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Refuge Cover Decreases the Incidence of Bite Wounds in Laboratory South African Clawed Frogs (*Xenopus laevis*)

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The *Guide for the Care and Use of Laboratory Animals* recommends environmental enrichment for all laboratory animals, including amphibians. In this study, we evaluated the effect of adding environmental enrichment in the form of acrylonitrile–butadiene–styrene (ABS) pipes as covered refuge for laboratory *Xenopus laevis* housed in 2 pond-style tanks (capacity, 300 l; stocking density, approximately 150 frogs/tank; dimensions, 1.3 × 1.8 × 1.3 m). Medical records from animals housed in these 2 ponds between 1 January 2001 to 31 December 2003 revealed the incidence of bite wounds to be 5.0%, 4.0%, and 5.0% annually, respectively, and indicated 2 episodes of cannibalism (in 2003). In January 2004, we added ABS pipes as refuge housing to these tanks and continued to monitor the number of bite wounds and cannibalism. Over the following 24 mo (1 January 2004 to 1 January 2006), the incidence of bite wounds declined to 0.3% and 0.7% annually, respectively; no episodes of cannibalism were reported. The results of this investigation indicate that environmental enrichment in the form of ABS pipes for refuge cover has a quantifiable beneficial effect on the physical and social wellbeing of laboratory *Xenopus laevis*.

Abbreviations: ABS, acrylonitrile–butadiene–styrene

Captive populations of the fully aquatic amphibian species *Xenopus laevis* are maintained in laboratory animal facilities around the world to provide scientists with a steady supply of oocytes, eggs, and embryos for biomedical research. The *Guide for the Care and Use of Laboratory Animals*[13] recommends environmental enrichment for all laboratory animals, including amphibians, and providing laboratory *Xenopus laevis* with environmental enrichment is now common practice. Typically, this enrichment includes adding refuge covers such as clay pots, plastic or earthernware pipes, cups, hollow aquarium logs, aquarium rocks, or polypropylene basket 'caves' to *Xenopus laevis* tanks.[4,9] Data on the effects of such enrichment for captive *Xenopus laevis* is sparse. One study reported a wide unexplained variation in the response of a small number of *Xenopus* to refuge cover, and several of the frogs refused to come out of the cover to eat.[8] Another study reported that environmental enrichment added to *Xenopus laevis* tanks had no detrimental effect but was not associated with a statistically significant effect, either positive or negative, on egg quality or egg production.[4]

To our knowledge, no report has addressed the effect of enrichment on density-linked indicators of stress in captive *Xenopus laevis*: bite wounds and cannibalism. We tested the hypotheses that refuge cover added to 2 of our densely stocked *Xenopus laevis* tanks would result in a decline in the number bite wound injuries and episodes of cannibalism.

Materials and Methods

Animals, housing, and husbandry. *Xenopus laevis* studied in this report were all sexually mature, adult female frogs (age, 2 to 3 y; snout to vent length, 7.5 to 9 cm; weight, 70 to 85 g) purchased from a single vendor (Nasco, Madison, WI). All frogs were housed in the same room in 1 of 2 pond-style 'holding tanks,' tanks A and B. These 2 tanks were dedicated to housing newly arrived and unmanipulated frogs until the animals were needed by the research laboratory for egg harvesting, as approved by Stanford University's institutional animal care and use committee. Typically, after a 1-wk acclimation period, frogs were given a priming dose of pregnant mare serum gonadotropin (dose, 100 to 800 IU; Sigma-Aldrich, St Louis, MO) in the dorsal lymph sac, followed by an injection of human chorionic gonadotropin (dose, 50 to 800 IU; Sigma-Aldrich) to induce egg laying 24 to 48 h later. Researchers then collected the eggs and returned the frogs to 'resting tanks' C and D, pond-style tanks as described earlier but with compartmentalized sections, thus allowing the frogs to rest in smaller groups (5 to 20 frogs/section; 50 to 75 frogs/tank). Frogs were rested for 3 to 4 mo and then returned back to tanks A and B, where they remained for 1 wk to 6 mo before they were used again for egg harvest. Most of the frogs were kept for 1 to 2 y before the quality and quantity of eggs produced naturally declined with the frog's age. As long as a frog remains in good health, the number of times eggs may be harvested during that period was unlimited, but given the size of this researcher's *Xenopus laevis* colony, most of the frogs were used for egg laying only once or twice a year. Sick, debilitated, or aged frogs (usually frogs 4 to 5 y of age with a snout-to-vent length of approximately 11 cm or greater) with investigator-confirmed diminished egg production (for example, the frog continually fails to produce eggs after priming, or 20% or more of the eggs are degenerate) are euthanized by intracelomic injection of 0.5 ml buffered 10% tricaine methane sulfonate solution.

