Journal of Environmental Biology **April 2006,** 27(2) 187-190 (2006)

Effect of nitrate-related compounds on growth, survival and hematological responses in tadpoles of the Cuban tree frog, *Osteopilus septentrionalis* (Boulenger)

F. Punzo[1] and S. Law[2]
[1] Department of Biology, Box 5F, University of Tampa, Tampa, Florida 33606, USA
[2] P.O. Box 546, Tampa, Florida 33606, USA

(Received: 16 April, 2004 ; Accepted: 08 February, 2005)

Abstract: *Experiments were conducted to assess the effects of nitrate-related compounds on survival, growth, and hematological responses in tadpoles of the Cuban treefrog, Osteopilus septentrionalis . Stage-25 tadpoles were exposed to a nitrate dilution series and exposed to distilled water (controls), 40 ppm nitrate, or 100 ppm nitrate. Survivorship was significantly higher for control animals as compared to those exposed to 40 and 100 ppm nitrate. Total blood Hb concentrations were not significantly altered by exposure to sodium nitrite, and a significant positive correlation was found between methemoglobinemia and nitrite concentration over the test range of 1.0 to 50.0 mg/l. Percentage Hb was significantly correlated with nitrite concentration. Percentage MHb for all treatment groups was significantly higher (18.4 to 45.3 %) than that of controls (5.4 %).*

Key words: *Growth, Hematological responses, Nitrate, Osteopilus.*

Introduction

Over the last three decades, scientists have drawn attention to the worldwide decline in amphibian populations (Barinaga, 1990; Punzo, 2005). These declines have been attributed to a combination of many factors including climate change (Alexander and Eischeid, 2001), parasitic infections (Kiesecker *et al.,* 2004), pathogens (Carey, 1993), habitat destruction (Delis *et al.,* 1996), exposure to ultraviolet radiation (Blaustein *et al.,* 1998), acid rain (Punzo, 1983, 2005; Alford and Richards, 2000) and other chemical industrial pollutants (Punzo, 1993; Kiesecker *et al.,* 2001), pesticides and herbicides (Punzo, 1976, 1997; Lips *et al.,* 2003), and exposure to nitrate / nitrite compounds associated with sewage effluents and the use nitrogen fertilizers in the agricultural landscape (Berger, 1989; Punzo, 2005).

Ammonia is typically oxidized to nitrate by aerobic chemotrophic bacteria (Sharma and Ahlert, 1977) which results in high nitrate concentrations in surface waters. Nitrate compounds are readily soluble in water, forming the nitrate ions (NO_3^-). Nitrate ions can be toxic to aquatic organisms including fishes (Westin, 1974) and amphibians (Hecnar, 1995). A major source of nitrates in aquatic habitats stems from the use of nitrogen-based fertilizers and subsequent runoff into surface waters.

Nitrate-related compounds can have a wide range of adverse effects on larval amphibians including an impairment of growth and developmental processes (Hecnar, 1995; Marco *et al.,* 1999), feeding (Kiesecker *et al.,* 2004), respiratory physiology (Huey and Beitinger, 1980), and have been associated with carcinogenesis (Westin, 1974). In addition, the toxicity of nitrate-related compounds has been demonstrated in other aquatic organisms including larvae of the caddis fly, *Cheumatopsyche pettiti* (Camargo and Ward, 1992),as well as numerous species of fish (Westin, 1974; Bogardi *et al.,* 1991).

