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Effects of nitrate on metamorphosis, thyroid and iodothyronine deiodinases expression in *Bufo gargarizans* larvae

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HIGHLIGHTS

- Nitrate caused mortality increase in *Bufo gargarizans* larvae.
- Nitrate could delay metamorphosis and increase body size.
- Nitrate decrease thyroid hormone contents in *Bufo gargarizans* tadpole.
- Nitrate caused partial colloid depletion in the thyroid gland follicles.
- Nitrate induced down-regulation of *Dio2* mRNA and up-regulation of *Dio3* mRNA.

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ABSTRACT

Chinese toad (*Bufo gargarizans*) tadpoles were exposed to nitrate (10, 50 and 100 mg/L NO_3–N) from the beginning of the larval period through metamorphic climax. We examined the effects of chronic nitrate exposure on metamorphosis, mortality, body size and thyroid gland. In addition, thyroid hormone (TH) levels, type II iodothyronine deiodinase (*Dio2*) and type III iodothyronine deiodinase (*Dio3*) mRNA levels were also measured to assess disruption of TH synthesis. Results showed that significant metamorphic delay and mortality increased were caused in larvae exposed to 100 mg/L NO_3–N. The larvae exposed to 100 mg/L NO_3–N clearly exhibited a greater reduction in thyroxine (T4) and 3,5,3′-triiodothyronine (T3) levels. Moreover, treatment with NO_3–N induced down-regulation of *Dio2* mRNA levels and up-regulation of *Dio3* mRNA levels, reflecting the disruption of thyroid endocrine. It seems that increased mass and body size may be correlated with prolonged metamorphosis. Interestingly, we observed an exception that exposure to 100 mg/L NO_3–N did not exhibit remarkable alterations of thyroid gland size. Compared with control groups, 100 mg/L NO_3–N caused partial colloid depletion in the thyroid gland follicles. These results suggest that nitrate can act as a chemical stressor inducing retardation in development and metamorphosis. Therefore, we concluded that the presence of high concentrations nitrate can influence the growth, decline the survival, impair TH synthesis and induce metamorphosis retardation of *B. gargarizans* larvae.

1. Introduction

Global decline in amphibian populations is associated with nitrogen pollution caused by human activity, including industrial discharges, release of sewage, fertilizers and pesticides use (Hamer et al., 2004). In aquatic ecosystems, nitrogen occurs as three common forms (ammoniumion [NH_4^+], nitrite [NO_2^-], and nitrate [NO_3^-]) (Nancy, 2002). Nitrate is generated naturally by biological oxidation in which NH_4^+ is oxidized in a two-step process ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) (Wetzel, 2001), thereby nitrate is the most stable and abundant form in waters. The major sources of nitrate are agricultural application of nitrogen-based mineral fertilizers and atmospheric deposition of nitrogenous compounds (Camargo and Alonso, 2006). Large amounts of nitrogen pollution happens in aquatic ecosystems (Holland et al., 2005), and keeps increasing in future (Gruber and Calloway, 2008; Ortiz-Santaliestra et al., 2012). Models predict that nitrate concentrations may exceed values as high as 34.5 mg/L NO_3–N in surface waters and 300 mg/L NO_3–N in ground waters (Camargo et al., 2005; Gu et al., 2013;

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Wu, 2011). Most polluted areas, the average level of NO_3–N in surface waters was over 100 mg/L (Rouse et al., 1999; Cao et al., 2012). Therefore, nitrate pollution in recent years has aroused widely public concerns.

Nitrate exerts its effects on growth and development through a number of physiological mechanisms associated with the hypothalamic-thyroid axis. Several studies have demonstrated that nitrate disrupts thyroid gland function. Excessive nitrate uptake may decline the thyroid hormones (TH) levels and cause goiter in rats (Zaki et al., 2004). Nitrate at high doses can competitively inhibit iodine uptake and induce hypertrophic alterations in the thyroid gland, as demonstrated in humans (Tajtakova et al., 2006; Ward, 2009). Moreover, increasing intake of dietary nitrate was associated with an increased risk of thyroid cancer in human (Ward, 2009). Similarly, nitrate also exerts its effects on amphibian metamorphosis through alteration of thyroid hormones signaling pathways (Edwards et al., 2006; Hinther et al., 2012). Additionally, nitrate can increase mortality and incidence of deformities, delay growth and development of amphibian larvae (Smith et al., 2005; Krishnamurthy et al., 2006; Griffs-Kyle and Ritchie, 2007; Ortiz-Santaliestra and Sparling, 2007; Ortiz-Santaliestra et al., 2012).

Amphibian metamorphosis provides a unique model to study TH disruption because a series of transcriptional programs such as intestine remodeling, hind-limb emergence and tail resorption, are completely regulated by TH (Buchholz et al., 2007). TH includes thyroxine (T4) and 3,5,3′-triiodothyronine (T3). The main bioactive form of TH is T3, which has up to ten times greater activity than T4 (Stilborn et al., 2013). The major product of thyroid gland is T4, which passes through the larva's circulatory system. The enzyme type II iodothyronine deiodinase (*Dio2*) converts T4 to the main bioactive hormone T3 via an outer-ring deiodination reaction in peripheral tissues, whereas type III iodothyronine deiodinase (*Dio3*) inactivates T4 and T3 by inner ring deiodination reaction (Brown and Cai, 2007). Therefore, *Dio2* and *Dio3* were thought to play an essential role in amphibian metamorphosis and thyroid hormone homeostasis.

