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Nitrate-Induced Morphological Anomalies in the Tadpoles of *Nyctibatrachus major* and *Fejervarya limnocharis* (Anura: Ranidae)

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Abstract: The present study examined the effects of nitrate on the tadpoles of 2 ranid frogs (*Nyctibatrachus major* and *Fejervarya limnocharis*) studied under laboratory conditions. Tadpoles of these species were exposed to ecologically relevant concentrations of nitrates (0, 100, 2500, and 5000 µg NO_3-N l^{-1}). Upon exposure tadpoles of *N. major* showed protruded mouth, swollen body and head, paralysis, lateral and vertical swimming, restlessness, and wriggling movement. Tadpoles of *F. limnocharis* showed swollen body and head, bent tail, paralysis, restlessness, depigmentation, and intestinal hemorrhage. In both species, abnormalities increased with the increase in nitrate concentration. However, compared to *F. limnocharis*, tadpoles of *N. major* were more sensitive as they died before completing their larval tenure. Based on the results of this experiment, it is hypothesized that tadpoles of *N. major* are more sensitive and vulnerable to nitrate contamination.

Key Words: Nitrate, abnormality, tadpoles, agriculture contamination

Introduction

The widespread decline of amphibian populations has caused alarm among biologists throughout the world. A number of possible reasons for the apparent decline including habitat loss, over harvest, UV-B radiation, global warming, diseases, and chemical contaminants of the environment have been analyzed (Dalton, 2002; Krishnamurthy and Hussain, 2004). Amphibians are very sensitive to agricultural and environmental contaminants. Pesticides, chemical fertilizers, and other chemicals used in agriculture are severe threats to amphibian populations (Rouse et al., 1999). Nitrogen is a major component of agricultural manure and occurs in the form of nitrate, nitrite, ammonium ion, and ammonia. Among these, nitrate is the least toxic, most stable, and soluble form (Rouse et al., 1999). Recent experimental studies carried out by Guillette and Edwards (2005), Edwards et al. (2006), and Orton et al. (2006) revealed that nitrate produced severe effects ranging from gross toxicity to subtle changes in physiology and development in amphibians.

In India, compared to data for 1950, the present fertilizer consumption in agriculture has increased 170-fold, amounting to 90.12 kg ha^{-1}. Most of this is straight nitrogen fertilizers (urea, sodium nitrate, and ammonium nitrate) or mixed fertilizers (NPK), decontrolled phosphates (di-ammonium phosphate, muriate of potash, and single superphosphate), and potassium fertilizers. These fertilizers constitute a source of nitrogen in agriculture. In Western Ghats in India, many amphibian species inhabit the agro-ecosystems that include plantations of coffee, tea, rubber, croplands of paddy, ragi, jowar, and other vegetables. Some aquatic and semiaquatic species of amphibians breed in the shallow

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water of paddy fields. In these agro-ecosystems, nitrogen fertilizers are being used intensively. However, there is no information on the effect of nitrate on tadpoles. Hence, the present work was carried out to determine the effects of nitrate on tadpoles of 2 species of frog, *Nyctibatrachus major* (Boulenger) and *Fejervarya limnocharis* (Gravenhorst), in Western Ghats. *N. major* lives in the forest streams of Western Ghats. The habitat requirement for this species includes low air (range: 18.70-27.90 °C) and water temperatures (range: 18.50-25.50 °C). They generally live under circum neutral water pH under low light penetration (pH range 5.90-7.90 and illuminosity range 32.0-9600 Lux) (Gururaja et al., 2003). The adult and breeding habitats of *N. major* are confined to stretches of shallow forest streams and they are absent in the same stream downstream traversing through open agricultural land. *F. limnocharis* lives in all semiaquatic habitats of Western Ghats with a wide range of habitat variables (Krishnamurthy, 2003). The adult prefers more open habitats and breeds in shallow water. These 2 frogs live in the same area and same stream traversing through a forest in its upstream section and then agricultural fields. *N. major* does not inhabit the agricultural area and is confined to upstream areas, while *F. limnocharis* is ubiquitous. The tadpoles of these 2 species were collected form Kuvempu Bioreserve (KBR, Long: 13°35'-13°40', Lat: 75°15'-75°20', altitude: 710 m msl) located in central Western Ghats. In KBR, the native forest is fragmented and converted to paddy fields. Nitrate fertilizers are used in these paddy fields as a synthetic fertilizer. Table 1 illustrates the basic information on these 2 species in relation to nitrate concentration in the study area. The nitrate concentration of habitat soil and water of *N. major* was low compared to that of *F. limnocharis.* Environmental concentration of nitrate content in the water after application of fertilizer in the paddy field was high (6.0 ± 0.981 mg NO_3-N l^{-1}).