The Cuban treefrog, *Osteopilus septentrionalis* (Boulneger) was introduced into Florida at the end of the 19th century and has continued to expand its range since its arrival (Ashton and Ashton, 1988). It is the largest treefrog in the U.S. and has proved to be a voracious predator, feeding on all of the native species of treefrogs in Florida as well as a number of species of ranid frogs, which has contributed to the decline of these native species (Moler, 1992). This treefrog breeds in many aquatic habitats in Florida including streams, creeks, rivers and retention ponds; and all of these habitats are subject to runoff from adjacent agricultural fields. Although studies have been conducted on the toxicity of nitrates to other amphibian species (Bogardi *et al.,* 1991; Hecnar, 1995), to our knowledge, no data are available on the effect of nitrates on introduced species, including *O. septentrionalis* . The purpose of this study was to assess the effect of nitrate-related compounds on survival, growth, and hematological responses in tadpoles of *O. septentrionalis* .

Materials and Methods

In Florida, the Cuban tree frog frequently breeds in temporary retention ponds adjacent to fields where crops are cultivated. Using dip-nets, we collected eggs and tadpoles of *O. septentrionalis* from three shallow ponds adjacent to soybean fields in Polk County, Florida during May of 2003. After hatching, tadpoles were placed individually in glass jars (10 l) and reared at a water temperature of 23± 0.2ºC, under a 12L: 12D photoperiod regime in Percival Model 85A environmental chambers (Boone, Iowa). Tadpoles were fed *ad libitum* on a commercial tadpole diet (Carolina Biological Supply, Burlington, North Carolina). For experiments on the effect of nitrate on growth and survival, tadpoles were exposed to various

Table – 1: Growth and mortality of stage 25 tadpoles of *Osteopilus septentrionalis* exposed to either distilled water (0, controls) or various nitrate solutions (40 and 100 ppm) over a 13 day period.

		Tatal body length[1]		
Day	**Treatment**	**0**	**40 ppm**	**100 ppm**
1		10.53 (0.81)	10.51 (0.48)	10.64 (0.73)
7		13.24 (0.85)	11.22 (0.63)	9.73 (0.58)
13		18.83 (2.04)	13.31 (0.58)	All dead
		Number	Surviving	(Out of 13)
		13	13	13
		13	8	4
		12	2	0

[1]Growth measured as total body length in mm, and growth data expressed as means (N=13 tadpoles/test group); values in parentheses represent ± S.E.

Table – 2: Hematological responses[1] for stage 25 tadpoles of *Osteopilus septentrionalis* following a 24 hr exposure to various nitrite concentrations.

Concentration (mg/l)	**Hemoglobin (g / 100 ml)**	**Methemoglobin (g / 100 ml)**	**Methemoglobin (%)**
Control	5.2 (0.7)	0.4 (0.07)	5.4 (2.4)
1.0	4.9 (0.9)	0.9 (0.04)	18.4 (3.5)
3.0	5.9 (1.4)	1.3 (0.3)	25.6 (5.2)
5.0	6.3 (0.9)	1.7 (0.4)	30.7 (3.9)
10.0	6.0 (1.1)	2.2 (0.4)	36.7 (6.2)
50.0	5.5 (0.6)	1.8 (0.4)	45.3 (7.3)

[1] Four replicates (N = 6 tadpoles/replicate) were conducted for each treatment group and controls. Data expressed as means, and numbers in parentheses represent (± S.E.).

nitrate concentrations in aged tap water of medium hardness (137 total hardness mg/l) and low chloride concentration (4.9 – 5.0 mg/l), and a pH of 7.2 – 7.3. Nitrate levels were monitored at 0, 12, and 24 hr intervals, and other water parameters (total hardness, ammonia, chloride, temperature, and pH) were monitored at 0 and 24 hr intervals.

Tadpoles were exposed to a nitrate-N-dilution series from an aqueous stock solution (1g / l) of ammonium nitrate fertilizer (American Cyanamid, St. Louis, Missouri). Animals were selected randomly and total body length (TBL) measured. Thirty-nine tadpoles, each measuring 10 to 11 mm, were then selected from this random sample. Tadpoles were randomly assigned to one of three groups (N = 13 / group): (1) tadpoles reared in distilled tap water (controls); (2) reared in 40 ppm nitrate; and (3) 100 ppm nitrate. These concentrations were chosen based on a range of recorded levels of nitrates in shallow ponds adjacent to agricultural landscapes in FL (Fernald and Patton, 1994). The pH of distilled water and nitrate solutions was monitored at 24 hr intervals and fluctuated between 6.43 and 7.22, with distilled water exhibiting the lowest pH values.