Bufo gargarizan is a wide distributed amphibian species throughout the agricultural landscapes in China. *B. gargarizan* has been used as a model animal for detecting chemicals effects in our laboratory (Xia et al., 2012; Zhao et al., 2013). Since the contribution of nitrate to alteration of thyroid hormones signaling pathways in amphibians is unknown, we use a multidisciplinary approach to evaluate the TH-disruption of nitrate on *B. gargarizan* larvae development. The aim of present study was to investigate the effects of environmentally-relevant concentrations of nitrate on the timings of metamorphosis, mortality, body size and body mass at metamorphosis in *B. gargarizan*. In addition, levels of T4 and T3 were measured to assess changes in hormone production of thyroid. Moreover, thyroid gland histological analysis was used as a biomarker for thyroid damages. Finally, RT-qPCR was utilized to determine whether nitrate affects thyroid hormones homeostasis by examining the *Dio2* and *Dio3* expression in peripheral tissues, including intestine, hind-limb and tail.

2. Materials and methods

2.1. Animal husbandry

Five amplexed pairs of adult *B. gargarizans* were captured in Qinling Mountains, Shaanxi Province, China (109°06′52″E, 34°00′56″N). Frogs were placed in one tank with shallow water (50 mm). After spawning, the adult frogs were taken from the breeding tank. Eggs were maintained in one aquarium of 50 cm × 20 cm × 10 cm with shallow water (50 mm) kept at 18 ± 1 °C under a 12 h light: 12 h dark light cycle.

2.2. $NaNO_3$ exposure experiment

Individuals were allowed to develop to stage G26 before chronic toxicity testing (Gosner, 1960). For the study of developmental changes in metamorphosis, larvae at stage G26 were randomly selected and transferred into aquariums (50 cm × 20 cm × 10 cm) with certain nitrate concentrations in 5 L dechlorinated water (well-exposed). The experimental control groups exposed to 5 L dechlorinated water only. Tests consisted of 50 individuals per each container with three replicate containers per exposure concentration including controls. Larvae were offered boiled vegetables during exposure to $NaNO_3$.

Stock solutions of 1000 mg/L NO_3–N were prepared weekly by adding 6.07 g $NaNO_3$ to 1L distilled water. The nominal concentrations of NO_3–N tested were 10, 50 and 100 mg/L, respectively. Reagent-grade $NaNO_3$ (Sigma, St. Louis, MO) was used to prepare stock solution. Each stock solution was electronically pipetted and well-mixed with certain volume of dechlorinated water to obtain the nominal concentrations, and then added to the aquarium. Additionally, exposed individuals were kept under the same experimental conditions (at 18 ± 1 °C under a 12 h light: 12 h dark cycle). In order to maintain the appropriate concentration of NO_3–N and water quality, fifty percent of test solutions volume was renewed daily and entirely replaced every 48 h. Dead larvae were removed, and survival were evaluated when renewing the solutions.

At metamorphic climax (stage G42: determined as forelimbs emergence), the larvae were euthanized and then measured every parameter (body mass, intestine mass, total body length, tail length and hind-limb length). The exposure ended when half of larvae in control groups completed metamorphic climax. Until the end of experiment, each individual was weighted once on an analytical balance having readability of nearest 0.001 g. Lengths of the larvae were measured to the nearest 0.01 mm by Tesa-Cal Dura-Cal Digital electronic calipers. In addition, the number of larvae initiating forelimb emergence was also recorded daily for each replicate aquarium.

Total chlorine in well-exposed tap water was measured using GDYS-101SN chlorine metre (Little Swan, China). Dissolved oxygen and pH in well-exposed tap water were measured using GDYS-201M multi parameter water quality analyser (Little Swan, China). PC300 waterproof portable meter (Clean, USA) was used to monitor water conductivity. Total organic carbon (TOC) was measured by a TOC analyser (TOC-5000A, Shimadzu, Japan). The experimental NO_3–N concentration was measured using an Aquarium Pharmaceuticals Inc. (Chalfont, PA) colorimetric aquarium nitrate test kit that was modified for use on a 96-well microplate. All tank samples, interassay variance samples, and standard curve was prepared by serial dilution of a $NaNO_3$ solution and range from 0 to 30 mg/L NO_3–N. Each microplate contained 50 μL of sample diluted in 100 μL distilled water (or 150 μL standard), 10 μL HCl solution, and 10 μL well-mixed sulfanilamide solution. The microplate was vortexed for 20 s prior to addition of the sulfanilamide and then again for 15–20 s after the sulfanilamide was added. The assay is very sensitive to over-mixing. The microplate was incubated at room temperature for 5–10 min and immediately read on a BioRad Benchmark Plus microplate spectrophotometer using a wavelength of 540 nm. It is very accordant with the lamber-bier law, sample concentrations were calculated based on a linear regression of the standard curve ($R^2 > 0.99$).

2.3. Gross anatomical observations on thyroid gland

Five larvae at G42 in both controls and NO_3–N treated groups were euthanized, and fixed with 4% paraformaldehyde. For gross morphology examination, the larvae transferred into 70% ethanol,

skin and interhyoideus muscle were removed to expose thyroid gland under an Anatomical Microscope (Optec, China). Specimens of each treatment were observed under a Zeiss Discovery V12 stereoscope equipped with Cannon 7D digital cameras.