The pond-style tanks described in this report are self-flushing dark-green opaque tanks (Figure 1) that are approximately 1.3 m wide, 1.8 m long, and 1.3 m tall and filled to a 300-l capacity (water depth, approximately 0.6 m) with dechloraminated,

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Figure 1. Tanks A and B, 300-l capacity pond-style tanks, each housing approximately 150 frogs, the tank populations studied in this report.

Figure 2. Acrylonitrile–butadiene–styrene (ABS) refuge pipes added to tanks A and B, December 31, 2003.

potable water. The average daily census for tanks A and B was approximately 150 frogs/tank (approximately 2 l water per frog, as stated in the recommendations of the National Academy of Science),[12] with approximately 15 new frogs directly introduced into each tank each month and approximately 15 frogs removed each month for egg harvest. After egg harvest, the frogs were returned to a resting tank (tanks C and D), where they were housed for approximately 3 mo before being returned to tank A or B.

All frogs were fed a commercial chow (Frog Brittle, Nasco) at 1 g/frog according to the vendor's recommendations, 3 times weekly at 0700, 3 h prior to an automated water drain and refill which replaced 100% of the water volume. Frogs were observed by the caretakers during the feeding to ensure that no food was left uneaten. To confirm that the frogs ate to satiation, approximately 50 g of chow was thrown into each tank after the feeding frenzy; this allotment of chow generally was ignored. If not, frogs were fed again (1 g chow/frog) the following day. Tank water temperature in this room was maintained at 16 to 21 °C by an inline water heater (Edstrom Industries, Waterford, WI). The water comes from the municipal water supply and is filtered through an inline charcoal filter system (US Filters Westates, Oakland, CA) located in the frog housing room. Organic debris and uneaten food were removed daily with a small pool vacuum (Aquatic Eco-systems, Apopka, FL). Water quality was monitored approximately once every 6 to 8 mo (more frequently if a mechanical problem or disease outbreak occurred) and was tested with a commercially available water analysis kit (Voluette Analytical Standards, Hatch Company, Loveland, CO). Parameters tested and maintained within the ranges considered to be safe for aquatic amphibians included: pH, 7.0 to 8.5; average hardness, 15 to 30° dGH; total chlorine, less than 0.01 mg/l; chloramines, less than 0.01 mg/l; ammonia, less than 0.25 mg/l; nitrite, less than 0.20 mg/l; nitrate, 0.00 to 50.0 mg/l; copper, less than 0.02 g/l; water fecal coliform counts, less than 2000/100 ml; conductivity, 300 to 100 µOHM; and dissolved oxygen, 8.00 to 9.00 mg/l. The light cycle in the room is 12 h on, 12 h off. Ambient temperature in the room is 23 to 25 °C.

The frogs described in this report were observed daily by animal caretakers who have been trained in recognizing illnesses, bite wounds, cannibalism, and water quality or tank mechanical problems. When such problems were identified, caretakers reported to the veterinarian, who established a medical record for the care and treatment of the sick animals and contacted the facility manager regarding mechanical problems in the tanks.

Introduction of refuge cover as environmental enrichment and data collection. Because we had the strong clinical impression that tanks A and B had an ongoing history of morbidities related to density-associated bite wounds, and cannibalism, a plan for environmental enrichment was instituted. On 1 January 2004, 2 pipes (1 T-shaped and 1 Y-shaped; diameter, approximately 5 cm; Figure 2) made of a thermoplastic resin (acrylonitrile–butadiene–styrene [ABS]; Plastic Pipe Fittings Association, Glen Ellyn, IL) were introduced into each of these tanks. Introduction of these 2 large pipes into the middle section of the tank (far from the float valves and outflow drainage pipes at each end of the tanks) offered sufficient coverage for as many as 6 to 10 frogs (which hid inside and underneath the pipes). In addition, we chose pipes of this size, number, and type because they were heavy and thus not easily pushed around the tank by the frogs to become lodged beneath the float valve mechanism (as opposed to polyvinyl chloride pipes, which are lighter and easily moved by the frogs). Further, ABS conforms to the requirements of the American Society for Testing and Materials for plastic materials in contact with foodstuff and water intended for human consumption. The ABS pipe is black, opaque, and can be washed in the animal facility's automatic washer without melting when water temperature of the final rinse reaches approximately 88 °C for 20 min. No detergents or disinfectants are used.

After the introduction of the ABS pipes, animals were observed twice daily by trained husbandry staff, as described earlier. No other changes were made to the tanks. Frogs with bite wounds were identified and reported to the veterinarians, who entered the finding on the daily morbidity log and provided clinical care.

