Captive-rearing state endangered crawfish frogs *Lithobates areolatus* from Indiana, USA

Rochelle M. Stiles[1]*, Marcy J. Sieggreen[2], Rebecca A. Johnson[2], Kevin Pratt[2], Mark Vassallo[2], Michael Andrus[2], Maya Perry[2], Jonathan W. Swan[1] & Michael J. Lannoo[3]

[1] *Indiana State University, Terre Haute, Indiana 47809, USA*
[2] *National Amphibian Conservation Center, Detroit Zoological Society, Royal Oak, Michigan 48067, USA*
[3] *Indiana University School of Medicine–TH, Terre Haute, Indiana 47809, USA*

SUMMARY

Crawfish frogs *Lithobates areolatus* inhabit the tallgrass prairie of the southeastern Great Plains and Mississippi Delta, and have recently been considered for US federal listing under the Endangered Species Act. Here we attempt to determine the feasibility of head-starting crawfish frog tadpoles, and establish captive-rearing protocols. Captive-rearing produced more juveniles from fewer egg masses than a natural wetland in each year from 2013–2015, and survivorship of captive-reared tadpoles exceeded that of wild tadpoles. However, high rates of malformations, partial cannibalism, disease, and predation were seen among frogs in some years, and we therefore refined protocols to reduce these issues.

BACKGROUND

Crawfish frogs *Lithobates areolatus* are on the cusp of US federal listing under the Endangered Species Act. Grassland and wetland destruction, as well as disease, are major forces contributing to range-wide rarity (Kinney *et al.* 2011, Engbrecht *et al.* 2013, Terrell *et al.* 2014a). However, crawfish frogs have a high reproductive potential, and grassland conservation efforts are providing habitat not previously available (Redmer 2000, Williams *et al.* 2012, Terrell *et al.* 2014b). These frogs are obligate upland crawfish burrow dwellers, and may have historically relied on bison wallows for breeding wetlands (Heemeyer & Lannoo 2012, Heemeyer *et al.* 2012). Previous work suggests that they are most vulnerable during their larval stage (Kinney 2011). To expedite the re-colonisation of crawfish frogs to restored grasslands within their historic range, head-starting and releasing tadpoles has been suggested as a cost effective approach (Stiles *et al.* 2014).

In 2013–2015 we removed 1–2 egg masses from a sink population in a wetland at Hillenbrand Fish and Wildlife Area-West (HFWA-W; Greene County, Indiana; Terrell *et al.* 2014b). We transported these eggs to the Detroit Zoological Society (DZS; Royal Oak, Michigan), where we raised tadpoles to pre-metamorphic stages. We then transported these tadpoles back to HFWA-W and released them near their natal wetland. This wetland was surrounded by a drift fence/pitfall trap array, allowing us to determine post-release survivorship.

ACTION

Detroit Zoological Society Set-up: We reared crawfish frogs from hatching to late larval stages (Gosner Stages 31–39; Gosner 1960) at DZS in a biosecure space isolated from the resident amphibian population. We built two rooms in the basement of a mammal building: one for storage, isolation, and staff clothing changes; the second for housing egg masses and tadpoles. We purchased all new supplies and tools, changed into scrubs and rubber boots, and used quaternary disinfectant footbaths to eliminate cross-contamination with any amphibian building on the grounds.

In 2013 we raised tadpoles in 380 L tanks (Figure 1; Rubbermaid® Stock Tanks; 132 x 79 x 64 cm; Rubbermaid Commercial Products LLC, Winchester, Virginia). Four wooden tables (335 x 122 cm plywood) raised 18 tanks off the ground (two tables held four tanks, two held five). We connected tanks on the same table with a recirculating system. Water overflowed from each tank through sponge-covered "standpipes" (which maintained ~ 265 L of water depth ~ 50 cm) into one 150 L sump that housed thermostat-controlled chillers and heaters, mechanical, biological and chemical filtration; and a recirculation pump. Water returned through a drip line that was set above each tank.

