EFFECTS OF NITROGEN AMMONIA AND MS-222 ON *XENOPUS LAEVIS* DEVELOPMENT, GROWTH, AND FORAGING BEHAVIOR

J. Kelly Byram[1,2] and Max A. Nickerson[3]

ABSTRACT

The anesthetic MS-222 (i.e., tricaine, Finquel) is widely used by biologists on amphibians in the field, even though field use of MS-222 on amphibians is not approved by the U.S. Food and Drug Administration (FDA). Previous studies have identified the impact of MS-222 on vision, olfaction, stress, heart, and liver, and have documented its lethality to certain microbes that commonly populate amphibian skin. We examined the potential impacts of "off-label" use of MS-222 on a model aquatic amphibian, the African clawed frog (*Xenopus laevis* Daudin 1802). Animals were exposed to an environmentally relevant concentration of nitrogen ammonia, a pollutant commonly found in U.S. waterways, and unbuffered MS-222 in a manner simulating typical field use of the drug. The animals' foraging success in the hour post-recovery was observed. MS-222 impacted foraging behavior, with animals exposed to MS-222 eating significantly more food pellets than the control animals ($P = 0.01$). Although an ANOVA revealed no statistically significant difference in the mean weight and length between the animals exposed to nitrogen ammonia and their controls, the group of animals exposed to nitrogen ammonia had an increased variance in weight and length, which may indicate population-level effects.

Key words: MS-222, tricaine, *Xenopus laevis,* nitrogen ammonia, ammonia, stress.

[1]Office of Research, Health Sciences Center, University of New Mexico, Albuquerque, NM 87131-0001 USA <jkbyram@salud.unm.edu>
[2]Previous address: Department of Wildlife Ecology and Conservation, University of Florida, University of Florida, Gainesville, FL 32611-0430 USA
[3]Florida Museum of Natural History, University of Florida, Gainesville, FL, USA 32611-7800 USA; <maxn@flmnh.ufl.edu>

Byram, J. K., and M. A. Nickerson. 2012. Effects of nitrogen ammonia and MS-222 on *Xenopus laevis* development, growth, and foraging behavior. Bulletin of the Florida Museum of Natural History 51(4):217–226.

INTRODUCTION

Naturally occurring ammonia (NH_3) plays an important role in aquatic environments. Aquatic animals excrete ammonia as a byproduct of metabolism and, it is suspected, as a disturbance pheromone powerful enough to elicit an avoidance reaction in tadpoles (Manteifel 2006). Eutrophication results from high levels of nitrogen (e.g., nitrates, nitrogen ammonia) or phosphorous compounds in an ecosystem, and eutrophication resulting from anthropogenic activities has been implicated as one factor in global amphibian decline (Nyström et al. 2007) and pathology (Johnson et al. 2007).

Studies of the effects of ammonia on embryos and larvae have revealed statistically significant findings. Jofre et al. (2000) studied the effects of concentrations of un-ionized NH_3 up to 2 mg/l on green frogs (*Lithobates clamitans* Latreille 1801) and leopard frogs (*L. pipien*s Schreber 1782). Hatching success declined when leopard frog embryos were exposed to concentrations of NH_3 greater than 1.5 mg/l, and the frogs were more likely to be deformed. Green frogs experienced similar impacts at a lower concentration of NH_3 (0.6 mg/l), and the green frogs displayed the additional problem of decreased tadpole survival and growth at this concentration.

MS-222 (i.e., tricaine, Finquel) is widely used by biologists in the laboratory and in the field to euthanize and sedate amphibians for safety and to minimize stress from handling and various procedures, but research indicates MS-222 may actually increase stress (Vethamany-Globus et al. 1977), impair vision (Hoffman & Basinger 1977; Bernstein et al. 1986), and affect the amphibian heart (Bartlett et al. 2004; Cakir & Strauch 2005; Bartlett et al. 2010) and liver (Wayson et al. 1976). Stress has been linked to immunosuppression (Belden & Kiesecker 2005), declines in reproduction potential and survival (Edgington et al. 2003; Barbeau & Guillette 2007), and synergistic toxicity with a pesticide (Relyea & Mills 2001). Additionally, the use of MS-222 can mask the parasitic load and may have affected the results of previous amphibian parasite studies as it anesthetizes parasites as well (Fedewa & Lindell 2005; Solis et al. 2007a).

