

QNZ exposure induces development toxicity and mechanisms of hatching inhibition in large-scale loach (*Paramisgurnus dabryanus*) embryos

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ABSTRACT

QNZ is a quinazoline-type NF- κ B inhibitor and is one of the hot anti-inflammatory drug candidates in recent years. With its development and application, QNZ will inevitably enter the aquatic environment posing a threat to aquatic organisms. To investigate the potential toxicity of QNZ in the early life stages of the organism, this study exposed embryos of large-scale loach (*Paramisgurnus dabryanus*) to 0, 20, 40, 60, and 80 nM of QNZ. The hatching of embryos was significantly inhibited and hatching time was delayed. We explored the mechanism of hatching delay and failure. The results suggested that QNZ exposure reduced the number of hatching gland cells (HGCs) and hatching enzyme activity. Also, the frequency of spontaneous movements was inhibited by interfering with the expression of genes related to the cholinergic system and skeletal muscle development. Further, QNZ exposure induces a series of morphological changes (spine deformation, pericardial edema, tail deformation, and yolk sac edema) in embryos and newly-hatched larvae, and finally increased the deformity rate and mortality rate of newly-hatched larvae. The information presented in this study will provide a scientific basis for further studies into the potential toxicity of QNZ on aquatic organisms.

1. Introduction

Pharmaceutical compounds pollution is one of the most concerning issues because they are designed to react with specific pathways at low concentrations, which can cause considerable changes in aquatic species (Boxall et al., 2012). In fact, pharmaceutical residues have been identified in ground and surface waters throughout the world with levels ranging from ng/L to μ g/L (Patel et al., 2019). This study focused on QNZ, 6-amino-4-phenoxyphenylethylamino quinazoline, which exhibits high anti-inflammatory activity by inhibitory effects on NF- κ B activity (Zhu et al., 2020). It has been found that QNZ could play a potential clinical application in limiting angiogenesis and metastasis of Human hepatocellular carcinoma (HCC) (Liu et al., 2017), in addition to its anti-inflammatory effects. Also, it is a promising anti-Huntington's disease (HD) drug (Nekrasov et al., 2016). With the massive development and application of QNZ (Grekhnev et al., 2022; Rasmi et al., 2020), will inevitably enter the aqueous environment like other drugs, studies on QNZ have only been seen in cellular and mammalian species (Zhu et al.,

2020; Liu et al., 2017; Nekrasov et al., 2016), and no reports have been made on its actual aquatic concentrations. However, as the most potent compound among NF- κ B inhibitors, QNZ has become one of the most sought-after anti-inflammatory and anti-cancer drug candidates in recent decades (Grekhnev et al., 2022). This makes the possibility of its occurrence in the aqueous environment greatly increased with hospital waste and patient excreta. In view of its permanence and interference, the possible disruptive effects on aquatic organism merit great attention.

According to relevant studies reported that anti-inflammatory drugs that act as NF- κ B inhibitors are harmful to certain aquatic species. For instance, Aspirin induced cardiovascular toxicity in zebrafish (Philip et al., 2018). Exposure to Salicylic acid (SA) in the early life stages of common carp, there are significant toxicity effects on their hatching, early ontogeny, morphology, and growth (Buntenkötter et al., 2016). Other studies have also revealed that the use of anti-inflammatory drugs poses a risk to aquatic ecosystems and organisms. So, does QNZ also cause adverse effects on aquatic organisms. This has not been reported yet. The absence of reliable data on the substance's toxicity makes it

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impossible to estimate the dangers connected with it, making it difficult to build regulatory procedures to deal with the associated environmental contamination. As a result, research into the potential harmful effects of QNZ on aquatic animals is crucial.

Hatching process is a very critical step during the development of fish, and normal hatching is the basis to ensure the healthy growth of fish. In both fish and amphibians, hatching is accomplished by the secretion of specific enzymes, known as hatch enzymes, from hatching gland cells (HGCs) (Yu et al., 2015). Hatching enzyme is an astaxanthin-like metalloproteinase with a zinc-dependent catalytic structural domain, a specific protein synthesized and secreted at specific stages of early embryonic development, highly specific for egg membranes and essential for oviparous animals (Yashwanth et al., 2016). However, fish in the hatching period are more vulnerable to mechanical and osmotic stresses, and compounds present in the environment, making changes in this process potentially lethal.

Fish are frequently utilized as toxicological model organisms in the assessment of contaminant toxicity in aquatic environments, because of its highly susceptible to low-dose contaminants in the water environment, and manifest through gene expression disturbances, growth, or metabolic abnormalities (Ma et al., 2018). The large-scale loach (*Paramisgurnus dabryanus*), as an excellent model to study potential effects induced by toxic substances in the aqueous environment, has excellent environmental stress endurance, abundant spawning capacity and long spawning cycle (Shang et al., 2021). Therefore, in the present study, the early developmental processes, growth and hatching mechanisms related indicators of this were evaluated using large-scale loach embryos as a model system aimed to explore the toxic effects of QNZ on large-scale loach embryos. And thus provide basic information to estimate the environmental risks relative to the respective compounds.

2. Materials and methods

2.1. Chemical preparation

NF- κ B inhibitor QNZ (purity > 99%) in this study was supplied from Apexbio Technology LLC (CAS No.545380–34–5, Houston, TX, USA). The test solutions were generated by dissolving in dimethyl sulfoxide (DMSO), with the final content of DMSO fixed at < 0.001% (v/v) in all the tests. The chemical structure of QNZ is shown in Fig. S1 of supplementary information.

2.2. Selection of concentrations

Considering the wide range of anti-inflammatory drug species and concentrations detected in the environment and the scarcity of reports on QNZ alone, we conducted acute toxicity experiments to determine the toxic concentrations on large-scale loach embryonic development. Based on preliminary analyses (Tab.S1–2), we opted to use QNZ concentrations ranging from 20 to 40–60 to 80 nM.

2.3. Experimental fish

Adult large-scale loaches (*P. dabryanus*), with an average weight of 13.0–15.0 g and an average length of 10.5–12.5 cm, were obtained from the Freshwater Aquaculture Institute of Henan Province, China. And the large-scale loaches are selected according to the ratio of 1:2 (10 females, 20 males) and transferred to the laboratory in time.

