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Antioxidant Defense System in Tadpoles of the American Bullfrog (*Lithobates catesbeianus*) Exposed to Paraquat

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Abstract.—**There is a great need for proactive approaches to avoid amphibian declines. We investigated the possibility that antioxidant stress markers might serve as a proactive measure of physiological stress in anuran tadpoles. Commercially purchased American Bullfrog tadpoles (*Lithobates catesbeianus*, Gosner stage 36–37) were subjected to 0- (control), 0.1-, 0.5-, 1.0-, and 2.0-mg/L paraquat for 24 h. Liver and muscle (tail clip) tissues were removed and analyzed for catalase, superoxide dismutase (SOD), general peroxidase, and glutathione reductase (GR) activities. In the controls, there was no significant difference in GR activity in tissues collected from the liver and the tail; however, peroxidase, SOD, and catalase activities ranged from two- to 20-fold higher in the liver than in the muscle tissue. Treatment with paraquat resulted in significant increases in SOD, general peroxidase, and GR activities in the liver tissue, whereas the high constitutively expressed catalase activity remained unchanged. GR activity also increased significantly in the muscle tissue when the tadpoles were treated with 2-mg/L paraquat, but the activities of the other three antioxidant enzymes did not vary significantly from the control values in this tissue regardless of the paraquat treatment. After 24 h of paraquat treatment, all tadpoles at all treatment levels were alive and appeared to be vigorous, suggesting that the bullfrog is very tolerant to paraquat toxicity. It is proposed that this tolerance is caused by the stress-induced increases of antioxidant enzyme activity such as SOD, general peroxidases, and GR, as well as, the high constitutive activity of catalase. These findings suggest that, with refinement and further studies, antioxidant markers may be important indicators of sublethal environmental stress in amphibians.**

Amphibian populations are declining on both local and global scales (Wheeler et al., 2002, Young et al., 2004; McCallum, 2007). The many factors implicated in these declines include climate change (Pounds et al., 1999), diseases (Green and Dodd, 2007; Fellers et al., 2007), habitat fragmentation and land use (Funk et al., 2005; Germano, 2006; Gray et al., 2007), and pollution (Ortmann, 1909; Rouse, 1999; Burgett et al., 2007). Despite their intolerance to environmental stressors relative to other organisms (see Burgett et al., 2007; Karraker, 2007), few investigations of the amphibian physiological stress response exist (McCallum and McCallum, 2006; McCallum and Trauth, 2007); thus, the amphibian antioxidant system remains among the most poorly understood of these responses (Storey, 1996).

Reactive oxygen species (ROS) such as superoxide, H_2O_2, and the hydroxyl radical adversely affect cellular proteins (Davies, 1987), DNA (Imlay and Linn, 1988), and membrane lipids (Fridovich, 1986). Under normal conditions, the enzymatic and nonenzymatic antioxidant defense systems protect cells against the cytotoxic effects of ROS. The primary enzymatic antioxidants include superoxide dismutase (SOD), glutathione reductase (GR), catalase, and general peroxidases. The superoxide anion is eliminated by SOD that catalyses the dismutation of superoxide radicals to H_2O_2 (Moody and Hassan, 1984; Stead and Park, 2000). Catalase catalyses the disproportionation of H_2O_2 to H_2O and O_2, and a variety of general peroxidases also catalyze the breakdown of H_2O_2 (Chang et al., 1984).

Only a few studies have investigated antioxidant responses in amphibians. Unlike mammals, which demonstrate consistently high rates of aerobic metabolism, amphibians and other poikliotherms are frequently confronted with long periods of anoxic conditions (Lutz and Storey, 1997). For example, many amphibians must hibernate, forage, and secure refuge in the anoxic sediments of lakes, ponds, and streams (Ultsch, 1989). Most amphibian antioxidant research has focused on the role of these compounds in recovery from long periods of anoxia during hibernation. Amphibians in this group have many biochemical adaptations for surviving oxygen deprivation (Storey et al., 1996). The Northern Leopard Frog (*Lithobates pipiens*) exposed to 30 h of anoxia demonstrated significant increases in levels of skeletal muscle and heart catalase, heart and brain Se-GPX, and

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brain GST. These levels returned to normal within 40 h of treatment. Liver GSSG/GSH-eq ratios significantly increased during anoxia (Hermes-Lima and Storey, 1996). Throughout reoxygenation and 30 h of anoxia, lipid peroxidation levels did not change and was associated with high antioxidant liver levels. (Hermis-Lima and Storey, 1996). Despite many studies on hibernation-related antioxidant adaptations, little information exists regarding the amphibian antioxidant enzyme responses to other sources of oxidative stress.

