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Arsenic impacted the development, thyroid hormone and gene transcription of thyroid hormone receptors in bighead carp larvae (*Hypophthalmichthys nobilis*)

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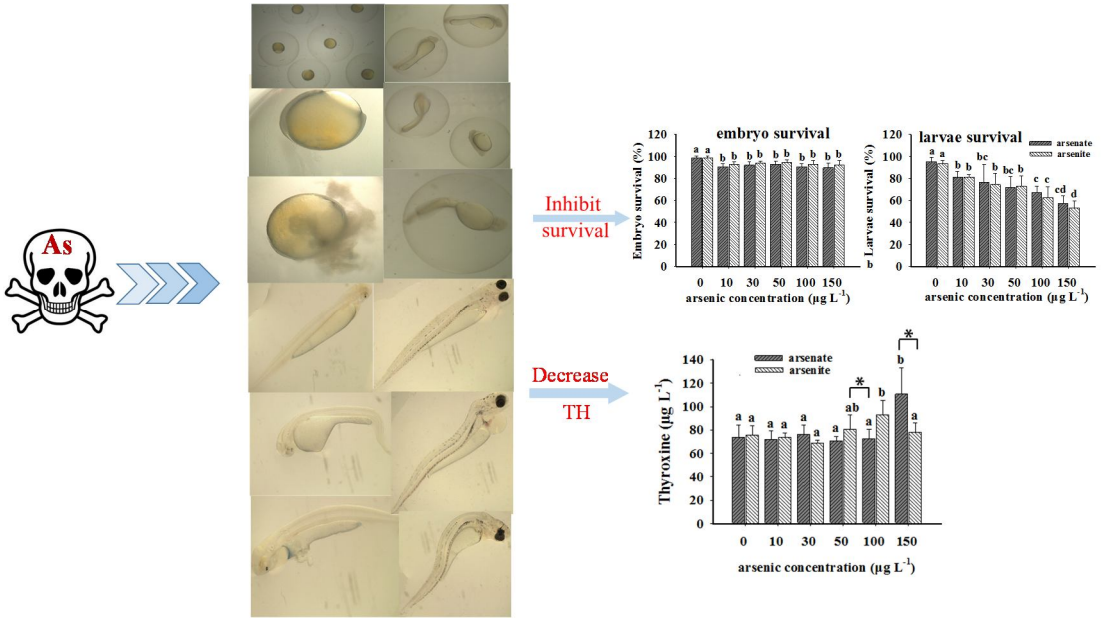

Highlight

- We tested arsenic toxicity on the embryos and larvae of bighead carp;
- We used low levels of AsV and AsIII from 10 to 150 μg L^{-1}
- Arsenic slightly impacted embryo survival, but greatly decreased larvae survival.
- Arsenic increased thyroid hormone thyroxine in fish larvae by23–50% .
- Arsenic reduced transcription of thyroid hormone receptors in fish larvae by 53–91%.

ABSTRACT

Arsenic (As) contamination in aquatic environment adversely impacts aquatic organisms. The present study assessed the toxicity of different As species and concentrations on bighead carp (*Hypophthalmichthys nobilis*) at early life stage, a major fish in Yangtze River, China. We measured the changes in embryo and larvae survival rate, larvae aberration, concentrations of thyroid hormone thyroxine, and transcription levels of thyroid hormone receptors (TRs) in fish larvae after exposing to arsenite (AsIII) or arsenate (AsV) at 0, 10, 30, 50, 100, or 150 μg L^{-1} for 78 h. As concentrations ≤150 μg L^{-1} had limited effect on embryo survival rate (6–8% inhibition), but larvae survival rate decreased to 53–57% and larvae aberration rate increased to 20–24% after As exposure. Moreover, thyroxine levels elevated by 23% and 50% at 100 μg L^{-1} AsIII and 150 μg L^{-1} AsV. Besides, AsIII and AsV decreased the transcriptional levels of TRα by 72 and 53%, and TRβ by 91 and 81% at 150 μg L^{-1} As. Our data showed that AsIII and AsV had limited effect on carp embryo survival, but they were both toxic to carp larvae, with AsIII showing more effect than AsV. As concentrations < 150 μg L^{-1} adversely influenced the development of bighead carp larvae and disturbed their thyroid hormone homeostasis.

Key words: arsenite; arsenate; bighead carp; early life stage; thyroid hormone receptors; thyroid hormone

1. Introduction

Arsenic (As) contamination is of environmental concern worldwide, which results from both natural processes and anthropogenic activities. Aquatic systems such as rivers and lakes have received discharge from domestic and industrial wastewaters for years. As a result, As contamination in aquatic environment is of concern in several regions around the globe. For example, elevated As concentrations in rivers and lakes have been reported, e.g., 150–180 μg L^{-1} in Yangzonghai Lake, China, 8–251 μg L^{-1} in Brahmaputra River, Bangladesh, and 125–145 μg L^{-1} in Ramgarh Lake, India [1–3]. It is known that As exists as arsenite (AsIII) or arsenate (AsV) in the aquatic environment, and both are toxic to aquatic organisms [4, 5].