Artificial insemination by intramuscular injection of HCG (human chorionic gonadotropin, 20–25 IU/g for females, half for males under the same body weight) into the base of the pectoral fin or dorsal fin of *P. dabryanus*. The collected fertilized eggs were randomly allocated into petri dishes containing different concentrations of QNZ, 200 ± 50 embryos in each petri dish and exposed to five conditions, were set up: a blank control (aerated distilled water), and four QNZ concentrations (nominal concentration: 20, 40, 60, and 80 nM), to explore the effects of

QNZ on embryonic development. Triplicate exposures were performed for each group. All related experiments have been approved by the Animal Ethical Committee of Henan Normal University (HNSD-SMKX-2221BS0428).

2.4. The large-scale loach embryo toxicity assay

Each group embryos were examined under a microscope, and the test data at typical time points were recorded including mortality rate, hatching rate, deformity rate, developmental stage, and delayed hatching. During the observation period, nine different endpoints of each group embryos/larvae were evaluated to see whether there were any abnormalities: namely body shape, somites, notochord, tail, fins, heart, face, brain, and pharyngeal arches/jaws, and eight larval specimens per group were used for scoring. At the same time, select the representative abnormal individuals for photos recording (Panzica-Kelly et al., 2010; Di Paola et al., 2022a). For additional reference, endpoints related to growth and developmental stage were assessed, including body length (at 96 hpf (hours post-fertilization), $n = 10$) and heart rates (at 96 hpf, $n = 9$). Triplicate exposures were performed for each group.

2.5. Scanning electron microscopy

Samples were taken during the hatching stage ($n = 5$). Specimens were preserved in 5% glutaraldehyde plus 2% paraformaldehyde in phosphate buffered saline (PBS). Before imaging, specimens were dehydrated gradually in an ethanol series (25%, 50%, 70%, 80%, 90%, 95%, and 100% $\times 3$). On the day of imaging, embryos were placed in amyl acetate for 1–2 h, critical point dried, gold/palladium sputter coated, and imaged on a Zeiss EVO 40vp scanning electron microscope (ZEISS, Germany).

2.6. Hatching enzyme activity

The protein levels of hatching enzyme were assessed via enzyme-linked immunosorbent assay (ELISA) made by Jingmei Biotechnology Co., Ltd. (Jiangsu, China). Thirty embryos were randomly collected and homogenized in a cold phosphate buffer saline (PBS) (pH=7.4) from each treatment group at a specific developmental period ($n = 3$ replicates), the supernatant was collected after centrifugation to detect the activities of hatching enzymes. And at the particular time, the fluid in the petri dishes of each treatment group was collected for further analysis. Enzyme activities were calculated according to manufacturer instructions.

2.7. Spontaneous movement in embryos

Transfer of embryos (20 hpf) to the 6-well plates for incubation were placed in a dissecting microscope (Leica, Germany) and let to acclimate for 5 min. Under the microscope, the number of muscle contractions was recorded for 1 min, including alternating tail curling and bending ($n = 5$). There were three replicates in each treatment group.

2.8. Gene expression analysis

Detection of expression of associated genes in embryos based on toxicity results. Total RNA was isolated from each set of embryos following the manufacturer's directions (RNA Extraction Kit, Invitrogen, CA, USA). The Trizol reagent was added to the samples (20 randomly selected embryos from surviving embryos per experimental group) and homogenized. After extraction with chloroform and isopropanol, the RNA precipitation was washed with ethanol. The concentration and quality of the isolated RNA were verified by 1% agarose-gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo scientific), respectively. cDNA was synthesized via the Prime Script reagent Kit (Takara, Japan). Primer sequence of related genes were designed by

DNAMAN (Tab.S3). The qRT-PCR amplifications were performed on a Roche Light Cycler96 Real Time PCR System (Roche, Switzerland) using the UltraSYBR Mixture (Toyobo, Tokyo, Japan). The qRT-PCR amplification condition was as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s; 65 °C for 60 s; 97 °C for 1 s, and 37 °C for 30 s. Each qRT-PCR analysis was carried out in triplicate and the relative expression levels of target gene were calculated with the $2^{-\Delta\Delta C_t}$ method. The relative level of the mRNA was standardized by β -actin (Di Paola et al., 2022a,b), which was used as the reference gene.

2.9. Statistical analysis

The results in this study were reported as mean $\pm$ standard deviation (SD). All experimental data were processed and analyzed using Origin 2019b and SPSS 16.0 software package. Prior to analysis, the normality and homogeneity of all data was checked (Kolmogorov-Smirnov test and Levene test). One-way analysis of variance (ANOVA) test and Tukey's post-hoc test was utilized to compare different treatments for significant differences. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Developmental toxicity

3.1.1. Mortality rates

Exposure at post-fertilization, the obvious effects of different concentrations of QNZ on the mortality rates of eggs (Fig. 1). As can be seen from Fig. 1a, the embryonic mortality rate of large-scale loach in the 20, 40, 60, and 80 nM QNZ treatment groups was significantly higher than that of the control group at 96 hpf ($p < 0.05$). And at the same treatment concentration, the mortality rate of the embryos increased with the prolongation of the exposure time (Fig. 1b).

3.1.2. Hatching rates

The average hatching rates of embryos (54 hpf) in the control and 20 nM QNZ exposure groups were $97.04 \pm 1.18\%$ and $95.62 \pm 1.05\%$, while the hatching rates of their embryos after 40, 60 and 80 nM QNZ treatment were significantly reduced to $87.12 \pm 0.73\%$ ($P < 0.01$), $79.28 \pm 1.98\%$ ($P < 0.01$) and $32.23 \pm 3.25\%$, respectively ($P < 0.01$, Fig. 2a). And the hatching inhibition of embryos hatching was exacerbated by QNZ treatment with increasing time (Fig. 2b).

