

Linking in-situ and ex-situ populations of threatened amphibians through genome banking

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Abstract

Genetic variation of the endangered Puerto Rican crested toad (PRCT; *Peltophryne lemur*) has dwindled over time in both wild and captive populations, leading to long-term sustainability issues for the recovery program. To address this challenge, we propose that PRCTs can be used as a model species to show how in-situ and ex-situ populations can be linked through sperm biobanking and gamete transfer, expanding genetic variability of both populations. Male toads ($n = 10$) in Guayanilla, Puerto Rico were administered human chorionic-gonadotropin (hCG) and luteinizing-hormone-releasing-hormone analog (GnRHa) to stimulate spermiation. Sperm was collected noninvasively from 9/10 males, cryopreserved in 10% trehalose with either 10% N,N-dimethylformamide (DMFA) or 10% dimethyl sulfoxide (DMSO), and transported in liquid nitrogen vapor to the National Amphibian Genome Resource Bank at Mississippi State University. Ultrasonography was used to identify females ($n = 3$) with mature oocytes for GnRHa-induced oviposition of eggs for in-vitro fertilization (IVF). Post-thaw sperm motility was 28% and 25% for sperm cryopreserved with DMFA or DMSO, respectively. Of the 9,672 eggs used for IVF, 4% ($n = 306/6,981$) were fertilized with frozen-thawed sperm, compared with 20% ($n = 525/2691$) fertilized with fresh sperm controls. Overall, 46 toadlets were produced from frozen-thawed sperm. After 4.5 months of headstarting, 14 juvenile toads produced from various genetic crosses using frozen-thawed sperm were released to new sites in the wild, introducing unique genetic representation and new founder lines. After 1.5 years, 24 adult toads produced using frozen-thawed sperm continue to thrive in the captive collection, and one of these males has now produced an F2 generation of offspring with 5,085 tadpoles released to the wild. This transformational study is the first to produce reproductively viable adult PRCTs using cryopreserved sperm from hormonally-induced wild males and captive females, introducing innovative methods that link in-situ and ex-situ populations of endangered amphibians to revolutionize genetic management.

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KEY WORDS

amphibian, assisted reproductive technologies, biobanking, cryopreservation, exogenous hormones, GnRH, hCG, in-vitro fertilization

1 | INTRODUCTION

The Puerto Rican crested toad (*Peltophryne lemur*; PRCT) is a critically endangered species native to the northern and southern coasts of Puerto Rico. While once abundant, only two viable populations on the southern coast remain due to population decline from habitat loss, extreme weather events, invasive species, and disease, with the northern population believed to be completely extirpated (Barber, 2008; Barber, personal communication, 2020). Consequently, genetic variation is waning rapidly, jeopardizing the possibility of a self-sustaining wild population under current conditions (Beauclerc, Johnson, & White, 2010). In order to reverse this trend, zoological institutions throughout the United States have collaborated under the Puerto Rican crested toad Species Survival Plan (SSP) to reintroduce tadpoles and toadlets back into their native ranges (Barber, 2017; USFWS, 2012), with more than 556,000 having been released as of 2020 (Barber, personal communication). This reintroduction program relies on captive breeding institutions to annually produce animals, and although wild population numbers are increasing, they have not yet become self-sustaining or grown to the point of delisting the species. Unfortunately, the genetic variation and sustainability of the captive population is also dwindling, meaning zoological institutions are releasing less gene diversity into reintroduced/repatriated populations.