The meager understanding of the anuran antioxidant system combined with the clear lack of accumulated knowledge about this important system stimulated us to conduct this investigation. Our objective was to investigate how the antioxidant systems of American Bullfrog tadpoles respond to sublethal oxidative stress due to paraquat exposure. We asked whether increasing concentrations of paraquat would increase the activity of four major antioxidant enzymes (SOD, catalase, peroxidases, and GR). We also asked whether liver and skeletal muscle antioxidant levels respond similarly to oxidative stress.

Materials and Methods

We chose American Bullfrog (*Lithobates catesbeianus*) tadpoles as an amphibian model for our study because their large range and wide use of habitats could make them a good indicator species for ecosystem health if the results were clear cut. This species is also large, reducing the number of tadpoles necessary to acquire necessary amounts of tissue for analyses. Finally, they are commercially available, making them an easily acquired model organism. We chose to use tadpoles because their aquatic lifestyle facilitates exposure regimes, and few previous studies exist which investigate the oxidative stress response in larval amphibian (Costa et al., 2008). American Bullfrog tadpoles (Gosner stage 36–37; Gosner, 1960) were purchased from Charles D. Sullivan Co., Inc. (Nashville, TN) and maintained communally in an aerated 20-L aquarium for two weeks. Tadpoles were fed pelleted tropical fish food ad libitum. Because these tadpoles originated from the wild, their exact age is not available. Three tadpoles were transferred to each of 15 clean 2-L glass containers, each filled with 1 L of fresh FETAX solution (ASTM, 1999) to avoid confounding interactions (Cairns and Cherry, 1990) and allowed to acclimate for 24 h. They were not fed during this portion of the experiment.

Paraquat (1,1′-dimethyl-4,4-bipyridinum dichloride; Gramaxone, Zeneca Laboratories) is a nonselective broad-spectrum herbicide known to induce an oxidative stress response via the elevation of superoxide levels (Day and Crapo, 1996). It was chosen as the stressor because it was a well-studied inducer of oxidative stress (except for in amphibians) that is known to impact amphibians in ways that are typically associated with oxidative stress (Dial and Bauer, 1984; Dial and Dial, 1987, 1995) and because it is regularly used by plant scientists as a model stressor (Shaaltiel et al., 1988; Gossett et al., 1996). We used a paraquat source solution of 37.05% paraquate dichloride to prepare a stock solution of 1,000 mg/L. Quantities of the stock solution were added to the containers to arrive at: 0, 0.1, 0.5, 1, and 2 mg/L. Each treatment was replicated three times. Twenty-four hours after the addition of the paraquat, the tadpoles were removed from the container and quickly anesthetized with 0.5% chloretone (1,1,1-trichloro-2-methyl-2-propanol [Wards Natural Sciences, Rochester, NY]; Ethridge, 1958). Liver and approximately 2 cm^2 of skeletal muscle (from tail) were removed from each tadpole while on ice. Livers and tail muscle from each of the three tadpoles in each container were pooled to form a replicate of each treatment. Upon removal, the tissues were immediately flash-frozen at $-70°C$ for subsequent protein extraction and antioxidant enzyme analyses.

Approximately 1 g of frozen tissue was homogenized in 3 mL of ice cold 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA with a few crystals of PMSF (phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 4°C at 17,000g for 20 min. Following centrifugation, 1 mL of the supernatant was centrifuge-desalted through a 10 mL bed of Sephadex G-25 (Sigma) (Anderson et al., 1992). A portion of the eluent was analyzed immediately for catalase activity, and the remainder was stored at $-70°C$ for subsequent analyses of SOD, peroxidase, and GR activities. Enzyme levels were detected via light spectrophotometry using a Beckman UV/visible Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and 1-mL cuvettes. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (Beers and Sizer, 1952). Peroxidase activity was measured by monitoring at 675 nm the H_2O_2-dependent oxidation of reduced 2,3′,6-trichloroindophenol (Nickel and Cunningham, 1969). Total SOD activity was assayed at 550 nm and was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of cytochrome C by superoxide generated by xanthine oxidase (Forman and Fridovich, 1973). GR activity was determined by monitoring the glutathione-dependent oxida-

tion of NADPH at 340 nm (Schaedle and Bassham, 1977).