3.1.3. Morphological abnormalities

Compared with the control group, as shown in Fig. 3A, the number of malformations in embryos and larvae was significantly increased in each treatment group ($p < 0.01$), and the morphology score showed significantly lower compared with the control group (Fig. 3B). When embryos were exposed to QNZ, developmental defects were seen (Fig. 3C). The morphological abnormalities present in this study were predominantly pericardial edema (PE), body malformations (BM) (including tail deformation, short tail and head malformation), yolk sac edema (YSE), and spinal curvature (SC), and the embryos in the 80 nM QNZ group were not properly developed and did not hatch (Fig. 3B). Meanwhile, we found that the body length of larvae was remarkably inhibited after exposure to 20, 40, 60, and 80 nM of QNZ for 96 hpf (Fig.S4a, $P < 0.05$), but there was no discernible difference in heartbeat (20 s) number of embryos between the treatment group and the control group (Fig.S4b, $P > 0.05$).

3.2. Embryo development

When 50% of the embryos in the control group reached the neural embryonic stage, the organ formation stage, and the hatching stage, the developmental stage of the embryos in the QNZ treatment groups was recorded and subsequently performing synchronicity analysis of embryonic developmental processes (Fig. 4). When 50% of the embryos in the control group reached the neural embryonic stage, the treatment groups were essentially at the proto-intestinal embryo stage (Fig. 4a), and for all periods tested thereafter, embryo development stage was significantly delayed in the treatment groups compared to the control group (Fig. 4b-c, $P < 0.05$). The specific data are detailed in Tab.S2–4. Moreover, we recorded the time taken for complete hatching of 50% of embryos in each treatment group. As shown in Fig. 5, the hatching period of embryos was significantly increased with higher concentrations of QNZ (0, 20, 40, and 60 nM). In the 80 nM QNZ exposure group, fully hatched embryos did not reach 50%.

3.3. The number of HGCs

To investigate the hatching inhibition by QNZ in embryo, we detected the number of HGCs. At 48 hpf, the microscopic image of the HGCs was compared with embryos that had been exposed to QNZ and those that had not, and it revealed that the number of HGCs in the treatment group's embryos was significantly lower than that of the control group, and that HGCs were virtually absent in the image of the 80 nM QNZ treatment group (Fig. 6a-e).

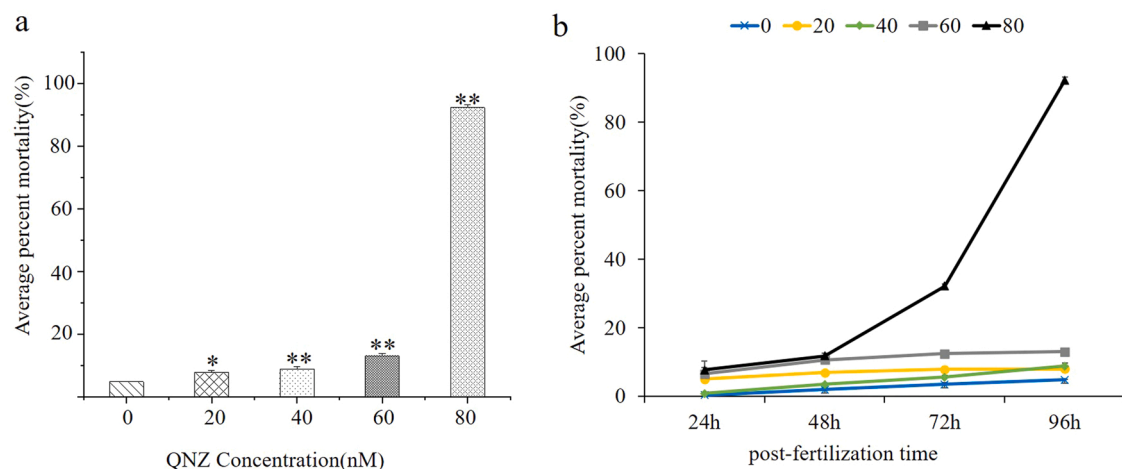


Fig. 1. The average mortality rate (a) and time (b) of the embryos after exposed to 0, 20, 40, 60, and 80 nM QNZ ($n = 3$). a show the average embryonic mortality rate in each group at 96 h; b displayed the trends in average mortality rate of the embryos at 24, 48, 72, and 96 h after exposure treatment. The values are presented as means $\pm$ SD (* $P < 0.05$, ** $P < 0.01$).

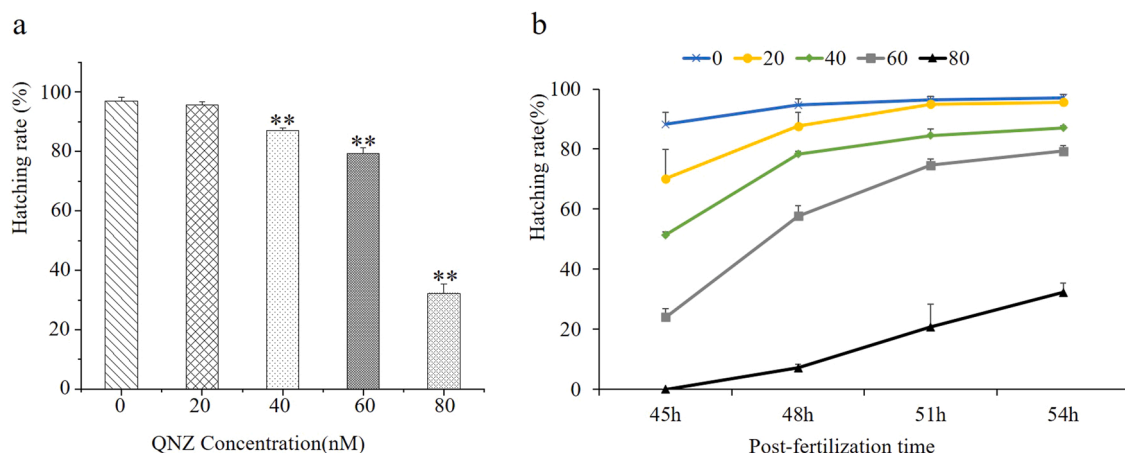


Fig. 2. Hatching rates (a) and time (b) of the embryos exposed to 0, 20, 40, 60, and 80 nM QNZ ($n = 3$). a show the ratio of the number of successfully hatched embryos to the total number at 54 h; b displayed the trends in average hatching rates of the embryos at 45, 48, 51, and 54 h after exposure treatment. Data are expressed as means $\pm$ SD from three independent experiments (* $P < 0.05$, ** $P < 0.01$).