PRCTs are distributed across 30 Association of Zoos and Aquariums (AZA) accredited institutions, housing 1,302 individuals descended from 48 founders. As of 2018, the entire captive population is maintained at a level of 96.98% genetic diversity (Table 1). The SSP predicts this level of genetic diversity will hold under current rates of population growth and continued import and release of individuals. However, the SSP also shows that only 16 of the 48 founders are actively contributing to the captive population's gene pool, with disproportionate gene representation in offspring (Table 1). Hence, while population growth may be high, the gene diversity is waning and unsustainable long-term. The SSP imports 10–20 founders every 4–8 years, with projections indicating that a minimum of 2 individuals imported every 4 years will maintain genetic diversity at 90% (Table 1). However, with one wild population fluctuating drastically per breeding event and the other population estimate being unknown, this current level of import may not be sustainable in the long term. If in-situ and ex-situ populations are linked via biobanking, high levels of genetic diversity are still achievable without animal removal.

While maintaining assurance colonies has become necessary for many threatened and endangered species, many complications arise threatening captive gene diversity. Reductions in population size, loss of fitness, genetic adaptation to captive settings, inbreeding depression, and

TABLE 1 Genetic summary table of current Puerto Rican crested toad captive population for calendar year (CY) 2018 (Puerto Rican Crested Toad [*Peltophryne lemur*] AZA Species Survival Plan Green Program, 2018)

Puerto Rican crested toad population genetic summary	CY 2018 values	Strategies for maintaining current levels of genetic diversity
Founders	48	Strategy 1: Remove Animals • Low removal (n=2–10 adults) • Low pairings in captivity, limited lifespan
Founder genome equivalents (FGE)	16.54	Strategy 2: Remove Tadpoles • Medium removal (10% egg clutch=1,000) • Many pairings in captivity, limited lifespan
Gene diversity (GD %)	96.98	Strategy 3: Remove ♂ Gametes • High removal (10 × 10 ⁶ sperm/male) • Thousands of crosses, long-term storage
Population mean kinship (MK)	0.0302	
Mean inbreeding (F)	0.0270	
Effective population size (Ne/N)	0.0857	
Percentage of known pedigree	95.8	
Years to 90% gene diversity	144	
Years to 10% loss of gene diversity	174	
Gene diversity at 100 years	94.5	
Gene diversity at 10 generations	94.9	

reduction of genetic variation all result from housing endangered species over many generations, threatening success of future reintroduction efforts (Frankham, 2008; Frankham, Ballou, Briscoe, & McInnes, 2010; Howell et al., 2020; Robert, 2009). To offset these challenges, relocating wild caught individuals into captivity has been a means to supplement dwindling founder stock in captive colonies (Beauclerc et al., 2010; Gagliardo et al., 2008; Swanson, Magarey, & Herrick, 2007). However, the removal of threatened individuals decreases natural breeding potential within the wild population, which is already at an unsustainable level. Furthermore, limited space and resources in zoological institutions dictates the number of individuals, as well as the number of species, that can be housed (Bishop et al., 2012; Howell et al., 2020). If gametes from live wild adult PRCTs could be removed, banked, and incorporated into the captive population through mixed wild: captive pairings, a new method of genetic management for threatened and endangered amphibian species could be developed, with the PRCTs serving as a model. Thus, linking in-situ and ex-situ populations through biobanking could be paramount to maintaining both high levels of genetic diversity along with sustainable population sizes in both groups (Howell et al., 2020; Ryder, 1995; Swanson et al., 2007; Wildt & Roth, 1997).

Amphibian Reproductive Technologies (ARTs) are typically used to intervene when animals fail to naturally reproduce and include procedures such as hormone therapy, in-vitro fertilization (IVF), and gamete cryopreservation (Clulow, Trudeau, & Kouba, 2014; Kouba, delBarco-Trillo, Vance, Milam, & Carr, ; Kouba & Vance, 2009). Historically, the primary means of sperm collection from male amphibians was through euthanasia and removal of the testes to create a macerate solution, which could be spread across eggs for IVF (Browne, Clulow, Mahony, & Clark, 1998; Shishova, Uteshev, Kaurova, Browne, & Gakhova, 2011; Upton, Clulow, Mahony, & Clulow, 2018). However, sperm collection from sacrificed animals is counterproductive to the maintenance of founder lines of endangered species. Another more sustainable method of sperm collection uses exogenous hormones to stimulate natural recruitment and release of spermatozoa (Kouba & Vance, 2009; Kouba, Vance, & Willis, 2009). Thus, sperm can be collected from live individuals following hormone therapy and used in IVF to produce new offspring or stored long-term.