Yangtze River Delta is the most industrialized and urbanized regions in China, due to the dense population and rapid economic growth in the area, various human activities have led to arsenic contamination in the river. Arsenic concentrations up to 21 μg L^{-1} in Nanjing section of Yangtze River has been reported [6]. With more economic development in the region, higher As level up to 150 μg L^{-1} has been estimated [7], which is the USEPA provisional guideline value [8]. Due to As toxicity, As concentrations < 150 μg L^{-1} may also have adverse effects on aquatic organisms [9, 10]. Considering As levels in Yangtze River has been increasing, it is important to understand the impact of As on aquatic organism. Bighead carp (*H. nobilis*), one of four major Chinese carps, is an important fish for human consumption in Yangtze River [11]. As a top predator in Yangtze River, it is more susceptible to As contamination than other aquatic organisms [12, 13]. It is known that fish at early life stages are more sensitive to As than adult fish because of their thinner epithelial layer combined with a relatively larger body surface to volume ratio, high metabolic rate, and limited mobility [14, 15]. Therefore, it is necessary to examine As toxicity on fish at early life stage.

In the recent years, much research has focused the effect of As on thyroid hormones in different animals. For example, Davey, et al. [16] demonstrated that 0.1–4.0 mg L^{-1} AsIII caused shrinkage in tadpole tail (*Xenopus laevis*) by influencing its thyroid hormone using an in vivo study. Allen and Rana [17] reported that content of thyroid hormone thyroxine in rats increased from 20 to 50 nmol L^{-1} after exposing to 40 mg kg^{-1} AsIII body weight. Mohanta et al. [18] manifested that thyroid hormone levels were significantly reduced in guinea pigs

(*Cavia porcellus*) after feeding food containing 50 mg kg^{-1} AsIII for 11 weeks. But limited information is available on fish. Although our previous study showed that AsIII significantly affects the thyroid hormone in zebrafish [19], no data are available regarding As effect on early life stage of fish. It is known that thyroid hormones including thyroxine (T4) and triiodothyronine (T3) play an important role in the growth and metabolism of fish, especially during transitory phase from embryonic to larvae [20, 21], so it is necessary to understand As effect on thyroid hormone in fish at early life stage.

To date, most studies have used high As concentrations (25–750 mg L^{-1}) to study As toxicity on fish [22, 23]. However, those As concentrations are far above the typical As concentrations (21–150 μg L^{-1}) in the aquatic environment. In addition, most studies only tested the effect of arsenate (AsV), with few studies include arsenite (AsIII) [19, 22]. To better understand the effects of As on fish, we used bighead carp at the early life stage as a test organism to determine the effects of different concentrations of AsV or AsIII (0, 10, 30, 50, 100, or 150 μg L^{-1}) on: 1) the development of bighead carp embryos and larvae, 2) the changes in thyroid hormone thyroxine levels in bighead carp larvae, and 3) the transcription of thyroid hormone receptor genes in bighead carp larvae. The data should shed light on the adverse impact of As on fish at early life stage in aquatic systems including Yangtze River.

2. Materials and methods

2.1 Experiment design

Arsenite ($NaAsO_2$, Sigma-Aldrich, ≥90%) and arsenate ($Na_2HAsO_4•7H_2O$, Sigma-Aldrich, ≥98%) were dissolved in Milli-Q water to make AsIII and AsV solutions. Based on the current (up to 21 μg L^{-1}) and projected As level (150 μg L^{-1}) in Yangtze River reported by Wu et al. [6] and Jing et al. [7]. AsIII and AsV concentrations of 0, 10, 30, 50, 100 and 150 μg L^{-1} were used, which are referred to as $AsIII_{10}$, $AsIII_{30}$, $AsIII_{50}$, $AsIII_{100}$, $AsIII_{150}$, AsV_{10}, AsV_{30}, AsV_{50}, AsV_{100}, and AsV_{150}. The water used in this experiment had the following characteristics: pH = 6.7 ± 0.5, dissolved oxygen = 6.4 ± 0.4 mg L^{-1}, conductivity = 0.252 ± 0.006 mS/cm, and total hardness = 194 ± 12.0 mg L^{-1} $CaCO_3$ [24]. During the experiment, 50 fertilized fish eggs at the middle blastula stage were placed in Petri dishes containing

different AsIII or AsV concentrations. To maintain constant AsIII and AsV concentrations, test solutions were replaced every 12 h. Water temperature and light were as following: 25 ± 1℃ and 14 light/10 dark photoperiod.