The following standard enzyme units were used to report results in this study. One unit of catalase and peroxidase was defined as the amount of enzyme to decompose 1 μmole of substrate/minute at 25°C, and one unit of GR was defined as the amount of enzyme required to reduce 1 μmole of substrate/min at 25°C. One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of cytochrome C by 50%. All data points are based on a mean of the measurements taken from the four replicates of each treatment. All data were subjected to a one-way analysis of variance, and an alpha level of 0.05 was used to assign significance.

We used MiniTab 13.0 Statistical Software (Minitab, Inc.) to analyze the data. An Anderson-Darling normality test was run on all data sets prior to analysis and the data were normalized for parametric analyses as required. Tail and liver enzyme responses were analyzed using analysis of variance (ANOVA) with a Tukey multiple comparisons test. Data were normalized using the normalize function in MiniTab.

Results

After 24 h of paraquat treatment, all tadpoles were alive and appeared to be vigorous, suggesting that the American Bullfrog tadpole is relatively tolerant to acute paraquat exposure. Tail GR levels significantly increased with increasing paraquat concentration and some treatments were significantly different (Table 1). Paraquat exposure of 2.0 mg/L elicited significantly higher GR production than any other treatments. No other differences were observed. Tail PER levels and CAT levels did not differ among treatments (Table 1). Tail SOD levels increased with increasing paraquat exposure (Table 1). The 0.1-mg/L treatment was significantly different from the 1.0-mg/L and 2.0-mg/L treatments. No other differences were observed.

Liver GR levels increased significantly as paraquat concentration increased (Table 1). There were no significant difference in GR levels between 0.5 and 1.0 mg/L, but all other treatment levels were significantly different. Liver PER levels increased with paraquat concentration (Table 1). The 0.1, 0.5, and 1.0 mg/L treatments did not induce significantly different liver PER levels. The control was significantly lower than all treatments except 1.0 mg/L. Liver PER levels were significantly higher in 2.0 mg/L than in all other treatments except 0.1 mg/L. Neither liver CAT levels nor liver SOD levels increased with paraquat concentration (Table 1). No other differences were observed.

TABLE 1. Tail and liver antioxidant enzyme responses (units/g fresh mass ± SE) after 24 h exposure to paraquat by 4-month-old tadpoles of the American Bullfrog (*Lithobates catesbianus*). GR = glutathione reductase, PER = peroxidase, SOD = superoxide dismutase, CAT = catalase. Means sharing letters within a column are not significantly different (Tukey post hoc test).

Paraquat (mg/L)	GR		PER		SOD		CAT	
	Liver	Tail	Liver	Tail	Liver	Tail	Liver	Tail
0	108 ± 11 a	127 ± 3 a	591 ± 55 a	261 ± 26	100 ± 0.6 a	21 ± 1.3 a,b	4,892 ± 727	290 ± 25
0.1	285 ± 11 b	117 ± 4 a	900 ± 33 b	325 ± 44	149 ± 1.7 a,b	25 ± 1.2 a,b	6,556 ± 483	269 ± 18
0.5	454 ± 14 c	134 ± 16 a	874 ± 47 b	211 ± 7	156 ± 6.7 b	21 ± 1.2 b	5,291 ± 510	191 ± 15
1.0	426 ± 8 c,d	137 ± 9 a	856 ± 28 a,b	265 ± 59	142 ± 6.7 a,b	19 ± 1.2 a	3,959 ± 377	201 ± 34
2.0	850 ± 34 d	173 ± 6 b	1,469 ± 172 c	341 ± 17	98 ± 11.2 a	17 ± 1.2 a	4,860 ± 709	212 ± 36
$F_{4,10}$	199.4	11.2	13.04	1.95	6.27	5.89	2.68	2.70
P	< 0.001	0.001	0.001	0.18	0.09	0.011	0.23	0.09