On 5 April 2013, we transported two egg masses (laid on 31 March) from HFWA-W to DZS. We placed each egg mass in one tank in separate systems. As tadpoles hatched, we distributed them to other tanks in the same system, where densities reached 3–6 tadpoles/L. We cared for the tadpoles daily. In the morning, we visually checked the health of eggs or tadpoles, documented water (maintained at 21 °C) and room temperatures, and confirmed all systems were functioning. We also fed tadpoles a diet of powdered spirulina algae mixed with a vitamin calcium supplement, sinking earthworm and spirulina algal pellets, earthworm and spirulina algal flakes, turtle brittle, fresh romaine, and periodically, kale leaves. In the evenings, we repeated the morning routine as well as cleaned the tanks. We changed 150–265 L of water per system by adjusting the standpipes to drain and adding fresh chlorine-free water with a

Figure 1. Crawfish frog *Lithobates areolatus* captive-rearing set up at Detroit Zoological Society in 2013.

* To whom correspondence should be addressed: rochelle.m.stiles@gmail.com

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Figure 2. Improved crawfish frog *Lithobates areolatus* captive-rearing set-up at Detroit Zoological Society in 2014–2015.

hose. We routinely checked ammonia, nitrite and nitrate levels on each system. To monitor growth, we measured total length and photographed one randomly selected tadpole once weekly.

In 2014–2015 we replaced the previous set-up with custom-designed shallow pools (Figure 2; ~ 190 L; 56 x 168 x 25 cm, filled to 20 cm) to increase surface and living area. We built elevated tables on top of each existing table and created four separated pools (using 3 x 25 cm wooden frames, 44 mm Firestone PondGard™ EPDM pond liners [Firestone Building Products, Indianapolis, Indiana], and 3 cm wooden slats to fastened down liners) on each tabletop. An additional upper shelf (90 cm above the lower level) created four more pools, for a total of 32 pools on the original four tables from 2013. We constructed pools with a slight slant to facilitate drainage to a bulkhead (2 cm diameter) connected to a sponge-covered standpipe (18 cm poly-vinyl-chloride [PVC] pipe glued to a threaded adapter), which drained into a 150 L sump below the tables. Upper- and lower-level pools were isolated from one another. Using PVC pipes, we created a "rain system" above each pool to automatically release fresh water eight times per day for 15 min intervals. We changed water daily using a syphon hose. A 2,275 L self-regulating reservoir system of carbon-filtered water provided an ample supply of fresh water for both rain and water changes. We regulated water temperature using a recirculation pump and chiller.

On 15 April 2014 we transported one egg mass (laid on 7 April) from HFWA-W to one 380 L tank (from 2013) filled to ~ 190 L and regulated by a temperature-controlled, recirculating system. We changed 30% of the water daily and maintained the temperature at 16 °C. As tadpoles hatched and grew, we increased the temperature to 20–21 °C. After 4 weeks, we moved tadpoles to 11 pools (density of ~ 2 tadpoles/L). We used the same morning and evening routines as 2013 to care for tadpoles. Additionally, we placed a 50 W incandescent spotlight at one end of each pool to offer a slightly warm, brightly lit area. These spotlights stayed on 12 hrs/day and increased local water temperatures to 24 °C.

On 27 March 2015 we transported three half egg masses from three different females (laid on 23 March). We used the same 380 L tank hatching system and 24 pools from 2014. After 4 weeks we began moving tadpoles gradually as follows: we moved one tadpole to each pool on day 1. If the previously-moved tadpoles still appeared healthy, we moved 20 tadpoles to each pool on day 2 and 100 tadpoles on day 3. Densities were < 0.6 tadpole/L. We increased the spotlights to 75 W, since 50 W bulbs only slightly increased the water temperature in 2014. We also increased the rain intervals from eight 15 min periods to eight 30 min periods per day. On 11 June we observed one tadpole with front limb development. We therefore converted the hatching tank into an enclosure for metamorphosing frogs. By the time of transport, 116 frogs had metamorphosed.