2.2 Embryo collection

Artificially fertilized eggs of bighead carp (*H. nobilis)* were obtained according to Chen, et al. [15]. Briefly, fertilized eggs were obtained by inducing ovulation of cultured brood stock with one male and one female being injected with (D-Ala6-Pro9-Net)-luteinizing hormone-releasing hormone analogue. The eggs were manually removed and artificially fertilized. Collected embryos were rinsed with embryonic rearing water and examined under anatomical lens (LAS EZ, Leica, Germany). Normally developing embryos were selected at the middle blastula stage (i.e., 5 hour post fertilization) and then transferred to plastic Petri dish (Nunc Plastics, Roskilde, Denmark) for AsIII and AsV treatments. Each incubation unit consisted of a glass Petri dish filled with 100 mL of a test solution.

2.3 Arsenic exposure experiment

It take ~30 h from embryo hatch into larvae, dead embryos were removed and counted every 8 h during these 30 h. Toxicological measurement included embryo survival, structural malformation and larvae survival. Malformations of crooked fish spine were defined as scoliosis and curvature of fish tail. Fish mortalities included coagulated embryos before hatching and dead larvae. To determine the effect of As on the content of thyroid hormone thyroxine in early life stage of bighead carp, newly hatched fish larvae were continued to expose to AsIII or AsV solutions for 48 h until carp embryo finished hatching. At the end of experiment, fish larvae were removed and stored at –80°C for subsequent assays. Each exposure experiment was replicated six times.

2.4 Analysis of thyroxine in fish larvae

Given thyroxine (T4) content secreted by thyroid gland in fish is more than 20 times of that triiodothyronine (T3), thyroxine was used as an indicator of thyroid hormones in this study. After exposing to AsIII or AsV for 78 h, fish larvae were removed to determine the changes in the thyroxine content. Thyroxine content was measured as whole-body homogenates with samples being kept on ice during the entire procedure. Whole larvae was

rinsed in 0.68% ice-cold physiological saline solution and then dried on filtrating paper. Samples were placed in Dounce homogenizer and homogenized after addition of physiological saline solution at tissue: solution of 1:9. Subsequently, whole larvae homogenates were centrifuged at 4000 *g* for 10 min at 4°C to remove cellular debris and cartilage fragments. The supernatant was removed and used for thyroxine content assays. The thyroxine levels were measured using enzyme link immune sorbent assay (ELISA) with a commercial kit for fish (Uscnlife, Wuhan, China). It was based on competitive binding enzyme immunoassay technique, with a detection limit of 0.3 μg L^{-1}.

2.5 RNA isolation and cDNA synthesis in fish larvae

After exposing to different concentrations of AsIII or AsV for 78 h, fish larvae were homogenized using a homogenizer with RNAiso Reagent (TaKaRa, Japan). Briefly, ~ 10 frozen larvae were homogenized in 1 mL of RNAiso Reagent with a homogenizer on ice. Homogenates were kept at 4ºC for 10 min and centrifuged at 12,000 *g* for 10 min at 4ºC. Supernatant was transferred into sterile centrifuge tubes with 0.2 mL of chloroform. After vortexing for 15 s, mixtures were kept at room temperature for 5 min and centrifuged at 12,000 *g* for 15 min at 4ºC. Aqueous supernatant was carefully removed into a new tube without disturbing the bottom phase and 0.5 mL of isopropanol was added and mixed. The mixtures were kept at room temperature for 10 min and centrifuged at 12,000 *g* for 10 min at 4ºC. The pellets were washed with 1 mL of 75% ethanol and centrifuged at 7500 *g* for 5 min at 4ºC before being air-dried for 5 min and finally resuspended in RNase-free water. RNA was measured by measuring the absorbance at 260/280 nm in a UV-Visible spectrophotometer (NanoDorp, ThermoFisher, USA). After remove genomic DNA using reagent (TaKaRa), RNA of 1 μg was subjected to cDNA synthesis with *SuperScript II* reverse transcriptase (TaKaRa, Japan). cDNA was stored at –80ºC for qPCR assays.