Discussion

The results of this study suggest that the American Bullfrog tadpole has a very active antioxidant defense system. In the liver tissue, the antioxidant enzyme activities were either constitutively high (Barabas et al., 1985; Dodge, 1994; Foyer et al., 1994) as in catalase or up-regulated when the tadpoles were subjected to oxidative stress as with GR, peroxidase, and SOD (Table 1) (see also Shaalteil et al., 1988; Perez-Campo et al., 1993; Foyer et al., 1997). As in other studies (Hermes-Lima et al., 1998; Lushchak et al., 2001), antioxidant enzyme activity in the liver was higher than in muscle tissue. When exposed to paraquat (oxidative stress), antioxidant enzyme activity in the liver ranged from two- to 20-fold higher than the activities observed in the muscle. Even in the controls (paraquat = 0 mg/L), GR was the only enzyme that did not exhibit higher activity in the liver than in muscle tissue (Table 1). These results suggest that the liver is more important than skeletal muscle for protecting American Bullfrog tadpoles against oxidative stress. Previously, Roundup Original® increased SOD and CAT activity in the liver; however, it reduced these activities in skeletal muscle after 48 h exposure to 1 ppm (Costa et al. 2008). Our results are similar to those from goldfish (*Carassius auratus*; Lushchak et al., 2001) and Northern Leopard Frogs (Hermis-Lima and Storey, 1998).

Because paraquat is known to exhibit its toxic effects through the production of superoxide (Day and Crapo, 1996), SOD plays an important and early role in the defense against oxidative stress induced by this herbicide. Even in the lowest paraquat treatment (0.1 mg/L), SOD activity in the liver was elevated by 50% relative to the control, whereas SOD activity in the tail muscle did not increase regardless of the paraquat treatment (Table 1). Elevated liver SOD activity has also been reported in anoxic/hypoxic Common Gartersnakes (*Thamnophis sirtalis*; Hermes-Lima and Storey, 1993), carp exposed to paraquat or extreme hypoxia (Vig and Nemscsok, 1989), and estivating snails (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998). Superoxide dismutase activity in the liver did not change significantly during anoxia and reoxygenation in goldfish (Luschchak et al., 2001), and SOD activity decreased by 30% in the liver of anoxic Red-Eared Turtles (*Trachemys scripta*; Willmore and Storey, 1997), but in both cases, the activity of this enzyme was expressed at constitutively high levels. A slight decrease in SOD activity was observed after freezing in the Wood Frog (*Lithobates sylvaticus*) (Joanisse and Storey, 1996), and in the Northern Leopard Frog (*Lithobates pipiens*), SOD activity remained at control levels during dehydration (Hermes-Lima and Storey, 1998). Liver SOD levels decreased dramatically in intravenously exposed adult *Lithobates esculenta* (Barabas et al., 1985). They observed a 40% reduction in SOD expression between the control and the LD50. This expression dropped another 50% in frogs inoculated with the LD100 for paraquat. The significant bell-shaped response of SOD activity in the American Bullfrog tadpoles begs the question of whether the American Bullfrog could be more tolerant of oxidative stress than these other three anuran species and whether high levels of paraquat deactivate SOD or overwhelm the SOD response.

The rapid dismutation of superoxide to H_2O_2 could leave the organism susceptible to damage from the highly toxic hydroxyl radical formed via the Fenton reaction (Hermes-Lima et al., 1994) if the enzymes that degrade hydrogen peroxide are not sufficiently active. Although catalase activity did not change significantly with paraquat treatment, it was constitutively expressed at very high levels in the liver of American Bullfrog tadpoles (>4 mmole H_2O_2 decomposed/min/g fresh mass), and relatively high levels in muscle tissue (>200 μmoles H_2O_2 decomposed/min/g fresh mass; Table 1). This higher catalase expression in the liver as compared to other organs is in agreement with other studies of lower vertebrates (Perez-Campo, et al., 1993; Hermes-Lima and Storey, 1993, 1995; Joanisse and Storey, 1996; Lushchak et al., 2001). High constitutive liver catalase activity has been proposed as an important component of the defense against oxidative damage in anoxic freshwater turtles (Willmore and Storey, 1997) and snakes (Hermes-Lima and Storey, 1993). In goldfish, catalase activity increased significantly during anoxia and reoxygenation in the liver but remained at control levels in the muscle tissue. In contrast, catalase activity did not change significantly from the values observed in the livers of dehydrated Northern Leopard Frogs belonging to the control group, although it increased 52% in muscle tissue (Hermes-Lima and Storey, 1998). Paraquat treatment failed to produce a significant change in catalase activity in the liver or the muscle tissue of American Bullfrog tadpoles, but the high constitutive levels of catalase in these tissues, especially the liver, may have been sufficient to prevent oxidative damage.