2.4. Histological measurements of thyroid gland

Five larvae exposed to 100 mg/L NO_3–N and controls were collected respectively, and were anesthetized in 0.03% tricaine methanesulfonate when reaching metamorphosis climax (G42). The whole body including thyroid gland was fixed in 4% paraformaldehyde, dehydrated through an ascending ethanol series and xylene and embedded in paraffin. Serial longitudinal sections at 6 μm thickness were cut, and stained with hematoxylin–eosin. The sections were observed and photographed under Nikon ECLIPSE 80 iMicroscope equipped with a computer and imaging system.

Sections in each sample were acquired from the central portions of the thyroid gland, and thyroid gland was observed pathological alterations such as hypertrophy, atrophy, follicular cell hypertrophy and colloid depletion. For assessing the toxic effects, we chose the largest thyroid gland section to provide an accurate reflection of thyroid gland.

2.5. Reverse transcription quantitative real-time PCR (RT-qPCR) analysis

Four larvae at stage G42 from controls and nitrate treatments were collected respectively. Tissues, including tail, hind-limb and intestine, were obtained from larvae. Tissues were treated with liquid nitrogen, and then stored at −80 °C for extracting total RNA. Total RNA from tissues were extracted using E.Z.N.A.TM tissue RNA Kit (Omega). RNA quality was confirmed by the ratio of O. D. absorbencies 260/280 nm and electrophoresis on a 1.5% agarose gel with ethidium bromide-stained. RNA quantification was measured through UV spectrophotometry at 260 nm using Nano Drop instrument (Thermo). Approximately 2 μg RNA was reverse transcribed into complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (BioRT Two Step RT-PCR Kit). The PCR amplifications were performed in 25 μl reaction mixture containing 100nM of dNTP mixture, 2 μl of cDNA solution as template, 10×PCR buffer (Mg^{2+}), specific primers, Taq mix DNA polymerase (Takara) and also distilled water. PCR was carried out as the following parameters: 94 °C × 3 min, 30 cycles of 94 °C × 30 s, 52 °C × 30 s, 72 °C × 1 min.

Primers for *B. gargarizans Dio2* and *Dio3* were designed using primer premier 5.0 software and were synthesized by Beijing Genomic Institute. The primers sequences are as follows: *Dio2* primers (sequence forward: 5′-GTCAATTTTGGATCAGCTACC-3′; sequence reverse: 5′-ATTGTCCATGCAGTCAG-3′), *Dio3* primers (sequence forward: 5′-CCAACACGGAGGTGGTGAT-3′; sequence reverse; 5′-TTGCAGGCGAGCCATGAAC-3′). *B. gargarizans* α-actin primers set as an internal control (sequence forward: 5′-GCTATG AACTGCCTGATGGTC-3′; sequence reverse: 5′-TGATGCTGTTGTAG GTGGTCT-3′), which generate a 133 bp product. The product size of *Dio2* and *Dio3* were 291 bp and 142 bp, respectively.

We investigated the differences in the expression levels of *Dio2* and *Dio3* mRNA among tissues from different nitrate doses and control larvae. Images of ethidium bromide-stained gels were captured by Gel DocTM XR^+ with Image LabTM software (BIO-RAD). For each tissue, the levels of *Dio2* and *Dio3* mRNA were normalized against the α-actin mRNA levels. The standard curve was used to check the specificity of PCR products. All samples were analyzed in triplicate and the mean value of these triplicate measurements were used for the calculations of the mRNA levels.

2.6. Thyroid hormones extraction and measurement

The whole-body thyroid hormones levels were measured in larvae at stage G42. Thyroid hormones were extracted and measured using Rat fT4 (Free Thyroxine) ELISA kit (Elabscience) and Rat fT3 (Free Triiodothyronine) ELISA kit (Elabscience). Approximately 5–6 larvae (1 g) for each treatment were minced to small pieces which will be homogenized in 9 mL PBS buffer with a glass homogenizer on ice. PBS buffer was prepared freshly. To further break the samples, we sonicated the suspension with a spasmodic ultrasonic disrupter or subject it to freeze–thaw cycles. Then centrifuge the homogenized samples at 5000*g* for 15 min at 4 °C. After centrifugation at 5000*g* for 15 min at 4 °C, the supernatants were collected for thyroid hormones measurement according to the manufacture's instructions. The detection limit was 0.94 ng/mL. Thyroid hormones levels are expressed as nanogram hormone per gram body weight. Intra-assay and inter-assay variations were below 10% in our study. No significant cross-reactivity or interference was observed for each kit.

2.7. Statistical analysis

Statistical analyses were performed with the software SPSS 16.0. One-Way Analysis of Variance (ANOVA) was used to detect the difference of mass (body mass and intestine mass), body size (total body length, tail length, and hind-limb), mortality and TH levels between controls and experimental treatments at metamorphic climax (stage G42). ANOVA was also used to compare statistically differences of *Dio2* and *Dio3* expression at mRNA levels within given tissues. Kaplan–Meier analysis was conducted on cumulative metamorphosis percent among the various treatment groups with monitoring days. The criterions were defined as statistically significant when $p \leqslant 0.05$ or as highly significant when $p \leqslant 0.01$ in all cases.

3. Results

3.1. Water quality

Dechlorinated tap water was used in present study and the quality was monitored during the experiment. Dissolved oxygen was about 6.0–7.5 mg/L. The pH ranged from 6.9 to 7.4. Conductivity, total chlorine and TOC was 196–268 μS/cm, 0.1–0.4 mg/L and 2.86–4.75 mg/L, respectively. Mean NO_3–N concentration in dechlorinated tap water was 2.392 mg/L (range, 2.21–2.42 mg/L). The mean experimental NO_3–N concentrations were 10.23 mg/L (range, 9.68–10.47 mg/L), 51.24 mg/L (range, 49.96–52.32 mg/L) and 101.28 mg/L (range, 99.82–102.31 mg/L).