Although marketed as a sedative appropriate for use in the field (Argent Chemical Laboratories undated; Western Chemical, Inc. undated), the U.S. Food and Drug Administration (FDA) specifically restricts the use of MS-222 on amphibians to the laboratory, stating "In other fish and cold-blooded animals, the drug should be limited to hatchery or laboratory use" (FDA 1998). Despite this clear restriction, the literature is replete with "off-label" use (i.e., use of a drug in a manner not approved by the FDA) of MS-222 in field studies of amphibian populations (Byram & Nickerson 2009). MS-222 is recommended for use on amphibians in veterinary and zoological publications (Gentz 2007) and for use in the field in government documents (Green 2001). As Crook and Whiteman (2006) stated in their own study of MS-222, "Typically the choice of anesthesia has been based on what other researchers have used rather than a critical evaluation of different methods."

The off-label use of MS-222 on amphibians in the field is potentially problematic because direct inquiry and anecdotal evidence point to field techniques very different from the suggested usage and safety guidelines, with field biologists often mixing the anesthetic powder with water from a local body of water, as instructed by the package insert provided by Argent Chemical Laboratories (undated) and Western Chemical, Inc. (undated). Additionally, anecdotal evidence indicates the MS-222 bath is often not buffered in the field (Nickerson pers. obs.). Use of unbuffered MS-222 creates the risk of amphibian death, since exposure to a pH below 4-5 can cause death in amphibians (Boutilier et al. 1992). Additionally, as pH decreases, herbicides become more toxic to amphibians (Edgington et al. 2003) and incidence of infection increases (Simon et al. 2002). Quality of the environmental water used for the anesthetic bath is an additional consideration. At the time of this study, the Argent directions for its MS-222 product marketed as "Finquel" read, "Do not use... water containing chlorine, heavy metals (copper, zinc, etc.), or other toxic contaminants" (Argent Chemical Laboratories undated), but with the

decline of water quality in aquatic habitats and the uncertainty of what chemicals and metals are in these habitats, there is the potential risk the anesthetic bath prepared in the field may be unsafe. Because of its use in the aquaculture industry on fish consumed as food for humans, copious research on MS-222's effects on fish is available, but research on its effects on amphibians—especially when it is used off-label—is comparatively scant. This study was designed to determine if MS-222 and nitrogen ammonia impact amphibians, both separately and in a typical field-preparation combination.

MATERIALS AND METHODS

African clawed frog (*Xenopus laevis* Daudin 1802) tadpoles ($n = 270$) from one clutch were purchased from Xenopus Express (Brooksville, FL) and distributed among four 10-gallon glass aquarium tanks: two control ($n = 136$) and two treatment ($n = 134$) tanks. Each tank was filled with 32 L tap water treated with 1 mL dechlorinator (Top Fin, Pacific Coast Distributing, Inc., Phoenix, AZ) for removal of chlorine, chloramine, and heavy metals. The two treatment tanks were dosed with nitrogen ammonia (NH_3-N; Ricca Chemical Company, Arlington, TX) to an environmentally relevant concentration of 0.5 mL/L, as indicated by concentrations of total nitrogen (TN) documented in the North Fork of White River (Quinlan & Phlips 2007; Solis et al. 2007b) and the Eleven Point River, Missouri (Solis et al. 2007b). We selected to use the nitrogen concentrations found in the North Fork of White River and the Eleven Point River because these rivers provide habitat for declined populations of endangered Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) and documented field use of MS-222 on *C. a. bishopi* has occurred at these sites (Byram & Nickerson 2009). Each 1.00 mL of NH_3-N contained 1.00 mg N and 1.216 mg NH_3. The concentration for the treatment environments was calculated based upon the nitrogen content of the NH_3-N. Concentration was verified by testing ammonia (NH_3) levels in the tanks (API, Inc., Chalfont, PA). Temperature, pH (5-in-1 strips, Hach Co., Loveland, CO), nitrite (nitrate/nitrite test strips, Hach Co., Loveland, CO), and nitrate (nitrate/nitrite test strips, Hach Co., Loveland, CO), were also monitored. The tanks were aerated with 10 cm air stones (Rolf C. Hagen Corporation, Mansfield, MA) run off of one air pump and adjusted to provide the same amount of aeration to each tank (by visual estimation). Tanks were neither heated nor cooled. Air and water temperature were monitored daily. Over the course of the study, the observed water temperature ranged between 16–23°C, but the average observed daily water temperature was usually 20–22°C.