2.6 Primer design and amplification of thyroid hormone receptors in fish larvae

The TRs gene sequences from zebrafish and carp for the primer design were retrieved from the NCBI database. TRs were aligned using DNAMAN (Version 7.0, USA). Two forward (TRα1, TRα2, TRα3; TRβ1, TRβ2, TRβ3) and two reverse primers (TRα1, TRα2, TRα3; TRβ1, TRβ2, TRβ3) were designed according to the nucleotide sequences of the

conserved regions, and used for further tests (Table 1). 25 μL of PCR reaction mixture contained the following: 2.5 μL 10 × PCR buffer, 2 μL dNTPs (2.5 mM each), 1 μL each 10 μL primer pair, 0.5 μL 5 U μL^{-1} Taq polymerase (TaKaRa, Japan), 1 μL DNA template (1-10 ng), and 18 μL PCR grade water. The optimized PCR was denatured for 10 min, followed by 40 cycle at 95ºC for 30 s, 60ºC for 45 s, and 72ºC for 1 min; after these cycles, the reaction mixtures were further incubated at 72ºC for 10 min. The PCR products were checked using 1.5% agarose gel eletrophoresis.

2.7 Quantification of gene expression by real-time polymerase chain reaction (RT-PCR)

Expression of the 2 target genes (TRα and TRβ) and internal control (β-actin) were measured by quantitative RT-PCR. All PCR reactions (20 μL) comprised 10 μL of SYBR Premix *Ex TaqII* (TaKaRa, Japan), 1 μL of cDNA, 10 pM of each forward and reverse primer (Table 2) and 7 μL of ultrapure water. The thermal cycling profile used was 95ºC for 30 s, 40 cycles of 94ºC for 5 s, and 60ºC for 30 s. Fluorescence yields obtained from 3 replicate reactions of each cDNA sample were analyzed using the Matercycler ep realplex (Eppendorf, Germany). The relative expression levels of different genes in bighead carp were calculated according to the $2^{-\Delta\Delta CT}$ method [25].

2.8 *Statistical analysis*

All data were presented as mean ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($\alpha = 0.05$). All statistical analyses were carried out with SigmaPlot (version 12.0; Systat Software Inc., San Joe, CA).

3 Results and Discussion

3.1 Arsenic was toxic to the embryo and larvae of bighead carp

Fish embryo and larvae are the most sensitive stage in the life cycle of a teleost, making them sensitive to low-level of environmental stress [11, 26]. Therefore, we tested the impact of 30 h exposure to different concentrations of AsIII or AsV (0, 10, 30, 50, 100 or 150 μg L^{-1}) on the development of bighead carp embryo. In our study, the fish embryos developed well in the control, with embryo survival rate exceeding 98%. However, the survival rates were slightly inhibited by As, decreasing to 92% at $AsIII_{150}$ and 90% at AsV_{150} ($p \leq 0.006$; Table 2, Fig. 1A), with dead embryos turning from transparent (Fig. 2A) into opaque (Fig. 2B) at 150

μg L^{-1} As. These results indicated that As affected the survival of fish embryos. This was because As was probably accumulated in the chorion in fish embryos, with some being in the perivitelline fluid and little being the embryo itself [27]. Li et al. [22] reported that at < 37.5 mg L^{-1}, As did not affect embryo survival rate in zebrafish during the embryonic stages (4–120 hour post fertilization). The different results are probably due to different fish species, which have different capacity to deal with As. In addition, As did not show significant effect on the hatching time of fish embryos, with all embryos taking ~30 h to develop into larvae (data not shown).

Some studies reported that fish larvae are 3–5 times more sensitive to contaminant than embryos [28, 29]. To better understand the As toxicity on early life stage of bighead carp, newly hatched larvae were continued to expose to AsIII or AsV for another 48 h. Compared with embryos, As showed more toxic effect on fish larvae. A significant concentration-response relationship between As concentration and larvae survival rate was observed, with larvae survival rates being decreasing to 53% at $AsIII_{150}$ and 57% at AsV_{150} ($p < 0.001$; Table 2, Fig. 1B). The results showed that As concentrations at ≤150 μg L^{-1} (USEPA provisional guideline value) significantly inhibited the survival rates of fish larvae. The fact that bighead carp larvae were more sensitive to As than embryo suggested that the chorion in fish embryos probably formed a protective barrier to protect embryos from As toxicity [30]. Moreover, the thinner epithelial layer coupled with a relatively large body surface area to volume ratio of fish larvae increased their sensibility to As [14].

Besides larvae survival rates, As also impacted the development of fish larvae (Figs. 1C & 2). The teratogenesis caused by As also showed a clear concentration-response relationship, with the aberration rate being increased from 0 to 24% (AsIII) and 0 to 20% (AsV) with increasing As concentration from 0 to 150 μg L^{-1} ($p < 0.001$; Table 2, Fig. 1C). Normal fish larvae showed well-developed head and tail region 24 h after post-hatching (Fig. 2C) whereas AsIII- and AsV-treated fish larvae showed rupture of pericardium (Fig. 2D), pericardium edema (Fig. 2E) and tail curvature (Fig. 2E). While normal fish larvae showed somites, well-developed head and tail region 48 h after post-hatching (Fig. 2F; red arrows), AsIII- and AsV-treated fish larvae showed morphological abnormalities (pericardial edema, Fig. 1G; and body arcuation, Fig. 2H). This may be due to the imbalance of osmotic