3.2. Mortality

Exposure to NO_3–N for as long as 78 days had a significant effect on mortality of *B. gargarizans*. Mortalities of groups exposed to the control, 10, 50, or 100 mg/L NO_3–N was 4.00 (±0.00)%, 4.67 (±0.67)%, 13.33 (±1.76)% or 24.67 (±0.67)%, respectively. Mortalities of groups exposed to 50 or 100 mg/L were significantly greater than mortalities of control groups, while no significant difference was observed in 10 mg/L groups.

3.3. Metamorphosis percent

Kaplan–Meier curves describing the relationships between cumulative metamorphosis percent and different NO_3–N concentrations are illustrated in Fig. 1A. Interestingly, the first individual to reach metamorphosis climax (stage G42) occured on the 57th

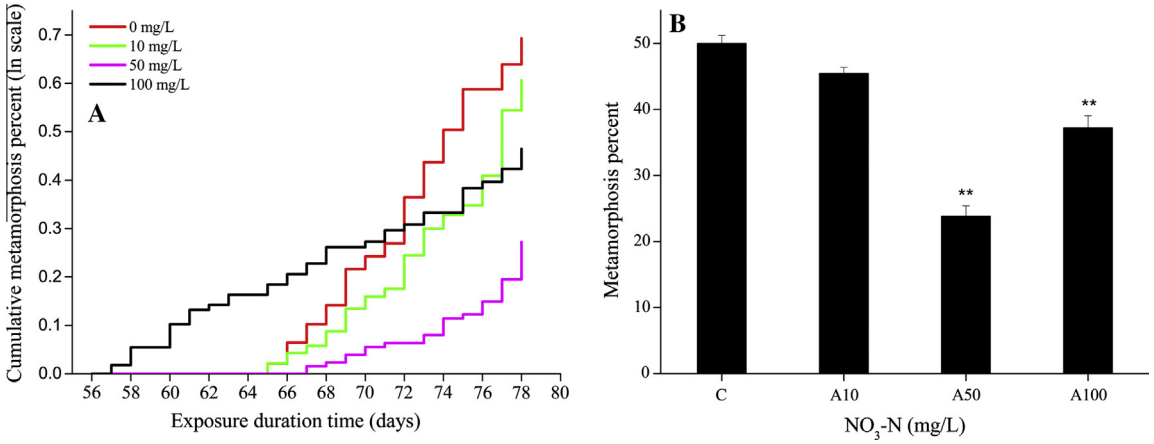

Fig. 1. (A) Time course of cumulative metamorphosis percent (ln scale) by the Kaplan–Meier analysis in *B. gargarizans* larvae exposed to control, 10, 50 and 100 mg/L NO_3–N concentrations. (B) Metamorphosis percent in *B. gargarizans* larvae exposed to control groups and different concentrations NO_3–N (A10, A50, A100) at the end of the experiment (on the 78th day). The data are presented as mean ± SE. Controls are denoted by C. * = Significantly different from control ($p \leqslant 0.05$). ** = Highly significant different from control ($p \leqslant 0.01$).

day of exposure in 100 mg/L NO_3–N. In comparison, the first larvae reaching metamorphosis climax initiated on the 65th day in both control and 10 mg/L NO_3–N groups. In addition, we found the first one reaching metamorphosis climax was on the 67th day of exposure in 50 mg/L NO_3–N, which was 10 days later than that of controls. At the end of the experiment (on the 78th day), the mean metamorphosis percent of larvae was 50% in control groups, while only 23.75% could be observed in 50 mg/L NO_3–N. However, the mean metamorphosis percent of larvae exposed to 10 and 100 mg/L NO_3–N were 45.23% and 37.01%, respectively. Exposure to 50 and 100 mg/L NO_3–N a highly significant increase of metamorphosis percent was detected (one-way ANOVA, $p \leqslant 0.01$) (Fig. 1B).

3.4. Body mass and Intestine mass of B. gargarizans larvae reared for stage G42

The only concentration of NO_3–N that generated significant body mass was 100 mg/L, which increased body mass up to 0.1752 g (one-way ANOVA, $p \leqslant 0.05$) (Fig. 2A). Exposure to 10 or 50 mg/L NO_3–N did not affect body mass (one-way ANOVA, $p > 0.05$). Our results demonstrate that intestine mass of larvae exposed to 100 mg/L NO_3–N was heavier than controls (one-way ANOVA, $p \leqslant 0.05$), but exposed to 10 or 50 mg/L NO_3–N were not significantly different from controls (one-way ANOVA, $p > 0.05$) (Fig. 2B).

3.5. Total body length, tail length and hind-limb length of B. gargarizans larvae reared for stage G42

Total body length, tail length and hid-limb length of larvae reaching metamorphic climax were recorded. Total body length of groups exposed to 100 mg/L NO_3–N was significantly greater than the control groups (one-way ANOVA, $p \leqslant 0.05$). For its part, 10 and 50 mg/L NO_3–N did not affect total body length of *B. gargarizans* larvae (one-way ANOVA, $p > 0.05$) (Fig. 3A). Tail length was significantly greater exposure to 10, 50 or 100 mg/L NO_3–N compared to the tail length of larvae exposed to the control (one-way ANOVA, $p \leqslant 0.05$) (Fig. 3B). We also found that hind-limb length was significantly increased in 10 and 100 mg/L NO_3–N than controls (one-way ANOVA, $p \leqslant 0.05$) (Fig. 3C).