Materials and Methods

Free-swimming and independently feeding tadpoles of *N. major* and *F. limnocharis* (stage 25; Gosner, 1960) were collected from a forest stream and shallow waters of a paddy field. All tadpoles used in this experiment were collected from the same spawn of the respective species. The tadpoles were kept in the laboratory for 3 days prior to the experiment. In the laboratory the tadpoles were maintained at 22 ± 3 °C. Later they were subjected to 5 different sub-lethal concentrations of nitrates comparable to those recorded in the field (Table 1). These were 0, 100, 1000, 2500, and 5000 µg NO_3-N l^{-1} prepared using sodium salt ($NaNO_3$) as a nitrate source. The sodium in $NaNO_3$ has been proved to be non-toxic at lower concentrations (Baker and Waights, 1993). Eight free feeding tadpoles of the same stage, length, and weight were exposed to each concentration separately in 1 l of nitrate solution in non-transparent polyethylene containers (18 cm × 14 cm × 8 cm: 2-l capacity). The nitrate solution in each container was changed every 48 h

Table 1. Basic environmental information of nitrate in relation to the 2 species of frogs selected for study.

Parameter	*F. limnocharis*	*N. major*
Tadpole habitat	Shallow waters	Forest streams
Concentration of nitrate in habitat water (mg l^{-1} ± SE)	0.53 ± 0.051	0.33 ± 0.033
Concentration of soil nitrate (mg 100 g soil ± SE)	0.16 ± 0.071	0.06 ± 0.029
Environmental concentration (water) of nitrate after fertilizer application (mg l^{-1} ± SE)	6.0 ± 0.981	-
LC_{50} values at 96 h of exposure*	7.61 mg l^{-1}	2.02 mg l^{-1}
95% lower and higher confidence limit of LC50 values*	4.4 -13.15 mg L^{-1}	0.81 - 4.91 mg L^{-1}
Larval period	28-36 days	92-108 days

*Personal observation

and all containers were constantly aerated to avoid the accumulation of nitrite and ammonia. Tadpoles of *F. limnocharis* were fed with aquatic vegetation, while decaying leaf litter was offered to *N. major* as food. Taking tadpoles in 0 nitrate concentration as a control, various morphological changes during larval life were recorded every 7 days until all tadpoles in the control metamorphosed. The abnormalities were recognized visually and by comparing the body size, shape, pigmentation, external structures, and movement pattern of tadpoles in various treatment groups with those tadpoles of the same developmental stage present in the control group. By measuring the head and body length and diameter, swollen head and body were identified. The depigmentation was recorded by visually comparing the density of black pigments on the tadpoles of the control and treatment groups.

Result

The Figure depicts combined percentage abnormality recorded for the tadpoles of the 2 species subjected to various nitrate concentrations. In the control, there were no abnormalities in either species. At 100 µg NO_3-N l^{-1} nitrate concentration, 12.5 ± 0.7% of total tadpoles of *N. major* showed abnormality. Thereafter, the abnormality reached its maximum in higher concentrations (range: 81.3%-93.8% of the total tadpoles). In *F. limnocharis* there were no abnormalities at 100 µg l^{-1}, but with the increase in nitrate concentration total abnormality reached 60% to 75% of the total tadpoles. The percentage abnormality was high among the tadpoles of *N. major*.

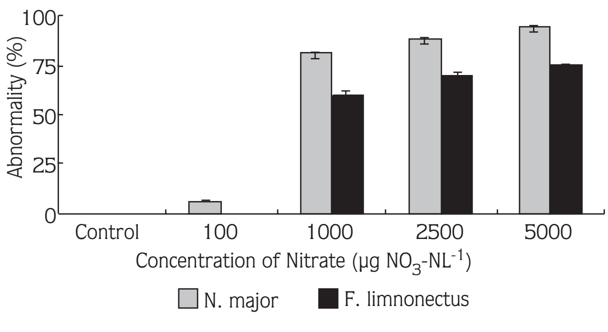

Figure. Combined percent abnormality exhibited by tadpoles of *N. major* and *F. limnocharis* at various concentrations of nitrate.