potential, leading to morphological abnormalities of fish larvae [31]. In addition, disruption of endocrine system in fish is another reason for As-induced morphological abnormalities [32]. The findings are consistent with those of Li et al. [22] who showed that higher AsIII concentrations (≥35 mg L^{-1}) induced impairments in cardiac and dorsal of zebrafish. In short, As had limited impact on fish embryos, but showed significant impact on the survival rate and development of bighead carp larvae. However, no significant difference was observed between AsIII and AsV on embryo survival, larvae survival, and larvae teratogenicity (Figs. 1 and 2).

Unlike previous studies focusing on As toxicity on fish at higher concentrations (up to 50 mg L^{-1}) [17, 22, 33], the present study showed As toxic effect of low concentrations of both AsIII and AsV (≤150 μg L^{-1}) on bighead carp at embryo and larvae stage. Our data showed that As ≤150 μg L^{-1} adversely affected the survival rate and development of fish larvae. Therefore, it is important to reduce As contamination in aquatic environment.

3.2 Arsenic increased thyroxine content and decreased gene transcription of TRs

Considering that As is a potential endocrine system disruptor [16], it probably disrupts the thyroid hormones in fish. In the present study, the effect of As on thyroid hormone in fish larvae was evaluated. After 78 h exposure to As, thyroid hormone thyroxine levels in fish larvae were 73.6–75.8 ng L^{-1} in the control. The thyroxine levels in AsIII-treated fish larvae showed significant increase at $AsIII_{50}$ and $AsIII_{100}$, which increased by 6.0% at $AsIII_{50}$, and 23% at $AsIII_{100}$ (p = 0.018; Table 2, Fig. 3A). However, at $AsIII_{150}$, thyroxine content was similar to the control.

Similar trend has also been observed in rats. Wu et al. [33] found that thyroxine levels in rats increased by 18% after exposing to 50 mg L^{-1} AsIII for 10 d. Allen and Rana [17] reported that 40 mg kg^{-1} AsIII gavaged to rats enhanced the thyroxine content from 20.1 to 52.0 nmoles L^{-1}. It is possible that rats strengthen their metabolism capacity by increasing thyroxine levels, resulting in more As being excreted out of their organisms. This is supported by Rana and Allen [34] who reported that elevated levels of thyroxine stimulated As excretion in urine in rats. In addition, Lam et al. [35] proposed that elevated levels of thyroxine improve the immune system in rats, decreasing the detrimental effect of

contaminants. In a similar fashion, increasing thyroxine probably improved the immune system in fish, helping fish to decrease the As toxicity.

Although AsV has lower toxicity than AsIII, some investigators showed that AsV also exerted toxic effect on thyroid gland in fish [36, 37]. In this study, AsV significantly elevated the thyroxine content in fish larvae at AsV_{150} ($p < 0.001$, Fig. 3A); however, compared with AsIII, AsV showed different results. AsV concentrations at ≤100 μg L^{-1} showed little effect, but thyroxine content in fish larvae increased by 50% at AsV_{150}. The data indicated that AsV was less toxic than AsIII as AsV showed thyrotoxicity only at ≥150 μg L^{-1}. The thyrotoxicity of AsV has also been reported by Liu et al. [36] in juvenile zebrafish, which were exposed to 1 mg L^{-1} AsV for 10 d. They suggested that AsV disrupts thyroid homeostasis via thyroid pathological changes. But in our study, low AsV concentrations at 150 μg L^{-1} showed thyrotoxicity on fish larvae. This was because fish larvae were more sensitivity than adult fish to counter As toxicity.

Thyroid hormones exert their effect on fish development primarily by binding to thyroid hormone receptors (TRs), which are encoded by two genes, TRα and TRβ [38]. In this study, after exposure to AsIII for 78 h, TRα and TRβ mRNA expression in bighead carp larvae were inhibited by AsIII at 10–150 μg L^{-1} and increasing AsIII didn't cause additional reduction in neither TRα nor TRβ ($P < 0.001$, Fig. 3BC). The most reduction in mRNA transcription of TRα was 72% at $AsIII_{150}$, and in mRNA transcription of TRβ was 89% at $AsIII_{100}$. Only at higher AsIII concentrations at 50 and 100 μg L^{-1} did it increase the thyroxine levels in fish larvae (Fig.3A), however, AsIII at the lowest concentration at 10 μg L^{-1} was equally effective in decreasing the transcriptional levels of TRs mRNA. It was possible that gene transcription of TRs was more sensitive to AsIII than thyroid hormone thyroxine. The decreased mRNA transcription of TRs was probably helped to withstand the hyperthyroidism caused by AsIII. This was similar with our previous study where we found that, after exposure to AsIII for 48 h, the augment of thyroxine levels in zebrafish decreased TRs transcriptional levels [19].