3.6. Effect of NO_3–N on thyroid gland in larvae of B. gargarizans

As depicted in Fig. 4A, the paired thyroid glands situated ventral to the hyobranchial skeleton, and medial to the geniohyoideus. The effects of NO_3–N on thyroid gland were conducted by histological analysis. In control groups, thyroid gland follicles were colloid filled and lined by a simple layer of tightly arranged follicular epithelial cells (Fig. 4B). Larvae in both control and NO_3–N treatments presented partial colloid depletion in thyroid gland follicles. However, the colloid depletion was more remarkable in 100 mg/L

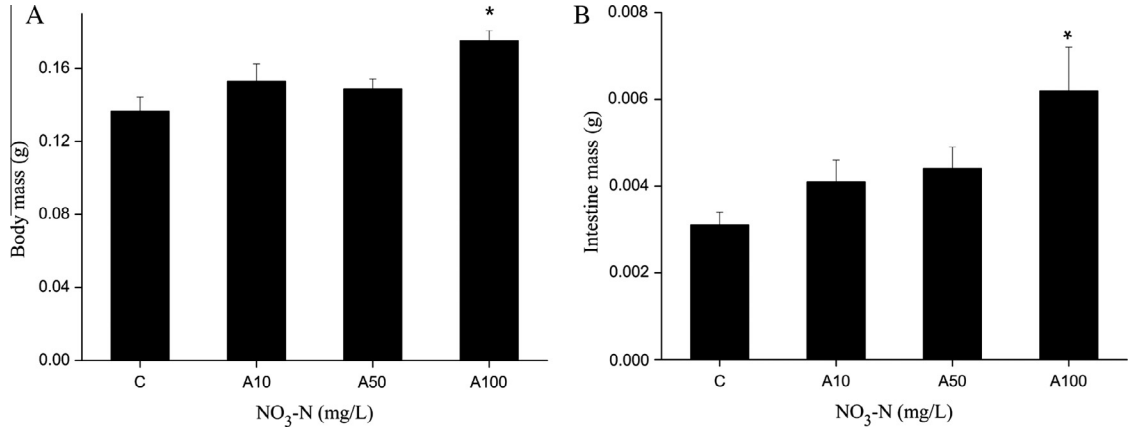

Fig. 2. (A) Body mass and (B) intestine mass of *B. gargarizans* larvae reared for stage G42 under control groups and different concentrations NO_3–N (A10, A50, A100). The data are presented as mean ± SE. Controls are denoted by C. The number indicated at the side of the "A" represents the concentration of NO_3–N in mg/L. * = significantly different from control ($p \leqslant 0.05$).

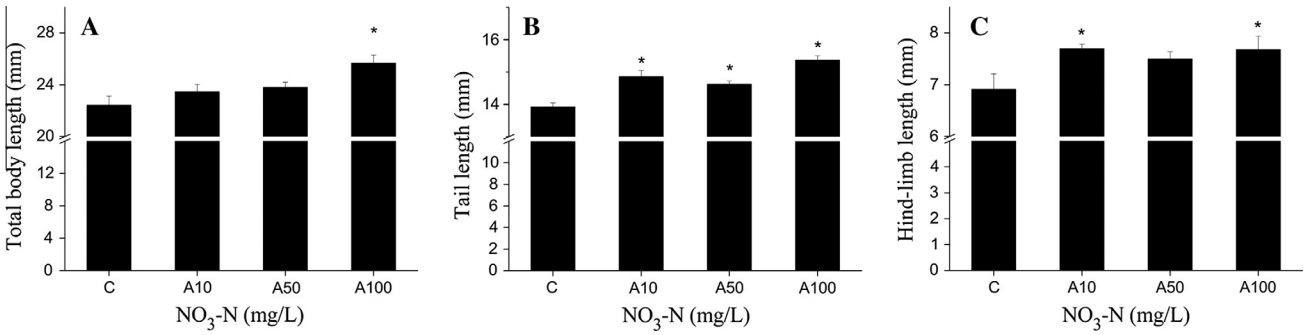

Fig. 3. (A) Total body length, (B) tail length and (C) hind-limb length of *B. gargarizans* larvae reared for stage G42 under control groups and different concentrations NO_3–N (A10, A50, A100). The data are presented as mean ± SE. Controls are denoted by C. The number indicated at the side of the "A" represents the concentration of NO_3–N in mg/L. * = significantly different from control ($p \leqslant 0.05$).