General peroxidases (the other group of enzymes that degrade hydrogen peroxide) activity increased almost twofold at the lower paraquat treatments (0.1, 0.5, and 1.0 mg/L) and almost threefold at the highest paraquat treatment (2 mg/L) in the tadpole liver (Table 1).

Literature reports on general peroxidase activity in the lower vertebrates are scarce, but there are numerous reports on the specific peroxidase, glutathione peroxidase (GPX), and GST, of which some isozymes exhibit peroxidase activity. GPX activity also increased significantly during freezing in the muscle tissue of Common Gartersnakes (Hermes-Lima and Storey, 1993) and in several tissues of Wood Frogs (Joanisse and Storey, 1996), but in the goldfish, GPX did not vary significantly from the control value in goldfish subjected to anoxia and reoxygenation; however, this enzyme was expressed at constitutively high levels in the goldfish liver (Lushchak et al., 2001). GST activity was also constitutively high in the goldfish liver and did not change significantly during anoxia and reoxygenation (Lushchak et al., 2001). In Northern Leopard Frogs, liver GPX increased significantly during dehydration (Hermes-Lima and Storey, 1998) and anoxia (Hermes-Lima and Storey, 1996), whereas GST levels remained unchanged in both the liver and muscle under either condition (Hermes-Lima and Storey, 1996, 1998). Neither GPX nor GST was measured in this study, but the induced increase in general peroxidase activity in the liver and the relatively high general peroxidase activity in the muscle tissue combined with the high constitutive catalase activity suggests that the American Bullfrog has an enhanced capacity for breaking down the H_2O_2 that would be generated during oxidative stress.

The ability to maintain the GSH/GSSG ration in favor of GSH for the reduction of oxidized ascorbate and other antioxidant roles is a critical component of the antioxidant defense system. The activity of GR, the enzyme primarily responsible for maintaining high GSH levels, increased approximately 2.6-, four-, and eightfold in the liver when the tadpoles were treated with 0.1-, 0.5-, or 1.0-, and 2.0-mg/L paraquat, respectively. GR activity also increased a significant 36% in the muscle tissue, when the tadpoles were subjected to the high paraquat (2 mg/L) treatment (Table 1). Hermes-Lima and Storey (1993) reported that constitutively high GR activity was maintained during anoxia in gartersnakes. GR activity remained at control levels in the hepatopancreas and foot muscle of estivating land snails (Hermes et al., 1998), but during anoxia and reoxygenation in goldfish, GR activity increased significantly in liver tissue and did not change significantly from the controls in muscle tissue (Lushchak et al., 2001). In the Northern Leopard Frog, control levels of GR activity were maintained in the liver during dehydration and rehydration, whereas in the muscle tissue, GR activity decreased significantly during dehydration and returned to control levels after rehydration (Hermes-Lima and Storey, 1998). In this study, that GR activity was elevated in both the liver and muscle tissues after exposure to paraquat suggests that the bullfrog has a greater capacity for the regeneration of GSH than the other anuran species, Northern Leopard Frog. The mechanism responsible for the upregulation of GR activity or the activities of the other antioxidant enzymes is, at the present, unknown, but studies with paraquat and salt tolerance in cotton (Gossett et al., 1996; Manchandia et al., 1999) suggest that the increases in the activities of these enzymes may be caused by the increased transcription of the genes responsible for encoding these specific proteins.

That SOD activity did not increase above the control level at 24 h of exposure to the highest paraquat treatment (2 mg/L) is a puzzle (Table 1). One possibility is that the 2mg/L-treatment is beyond the threshold at which the tadpole can mount an effective defense against oxidative stress, and the tadpoles may have eventually died had the experiment been carried out for a longer period of time. Alternatively, it may be that at the high concentration of paraquat, the nonenzymatic quenching of superoxide was sufficient to provide protection against oxidative stress. GR activity increased eightfold in the liver and 52% in the muscle tissue (Table 1) when the tadpoles were exposed to 2-mg/L paraquat. These high levels of GR activity could have produced sufficient GSH to keep the ascorbate pool in the reduced form, which can directly scavenge superoxide radicals (Foyer et al., 1991). Hence, the superoxide scavenging capacity of reduced ascorbate combined with the induced increase in peroxidase activity (threefold, Table 1) and the high constitutive activity of catalase (Table 1) may have been sufficient to protect the tadpoles from the cytotoxic effects of paraquat.