In addition, AsV also significantly suppressed TRα and TRβ transcription ($p \leq 0.048$; Table 2, Figure 3BC). We hypothesized that the down-regulation of TRs was probably because fish larvae decreased TRs transcription to counteract thyroid hormone disorder caused by As. Similar result was reported by Liu et al. [39] who showed that, after exposing

to triadimefon for 114 h, zebrafish decreased mRNA transcription of TRs to counter increased thyroxine content. Davis et al. [40] also demonstrated that thyroxine can affect the transcriptional activity of TRs by promoting serine phosphorylation of the protein.

Compared to AsIII, the impact of AsV was irregular and less in magnitude, with the most reduction of TRα transcription being 53% at AsV_{50} and TRβ transcription being 81% at AsV_{100} (Fig. 3BC). The differences of TRs mRNA transcription between AsIII and AsV was probably attributed to their different toxic mechanism. Regarding the different toxic mechanism between AsIII and AsV, previous studies showed that AsIII and AsV exert toxicity via different pathways. AsIII exerts toxicity via three possibilities: (a) binding to sulfhydryls thereby damaging proteins and enzymes, (b) causing oxidative stress from production of reactive oxygen species, and (c) inducing nucleophilicity via depletion of *S*-adenosylmethionine. With respect to AsV, due to its structure similarity with phosphate, it can interfere with oxidative phosphorylation by forming an unstable arsenate ester, impacting production of ATP. Moreover, oxidative stress caused by AsV has also been contributed to its toxicity [41, 42].

4. Conclusions

In this study, we showed As concentrations at ≤150 μg L^{-1} had limited effect on embryo survival of bighead carp (6–8% inhibition), but they significantly inhibited the survival rate and increased the aberration rate of fish larvae. This is the first study showed that both AsIII and AsV at low concentrations (μg L^{-1} in this study vs. mg L^{-1} in previous studies) caused adverse impact on bighead carp larvae. The formation of a protective barrier by embryo's chorion probably protected embryo from As toxicity. In addition, higher permeability of fish larvae than embryos increased their sensitivity to As toxicity. During the development stage from embryos to larvae, bighead carp suffered from As toxicity, showing impairments in cardiac and dorsal. Besides, As interfered with thyroid hormone homeostasis in bighead carp larvae by increasing thyroid hormone levels and reducing TRs mRNA transcriptional levels. This observation manifested that As concentrations at 10–150 μg L^{-1} in aquatic system such as Yangtze River probably adversely influence the survival and development of bighead carp larvae. If As level continues to increase, survival of bighead carp larvae maybe adversely

impacted. These results showed that As caused adverse impact on fish at early life stage and it is important to control As contamination in aquatic systems.

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Table 1. Real-time PCR primers used in this experiment.

Name		Sequence
β-actin	Forward	5’- TTCCAGCCATCCTTCTTG-3’
	Reverse	5’- ACCACCAGACAATACAGT-3’
TRα	Forward	5’- CAAGTTCCTACCAGAAGACA-3’
	Reverse	5’- GATGGCAGGCGTAATGAT-3’
TRβ	Forward	5’- TTGTGAGGACCAGATCAT-3’
	Reverse	5’-GTTCAGTGTCAGAGTCTC-3’

Table 2. One-way ANOVA of the effect of arsenite (AsIII) and arsenate (AsV) on embryo survival, larvae survival, larvae abnormality, thyroid hormone thyroxine contents and gene expression of thyroid hormone receptors in bighead carp larvae.

Parameters	Source of variation	DF	SS	MS	F	P
Embryo survival	AsIII	5	0.015	0.003	4.086	0.006
	AsV	5	0.033	0.007	6.809	<0.001
Larvae survival	AsIII	5	0.596	0.119	20.12	<0.001
	AsV	5	0.497	0.010	11.93	<0.001
Larvae aberration rate	AsIII	5	0.206	0.041	12.39	<0.001
	AsV	5	0.153	0.031	17.57	<0.001
Thyroxine	AsIII	5	1363	272	3.697	0.018
	AsV	5	4754	951	6.864	<0.001
TRα	AsIII	5	1.096	0.219	25.93	<0.001
	AsV	5	0.667	0.133	3.159	0.048
TRβ	AsIII	5	1.982	0.396	48.22	<0.001
	AsV	5	2.441	0.488	25.45	<0.001