At approximately 55 days of development, animals began to exhibit aggressive behavior and water quality had begun to decline, even though the water in the tanks was changed daily, so the animals were separated into individual habitats. Only animals that had reached a stage where they could eat food pellets (Nieuwkoop-Faber stage 64 or greater) moved on to the next stage of the protocol. These juveniles were given a unique identification number and placed in individual one-gallon bowls marked with the animal's identification number. The remaining animals that had not metamorphosed did not continue in the protocol.

Measurements

Development and growth were monitored throughout the study. Tadpoles were randomly selected from the tanks for measurement and staging at several points of the protocol by scooping or netting in a variable fashion throughout the tank areas. The tadpoles were measured with a ruler to the nearest 1.0 mm and weighed to the nearest 0.1 g using an Ohaus ProScout Scale (Ohaus Corporation, Parsippany, NJ). They were then viewed under a light microscope for staging according to the Nieuwkoop-Faber table (Nieuwkoop & Faber 1994).

Once the animals metamorphosed and were in their individual bowls, they were examined, weighed to the nearest 0.1 g using an Ohaus ProScout Scale (Ohaus Corporation, Parsippany, NJ), and snout-vent length (SVL) was measured to the nearest 1.0 mm using a ruler 3 times (days 55, 96, 112–113, and 130–131) during the water change process to minimize stress. Dimorphism

is not pronounced enough to reliably sex young juveniles without dissection, so data on sex were not collected.

Environment

Bowls were housed in numerical order on shelves and unused bench space throughout the laboratory. Water changes, first partial and then full, were performed on a regular basis with room-temperature tap water treated with Top Fin Tap Water Dechlorinator (Pacific Coast Distributing, Inc., Phoenix, AZ) for removal of chlorine, chloramine, and heavy metals.

Temperature was not maintained on an individual basis and was dictated by the ambient temperature of the laboratory space. Laboratory windows provided filtered light for the animals, which was dictated by local weather patterns, and exposure to artificial fluorescent lighting was regular and incidental to periods of human occupation of the laboratory, which could not be regulated, since multiple researchers on variable schedules had access to adjacent laboratory space.

Diet

Tadpoles were fed a liquid diet of tadpole powder (Xenopus Express, Brooksville, FL) prepared with filtered tap water (Brita faucet mount filtration system, Brita Products Company, Oakland, CA). The solution was mixed well and shaken as needed to assure the powder remained suspended and distribution of nutrition was equivalent across the tanks. Additionally, food was added to the tanks in a uniform fashion but in a random order determined using a random number generator (www.random.org), assuring that any variability in food distribution related to suspension of food was distributed evenly over time across the tanks.

After the animals metamorphosed and were separated to individual habitats on day 55, animals were fed 3/32" (2.38 mm) floating frog food pellets (Xenopus Express, Brooksville, FL) on a regimented schedule.

MS-222 Treatments

Half of the nitrogen ammonia-exposed animals and half of the control animals were randomly assigned to the MS-222 treatment group by using a random number generator (www.random.org) to pick animal numbers. The remaining 50% of the animals were assigned to the control group.

The anesthetic solution of 1 L tap water at 23.3°C dechlorinated with Top Fin Tap Water Dechlorinator (Pacific Coast Distributing, Inc., Phoenix, AZ), 0.50 mg NH_3-N, and 1 g tricaine powder (Finquel, Argent Labs, Redmond, WA) for the NH_3-N-exposed animals was prepared without buffer as is often true in the field. The anesthetic solution for the control group (non-nitrogen ammonia) was prepared according to instructions, with 1 L tap water at 23.3°C dechlorinated with Top Fin Tap Water Dechlorinator (Pacific Coast Distributing, Inc., Phoenix, AZ), 1 g tricaine powder, and1 g baking soda (buffer). Over the course of days 112 and 113, animals were removed from their individual tanks, weighed, measured, and then placed in the anesthetic bath. Animals were kept in the anesthetic bath until they stopped swimming, at which point they were retrieved and gently placed on their backs on the researcher's palm. If the animal attempted to right itself, it was placed back in the anesthetic bath and carefully monitored. When the animals ceased attempting to right themselves in the researcher's hand, they were considered anesthetized. The time it took for the animals to reach the desired anesthetic plane was not recorded, and it varied widely from animal to animal. Typically animals ceased righting themselves within 3–10 minutes.

Then half of the animals (half anesthetized, half not anesthetized) had a small web clip performed. The web clipping control group was handled for a duration and in a fashion mimicking the handling of the web clipping group to control for handling stress.