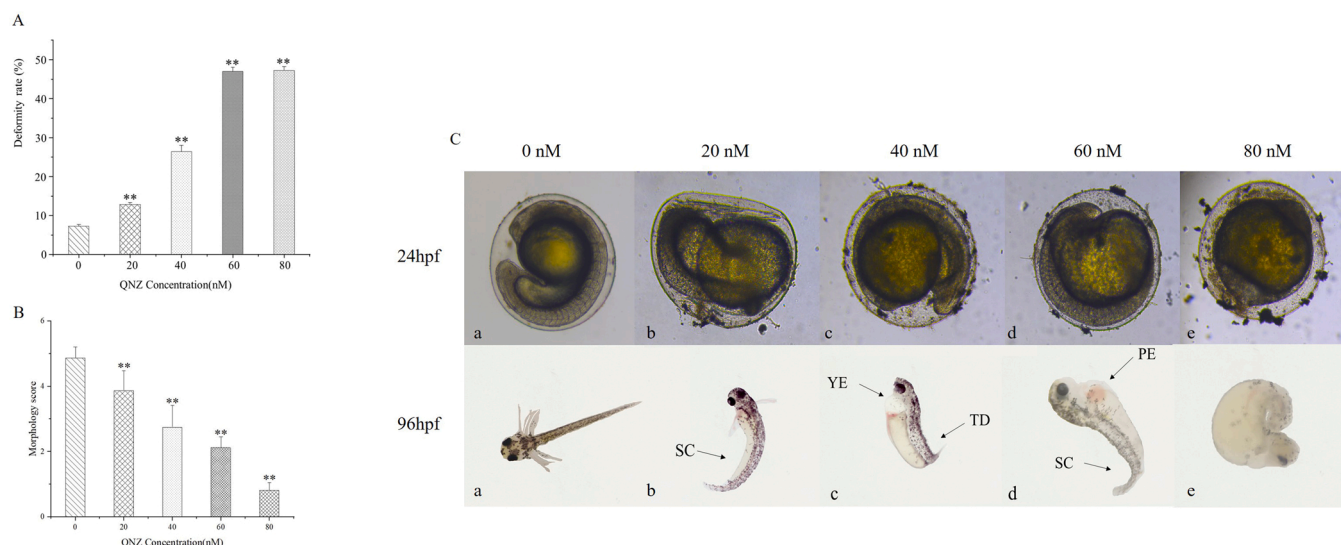


Fig. 3. Deformity rates of newly-hatched larvae (A, $n = 3$), morphology score (B, $n = 8$) and morphological abnormalities (C) after exposure to 0, 20, 40, 60, and 80 nM QNZ. A: Deformity rates of newly-hatched larvae at 96 h. B: Morphology score of newly-hatched larvae at 96 h. Data are expressed as means $\pm$ SD (* $P < 0.05$, ** $P < 0.01$). C: Abnormal individuals present in each treatment group, including spinal curvature (SC); c: larvae with yolk sac edema (YSE) and tail deformation (TD); d: larvae with spinal curvature (SC) and pericardial edema (PE).

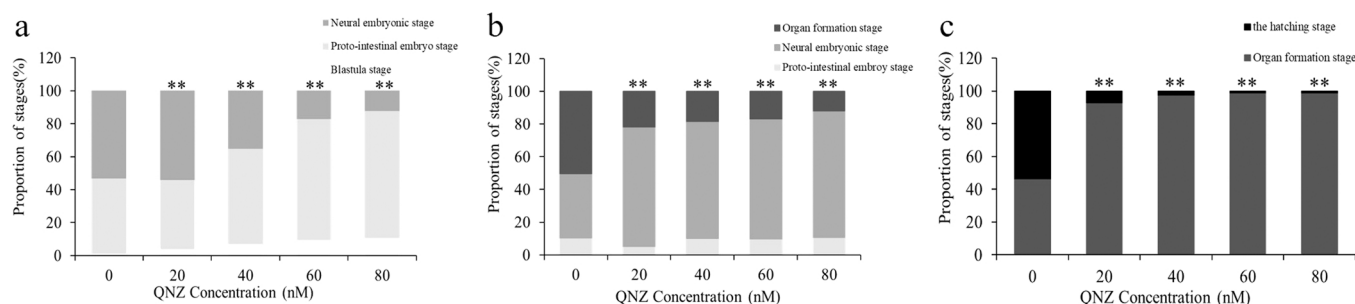


Fig. 4. Synchronicity analysis of embryonic developmental processes that proportion of embryonic developmental stages following exposure to each QNZ concentration (0, 20, 40, 60, and 80 nM). a showed the percentage of embryos in each of the other QNZ treatment groups at the developmental stage when 50% of the control group reached the neural embryonic stage. b showed the percentage of embryos in each of the other QNZ treatment groups at the developmental stage when 50% of the control group reached the organ formation stage. c showed the percentage of embryos in each of the other QNZ treatment groups at the developmental stage when 50% of the control group reached the hatching stage. Asterisks (*) are considered to be remarkable differences compared with the control (* $P < 0.05$, ** $P < 0.01$).

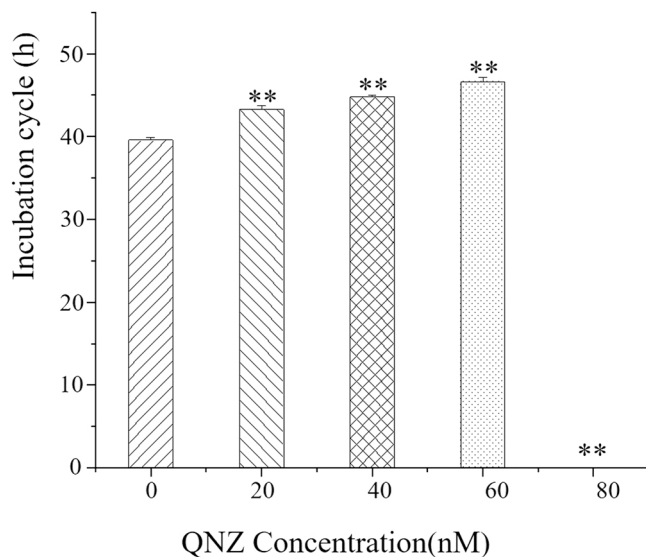


Fig. 5. The time to complete hatching of 50% of embryos in each group (0, 20, 40, 60, and 80 nM QNZ) ($n = 3$). The values are presented as means $\pm$ SD (* $P < 0.05$, ** $P < 0.01$).