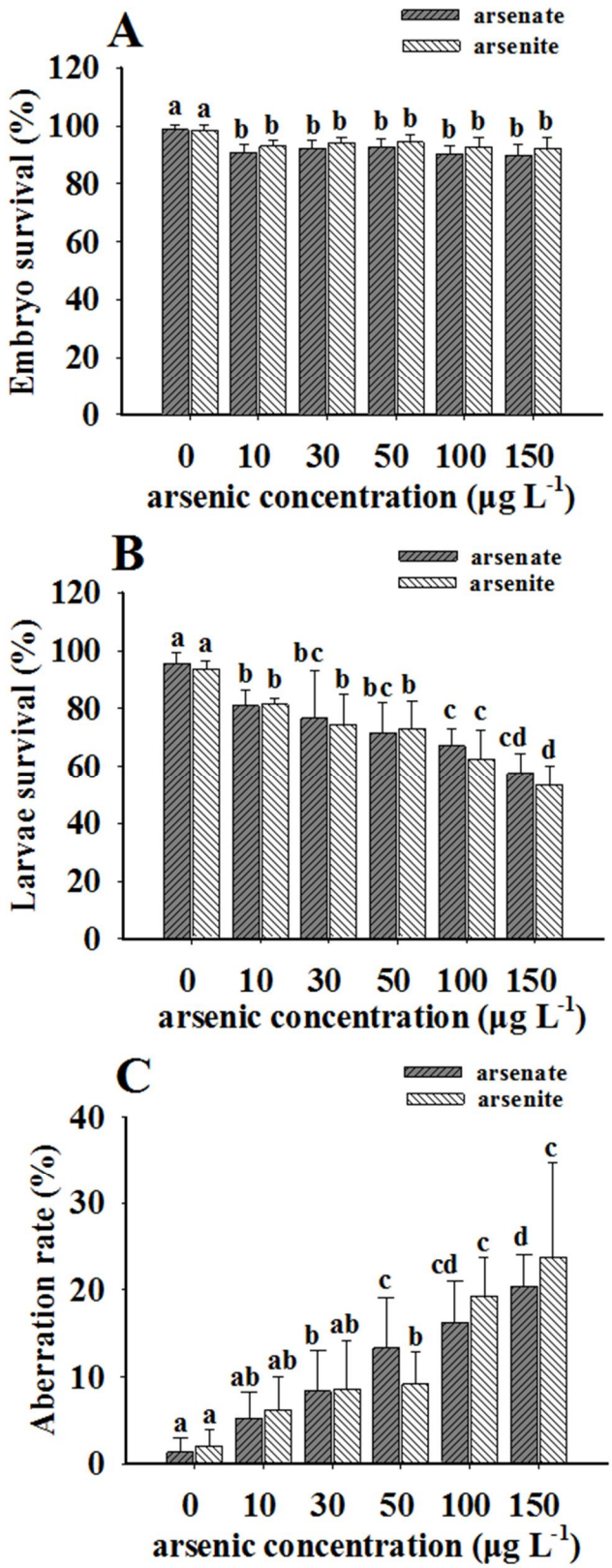

Figure 1. Effect of different arsenite and arsenate concentrations on embryos survival (A), larvae survival (B), and larvae aberration rates (C). Vertical bars represent ± SE, and different letters denote significant difference at p <0.05 (n = 6).