Anesthetized animals were then placed directly in a recovery bath. For animals in the nitrogen ammonia group, the bath was a mixture of 1 L 23.3°C dechlorinated tap water and 0.50 mg NH_3-N. This was the same concentration of nitrogen ammonia they had lived in since the beginning of the study. For the control animals, the bath omitted

the NH_3-N. Periodically, water was gently agitated by hand to create a flow of water on the skin of the animals to facilitate recovery. Once the animals recovered gross motor skills and were able to right themselves on the researcher's palm, they were returned to their individual tanks, which had been cleaned and refilled with water in the interim. The tank was then placed in a lighted observation area and the time noted, and 8 pieces of the same brand and size floating food pellets, fed throughout the study, added to the water. Animals were observed intermittently from a distance of approximately 0.3 to 1.3 meters to assure their safety and recovery. At one hour post-treatment, the number of food pellets eaten was noted, uneaten food pellets were removed from the tank, and the animal's tank returned to its assigned place in the laboratory space.

Animals were weighed and measured on days 130 and 131. This ended the protocol, and all study animals were adopted through the University of Florida Animal Care Service's laboratory animal adoption program.

Statistical Analyses

Stage, total length, SVL, weight, and consumption data were compared for the treatment and control groups. The parametric day 55 weight data were analyzed with a *t*-test, while the other data did not meet the assumptions of parametric analysis and were analyzed with a nonparametric ANOVA (Kruskal-Wallis).

Analysis was complicated by the mortality of treatment subjects due to causes unrelated to the experimental protocol. A graduate student with IACUC certification and years of experience working with animals, who was briefly employed as a lab assistant, overdosed some of the treatment animals with NH_3-N, presumably by a factor of two, which proved lethal. Therefore, the sample sizes of the groups for the measurement data of days 130–131 were not equal (see Table 1). Post-treatment (MS-222) growth (weight and SVL) were calculated by subtracting day 112 and 113 data from day 130 and 131 data. All statistical analyses were performed with alpha = 0.05 using SAS software (v. 9.1, SAS Institute, Inc., Cary, NC).

RESULTS

One variable not being measured for analysis but whose outcome is worthy of note is the pH of the anesthetic baths. The pH of the anesthetic baths were measured as a matter of safety, since Boutilier et al. (1992) report that a pH below 4–5 can cause death in amphibians. While the buffered MS-222 baths were pH 7 (neutral), the pH dropped to 4–5 (acidic) in the unbuffered baths.

Development and Growth

No statistically significant differences were indicated in stage (P value range = 0.22 – 0.87, n = 20; Fig. 1) or total length (P value range = 0.18 – 0.79, n = 20; Fig. 2) during metamorphosis between the nitrogen ammonia treatment group and the control group. Nor was there a statistically significant difference between the nitrogen ammonia treatment group and the control group at the end of the study (i.e., days 130–131) in SVL (P = 0.17, n = 136, χ^2 = 1.88, df = 1; Fig. 3) or weight (P = 0.24, n = 136, χ^2 = 1.40, df = 1; Fig. 4).

Animals anesthetized with unbuffered MS-222 exhibited no difference in growth in terms of weight or SVL compared to control subjects 18

Table 1. Summary of sample sizes for days 130–131 for the nitrogen ammonia treatment (NH_3-N) and control groups.

	Web-Clipped		Control	
	NH_3-N	Control	NH_3-N	Control
MS-222	17	18	16	19
Control	10	20	16	20

days after treatment ($P = 0.16$, $n = 136$, $\chi^2 = 1.93$, df = 1 and $P = 0.47$, $n = 136$, $\chi^2 = 0.53$, df = 1, respectively). Weight was likewise unaffected by the web clipping ($P = 0.23$, $n = 130$, $\chi^2 = 1.41$, df = 1), but web-clipped animals did show a statistically significant increase in SVL compared to the unclipped animals ($P = 0.01$, $n = 130$, $\chi^2 = 6.48$, df = 1).

Foraging

The animals treated with MS-222 ate significantly more food pellets in the recovery hour after treatment than did the control group ($P = 0.01$, $n = 149$, $\chi^2 = 6.1$, df = 1). Within-group analysis of the animals who received MS-222 treatment revealed no difference in food pellet consumption between the animals whose webbing was clipped and their controls ($P = 0.09$, $n = 149$, $\chi^2 = 2.95$, df = 1), nor was there any difference between the animals raised in 0.50 mg/L concentration of nitrogen ammonia and their controls ($P = 0.70$, $n = 149$, $\chi^2 = 0.15$, df = 1).