Subjects chosen for testing were reared individually in glass jars containing 500 ml of either distilled water or nitrate solution. Jars were placed on plastic trays in a blocked array to minimize any position effects. Test temperature and photoperiod regime were the same as those described above. Distilled water or nitrate solutions were replaced after 7 and 13 days, and the total body length (TBL) and weight of each tadpole were measured on these days. Subjects were fed on days 0, 7 and 13, to ensure that any observed mortality could not be attributed to food deprivation. Jars were monitored every 24 hr to count and remove dead subjects. Experiments were terminated after 13 days.

For experiments on hematological responses, stage 25 tadpoles (Gosner, 1960) were exposed to nitrite concentrations of 1, 3, 5, 10 or 50 mg/l, as well as 0 mg/l (controls). Tadpoles (4 replicates, N = 6 tadpoles / replicate) were exposed to each treatment and control group. All experiments were conducted using aerated water of medium hardness (141 total hardness mg/l), low chloride (5.0 mg/l), and pH of 7.3. Nitrite levels and other water parameters were monitored as described previously for experiments on growth and survivorship. Reagent grade sodium nitrite was used as the nitrite source (Sigma Chemical Co., St. Louis, Missouri). After an exposure period of 24 hr, blood was collected from each tadpole using heparinized capillary tubes (Carolina Biological Supply, Burlington, North Carolina). Total hemoglobin was determined using the cyanomethemoglobin method (Hainline, 1958), and blood hemoglobin determined using the photoelectric method of Evelyn and Malloy (1938).

All statistical analyses followed procedures described by Zar (1984). Because a Bartlett's test showed homogeneity of variances, data on growth and survival were analyzed using analysis of variance (ANOVA). Tukey tests were used to analyze for differences between treatment means. Data on survivorship were analyzed using a Chi Square test. Data on hematological parameters were analyzed using a best-fit regression method.

Results and Discussion

Growth of tadpoles as reflected by change in body length in response to ammonium nitrate is shown in Table 1. An analysis of variance (ANOVA) of body length at 7 days showed a significant effect of blocks ($F = 3.14$, $p < 0.05$). There was also a significant effect of treatments ($F = 81.04$, $p < 0.001$) when block effects were removed. Comparison of treatment means using Tukey tests showed that control subjects were significantly larger than those exposed to 40 ppm nitrate ($q = 10.11$, $p < 0.05$), and subjects exposed to 40 ppm nitrate were significantly larger than those exposed to 100 ppm nitrate ($q = 7.05$, $p < 0.05$). These results are in agreement with those reported for the hylid frog, *Litoria caerulea* (Baker and Waights, 1994), which showed decreased rates of larval growth and differentiation and a concomitant increase in mortality when exposed to water containing nitrate concentrations of 40 and 100 mg/l. Tadpoles of the toad, *Bufo bufo* exhibited reduced growth rates and increased mortality when exposed to sodium nitrite under laboratory conditions (Baker and Waights, 1993).

Survivorship of tadpoles is shown in Table 1. We conducted a Chi Square analysis on number of tadpoles surviving on day 13, and pooled data from both nitrate treatment groups. Survivorship was significantly higher for tadpoles exposed to distilled water as compared to animals exposed to nitrate ($X^2 = 26.12$, $p < 0.01$, with Yate's correction).