The bullfrog tadpoles appear to have some tolerance to paraquat toxicity because 100% of the tadpoles were alive and vigorous after 24 h of exposure to the highest paraquat treatment (2 mg/L), which is well above the published LC 50 for other anuran species (USFWS, 1990). However, determination of an LC50 or LD50 for bullfrog larvae has not been conducted, and it is unknown how these results might vary as they pass through the highly complex life cycle characteristic of anurans.

Our data provide baseline evidence that the antioxidant system in bullfrog tadpoles and potentially other species of amphibians may serve as a useful tool in field studies. It is important that field tools be developed to assist in conservation monitoring and restoration; however, further development of this tool is necessary. Problems that must be confronted include the following: (1) a complete evaluation

of oxidative stress tolerance to paraquat or another oxidative stressor to establish a system of responses; (2) evaluation of a wider selection of enzymes and enzyme activity to determine whether any key components correlate between muscle and liver antioxidant production; (3) evaluation of how antioxidant production responds through key developmental stages of anurans; and (4) evaluation of interspecific variation in antioxidant responses. Undoubtedly, the use of these enzymes as an environmental indicator of amphibian stress has potential necessitating further in-depth studies of the physiologic mechanism involved.

Literature Cited

Anderson, J. V., B. I. Chevone, and J. L. Hess. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. Plant Physiology 98:501–508.

ASTM. 1999. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). Designation, E 1439–98.

Barabas, K., L. Szabo, and B. Matkovics. 1985. Effects of Gramoxone and cooling on the antioxidant enzymes of frog (*Rana esculenta*). Acta Biologica Hungarica 35(3–4):295–300.

Beers, R. R., and I. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. Journal of Biological Chemistry 195:133.

Burgett, A. A., C. D. Wright, G. R. Smith, D. T. Fortune, and S. L. Johnson. 2007. Impact of ammonium nitrate on Wood Frog (*Rana sylvatica*) tadpoles: effects on survivorship and behavior. Herpetological Conservation and Biology 2:29–34.

Cairns, J., Jr., and D. S. Cherry. 1990. The significance of water formulation in toxicity testing. Environmental Auditor 1(4):183–185.

Chang, H., B. Z. Siegel, and S. M. Siegel. 1984. Salinity induced changes in isoperoxidase in taro, *Colocasia esculenta*. Phytochemistry 23:233–235.

Costa, M. J., D. A. Monteiro, A. L. Oliveira-Neto, F. T. Rantin, and A. L. Kalinin. 2008. Oxidative stress biomarkers and heart function in bullfrog tadpoles exposed to Roundup Original. Ecotoxicology 17(3):153–163.

Davies, K. J. A. 1987. Protein damage and degredation by oxygen radicals I. General aspects. Biological Chemistry 262:9895–9901.

Day, B. J., and J. D. Crapo. 1996. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced lung injury in vivo. Toxicology and Applied Pharmacology 140:94–100.

Dial, C., and N. A. Dial. 1987. Lethal effects of diquat and paraquat on developing frog embryos and 15-day-old tadpoles, *Rana pipiens*. Bulletin of Environmental Contamination and Toxicology 38: 1006–1011.

———. 1995. Lethal effects of the consumption of field levels of paraquat-contaminated plants on frog tadpoles. Bulletin of Environmental Contamination and Toxicology 55:870–877.

Dial, N. A., and C. A. Bauer. 1984. Teratogenic and lethal effects of paraquat on developing frog embryos (*Rana pipiens*). Bulletin of Environmental Contamination and Toxicology 33:592–597.

Dodge, A. 1994. Herbicide action and effects on detoxification processes. *In* C. H. Foyer and P. M. Mullineaux (eds.), Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants, pp. 219–236. CRC Press, Boca Raton, FL.

Ethridge, R. E. 1958. Methods for preserving amphibians and reptiles for scientific study. Available at http://www.ummzumich.edu/herps/herpinstr.html. Last accessed 5 July 2006.