Transport: Before hind limbs fully developed, we transported tadpoles from DZS back to HFWA-W on 25 June 2013, after 81 days of development. We loaded tadpoles into 76 L plastic totes (Rubbermaid®), with two-thirds air and one-third water (~ 25 L; ≤ 7 tadpoles/L), lined with plastic bags (4 mm thick, 38 x 38 x 56 cm; Pentair Aquatic Ecosystems) and closed with rubber bands. We transported totes with a cargo van; the trip was nearly 400 miles and lasted approximately 6 hours. The following years, on 9 July 2014 (85 days) and 22 June 2015 (85 days), we transported tadpoles with the same totes. In 2015 we also transported recently metamorphosed frogs in portable plastic terrariums with enough water to keep animals moist.

Hillenbrand Fish and Wildlife Area-West: We transferred tadpoles to enclosures upon arrival to HFWA-W, since the most reliable method for permanently marking frogs is toe-clipping after metamorphosis (Dodd 2010). In 2013 tadpoles completed development in a mesh enclosure made from three holding boxes (122 x 122 x 366 cm, Ace Knotless Holding Box, stock number NHB1-12, Memphis Net and Twine, Memphis, Tennessee) sewn end to end, protected by chicken wire and wire mesh screening, and secured by wooden poles (5 x 5 x 183 cm)

Figure 3. Wetland enclosure where captive-reared crawfish frog *Lithobates areolatus* tadpoles completed development in 2013.

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Table 1. A comparison of crawfish frogs *Lithobates areolatus* captive-reared at Detroit Zoological Society (Royal Oak, Michigan) with conspecifics that developed in a natural wetland at Hillenbrand Fish and Wildlife Area-West (Greene County, Indiana) in 2013–2015. We calculated the number of wild egg masses from the number of spent females that crossed our drift fence and pitfall traps encircling the natural wetland (Terrell *et al.* 2014b). We estimated the number of wild hatchlings based on adult females' length, the regression established by Redmer (2000) correlating female size and eggs per mass, and an estimated hatchling rate of 98% (Kinney 2011).

	2013		2014		2015	
	Wild	**Captive-reared**	**Wild**	**Captive-reared**	**Wild**	**Captive-reared**
Estimated count						
Egg masses	52	2	37	1	22	1
Hatchlings	215,807	8,774	191,376	4,971	120,727	2,449
Tadpoles transported	-	3,051	-	4,951	-	2,409
Juveniles	8	519	844	2,999	0	1,688
Average juvenile size						
Snout-vent length (mm)	33	29	30	30	-	29
Mass (g)	2.9	2.2	2.5	3.1	-	3.3
Survivorship						
Hatching–transport	-	34.8%	-	99.6%	-	98.4%
Transport–metamorphosis	-	17.0%	-	60.6%	-	70.1%
Hatching–metamorphosis	< 0.1%	5.9%	0.4%	60.3%	0%	68.9%
Malformations						
Count	0	359	1	1,123	-	463
Rate	0%	69.2%	0.1%	37.6%	-	27.4%
Estimated cost						
Total	$0	$16,000	$0	$25,000	$0	$12,000
Cost/juvenile	$0	$31	$0	$8	-	$7

and insulation supports (61-cm, Item #12324; Southeastern Wire Fabricators Inc., Hemingway, South Carolina; Figure 3). We attached mesh wings (0.3 cm thick, 61 cm wide, Ace Knotless Netting mesh, stock number 1001-2, Memphis Net and Twine) to the enclosure to guide metamorphosed frogs as they exited and emerged onto land, where we captured them using a drift fence and pitfall trap array (see Kinney 2011). In response to shrew predation, we augmented our drift fence array with mammal-proof wire mesh screening (5 mm diameter) and transferred the remaining tadpoles to 380 L aquatic mesh cages (Reptariums®, Apogee, Dallas, Texas; Figure 4) positioned in the shallows of the wetland.

