Walters et al., 2016). These enzymes are considered to be appropriate biomarkers for various stresses, detectable before hazardous effects occur in the red swamp crayfish, *Procambarus Clarkii* (Jiang et al., 2014). Chang et al. (2012) reported that ZnO NPs are highly reactive and induce ROS production at the relatively lesser concentration of 10 mg/L in aquatic organisms. Vogt et al. (2018) confirmed that toxic materials in crustaceans have actions on ROS production inducing cytochrome P450 system disruptions on steroid synthesis and reproduction mechanisms. Stress assays for different contaminating substances and especially NPs are useful to assess environmental risks, usually in evaluating endpoint variables such as mortality rate, egg survival, embryonic development, and reproductive performance (LeBlanc, 2007; Rodrigues and Pardal, 2014). Results from the present study not only confirm results from previous studies, but also emphasize that ZnO-NPs have marked effects on *M. rosenbergii* reproductive performance, antioxidant enzyme activities and CHH secretion modulation. This species, as a freshwater decapod crustacean, therefore, is an appropriate specimen for studying reproductive effects of nano-materials in the freshwater habitat of this species.

5. Conclusion

Aquatic environments are the ultimate destination for accumulation of NPs and the study of the effects of these materials on the reproductive and physiological responses of aquatic organisms in these environments is very important. The results from the present study indicated that ZnO-NPs have marked effects on reproductive performance, offspring development, CHH release from the X-organ into the hemolymph and anti-oxidant enzymes activities in the freshwater prawn, *Macrobrachium rosenbergii*. The findings provide evidence that CHH release from the X-organ into the hemolymph and also SOD and catalase enzymes activities increase in a dose-response manner to ZnO-NPs in *M. rosenbergii*. Monitoring of ZnO-NPs in freshwater environments is essential for evaluating reproduction performance of aquatic species and for protection of the ecosystem in which these species reside.

**CRediT authorship contribution statement**

K. Rezaei Tavabe: Funding acquisition, Conceptualization, Writing - review & editing. B. Samadi Kuchaksaraei: Investigation, Writing - original draft. S. Javanmardi: Investigation, Formal analysis.

**Declaration of Competing Interest**

The authors declare that they have no conflict of interest.

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