3.4. The activity of hatching enzyme

Since there was no significant change in hatching rate in the 20 nM QNZ treatment group, we assayed hatching enzyme activity in embryos exposed to 40, 60, and 80 nM QNZ, with the results shown in Fig. 7a. In all treatment groups, the activities of hatching enzymes showed a trend of higher followed by lower with the development of embryos. However, at the same developmental stage, the activities of the hatching enzyme in embryos had significant changes in different QNZ concentrations. At the muscle effect stage, the 80 nM of QNZ group was significantly inhibited in the hatching enzyme activities of embryos compared with the control group ($P < 0.05$). Additionally, all QNZ treatment groups significantly reduced the activities of the hatching enzyme at the hatching stage in comparison to the control group ($P < 0.05$).

The activity of hatching enzymes in the hatching fluid of embryos was measured in this experiment. The results are shown in Fig. 7b that the activities of the hatching enzyme were no statistical difference in the hatching fluid. Only the high concentration group showed the activity of its hatching enzyme was notably inhibited at the 12 h post-hatching ($P < 0.05$).

3.5. Spontaneous tail movement

In Fig. 8, the quantified tail-wagging counts are shown. Following exposure, the average tail-wagging rates of the embryos in the 20 nM QNZ group ($P < 0.05$), the 40 nM QNZ group ($P < 0.05$), the 60 nM QNZ group ($P < 0.05$), and the 80 nM QNZ group ($P < 0.05$) were 4, 3.8, 2.6, and 2 times per minute, respectively. These rates were significantly lower than those in the control group (5.8 times per minute).

3.6. Gene transcription levels

Among the genes tested, the mRNA levels of the genes (*Mef2c*, *BMP4*, *MyoD*, *Myogenin* and *Myf5*) related to the growth and development of skeletal muscle was variably affected after exposure to QNZ and the heat map of which was shown in Fig. 9. In the hatching stage, *Mef2c*, *MyoD* and *Myogenin* were remarkably down-regulated in the QNZ-treated group (40, 60, 80 nM, $P < 0.05$) compared with those in the control group. At the organ formation stage, 40 and 80 nM QNZ-treated significantly reduced the mRNA level of *BMP4* and *Myf5* ($P < 0.05$) in contrast to the control group ($P < 0.05$), while the *BMP4* was

remarkably up-regulated at the hatching stage in the 40 nM treatment group ($P < 0.05$).

The expression levels of genes related to embryonic hatching were detected. From Fig. 9, we can see that the mRNA levels of *HCE*, *LCE*, *Ctslb*, *klf4*, and *CAP1* were remarkably down-regulated after QNZ exposure during the hatching stage, compared with control embryos ($P < 0.05$). There was a distinct discrepancy in the expression level of *cd63* in the 40 nM and 60 nM treated group in contrast to those in control at the organ formation phase, ($P < 0.05$). Compared with the control, the *Foxp16* gene was remarkably up-regulated in the 40 nM QNZ group ($P < 0.05$), but we detected the striking suppression in its transcription in the 80 nM treated group during the hatching stage ($P < 0.05$).

The heat map representing the gene expression (*ache*, *ChAT*, and *ACHA7*) in embryo on the cholinergic system is shown in Fig. 9. Only in the hatching stage following QNZ exposure did the expressions of *ChAT* and *ache* significantly decrease when compared to the control group ($P < 0.05$). In the meantime, no extreme discrepancy was noticed when the embryos at other periods all the QNZ concentrations; however, after exposure in the 60 and 80 nM QNZ groups in the hatching stage, the mRNA level of *ACHA7* was lower than the control group ($P < 0.05$).

4. Discussion

The hatching process is an essential part of the development of oviparous animals. The process is influenced by many different endogenous and environmental factors and has been shown to be sensitive to a wide range of chemical agents (Wu et al., 2021; Zheng et al., 2020; De la Paz et al., 2017; De la Paz et al., 2017). It is usually evaluated in bioassays to determine the toxicity of different agents on early fish development. In our study, the 40, 60, and 80 nM QNZ-treated groups significantly reduced the embryo hatching rate at all time periods of observation. Also, hatching of large-scale loach embryos was further inhibited with increasing time. During embryonic development, three typical developmental periods were selected, and the synchronicity of embryonic developmental processes in each group and the time required to reach half hatching were examined by different statistical methods, all results showing that QNZ delayed embryos hatching. In this study, QNZ postponed the hatching time and induced hatching failure of the embryos. The fundamental mechanisms of the harmful effects of QNZ-induced hatching inhibition, however, remain a mystery.

Fish hatching is largely dependent on the digestion of egg membranes by hatching enzymes. Hatching enzymes hydrolyze the glycoproteins that make up the radial band of the egg membrane, causing swelling of the egg membrane and the appearance disintegration of the egg membrane (Bond and Beynon, 1995). Hatching enzymes are synthesized in the HGCs during embryonic development, and are produced and released from the HGCs during the larval hatching (Yashwanth et al., 2016). Therefore, the synthesis and secretion of hatching enzymes are largely influenced by the formation and migration of HGCs. The amounts, distribution and morphology of HGCs in the chorion are all factors that influence the success of embryo hatching (Dananjaya et al., 2020). And HGCs are identified morphologically by the microvilli on their apical surface (Yoshizaki and Yamamoto, 1979). At 48 hpf, imaging HGCs under scanning electron microscope and it showed that exposure to QNZ could cause significant abnormalities in HGCs on the surface of embryos. In addition, the current studies found that the expression of multiple specific genes (*ctslb*, *Foxp1b*, *cd63*, *klf4*, and *CAP1*) can be a marker to assess the formation and migration of the HGCs (Gardiner et al., 2005; Michael et al., 2011). The transcriptional disorders of these genes would disrupt the formation of hatching enzymes, which would ultimately result in the zebrafish failing to complete the hatch (Gardiner et al., 2005; Michael et al., 2011; Wu et al., 2021). In this work, QNZ exposure interferes with the normal expression of this series of genes, and the abnormal transcription of these genes is consistent with the earlier studies.