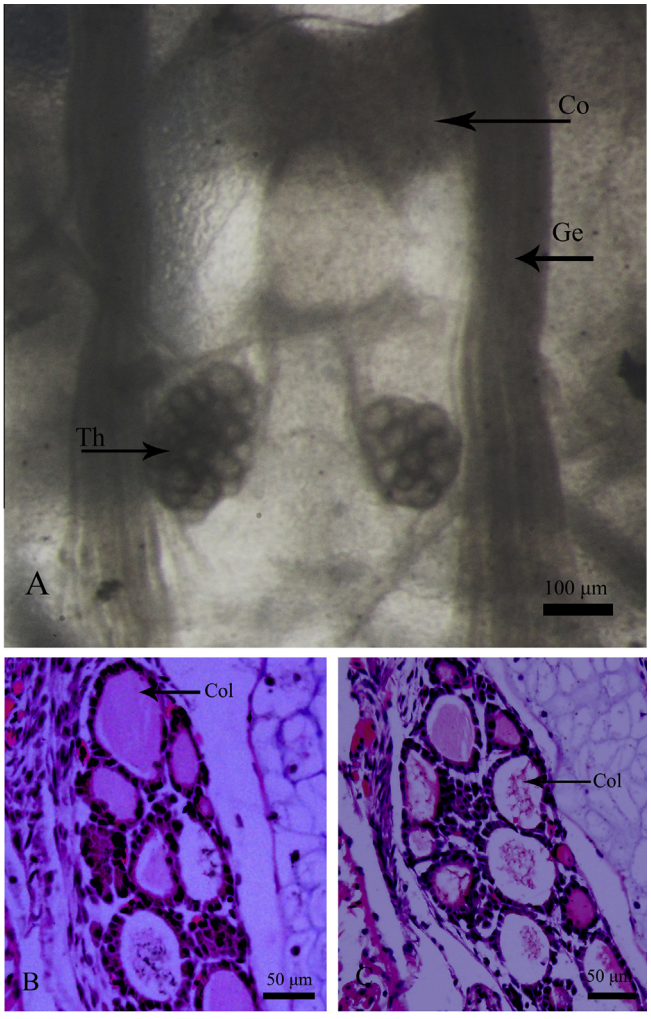

Fig. 4. (A) Gross morphology of thyroid glands examined at stage G42 of *B. gargarizans* larvae in control groups. Thyroid gland (*arrow*) was located on both sides of urobranchial cartilage. Scale bar for A is 100 μm. Ge. Geniohyoideus; Co. Copula II. Comparative thyroid gland histology of *B. gargarizans* larvae exposed to control groups (B) and 100 mg/L NO_3–N groups (C) at stage G42. Each scale bar corresponds to 50 μm. Th. Thyroid gland; Col. Colloid.

NO_3–N treatment compared with the control (Fig. 4C). Histological analysis also shows that exposure to all NO_3–N concentrations did not affect any remarkable lesions of thyroid gland size, such as atrophy or hypertrophy.

3.7. Whole-body TH levels

As shown in Fig. 5, concentration of 100 mg/L NO_3–N caused a highly significant reduction in both T4 and T3 levels (one-way ANOVA, $p \leqslant 0.01$) whereas, 10 mg/L NO_3–N did not affect T4 and T3 levels (one-way ANOVA, $p > 0.05$). Moreover, reduced T4 levels also occurred in 50 mg/L NO_3–N (one-way ANOVA, $p \leqslant 0.05$) (Fig. 5A). The T3/T4 ratio measured in control and 10, 50, or 100 mg/L NO_3–N treatments at G42 was 1.164, 1.717, 1.151, and 1.213, respectively.

3.8. Effect of NO_3–N on Dio2 and Dio3 expression

To determine the importance of the intracellular conversion of TH, the relative expressions of *Dio2* and *Dio3* mRNA in three tissues of larvae were investigated by RT-qPCR during metamorphosis climax. In intestine, there was a significant decrease of *Dio2* mRNA levels with larvae exposed to different concentrations of NO_3–N (one-way ANOVA, $p \leqslant 0.05$). Exposure to 100 mg/L NO_3–N significantly reduced *Dio2* mRNA levels in hind-limb (one-way ANOVA, $p \leqslant 0.05$), while exposure to 10 or 50 mg/L NO_3–N *Dio2* mRNA levels did not affected in hind-limb (one-way ANOVA, $p > 0.05$). Although tail resorption is an important indicator of amphibian metamorphosis, *Dio2* mRNA levels did not have significant difference between controls and experimental concentrations in tail at the metamorphosis climax (one-way ANOVA, $p > 0.05$) (Fig. 6A).

In both intestine and hind-limb, exposure to 50 and 100 mg/L NO_3–N a significant increase of *Dio3* mRNA levels was detected (one-way ANOVA, $p \leqslant 0.05$ for 50 mg/L NO_3–N in intestine; $p \leqslant 0.01$ for 50 mg/L NO_3–N in hind-limb; $p \leqslant 0.01$ for 100 mg/L NO_3–N). Exposure to 100 mg/L NO_3–N resulted in a significant rise in *Dio3* mRNA levels in tail at the metamorphosis climax (one-way ANOVA, $p \leqslant 0.01$) (Fig. 6B).

4. Discussion

Exposure to 50 and 100 mg/L NO_3–N caused significant mortalities in *B. gargarizans* larvae. Our results showed that NO_3–N can be seriously hazardous for amphibian survival, as has been suggested previously (Geoffrey et al., 2006; Garcia-Munoz et al., 2011). Though there is no report regarding nitrate converts to nitrite in the gut of tadpoles, some mechanisms for nitrate toxicity in fish and prawns have been proposed. The main toxic effect of nitrate on aquatic animals is due to the blockade of the oxygen-carrying capacity of hemoglobin, which may induce methaemoglobinaemia. In the gut, bacteria convert nitrate to nitrite, which competes with oxygen and binds to hemoglobin (Cheng and Chen, 2002; Jensen, 2003). Thereby, the oxygen-carrying capacity of the hemoglobin