Roughly the same percentage of animals in the MS-222 treatment and control groups ate all of the food pellets offered: 22.9% of the MS-222 group and 19.4% of the control group. However, the percentage of animals that ate nothing varied

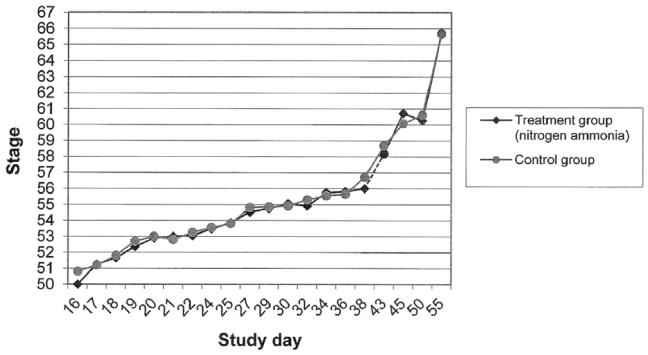

Figure 1. Mean Nieuwkoop-Faber developmental stage of African clawed frogs (*Xenopus laevis*) subjected to nitrogen ammonia treatments and no treatment (i.e., control) by study day.

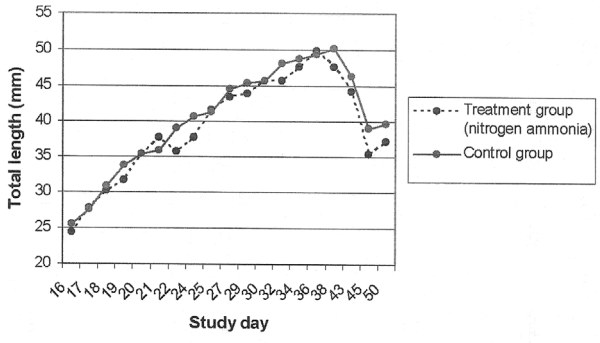

Figure 2. Mean total length and SVL of African clawed frogs (*Xenopus laevis*) subjected to nitrogen ammonia treatments and no treatment (i.e., control) by study day. As tadpoles develop into juveniles and their tails begin to disappear, their length decreases. Once the tail is gone, the juveniles continue to grow and their length begins to increase once again.

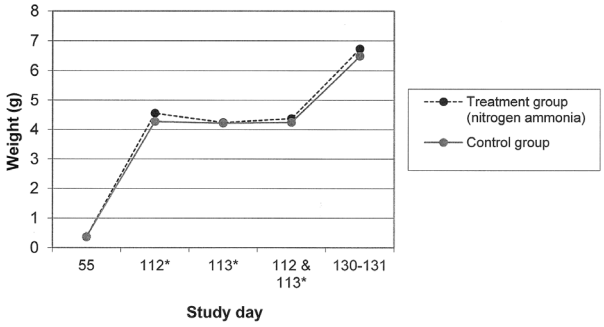

Figure 3. Mean total weight of African clawed frogs (*Xenopus laevis*) subjected to nitrogen ammonia treatments and no treatment (i.e., control) by study day. *Since half the animals were weighed on day 112 and half were weighed on day 113, the mean weights for days 112 and 113 are reported separately and in combination (112 & 113).

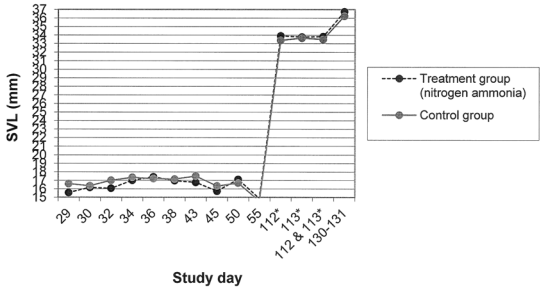

Figure 4. Mean snout-vent length (SVL) of African clawed frogs (*Xenopus laevis*) subjected to nitrogen ammonia treatments and no treatment (i.e., control) by study day. *Since half the animals were measured on day 112 and half were measured on day 113, the mean SVL for days 112 and 113 are reported separately and in combination (112 & 113).