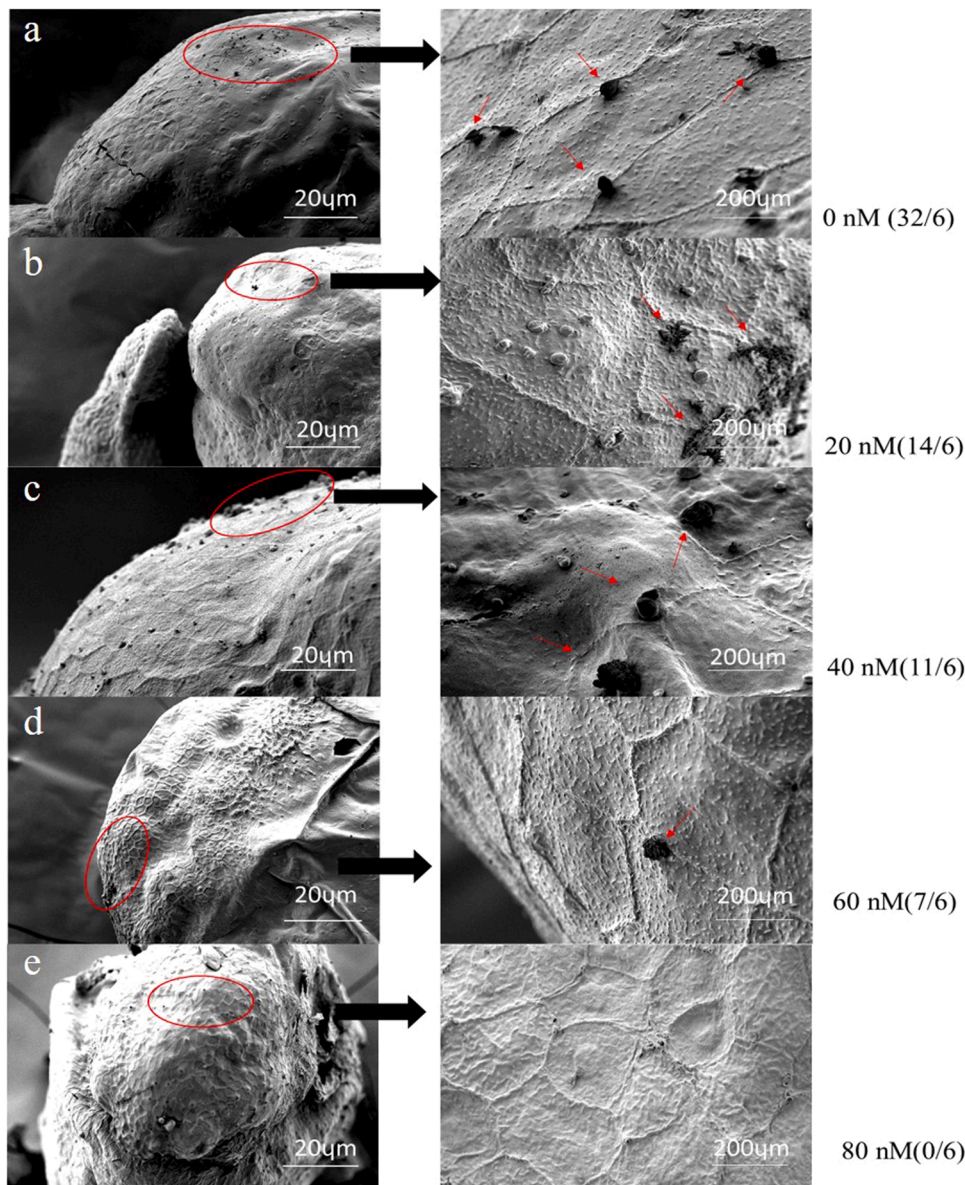


Fig. 6. Scanning electron micrographs showing quantity and morphology of hatching gland cells (HGCs) in large-scale loach embryos after QNZ treatment. The red circle refers to the area of greatest hatching gland density, and the red arrows point to hatching gland cells (HGCs). All cells > 50% within the rectangle were counted. The control group counted three plots with a total of 16 cells; six plots were counted for the 20 nM QNZ treatment group, with a total of 14 cells; six plots were counted for the 40 nM QNZ treatment group, with a total of 11 cells; six plots were counted for the 60 nM QNZ treatment group, with a total of 7 cells; and six plots were counted for the 80 nM QNZ treatment group, with a total of 0 cells.

Hatching enzymes are divided into two primary classes based on their gene structure and evolutionary history. These classes are known as high choriolytic enzymes (HCE) and low choriolytic enzymes (LCE) (Zhang et al., 2021). The chorionic proteins are entirely solubilizing by the cooperative action of both enzyme classes (Sano et al., 2014). Our results clearly showed that the mRNA level of *HCE* and *LCE* was significant down-regulation following exposure to QNZ at the hatching stage. Moreover, the activity of hatching enzymes largely determines the success of hatching (Yashwanth et al., 2016; Wu et al., 2021). When embryos are exposed to environmental contaminants for a long period of time, their hatching enzyme activity is inhibited, ultimately causing a decrease in the hatching rate of zebrafish embryos (Yashwanth et al., 2016; Wu et al., 2021). The location of the presence of the hatching enzyme after secretion is uncertain, it may be present within the chorionic membrane or may be released outside the embryo due to chorionic membrane cleavage. Therefore, during several typical developmental stages, the activity of hatching enzymes was measured at two locations in the embryo and in the embryo incubation solutions. The results suggest that the activity of hatching enzymes in the embryo itself, which is also supported by the results of hatching rates, were suppressed in the different concentration of QNZ during the muscle effect stage, the

hatching stage and the 12 h post-hatching. Based on the aforementioned findings, it was reasonable to draw the conclusion that QNZ ultimately prevents embryos from hatching by interfering with HGCs formation, the production, secretion, and activity of hatching enzymes.

