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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,