Review of the medical records and data collection. Medical records for the frogs housed in tanks A and B between 1 January 2001 through 31 December 2003 (during which no pipes were in the tanks) were reviewed and followed for the next 24 mo (1 January 2004 to 1 January 2006) after the ABS pipes were added. Information was gathered on the number of monthly cases with a confirmed diagnosis of bite wounds on the feet, legs, or axilla and of cannibalism. The incidence of bite wounds (for example, the number of new cases of bite injuries per month) for both tanks combined was determined and expressed as a percentage of the population (approximately 150 frogs/tank; thus a total of approximately 300 frogs in this study population).

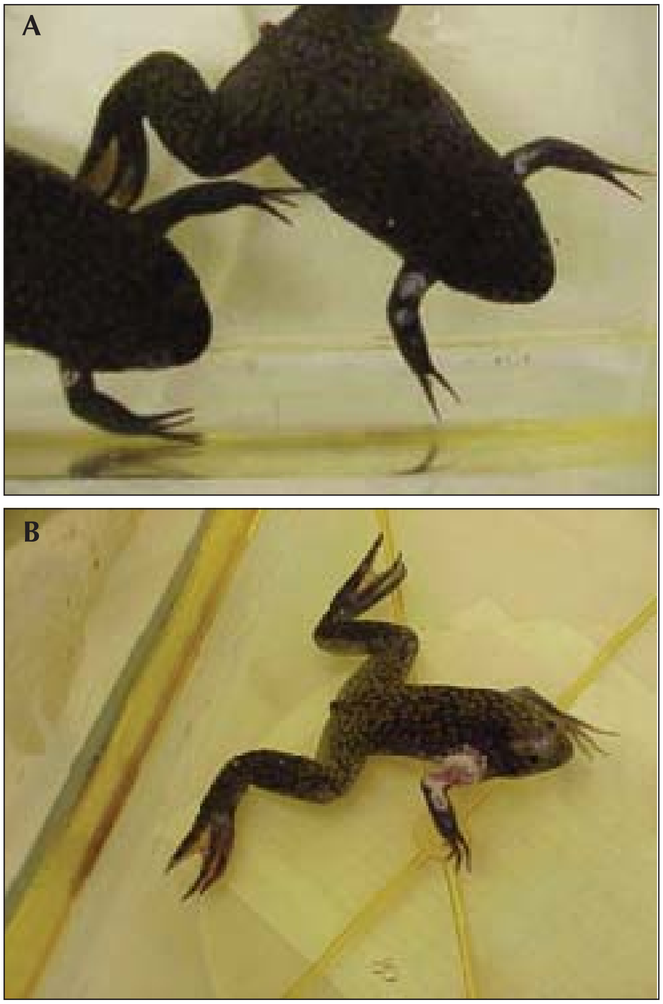

Figure 3. Bite wounds typical of the injuries reported from tanks A and B. A) Healed bite wounds on the axilla of 2 adult female South African Clawed frogs (*Xenopus laevis*) from tanks A and B. B). A bite wound on the arm of a small, adult female South African Clawed frog (*Xenopus laevis*) euthanized due to the severity of the lesion.

Results

Between 1 January 2001 and 31 December 2003, before the addition of the pipes, 41 frogs from the total population of approximately 300 frogs sustained bite wounds, and 9 (22%) of those 41 affected frogs were euthanized due to the severity of the injury (Figure 3 A and B); 4 of the euthanized frogs came from tank A and 5 from tank B. Frogs with minor bite wounds (for example, no signs of muscle or bone trauma, still able to swim and use the arm) were managed by placement in clear, polycarbonate cages (50 × 40 × 20 cm; 1 to 2 frogs per cage) filled with 1 to 2 l isotonic saline for 10 to 14 d until the wounds had healed. Frogs housed in polycarbonate cages were fed as described for pond-style housing, and the water changed daily, 2 h after feeding. Recovered frogs were returned to regular housing.

In 2003, 2 frogs (1 each from tanks A and B) were found dead with their hindlegs cannibalized. The incidence of bite wounds during 2001, 2002, and 2003 was 5.0%, 4.0% and 5.0%, respectively. After the pipes were added in 2004, the incidence decreased to 0.3%, and 0.7% during 2004, 2005, and 2006, respectively (Figure 4); there were no reports of cannibalism. During every observation period, frogs were noted to be hiding inside and beneath the pipes (Figure 5).