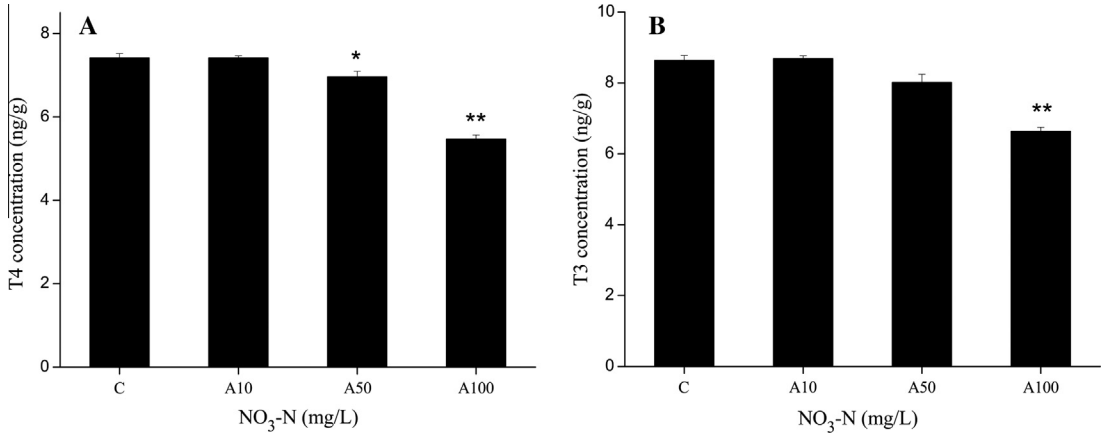

Fig. 5. Whole-body T4 (A) and T3 (B) contents in *B. gargarizans* larvae exposed to control groups and different concentrations NO_3–N (A10, A50, A100) at stage G42. The data are presented as mean ± SE. Controls are denoted by C. * = significantly different from control ($p \leqslant 0.05$). ** = highly significant different from control ($p \leqslant 0.01$).

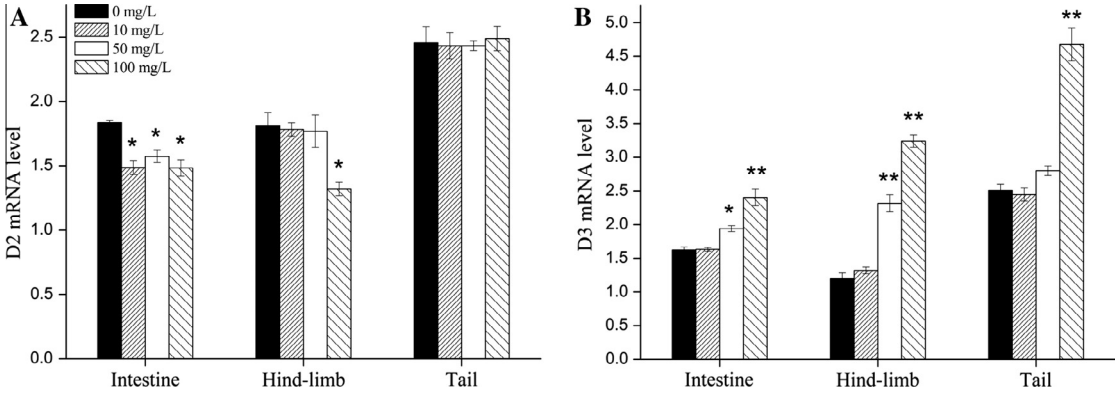

Fig. 6. Tissue-specific *Dio2* (A) and *Dio3* (B) mRNA levels of larvae at stage G42, determined using RT-PCR assay. The data are presented as mean ± SE. * = significantly different from control ($p \leqslant 0.05$). ** = highly significant different from control ($p \leqslant 0.01$).

is inhibited, causing hypoxia and ultimately death (Camargo and Alonso, 2006).

In present study, exposure to 50 and 100 mg/L NO_3–N for 78d resulted in a significant decrease in metamorphosis percentage. Metamorphosis percent is a strong and easily quantifiable characteristic of larvae in responses to chemicals (Brodeur et al., 2013; Higley et al., 2013; Zhao et al., 2013). Thyroid hormones are primary regulators of amphibian metamorphosis (Miyata and Ose, 2012). T4 is the primary circulating, but inactive form of thyroid hormones. T4 is converted to active T3 by *Dio2*, an enzyme specific to T3 target cells. In contrast, *Dio3* inactivates T4 and T3 in target cells. The timing of metamorphosis is therefore a combined function of T3/T4 synthesis and differential *Dio2*/*Dio3* expression (Huang et al., 2001). We suggested two main hypotheses: (1) Nitrate treatments decreased the percentage of amphibian metamorphosis in term of declines in T3 and T4 secretion in thyroid gland; and (2) high-dose NO_3–N reduced the level of T3 and T4 in target tissues resulted from increased *Dio3* expression and/or decreased *Dio2* expression.