In the present study, 11 clearly visible morphological and behavioral abnormalities were recorded. The morphological abnormalities include protruded mouth (stiff protruded snout projected anteriorly), swollen head (bulging of the tadpole head at a ratio of 1:1.8 compared to normal tadpoles) and body (sac-like swollen trunk filled with fluid and having a size of 1:2 compared to normal tadpoles), bent tail (curvature of the tail), and depigmentation. The behavioral abnormalities include lateral swimming (lateral line facing the surface of the water), vertical swimming (frequent vertical swimming from bottom to surface of water), wriggling movement (circular, abrupt clockwise and anti-clockwise movement), restlessness (continuous swimming without an intermittent break), and paralysis (inactive and exhibiting simulation of death). In addition, some of the tadpoles also exhibited depigmentation and externally visible intestinal hemorrhage.

Tables 2 and 3 present various abnormalities recorded in different concentrations of nitrate among the tadpoles of *N. major* and *F. limnocharis*, respectively. Swollen head and body and paralytic posture are the common abnormalities in both species. Apart from these, among the tadpoles of *N. major*, protruding mouth, lateral and vertical swimming, wriggling movement, and restlessness are the prominent observations. In tadpoles of *F. limnocharis*, depigmentation, bent tail, and intestinal hemorrhage are the exclusive abnormalities observed under nitrate treatment. Among the 6 abnormalities recorded in *F. limnocharis*, except paralytic posture, all abnormalities were morphological. In *N. major*, behavioral abnormalities were prominent and all tadpoles subjected to higher nitrate concentrations (1000 µg NO_3-N l^{-1} to 5000 µg NO_3-N l^{-1}) developed prominent abnormalities and died sporadically after day 56. Other tadpoles subjected to 0 and 100 µg NO_3-N l^{-1} metamorphosed into froglets after day 92. Similarly, in *F. limnocharis*, all tadpoles were found to be dead at higher concentrations of nitrate between days 28 and 35 of the experiment. However, at low and zero concentrations tadpoles metamorphosed into froglets after day 28. Table 4 presents percent relevance of different abnormalities in different concentrations of nitrate among the tadpoles of *N. major* and *F. limnocharis*. Paralysis appeared to be maximum in both species.

Table 2. Abnormalities recorded in tadpoles of *Nyctibatrachus major* during the exposure to various nitrate concentrations over 56 days. The letters indicate the corresponding abnormality listed in the footnote.

Days	Concentration of nitrate (μg NO_3-N l^{-1})				
	Control	100	1000	2500	5000
0	-	-	-	P	P
7	-	-	Pm, Sb, Sh	P, R, Sh	P, Sb, Sh
14	-	-	P, Sh	Ls, P, R, Sb, Vs	Ls, P Sh, Vs
21	-	P	P, Sh, Vs, Wm	P, Sb, Sh	P, Sb
28	-	-	Ls	Ls, Sb	
35	-	-	Sh	Sb	
42	-	-	P	Sb	
49	-	-	P	Sb	
56	-	-	All are dead	All are dead	All are dead

Notes: Ls = Lateral Swimming, P = Paralysis, Pm = Protruded mouth, R = Restlessness, Sb = Swollen body, Sh = Swollen head, Vs = Vertical Swimming, Wm = Wriggling movement

Table 3. Abnormalities recorded in tadpoles of *Fejervarya limnocharis* during exposure to various nitrate concentrations over 28 days. The letters indicate the corresponding abnormality listed in the footnote.

Days	Concentration of nitrate (μg NO_3-N l^{-1})				
	Control	100	1000	2500	5000
0	-	-	-	P	P
7	-	-	Sb,	Sh, Dp, Bt	Bt, Dp, Sh
14	-	-	P, R	P, Sb,	Sb, P
21	-		P, Bt, Dp	R, Dp, Ih, P	P, Ih, Bt, P
28	-	-	Ih, P	Ih, P	Ih, P
35	Metamorphosed	Metamorphosed	All are dead	All are dead	All are dead

Notes: Bt = Bent Tail, Dp = Depigmentation, Ih = Intestinal Hemorrhage, P = Paralysis, R= Restlessness, Sb = Swollen Body, Sh = Swollen head

Discussion

Intensified fertilizer application and sewage production has increased the nitrate contamination in aquatic systems (Edwards et al., 2006). The major anthropogenic sources of nitrate contamination in water of agricultural areas are nitrogen-based fertilizers and animal wastes (Goolsby et al., 1991). The present-day agriculture practice involves extensive use of chemical fertilizers and these agrochemicals pollute amphibian habitats (Johansson, 2004). However, nitrate's role in the decline of amphibian populations remains unclear, although studies suggest that nitrate exposure affects larval development (Edwards et al., 2006). Nitrate and other chemicals are known to produce severe effects on larval growth and development of amphibians. The effect ranges from gross toxicity to more subtle changes in physiology and development (Guillette and Edwards, 2005; Orton et al., 2006). The nitrates showed chronic effects on physical and behavioral functions of the tadpoles. They include bent tail, body swelling and bulging, head deformities, and digestive system deformities, associated with severe weight loss and high

Table 4. Percentage relevance of each type of abnormality recorded among tadpoles of *N. major* and *F. limnocharis* at various nitrate concentrations. Values in parentheses indicate number of tadpoles with anomalies.