In 2014–2015 tadpoles completed development in 380 L Reptariums® and 285 L custom mesh cages (Figure 4). These custom cages were a cost-effective alternative, made from mesh fabric (3 mm nylon hex-mesh, Model #F03A-NY SP-HEXM-M003-ZS, AH&H Specialized Textile Outfitters, Sealy, Texas) and PVC pipes and elbows (1.3 cm diameter, 600 PSI Schedule 40 PVC Pressure Pipe, Item #23966; 1.3 cm diameter, 90-degree Schedule 40 side outlet elbow, Item #315498; Lowe's, Mooresville, North Carolina). We decreased the density of tadpoles from 0.33 tadpole/L in 2014 to 0.25 tadpole/L in 2015. We fed tadpoles frozen chopped spinach as needed in 2014 and switched to spirulina algal pellets ("W.T.A. Select" spirulina wafers, Wet Thumb Aquatics; algae wafers, Hikari Tropical, Himeji, Japan) in 2015.

After metamorphosis, we measured snout-vent length, weighed, and marked (toe-clipped) juveniles, documented malformations, and swabbed a subset for disease. We then released these juveniles near their natal wetland. We also monitored the metamorphosis of naturally-reared tadpoles using a drift fence paired with pitfall traps encircling a wetland at HFWA-W with the highest natural recruitment (Nate's Pond; Terrell *et al.* 2014b). To calculate survivorship data for these wild tadpoles, we first estimated the number of deposited egg masses from the number of spent females that exited the wetland. Next, we estimated the number of eggs per mass using snout-vent length of each female and a regression established by Redmer (2000). This regression correlates an adult crawfish frog female's length with the number of eggs in an egg mass laid by that female. Lastly, we estimated 98% of eggs hatched based on previous work (Kinney 2011). Here, we present data on wild juveniles for comparison with captive-reared frogs in 2013–2015.

CONSEQUENCES

Captive-rearing produced more juveniles from fewer egg masses than the natural wetland at HFWA-W (Table 1; Nate's Pond; Terrell *et al.* 2014b). Wild and captive-reared juveniles were similar in size across all years; however, malformations were more common among captive animals.

Figure 4. Aquatic mesh cages where captive-reared crawfish frog *Lithobates areolatus* tadpoles completed development in 2014–2015. A 380 L Reptarium® is on the left, a 285 L custom cage on the right.

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Table 2. Malformations of metamorphosed crawfish frogs *Lithobates areolatus* captive-reared at Detroit Zoological Society (Royal Oak, Michigan) in 2013–2015. Reduced/absent limbs refer to brachydactyly, ectrodactyly, or ectromelia, likely caused by partial cannibalism. Joint immobility most often refers to the tibiofibular and femur joint (knee); however, in severe cases, the hip joint was disarticulated (see radiographs in Lannoo 2008). Malformation types follow Meteyer (2000) and Lannoo (2008).

Malformation	2013	2014	2015	Total
Scoliosis	0	613 (54.3%)	59 (12.7%)	672 (34.5%)
Reduced/absent limbs	345 (96.1%)	180 (16.0%)	61 (13.2%)	586 (30.1%)
Joint hypomobility	0	200 (17.7%)	325 (70.2%)	525 (26.9%)
Scoliosis & reduced/absent limbs	7 (1.9%)	52 (4.6%)	4 (0.9%)	63 (3.2%)
Joint hypomobility & reduced/absent limbs	2 (0.6%)	38 (3.4%)	5 (1.1%)	45 (2.3%)
Scoliosis & joint hypomobility	0	37 (3.3%)	8 (1.7%)	45 (2.3%)
Scoliosis, joint hypomobility, & reduced/absent limbs	0	8 (0.7%)	0	8 (0.4%)
Gill slit exposed & reduced/absent limbs	2 (0.6%)	0	0	2 (0.1%)
Gill slit exposed	1 (0.3%)	0	0	1 (< 0.1%)
Brachygnathia	1 (0.3%)	0	0	1 (< 0.1%)
Microcephaly & reduced/absent limbs	1 (0.3%)	0	0	1 (< 0.1%)
Syndactyly	0	0	1 (0.2%)	1 (< 0.1%)
Total	359	1,128	463	1,950