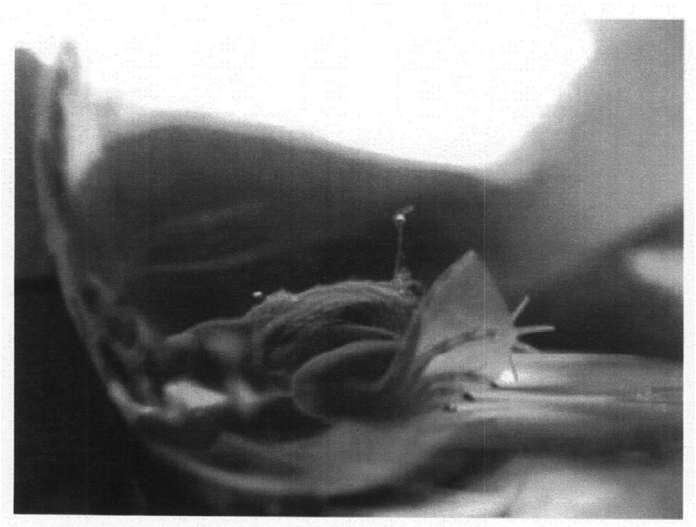

Figure 5. Reaction of African clawed frog (*Xenopus laevis*) to treatment with buffered MS-222. This animal had no visible skin problems or defects prior to treatment with MS-222, but exhibited bubbling and sloughing in the hour post-treatment.

greatly between the two groups. Only 10% of the MS-222 animals ate nothing, while nearly one-third (31.9%) of the control animals did not consume any food pellets. Among the unanesthetized animals, web clipping did not impact foraging ($P = 0.36$, $n = 149$, $\chi^2 = 0.82$, df = 1).

DISCUSSION

This study found unbuffered MS-222 dropped to pH 4–5, while the buffered MS-222 bath remained a neutral pH 7. This result should be noted by researchers preparing MS-222 in the field without buffer, since a pH under 4–5 can result in death in amphibians (Boutilier et al. 1992). Additionally herbicides become more toxic to amphibians (Edgington et al. 2003) and incidence of infection increases (Simon et al. 2002) with a decrease of pH. Figure 5 is a photo of one frog taken during the recovery hour after its treatment with *buffered* MS-222 and gentle handling with gloved hands. Even with gentle handling and a buffered, MS-222 bath, the visible white, cottony layer that formed on the frog's skin indicates trauma.

Development and Growth

Extrapolating the effects of sublethal, environmentally relevant concentrations of chemicals from published studies using high concentrations of chemicals can be difficult. Orlando and Guillette (2001) suggest that examining data from pollution-exposed populations in terms of central tendency (e.g., ANOVA) without studying the accompanying variance can result in Type II errors because the variance is indicative of the variation in individual responses to contaminant exposure in the population. This study did not find a statistically significant effect of MS-222 or an environmentally relevant concentration of nitrogen ammonia, either separately or in concert, on the growth and development of *Xenopus laevis* in the laboratory in terms of progression through the stages of development, weight, and length. However, Orlando and Guillette (2001) posit that an early indicator of disruption in a population may be increased phenotypic variance. Therefore, further exploration of the variance of these measures may be a prudent next step in exploring possible impacts of sub-lethal concentrations of nitrogen ammonia.

These results should remain in the context of this research design, i.e., a single dosage with great attention paid to each animal under anesthesia and immediate removal from the bath upon sedation. Dosage and length of exposure are two variables that figure predominantly in the stress response of

animals to MS-222 exposure, and the vast range of susceptibility to MS-222 that seems to vary by species and perhaps be modulated by repeated exposure (Zuccarelli & Ingermann 2005) should be noted. Researchers in the field should realize that, especially when working with some species with limited populations, they are likely working with individuals who have been studied by other researchers and may have previously been exposed to MS-222. This repeated exposure could result in variations in susceptibility to MS-222, so care and individual attention should be given to each animal during the sedation process to avoid possible over-sedation (i.e., death). Additionally, researchers should consider the potential changes in the stress response of animals that are repeatedly exposed to MS-222.

Foraging

There has been some debate about direct biochemical measures of stress. Welker et al. (2007) conclude that hyperglycemia may not be regulated by cortisol alone, although previous research, like Vethamany-Globus et al. (1977) used blood glucose levels to measure the stress response. Therefore, behavioral indicators may inform our interpretation of the research and assist in the development of appropriate measures. For example, some studies have used differences in eating patterns to differentiate between the level of stress and pain. Carr et al. (2002) summarize the research on the relationship between stress and eating patterns across a range of animals, with chronic or severe stress typically inducing anorexia, and relatively minor stress (tail-pinching in rats) causing overeating in response. In our study, therefore, we measured the number of food pellets animals ate in the recovery hour after treatment with MS-222 in order to measure the stress response to the exposure to MS-222. We found that the animals treated with MS-222 ate significantly more food pellets in the recovery hour after treatment than did the control group. A clear difference between the two groups existed in the exhibition of short-term anorexic behavior, with only 10% of the MS-222 exposed animals refusing food compared to food refusal by nearly one-third (31.9%) of the control animals, but this appeared to be a short-term behavior, since long-term anorexic behavior would have subsequently resulted in significantly smaller weight and length measures in the control group.