Abnormality	*N. major* (n = 8) Nitrate concentration (µg NO_3N l^{-1})				*F. limnocharis* (n = 8) Nitrate concentration (µg NO^3N l^{-1})		
	100	1000	2500	5000	1000	2500	5000
Bent tail					12.5 (1)	12.5 (1)	25 (2)
Depigmentation					12.5 (1)	12.5 (1)	12.5 (1)
Intestinal hemorrhage					12.5 (1)	12.5 (1)	25 (2)
Lateral Swimming		12.5 (1)	25 (2)	12.5 (1)			
Paralysis	12.5 (1)	25 (2)	25 (2)	25 (2)	25 (2)	25 (2)	12.5 (1)
Protruded mouth		12.5 (1)					
Restlessness			12.5 (1)		12.5 (1)	12.5 (1)	
Swollen body		12.5 (1)	12.5 (1)	25 (2)	12.5 (1)	12.5 (1)	12.5 (1)
Swollen head		25 (2)	12.5 (1)	12.5 (1)		12.5 (1)	12.5 (1)
Vertical Swimming			12.5 (1)	12.5 (1)			
Wriggling movement		12.5 (1)					

mortality (Hecnar, 1995). Ammonium nitrate has shown significant direct toxicological effects on behavioral and survivorship in tadpoles of the wood frog *Rana sylvatics* (Burgett et al., 2007). In the present study, tadpoles of both species showed prominent anomalies of swollen head and body. However, tadpoles of *N. major* showed unique abnormalities related to behavior and none of the tadpoles at higher concentrations survived. The larval period of *F. limnocharis* was short compared to that of *N.* major (Table 1). Obviously, when these tadpoles live in contaminated water, the possibility of long-term exposure becomes high for *N. major*. However, the present observation reveals that the tadpoles of *N. major* die before they complete half of their larval period (days) and before the appearance of prominent morphological abnormalities that appeared in *F. limnocharis* (Tables 2 and 3). In contrast to this, although the tadpoles of *F. limnocharis* have a shorter larval life (28-36 days), they showed morphological abnormalities and survived until the other tadpoles in the control and low nitrate concentration metamorphosed. However, at this stage they failed to complete various stages of development. Wood frogs in ephemeral pools with nitrite accumulation showed delayed metamorphosis (Griffis-Kyle, 2007). Therefore, the delayed metamorphosis recorded in *F. limnocharis* in the present study could be attributed to high nitrate concentrations. Survival of Chorus frog and Leopard frog tadpoles was significantly decreased after exposure to 10 mg NO_3-N l^{-1} (Hecnar, 1995). In the present study, a drastic reduction in survival was recorded below 5 mg NO_3-N l^{-1}, which is 50% less than those recorded for Chorus and Leopard frogs. Therefore, nitrate in the field could reduce the mass of larval population or induce anomalies that could be detrimental to these 2 species.

Toad tadpoles exposed to 11 or 23 mg NO_3-N l^{-1} showed unusual swimming patterns and deformities, including missing or deformed forelimbs and toes (Xu and Oldham, 1997). Cascades frogs (*Rana cascadae*) exposed to 3.5 mg NO_2-N l^{-1} metamorphosed more slowly than the controls and emerged less developed from the water at the same time as the controls (Marco et al., 1999). These examples suggest that sensitivity to nitrate varies among species and development stages. Similarly, tadpoles of *N. major* and *F. limnocharis* showed differences in response to various concentrations of nitrate. Based on differential response of tadpoles under treatment and the types of the anomalies they developed, it could be hypothesized that species requiring a specific range of habitat are more vulnerable to nitrate contamination. Further, it could be inferred that nitrate is toxic to tadpoles of both species and, given the chance that the observations revealed in the laboratory occurred in natural conditions, then nitrate could act as a major factor causing the depletion of larval population and leading to a decline in the frog population in the agro-ecosystem of Western Ghats.

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