Fellers, G. M., D. F. Bradford, D. Pratt, and L. L. Wood. 2007. Demise of repatriated populations of Mountain Yellow-Legged Frogs (*Rana muscosa*) in the Sierra Nevada of California. Herpetological Conservation and Biology 2:5–21.

Forman, H. J., and I. Fridovich. 1973. Superoxide dismutase: a comparison of rate constants. Archives of Biochemistry and Biophysics 158:396–400.

Foyer, C. H., M. Lelandais, E. A. Edwards, and P. M. Mullineaux. 1991. The role of ascorbate in plants: interactions with photosynthesis and regulatory significance. *In* E. Pell and K. Steffen (eds.), Active Oxygen Oxidative Stress and Plant Metabolism, pp. 131–144. Proceedings of the 6th Annual Penn State Symposium in Plant Physiology, St. Louis, MO.

Foyer, C. H., P. Descourviers, and K. J. Kunert. 1994. Protection against oxygen radicals: an important defense mechanism in transgenic plants. Plant Cell and Environment 17:507–523.

Foyer, C. H., H. Lopez-Delgado, J. F. Dat, and I. M. Scott. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Plant Physiology 100:241–254.

Fridovich, I. 1986. Biological effects of the superoxide radical. Archives of Biochemistry and Biophysics 247:1–11.

Funk, W. C., A. E. Greene, P. S. Corn, and F. W. Allendorf. 2005. High dispersal in a frog species suggests that it is vulnerable to habitat fragmentation. Biology Letters 1:13–16.

Germano, D. J. 2006. Habitat characteristics of sites with Yellow-Blotched Salamanders (*Ensantina eschscholtzii croceator*). Herpetological Conservation and Biology 1:121–128.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae. Herpetologica 16:183–190.

Gossett, D. R., S. W. Banks, E. P. Millhollon, and M. C. Lucas. 1996. Antioxidant response to NaCl in a control and an NaCl-tolerant cell line grown in the presence of paraquat, buthionine sulfoximine, and exogenous glutathione. Plant Physiology 112:803–809.

Gray, M. J., L. M. Smith, D. L. Miller, and C. R. Bursey. 2007. Influences of agricultural land use on *Clinostomum attenuatum* metacercariae prevalence in southern Great Plains amphibians, U.S.A. Herpetological Conservation and Biology 2:23–28.

Green, D. E., and C. K. Dodd. 2007. Presence of amphibian chytrid fungus *Batrachochytrium dendrobatidis* and other amphibian pathogens and warm-water fish hatcheries in Southeastern North America. Herpetological Conservation and Biology 2:43–47.

Hermes-Lima, M., and K. B. Storey. 1993. Role of antioxidants in the tolerance of freezing and anoxia by garter snakes. American Journal of Physiology–Regulatory Integrative and Comparative Physiology 265:R646–R652.

———. 1995. Antioxidant defenses and metabolic depression in pulmonate land snails. American Journal of Physiology 265:R1386–R1393.

———. 1996. Relationship between anoxia exposure and antioxidant status in the frog *Rana pipiens*. American Journal of Physiology 265:R918–R925.

———. 1998. Role of antioxidant defenses in the tolerance of severe dehydration by anurans. The case of the Leopard Frog, *Rana pipiens*. Molecular and Cellular Biochemistry 189:79–89.

Hermes-Lima, M., E. M. Wang, H. H. Schuman, K. B. Storey, and P. Ponka. 1994. Oxidative degradation of deoxyribose catalyzed by Fe(III)-EDTA complex. Kinetic aspects and potential usefulness for sub-micromolar iron measurements. Molecular and Cellular Biochemistry 137:65–73.

Hermes-Lima, M., J. M. Storey, and K. B. Storey. 1998. Antioxidant defense and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comparative Biochemistry and Physiology B Biochemistry and Molecular Biology 120:437–448.

Imlay, J. A., and S. Linn. 1988. DNA damage and oxygen radical toxicity. Science 240:1302–1309.

Joanisse, D. R., and K. B. Storey. 1996. Oxidative damage and antioxidants in *Rana sylvatica*, the freeze tolerant Wood Frog. American Journal of Physiology 113:23–35.

Karraker, N. E. 2007. Are embryonic and larval Green Frogs (*Rana clamitans*) insensitive to road deicing salt? Herpetological Conservation and Biology 2:35–41.