The lack of difference in foraging between the web-clipped and unclipped animals indicates that being web-clipped during handling was no more stressful than the handling alone. This is in line with the findings of recent research (e.g., Kinkead et al. 2006; Langkilde & Shine 2006). However, the greater length of web-clipped animals versus their controls indicates that web-clipping did impact the animals, although we noticed no difference in foraging and the web clips healed without incidence. Additionally, since there was no statistically significant difference in weights between the web-clipped animals and their controls, the increase in length without an increase in weight versus the control group may indicate a resulting difference in phenotype (i.e., a frog with a longer, thinner appearance) resulting from the web-clipping experience. This possibility is worth further investigation, as a change in phenotype may be an indicator that populations often subjected to research and clipping are experiencing a measurable impact from this research technique.

ACKNOWLEDGMENTS

The authors thank the Cryptobranchid Interest Group, whose grant helped to fund this research, and the Reptile and Amphibian Conservation Corps for its support. The comments of the editor and the anonymous reviewer on the manuscript were greatly appreciated by the authors. This work was done with IACUC approval (project E976).

LITERATURE CITED

Argent Chemical Laboratories. undated. Finquel MS-222 (instruction sheet). Argent Chemical Laboratories, Redmond.

Barbeau, T. R., & L. J. Guillette Jr. 2007. Altered endocrine parameters in female frogs (*Xenopus laevis*) exposed to environmentally relevant concentrations of nitrate. Journal of Herpetology 44:590–596.

Bartlett, H. L., R. B. Escalera II, S. S. Patel, E.

W. Wedemeyer, K. A. Volk, J. L. Lohr, & B. E. Reinking. 2010. Echocardiographic assessment of cardiac morphology and function in *Xenopus*. Comparative Medicine 60(2):107–113.

Bartlett, H. L., T. D. Scholz, F. S. Lamb, & D. L. Weeks. 2004. Characterization of embryonic cardiac pacemaker and atrioventricular conduction physiology in *Xenopus laevis* using noninvasive imaging. American Journal of Physiology Heart and Circulatory Physiology 286:H2035–H2041.

Belden, L. K., & J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp. trematode cercariae. Journal of Parasitology 91(3):686–688.

Bernstein, P. S., B. S. Fulton, & R. R. Rando. 1986. Mechanism of action of aromatic amines that short-circuit the visual cycle. Biochemistry 25: 3370–3377.

Boutilier, R. G., D. F Stiffler, & D. P Toews. 1992. Exchange of respiratory gases, ions, and water in amphibious and aquatic amphibians. Pp. 84 *in* M.E. Feder & W.W. Burggren, eds., Environmental Physiology of the Amphibians. University of Chicago Press, Chicago.

Byram, J. K., & M. A. Nickerson. 2009. The Use of Tricaine (MS-222) in Amphibian Conservation. Paper No. 1 in the Occasional Papers in Reptile and Amphibian Conservation Series. Reptile and Amphibian Conservation Corps, Gainesville.

Cakir, Y., & S. M. Strauch. 2005. Tricaine (MS-222) is a safe anesthetic compound compared to benzocaine and pentobarbital to induce anesthesia in leopard frogs (*Rana pipiens*). Pharmacological Reports 57:467–474.

Carr, J. A., C. L. Brown, R. Mansouri, & S. Venkatesan. 2002. Neuropeptides and amphibian prey-catching behavior. Comparative Biochemistry and Physiology Part B 132:151–162.

Crook, A. C., & H. H. Whiteman. 2006. An evaluation of MS-222 and benzocaine as anesthetics for metamorphic and paedomorphic tiger salamanders *Ambystoma tigrinum nebulosum*). American Midland Naturalist 155:417–421.

Edgington, A. N., G. R. Stephenson, P. M. Sheridan, D. G. Thompson, & H. J. Boermans. 2003. Effect of pH and Release® on two life stages of four anuran amphibians. Environmental Toxicology and Chemistry 22(11):2673–2678.

Fedewa, L. A., & A. Lindell. 2005. Inhibition of growth for select Gram-negative bacteria by tricaine methane sulfonate (MS-222). Journal of Herpetological Medicine and Surgery 15:13–17.