Survivorship: Survival rates of captive-reared tadpoles exceeded those of wild tadpoles. Overall survivorship (hatching to metamorphosis) for captive tadpoles was 1,600 times greater (5.9%:0.004%) in 2013 and 140 times greater (61.8%:0.4%) in 2014 than the survivorship for wild tadpoles. In 2015, the natural wetland produced no juveniles from 22 egg masses, while 1,688 (70.1%) juveniles were reared from collectively one-and-a-half (i.e., three half masses from three females) egg masses in captivity.

Malformations: Malformation rates were high initially (69.2% in 2013), but by releasing younger tadpoles (prior to Gosner Stages 31–39; Gosner 1960), we reduced malformation rates (27.4% in 2015). Across years, most malformed, captive-reared juveniles had scoliosis (34.5%), reduced/absent limbs (i.e., brachydactyly, ectrodactyly, or ectromelia; 30.1%), or joint immobility (26.9%; Table 2). We also observed exposed gill slits, brachygnathia (shortened lower jaw), microcephaly (shortened upper jaw), syndactyly (fusion of digits), and a combination of these malformations. In particular, early release led to a reduction in malformations related to partial cannibalism (e.g., brachydactyly, ectrodactyly, or ectromelia) from 96.1% of all malformed juveniles in 2013 to 13.2% in 2015 (Table 2).

Disease: Crowding from captive-rearing increases the potential for disease. In 2014, after their release, tadpoles acquired a ranavirus infection that reduced their survivorship to 60.6%, as follows. Ten days after transport (tadpoles in captivity showed no clinical signs of infection), tadpoles from a range of developmental stages became lethargic and began dying. Deaths were associated with abdominal and limb edema, hemorrhaging around mouths and limbs, and skin ulcers. Ranavirus was confirmed visually (histological sections) and with Taqman real-time PCR (by the Wildlife Disease Laboratories at the Institute for Conservation Research, San Diego Zoo Global).

Predation: Increased juvenile densities also increase predation risk. In 2013, as juveniles exited the enclosure and approached the drift fence, northern short-tailed shrews *Blarina brevicauda* preyed on the crawfish frogs. Shrew predation peaked from 8–10 July with 85 deaths. Predation decreased following our drift fence modifications (addition of mammal-proof wire mesh screening) and transport of tadpoles to smaller mesh cages.

DISCUSSION

Prior to the present work, crawfish frogs have never been raised in captivity. While ranid tadpoles are relatively easy to raise (Wind 2002), crawfish frogs present a challenge due to their tendency towards hindlimb cannibalism, a characteristic of species inhabiting seasonal wetlands (Hoff *et al.* 1999). We were able to decrease cannibalism and increase survivorship over the span of our three year project due to several alterations. First, we built custom, shallow pools to raise tadpoles in 2014–2015. These basins were more effective and required less labour to maintain than the deep 380 L tanks used initially in 2013. Their shallow depth aided us in monitoring tadpole health, feeding appropriate amounts, and allowed us to safely siphon dirty water, waste, and leftover food. Additionally, we suggest releasing tadpoles directly into wetlands rather than into mesh cages. Cages concentrated tadpoles and likely contributed to the 2014 ranavirus die-off.

We have learned enough to recommend the captive-rearing techniques described here to organizations wishing to increase the number of crawfish frog populations in regions across their historic range. We do not advise using captive-rearing of tadpoles to augment existing populations. Rather we feel that captive-rearing is the most biologically realistic and cost-effective method to establish new populations. Further, since natural tadpole mortality is so high, there is almost no cost to host populations. Captive-rearing can be combined with ecological factors (e.g. removing tadpole predators and competitors, increasing upland burrow availability for juveniles using artificial means) to promote success of these programmes.

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