Results on hematological parameters indicated that various nitrite levels had no significant effect on total hemoglobin (Hb) concentrations (Table 2). However, a positive relationship was found between nitrite concentration and methemoglobinemia over the nitrite concentration range of 1.0 to 50.0 mg/l. The percentage methemoglobin (MHb) was significantly correlated to nitrite concentration ($p < 0.01$), with a best-fit regression model of: $\log_{10}$ MHb (% total Hb) = 1.403 + 0.448 $\log_{10} NO_2^-$ (mg/l). Standard errors for the intercept and slope were 0.0311 and 0.0185, respectively, with a coefficient of determination of 0.87. The percentage of MHb from all treatment groups were significantly different from controls which had a mean of 5.4% MHb (Table 2, $F = 16.72$, $p < 0.05$). Previous research has shown that exposure to nitrites can cause a rapid conversion of Hb to MHb which is incapable of binding to oxygen, thereby reducing the oxygen supply to tissues and cells (Bogardi *et al.,* 1991).

Results of these experiments clearly show that significant mortality of tadpoles of the Cuban treefrog can be expected to occur when these animals are found in surface waters containing nitrate-related compounds. In addition to the death of some subjects, observations of tadpoles during testing indicated that animals exposed to 40 ppm nitrate were more sluggish in their general movements as compared to control subjects. Slower swimming speed may result in an impaired ability to escape from mobile predators as well as a reduction in the size of food-containing patches that could be searched in a given period of time. Thus, this introduced species that has been implicated in declining numbers of some native amphibian species in Florida, exhibits a sensitivity to nitrate / nitrite contamination that is in general agreement with that reported for several species of frogs and toads native to the southeastern U. S. (Berger, 1989; Oldham and Hilton-Brown, 1992) as well as Europe, Asia, and South America (Berger, 1989; Bogardi *et al.,* 1991; Alford and Richards, 2001).

Acknowledgments

We thank R. Dalela, L. Humphreys, and anonymous reviewers for comments on an earlier draft of the manuscript, E. Pedrosa for assistance in maintaining animals in captivity, and A. Jenzarli for consultation on statistical analyses. A Faculty Development Grant to FP from the University of Tampa made much of this work possible.

References

Alexander, M.A. and J.K. Eischeid: Climate variability in egions of amphibian declines. *Conserv. Biol.,* **15,** 930-942 (2001).

Alford, R.A. and S.J. Richards: Global amphibian declines: a problem in applied ecology. *Annu. Rev. Ecol. Syst.,* **31,** 133-165 (2000).

Ashton, R.E. and P.S. Ashton: Handbook of reptiles and amphibians of Florida, Part 3. The amphibians. Windward Publ, Miami, Florida (1988).

Baker, J. and V. Waights: The effect of sodium nitrate on the growth and survival of toad tadpoles (*Bufo bufo*) in the laboratory. *Herpetol. J.,* **3,** 147-149 (1993).

Baker, J. and V. Waights: The effects of nitrate on tadpoles of the tree frog *Litoria caerulea*. *Herpetol. J.,* **4,** 106-108 (1994).

Barinaga, M.: Where have all the froggies gone? *Science,* **247,** 1033-1034 (1990).

Berger, L.: Disappearance of amphibian larvae in the agricultural landscape. *Ecol. Internat. Bull.,* **17,** 65-73 (1989).

Blaustein, A.R, J.M. Kiesecker, D.P. Chivers, D. Hokit, A. Marco, L.K. Belden and A. Hatch: Effects of ultraviolet radiation on amphibians: field experiments. *Amer. Zool.,* **38,** 799-812 (1998).

Bogardi, I., R.D. Kuzelka and W.G. Ennega: Nitrate contamination: Exposure, consequence and control. NATO ASI Series G, Vol. 30 (Ecological Sciences), Springer, New York (1991).

Camargo, J.A. and J. Ward: Short-term toxicity of sodium nitrate to non-target fresh water invertebrates. *Chemosphere,* **24,** 23-28 (1992).

Carey, C.: Hypothesis concerning the causes of disappearance of Boreal toads from the mountains of Colorado. *Conserv. Biol.,* **7,** 355-362 (1993).