Additionally, the results also demonstrated that there were significant and dose-dependent effects on both mass and body size of *B. gargarizans* larvae reaching metamorphic climax. In a similar experiment on bullfrogs, it found that body mass at metamorphosis increased with added nitrate (Smith et al., 2005). Our result is consistent with bullfrog study. However, a negative effect of nitrate exposure on larval mass and total length has been observed (Krishnamurthy et al., 2006; Garcia-Munoz et al., 2011). Moreover, some other studies observed no significant effects of nitrate on body size (Hecnar, 1995). Although the potential mechanism is not clear, it seems that increased mass and body size at metamorphosis may be correlated with longer developmental time (Brodeur et al., 2013). Increased body size may enhance the tolerance of amphibian to nitrate (Schuytema and Nebeker, 1999). Additionally, *B. gargarizans* breed mostly in temporary habitats, where the metamorphosis must be completed rapidly to escape drying habitats. As long larval period is generally assumed to confer poor fitness (Wilbur and Collins, 1973; Werner, 1986), the impacts of NO_3–N may cause a more dangerous threat to amphibian metamorphosis. Together with increasing size at metamorphosis, NO_3–N between 10 and 100 mg/L concentrations also caused significantly longer hind-limb length and tail length. The current study is the first to report an increase of hind-limb length and tail length at metamorphosis in larvae exposed to NO_3–N.

The thyroid gland synthesizes mainly T4 and releases it into the circulation. T4 is converted to T3 in peripheral tissues. Many investigations have demonstrated that NO_3–N could induced a significant decrease in T3 and T4 levels and might be considered as a goitrigenic factor in rat (Zaki et al., 2004; Mukhopadhyay et al., 2005). Our report clearly observed that treatment with 100 NO_3–N can significantly decline T4 and T3 levels, which is consistent with all the above results. However, our result also showed that exposure to 100 mg/L NO_3–N could increase the T3/T4 ratio. At metamorphic climax, which corresponds with the emergence of forelimbs (G42), there is a corresponding peak in the primary circulating thyroid hormone, T4. T4 contains iodine, and nitrate can impair the formation of thyroid hormones through the inhibition

of iodine uptake and transport (Boas et al., 2012). We proposed that high dose nitrate decreased T4 levels more sharply than T3 levels, leading to increased T3/T4 ratio.

It was recognized that thyroid histology applied as a sensitive and important diagnostic end point for detecting a chemical's effect to interact with T3 and T4 synthesis (Miyata and Ose, 2012; Saka et al., 2012). Morphological lesions in the thyroid gland were characterized as hypertrophy, atrophy, colloid depletion, hypertrophy and hyperplasia of follicles (Grim et al., 2009). In present study, 100 mg/L NO_3–N resulted in partial colloid depletion in thyroid follicles. Colloid depletion caused by nitrate may be associated with reduction of T3 and T4 levels, which can result in a toxic developmental effect. There is a primary mechanism by which excessive ingested nitrate may have negative effects on the thyroid gland. Because nitrate is a competitive inhibitor of the sodium/iodine symporter (NIS) on the surface of thyroid follicles, nitrate will decrease the active transport of iodine into the thyroid (Tonacchera et al., 2004; Pearce and Braverman, 2009). Iodine uptake inhibition linked to colloid depletion of the thyroid follicles. Nitrate inhibits iodine uptake by the colloid depletion of the thyroid follicles, thus impairing T3 and T4 synthesis (Boas et al., 2012; Miyata and Ose, 2012).

Exposure to 100 mg/L NO_3–N might decrease TH production in peripheral target tissues through alteration the expression of *Dio2* and *Dio3*. *Dio2* and *Dio3* are found in several peripheral target tissues in amphibians, and the levels of their expression are responsible for regulating the local levels of TH in peripheral tissues (Gereben et al., 2008; Bouzaffour et al., 2010; Itoh et al., 2010). Here we measured relative *Dio2* and *Dio3* mRNA transcript levels to determine whether NO_3–N has any effects on *Dio2* and *Dio3* expression. At 100 mg/L, *Dio2* mRNA levels were significantly decreased in intestine and hind-limb, whereas *Dio3* mRNA levels were increased in intestine, hind-limb and tail. Consequently, it seems very probable that exposure to the highest NO_3–N concentration limit the TH production in intestine and hind-limb through both low level expression of *Dio2* and/or high level expression of *Dio3*. However, NO_3–N limit the TH production in tail only by increased *Dio3* expression. Although intestine remodeling, hind-limb emergence and tail resorption during metamorphosis are regulated by TH, intestine and hind-limb regulated by TH to grow and differentiate, and tail was induced by TH to resorb (Brown and Cai, 2007). The reason for those differences in different tissues responses is not clear, but one possibility is that different tissues in an organism have different sensitivity to nitrate.

Increasing body size at metamorphosis might also have been the result of less TH availability in peripheral tissues. Because hind-limb length and tail length are morphological indicators of metamorphosis development (Miyata and Ose, 2012), the lack of TH availability may engender hind-limb growth maintained and tail resorption delayed, the larvae will develop into grossly deformed giants (Rot-Nikcevic and Wassersuq, 2004). In addition, low levels of TH will induce hind-limb growth, whereas higher concentration TH is required for tail resorption (Furlow and Neff, 2006). Therefore, the increasing body size at metamorphosis in larvae exposed to NO_3–N may be a direct consequence of the increase in hind-limb and tail.

5. Conclusion

In conclusion, exposure to NO_3–N had a negative effect on *B. gargarizans* larvae, showed by increased mortality and significant metamorphic retardation. Effects of nitrate on metamorphosis of *B. gargarizans* might be due to effects of TH synthesis. In addition, high dose of NO_3–N depleted partial colloid in the thyroid follicles and impaired T3 and T4 synthesis. Moreover, NO_3–N can decrease *Dio2* mRNA levels and increase *Dio3* mRNA levels in peripheral tissues. Taken together, NO_3–N might induce a considerable reduction of TH levels and alteration of deiodinases mRNA levels in peripheral tissues, thus resulting in retarded metamorphosis and an increase body size at metamorphosis.

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