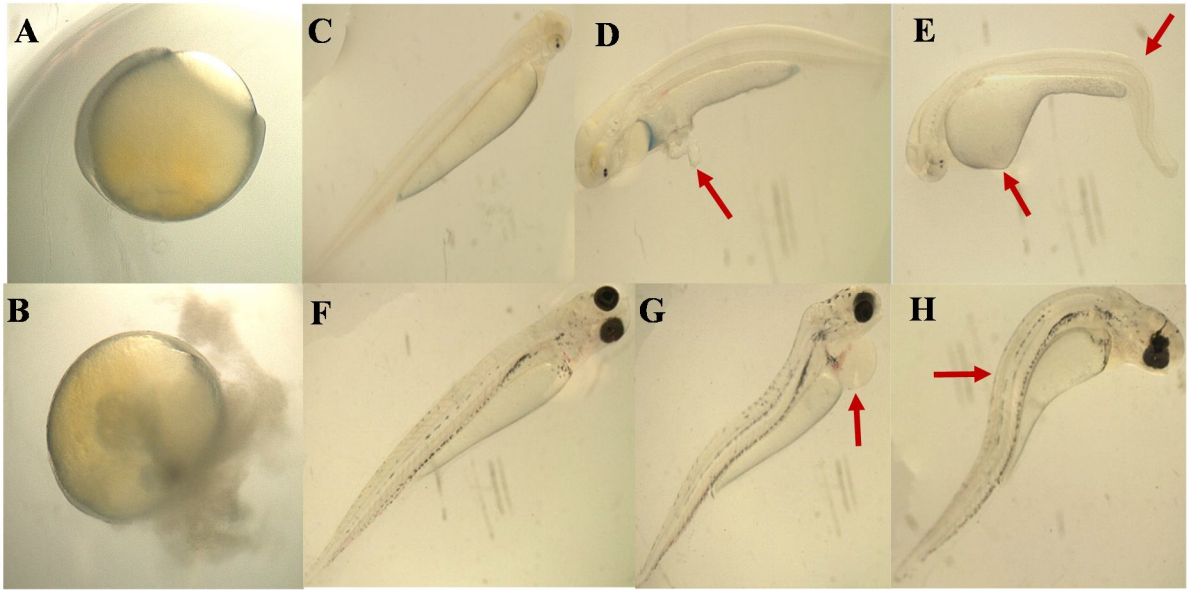

Figure 2. Changes in morphologies of bighead carp embryo and larvae after being exposed to arsenite (AsIII) or arsenate (AsV) for 78 h. Control embryo chorion was intact and transparent (A), and the chorion of AsIII- and AsV-treated embryo was opaque, irregular and fractured (B). 24 h after post-hatching, normal larvae showed clearly well-developed head and tail region (C; red arrows), whereas AsIII- and AsV-treated larvae showed rupture of pericardium (D; red arrows), pericardium edema (E; red arrows) and tail curvature (F; red arrow). 48 h after post-hatching, normal larvae clearly showed somites and well-developed head and tail region (G; red arrows), whereas AsIII- and AsV-treated larvae showed pericardial edema (H; red arrows).

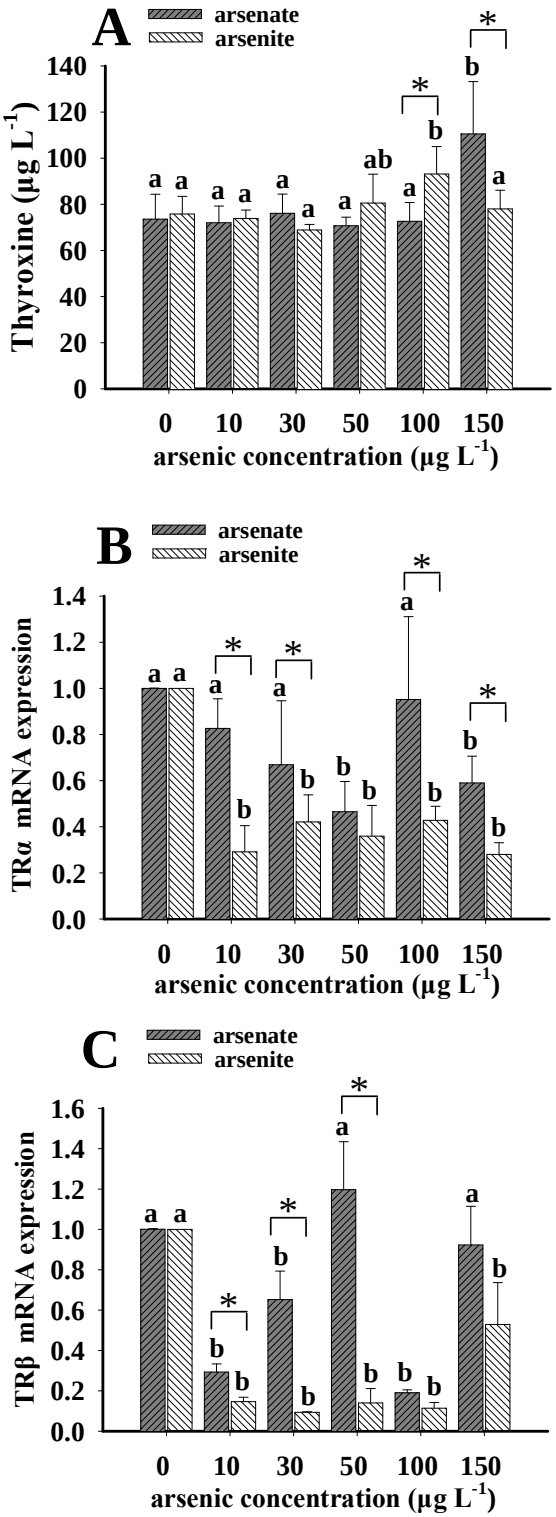

Figure 3. Changes in thyroid hormone thyroxine levels (A) and mRNA transcriptional levels of thyroid hormone receptor TRβ (B) and TRα (C) of bighead carp larvae after being exposed to different arsenite or arsenate concentrations. Vertical bars represent ± SD, different letters denote significant difference for a given As species and * denotes significant difference for a given As concentration at p <0.05 (n =3–4).