Delis, P.R., H.R. Mushinsky and E.D. McCoy: Decline of some west-central Florida anuran populations in response to habitat degradation. *Biodiver. Conserv.,* **5,** 1579-1595 (1996).

Evelyn, K.A. and H.T.: Malloy: Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.*, **126,** 655-662 (1938).

Fernald, E.A. and D.J. Patton: Water respurces Atlas of Florida. Florida State Univ Press, Tallahassee, Florida (1994).

Gosner, K.L.: A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica,* **16,** 183-190 (1960).
Hainline, A.: Hemoglobin. *In:* Standard methods of clinical chemistry (*Ed:* D. Seligson). Academic Press, New York, pp. 49-60 (1958).
Hecnar, S.J.: Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environ. Toxicol. Chem.,* **14,** 2131-2137 (1995).
Huey, D.W. and T. Beitinger: Hematological responses of larval *Rana catesbiana* to sublethal nitrite exposures. *Bull. Environ. Contam. Toxicol.,* **25,** 574-577 (1980).
Kiesecker, J.M., A.R. Blaustein and L.K. Belden: Complex causes of amphibian population declines. *Nature,* **410,** 681-684 (2001).
Kiesecker, J.M., L.K. Belden, K. Shea and M.J. Rubbo: Amphibian decline and emerging disease. *Amer. Sci.,* **92,** 138-147 (2004).
Lips, K.R., J.D. Reeve and L.R. Witters: Ecological traits predicting amphibian population declines in Central America. *Conserv. Biol.,* **17,** 1078-1088 (2003).
Marco, A., C. Quilchano and A.R. Blaustein: Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. *Environ. Toxicol. Chem.,* **18,** 2836-2839 (1999).
Moler, P.E.: Rare and endangered biota of Florida. Vol. III. Amphibians and reptiles. Univ Florioda Press, Gainesville, Florida (1992).
Oldham, R.S. and D. Hilton-Brown: Effect of agricultural fertilizers on amphibians. Contract Report to Nature Conservancy Council. No. F72-15-05, Washington, DC (1992).
Punzo, F.: Organochlorine insecticide residues in amphibians and reptiles from Iowa and lizards from the southwestern United States. *Bull. Environ. Contam. Toxicol.,* **21,** 842-848 (1976).
Punzo, F.: Effects of environmental pH and temperature on embryonic survival capacity and metabolic rates in the smallmouth salamander, *Ambystoma texanum*. *Bull. Environ. Contam. Toxicol.,* **31,** 467-473 (1983).
Punzo, F.: Ovarian effects of a sublethal concentration of mercuric chloride in the river frog, *Rana heckscheri* (Anura: ranidae). *Bull. Environ. Contam. Toxicol.,* **50,** 385-391 (1993).
Punzo, F.: The effects of azadirachtin on larval stages of the oak toad, *Bufo quercicus* (Holbrook). *Florida Scient.,* **60,** 158-164 (1997).
Punzo, F.: The potential impact of pesticides, heavy metals, and growth regulators on Florida. amphibians. *In :* Status and conservation of Florida amphibians and reptiles (*Eds:* W. Meshaka and K. Babbitt). Krieger Press, Gainesville, Florida, pp. 134-146 (2005).
Sharma, B. and R.C. Ahlert: Nitrification and nitrogen removal. *Water Res.,* **11,** 897-925 (1977).
Westin, D.T.: Nitrate and nitrite toxicity to salmonid fishes. *Prog. Fish Cult.,* **36,** 86-89 (1974).
Zar, J.H.: Biostatistical analysis. 2nd. ed. Prentice Hall, Englewood Cliffs, New Jersey (1984).

Correspondence to:
Dr. Fred Punzo
Department of Biology, Box 5F
University of Tampa, Tampa
Florida 33606, USA
E-mail: fpunzo@ut.edu
Tel.: +1-813-253-3333