Since the digestion of chorion by hatching enzymes is limited to the inner layer of chorion (radial band), to accomplish hatching, embryos also need to break the weakened chorionic membrane with its own tail movements (De et al., 2017). Our results suggest the presence of QNZ results in the numbers of tails spontaneous movements of large-scale loach embryos were remarkably decreased. In early growth stage, the cholinergic system is necessary for neurons and muscle development in larvae and has been regarded as a major component influencing the larvae's spontaneous tail movement (Serrera-Figallo et al., 2020). Thus, transcriptional profiles of cholinergic system related genes were characterized. The results showed the mRNA transcript levels of *ache*, *ChAT* and *ACHA7* were all inhibited to different degrees after treatment with QNZ. We believe that QNZ treatment suppresses the expression of genes related to the cholinergic system in large-scale loach embryos, and that these abnormalities are consistent with changes in tail spontaneous movements. It suggests the change in spontaneous movements might be caused by the cholinergic system changing.

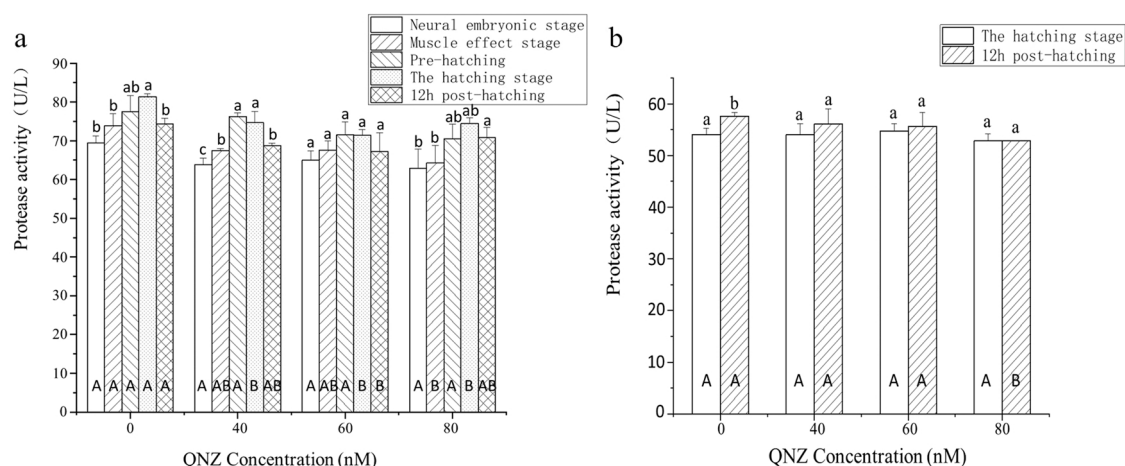


Fig. 7. The activity of hatching enzyme of embryos in the all QNZ treatment groups (40, 60, and 80 nM QNZ) and the control group (n = 3). a show the hatching enzyme activity of the embryo itself. b shows the hatching enzyme activity in the embryo incubation fluid. Each bar represents mean $\pm$ SD. Uppercase letters and lowercase letter above error bars represent differences between and within groups, respectively. They signify difference as follows: the same letters refer to no significant difference ($p > 0.05$), and different letters refer to a significant difference ($p < 0.05$) in each group.

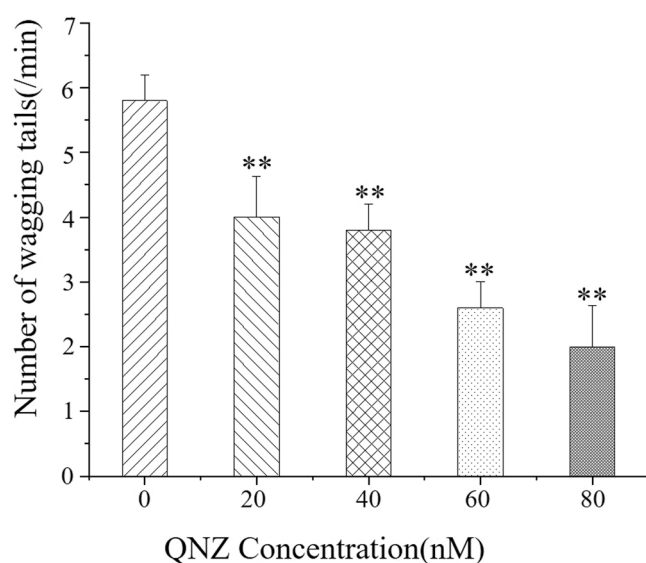


Fig. 8. Spontaneous tail movements of embryos before hatching after exposure to QNZ (n = 5). Data are expressed as means $\pm$ SD from three independent experiments (* $P < 0.05$, ** $P < 0.01$).

Another purpose for delay or failure to hatching might be due to skeletal muscle developmental aberrations, which may also limit the spontaneous movements of developing embryos (Bergstrom and Tapscott, 2001). Multiple distinct genes control the process of skeletal muscle growth (Bergstrom and Tapscott, 2001; Wu et al., 2021). Dysregulation of *Mef2c* influences normal bone development in mice, with osteoporosis and eventual embryo hypoplasia, which confirms the importance of it in skeletal muscle growth (Hinits and Hughes, 2007). In the large-scale loach embryos, our findings demonstrated that the mRNA level of *Mef2c* was down-regulated significantly following exposure to QNZ at the hatching stage. Myogenic regulatory factors (MRFs), which include *MyoD*, *Myf5*, and *myogenin* and can transform non-muscle cell types into myoblasts, are essential for the development of skeletal muscle (Sassoon, 1993). In the MRFs, *MyoD* and *Myf5* have a critical role in the determination of muscle, regulating the transcription of myofibrillar genes that promote sarcomere formation and muscle contraction (Shintaku et al., 2016). The reduction of the *MyoD* expression levels was observed in exposure embryos at the hatching stage, moreover, the expression

levels of the *Myf5* is significant decrease in 40 and 80 nM QNZ at the organ formation stage. Consistent with the result of this present study, the expression of related genes was also similarly affected after exposure to cyhalofop-butyl and 2-methyl-4-chlorophenoxy acetic acid (MCPA-Na) (Sun et al., 2021). *Myogenin* acts as differentiation factors play vital roles in mediating differentiation and maturation of myocytes (Hasty et al., 1993). This study found that *Myogenin* expression level also was distinct down-regulation in exposure groups. As a member of the *BMP* family, *BMP4* functions as an osteogenic bone inducer and in the development of the skeleton (Su and Dong, 2018). When transcription of *BMP4* is repressed during fin regeneration in zebrafish, it leads to abnormal scleroblast differentiation and function and disrupts fin growth (Smith et al., 2006). In the present experiment, the mRNA levels of *BMP4* at the organ formation stage were significantly down-regulated after exposure to QNZ at 40 and 80 nM compared to normal embryos. Combining the aforementioned results, it was logical to believe that the inhibition of spontaneous movements in embryos were attributed to abnormal development of the cholinergic and skeletal muscle systems, which eventually also leads to inhibition of hatching in large-scale loach embryos.