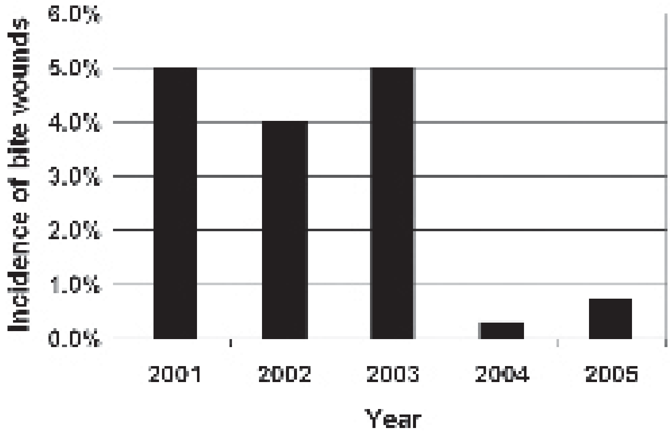

Figure 4. Histogram showing the incidence of bite wounds before and after the addition of ABS pipes.

Discussion

Given previously published review of the experimental data[6] suggesting that captive reptile and amphibian species do benefit from environmental enrichment, we sought to determine whether providing refuge cover to captive *Xenopus laevis* in our research animal facility would decrease the number of bite wounds and reports of cannibalism. Both of these parameters are readily quantifiable and therefore were measured to determine the effect of adding environmental enrichment in the form of refuge escapes to the frog tanks. Our data indicate a noteworthy decline in the incidence of bite wounds and no further reports of cannibalism after the addition of the ABS pipes to densely populated frog tanks. To our knowledge, this report is the first to document a clear benefit of refuge cover for laboratory *Xenopus laevis*: a decrease in bite wounds and cannibalism.

During the past decade, increased concern for the psychologic well-being of laboratory animals has led to the concept of environmental enrichment. Various approaches to enrichment strategies, including contact with conspecifics, interaction with other species, and enhancement of the physical characteristics of the captive environment have been applied to mammals but less consistently so to aquatic amphibians. This difference is due, in part, to the unique behavioral and physiologic response of amphibians to stress, and because amphibians are an evolutionarily distant species, in which stress-behavior is less familiar and therefore less recognizable to humans.[6] In addition, objectively quantifying captive amphibian stress by studying parameters used in mammalian stress studies (for example, plasma corticosteroid measurements and growth rates) can be difficult, because amphibian taxa often lack baseline data from wild populations for comparison. In addition, species-specific captive amphibian responses to stress are markedly different from captive mammals', and behavioral data from amphibian enrichment studies thus can be difficult to interpret. For example, plasma corticosteroid levels reflecting presumably low-stress conditions in a free-ranging ranid frog are much higher than the levels that mammals display.[6]

Healthy captive amphibians establish social hierarchies.[2,3,6] Studies addressing the establishment of social hierarchies specifically in captive *Xenopus laevis* are lacking, but space-restricted aquatic housing tanks into which new individuals constantly are introduced, removed, and then reintroduced are prime environments for continued efforts of the dominant conspecifics to establish ruling in the social hierarchy. In addition, bite wounds and cannibalism in captive *Xenopus laevis* may be the result of

Figure 5. ABS pipe with *Xenopus laevis* hiding inside and beneath.

their natural predatory behavior and the absence of refuge cover and an escape route for the subordinate animals.

We considered the possibility that inadequate food supply contributed to the occurrence of cannibalism in tanks A and B. However, cannibalism is a normal behavior in *Xenopus laevis*[5,18] and a common density-dependent regulatory mechanism in free-ranging populations.[15,17] Eggs, live tadpoles, and froglets are part of the normal diet of adult wild *Xenopus laevis*.[7,10,11] Cannibalism by larger conspecifics on smaller, debilitated, or subordinate conspecifics is well documented in captive amphibians, even in the presence of sufficient food.[1,14,17] Frog farmers raising *Rana* spp. with mixed class sizes were amongst the first to recognize the phenomenon.[16] To ensure that the frogs in tanks A and B were receiving sufficient food, they were observed daily when fed. After the feeding frenzy, an additional approximately 50 g of chow was added to each tank. This additional food went uneaten and was considered a sign of a sufficient food supply and satiation. As a result of our findings, we make efforts to segregate the occasional small frogs (for example, those that are 2 cm or more smaller than the rest of their shipment group) from the rest of the population.

The space needs for laboratory *Xenopus laevis* are unknown, but wild *Xenopus laevis* are rarely seen touching each other.[18] In their natural habitats (murky, still, small bodies of brackish water), this species escapes predators by hiding in mud and in or around natural elements that provide refuge cover (thick vegetation, logs, rocks, and so forth).[18] Space constraints on captive aquatic species housed in tanks may make these preferences impossible. Adding refuge cover to the frog tanks provides a means of escape for subordinate animals when they cannot otherwise put a physical distance between them and an aggressor.

As a result of our findings, we have added ABS to all pond-style tanks in the animal facility and make every attempt to house the smallest frogs in separate groups. Since we have instituted this policy, the yearly incidence of bite wounds has declined dramatically and there have been no reports of cannibalism.

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