Food and Drug Administration (FDA). 1998. 21 CFR 529.2503 Tricaine methanesulfonate. www.absc.usgs.gov/research/vet/policies/21CFR530.9VL. Accessed June 2008.

Gentz, E. J. 2007. Medicine and surgery of amphibians. ILAR Journal 48(3):255–259.

Green, D. E. 2001. Anesthesia of amphibians in the field. USGS Standard Operating Procedure. ARMI SOP No. 104.

Hoffman, R., & S. Basinger. 1977. The effect of MS-222 on rhodopsin regeneration in the frog. Vision Research 17:335–336.

Jofre, M. B., M. L. Rosenshield, & W. H. Karasov. 2000. Effects of PCB 126 and ammonia, alone and in combination, on green frog (*Rana clamitans*) and leopard frog (*R. pipiens*) hatching success, development, and metamorphosis. Journal of the Iowa Academy of Science 107(3–4):113–122.

Johnson, P. T. J., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, & S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences 104(40):15781–15786.

Kinkead, K.E., J. D. Lanham, & R. R. Montanucci. 2006. Comparison of anesthesia and marking techniques on stress and behavioral responses in two *Desmognathus* salamanders. Journal of Herpetology 40: 323–328.

Langkilde, T., & R. Shine. 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. Journal of Experimental Biology 209:1035–1043.

Manteifel, Y. B. 2006. Tadpoles of the grass frog (*Rana temporaria*) and moor frog (*Rana arvalis*) avoid zones with elevated ammonia

concentration. Doklady Biological Sciences 411:458–460.

Nieuwkoop, P. D., & J. Faber. 1994. Normal Table of *Xenopus laevis* (Daudin). Garland Publishing, Inc., New York

Nyström, P., J. Hansson, J. Månsson, M. Sundstedt, C. Reslow, & A. Broström. 2007. A documented amphibian decline over 40 years: possible causes and implications for species recovery. Biological Conservation 138:399–411.

Orlando, E. F., & L. J. Guillette Jr. 2001. A re-examination of variation associated with environmentally stressed organisms. Human Reproduction Update 7(3):265–272.

Quinlan, E. L., & E. J. Phlips. 2007. Preliminary investigation of periphyton in the North Fork of the White River, Missouri. Unpublished report.

Relyea, R.A., & N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings of the National Academy of Sciences 98(5):2491–2496.

Simon, M. P., I. Vatnick, H. A. Hopey, K. Butler, C. Korver, C. Hilton, R. S. Weimann, & M. A. Brodkin. 2002. Effects of acid exposure on natural resistance and mortality of adult *Rana pipiens*. Journal of Herpetology 36(4):697–699.

Solis, M. E., J. M. Bandeff, & Y. W. Huang. 2007a. Hematology and serum chemistry of Ozark and eastern hellbenders (*Cryptobranchus alleganiensis*). Herpetologica 63:285–292.

Solis, M. E., C. C. Liu, P. Nam, D. K. Niyogi, J. M. Bandeff, & Y. W. Huang. 2007b. Occurrence of organic chemicals in two rivers inhabited by Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). Archives of Environmental Contamination and Toxicology 53:426–434.

Vethamany-Globus, S., M. Globus, & I. Fraser. 1977. Effects of tricaine methane sulfonate (MS-222) on blood-glucose levels in adult salamanders (*Diemictylus viridescens*). Experientia 33:1027.

Wayson, K. A., H. Downes, R. K. Lynn, & N. Gerber. 1976. Studies on the comparative pharmacology and selective toxicity of tricaine methanesulfonate: metabolism as a basis of the selective toxicity in poikilotherms. The Journal of Pharmacology and Experimental Therapeutics 198(3):695–708.

Welker, T., C. Lim, M. Aksoy, & P. Klesius. 2007. Effect of buffered and unbuffered tricaine methanesulfonate (MS-222) at different concentrations on the stress responses of channel catfish (*Ictalrus punctatus* Rafinesque). Journal of Applied Aquaculture 19(3):1–18.

Western Chemical, Inc. undated. Tricaine-S. http://www.wchemical.com/Assets/File/tricaineS_instructions.pdf. Accessed May 2012.

Zuccarelli, M. D., & R. L. Ingermann. 2005. Influence of neutralizing agents on the anaesthetic efficacy of tricaine on *Oncorhynchus mykiss* (Walbaum) fry. Aquaculture Research 36:933–935.