Fish hatching is a critical developmental turning point in the fate of the individual that signals the end of embryonic development and the start of the larval stage (Gilbert, 2000). Larvae may appear dysplasia due to abnormal expression of skeletal muscle-related genes in the development stage of embryos. A key indicator for assessing the teratogenicity of chemicals is dysplasia (Zheng et al., 2020). In the present study, the morphological abnormalities that occurred upon exposure to QNZ were mainly body deformities, including spinal curvature and tail deformation, which was supported by the results of the expression of related genes on the skeletal muscle up-(*BMP4*) and down-regulation (*Mef2c*, *MyoD*, *Myf5*, *Myogenin* and *BMP4*) at different development times. In addition, we also observed pericardial edema and yolk sac edema in the experimental groups. Among the tested genes, *Mef2c* and *BMP4* are also involved in the heart development as a key regulator (Hinits and Hughes, 2007; Bajoghli et al., 2007). The abnormal expression of these genes may be one of the main reasons for the appearance of cardiac malformation. In this study, QNZ has an effect on heart development of large-scale loach embryos, but is not related to heartbeats of larvae, and the details require further studies. As the only nutrient provider for the embryo, edema in the yolk sac will lead to the embryo being unable to absorb nutrients properly, thus delaying or inhibiting the growth of larvae (Nguyen et al., 2021). In this study, exposure to QNZ at the concentrations of 20, 40, 60, and 80 nM resulted

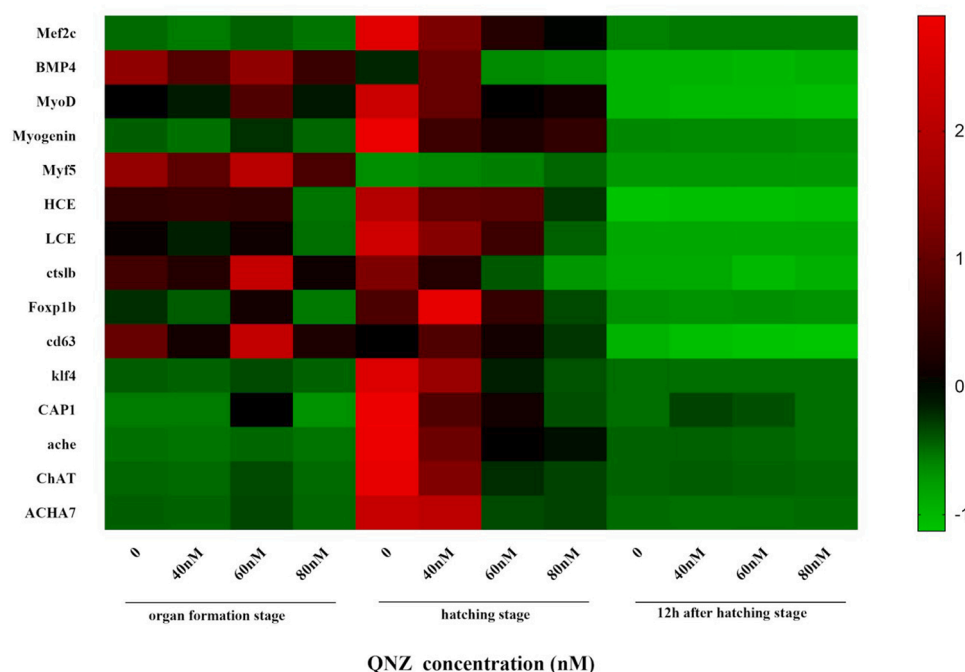


Fig. 9. Heat map of expression levels of genes related to hatching enzyme, muscle development, and cholinergic system of embryos. Data are expressed as means $\pm$ SD. The specific values in the heat map were derived from the fold change of gene expressions converted by log base 2.

in a profound reduction in the body length of large-scale loach larvae, which was supported by the presence of yolk sac malformations. In addition, QNZ also caused an increase in the total malformation rate and morphology score during early developmental stages of fish. The various deformities can disrupt the normal function of various parts of larvae, which in turn prevents the embryo from growing properly. In the present experiment, the presence of a large number of deformed larvae eventually led to a substantial increase in mortality in each experimental group.

In conclusion, our findings indicated that the hatching delay and hatching inhibition of large-scale loach embryos induced by QNZ exposure. The alteration of the number of HGCs, the reduction of hatching enzyme activity, and aberrant skeletal muscle and cholinergic system induce inhibition of embryonic spontaneous movements were some of the main causes of the inhibition of embryonic hatching caused by exposure to QNZ. Additionally, combined with the expression of associated genes result, the existence of QNZ could had adverse influence on growth and even led to their death, to large-scale loach embryos. The above results indicated QNZ induces early developmental toxicity in large-scale loach embryos, but in view of the complexity of embryo development, the more comprehensive understanding of molecular mechanism needs to be further explored.

CRediT authorship contribution statement

Xiaohua Xia, Xiaoyu Ma: Conceptualization, Methodology, investigation, Data curation, Writing – original draft. **Ning Liang:** Formal analysis, Validation, Review. **Xiangyu Duan:** Visualization, Software. **Songyun Wang:** Methodology, Investigation. **Wanwan Guo:** Visualization, Software. **Zhongjie Chang:** Read and revised the manuscript, Review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2023.114663](https://doi.org/10.1016/j.ecoenv.2023.114663).

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