Lushchak, V. I., L. P. Lushchak, A. A. Mota, and M. Hermes-Lima. 2001. Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. American Journal of Physiology 280:R100–R107.

Lutz, P. L., and K. B. Storey. 1997. Adaptations to variations in oxygen tension by vertebrates and invertebrates. *In* W. H. Dantzler (ed.), Handbook of Physiology. Section 13. Comparative Physiology. Vol. 2, pp. 1479–1522. Oxford University Press, Oxford.

Manchandia, A. M., S. W. Banks, D. R. Gossett, B. A. Bellaire, M. C. Lucas, and E. P. Millhollon. 1999. The influence of α-amanitin on the NaCl-induced upregulation of antioxidant enzyme activity in cotton callus tissue. Free Radical Research 30:429–438.

McCallum, M. L. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology 41:483–491.

McCallum, M. L., and J. L. McCallum. 2006. Publication trends of natural history and field studies in herpetology. Herpetological Conservation and Biology 1:62–67.

McCallum, M. L., and S. E. Trauth. 2007. Physiological trade-offs between immunity and reproduction in the Northern Cricket Frog (*Acris crepitans*). Herpetologica 63:269–274.

Moody, C. S., and H. M. Hassan. 1984. Anaerobic biosynthesis of the manganese-containing superoxide dismutase in *Escherichia coli*. Journal of Biological Chemistry 259:12821–12825.

Nickel, R. S., and B. A. Cunningham. 1969. Improved peroxidase assay method using leuco 2,3′,6-trichloroindophenol and application to comparative measurements of peroxidase catalysis. Annals of Biochemistry 27:292–299.

Ortmann, A. E. 1909. The destruction of fresh-water fauna in western Pennsylvania. Proceedings of the American Philosophical Society 48:90–110.

Perez-Campo, R., M. Lopez-Torres, C. Rojas, S. Cadenas, and G. G. Barja. 1993. A comparative study of free radicals in vertebrates—I. Antioxidant enzymes. Comparative Biochemistry and Physiology 105:749–755.

Pounds, J. A., M. P. L. Fogden, and J. H. Campbell. 1999. Biological response to climate change on a tropical mountain. Nature 398:611–615.

Rouse, D. J., C. A. Bishop, and J. Struger. 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. Environmental Health Perspectives 107:799–803.

Schaedle, M., and J. A. Bassham. 1977. Chloroplast glutathione reductase. Plant Physiology 59: 1011–1012.

Shaalteil, Y., A. Glazer, P. F. Bocion, and J. Grexxel. 1988. Cross tolerance to herbicidal and environmental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. Pesticide Biochemistry and Physiology 31:13–20.

Stead, D., and S. F. Park. 2000. Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress. Applied Environmental Microbiology 66:3110–3112.

Storey, K. B. 1996. Oxidative stress: animal adaptations in nature. Brazilian Journal of Medical and Biological Research 29:1715–1733.

Storey, K. B., D. D. Mosser, D. N. Douglas, J. E. Grundy, and J. M. Storey. 1996. Biochemistry below 0°C: nature's frozen vertebrates. Brazilian Journal of Medical and Biological Research 29:283–307.

Ultsch, G. R. 1989. Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. Biological Review 64:435–516.

USFWS (United States Fish and Wildlife Service). 1990. Paraquat Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review. Biological Report 85(1.22). U.S. Department of the Interior, Washington, DC.

Vig, E., and J. Nemcsok. 1998. The effect of hypoxia and paraquat on the superoxide dismutase activity in different organs of carp, *Cyprinus carpio*. Journal of Fish Biology 35:23–25.

Wheeler, B. A., M. L. McCallum, and S. E. Trauth. 2002. Abnormalities in the Ozark Hellbender, *Cryptobranchus alleganiensis bishopi*. Journal of the Arkansas Academy of Science 56:250–252.

Willmore, W. G., and K. B. Storey. 1997. Antioxidant systems and anoxia tolerance in freshwater turtles, *Trachemys scripta elegans*. Molecular and Cellular Biochemistry 170:177–185.

Young, B. E., S. N. Stuart, J. S. Chanson, N. A. Cox, and T. M. Boucher. 2004. Disappearing Jewels: The Status of New World Amphibians. NatureServe, Arlington, VA.

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