Genetic resource banks store cryopreserved sperm for an indefinite period of time. Frozen sperm can then be used in reproductive efforts to introduce old founder lines, new genetic variation, and build population numbers (Browne & Figiel Jr., 2011; Howell et al., 2020; Kouba et al., 2013). Thus far, cryopreservation has been used mainly in laboratory settings to store gametes of captive individuals held in assurance colonies (Browne

et al., 1998; Poo & Hinkson, 2019; Shishova et al., 2011). However, cryopreservation protocols are now being developed to collect and freeze sperm from wild amphibians and incorporate the sperm through IVF into the captive collection for increased genetic variation instead of removing reproductively viable individuals. Unfortunately, cryopreserving sperm in the field comes with a new array of challenges in managing equipment, personnel, and regulatory compliance. Here we overcome these challenges and highlight how ART and cryopreservation technologies can establish genetic linkages between in-situ and ex-situ amphibian populations to produce new individuals through diverse genetic crosses.

The purpose of this study was to develop a protocol for linking in-situ and ex-situ populations of the Puerto Rican crested toad by biobanking exogenously induced sperm from wild males and conducting IVF, rather than live animal incorporation. The Conservation Physiology Lab at Mississippi State University partnered with Fort Worth Zoo (FWZ), the Puerto Rican Department of Natural and Environmental Resources (PRDNER), and the US Fish and Wildlife Service (USFWS) to induce, collect, and evaluate sperm from wild PRCT males; cryopreserve and transfer that sperm to the National Amphibian Genome Resource Bank for long-term storage; and create unique genetic pairings by hormonally-inducing captive females and conducting IVF using the biobanked sperm of wild males.

2 | METHODS

2.1 | Animals

Male PRCTs were collected in June 2018 from Guayanilla, Puerto Rico, which represents a newly discovered site and a source of underrepresented genes for the captive population, as only a few animals have been imported from this location in 2012 and 2014. Males were located at night and collected from the surface of karst formations or coerced out by simulating rainfall with misters (Figure 1). Once collected, males were held in soil-filled terrariums for no longer than 24 hr. Prior to hormone administration, male toads were transferred to individual plastic tubs with 1–2 cm water for the duration of sperm collection to encourage urine production. Animal collection was authorized through the FWZ-USFWS permit #TE121400-7 and covered under IACUC #17-H001 at FWZ.

Female PRCTs at FWZ were housed ($n = 6$) in 15-gal tanks equipped with a mesh-wrapped eggcrate, raised false bottom, and a 25 × 15 cm hiding cover. Tanks were inclined to allow for a ~2 cm pool of reconstituted reverse osmosis (R-R/O) water, creating a 1:4



FIGURE 1 Karst, or rock, formations are where Puerto Rican crested toads are found in the wild. Toads were either collected from the top of rocks or encouraged out by simulating rainfall with misters. Picture taken by Carlos Pacheco

water to land ratio. Adult toads were kept on a 12-hr light cycle year-round, with a temperature gradient of 23.3–27.8°C and basking areas at ~32.2°C. Adults were fed gut-loaded and vitamin dusted crickets 3× weekly and a pinkie-mouse monthly.

2.2 | Hormone administration and ultrasound analysis

Field caught males ($n = 10$) were administered hormones as a cocktail of 10 IU/g body weight (BW) of human chorionic gonadotropin (hCG) + 0.4 µg/g BW of luteinizing hormone releasing hormone analog (GnRHa) intraperitoneally ($n = 7$), or as a single 10 µg dose of GnRHa given nasally ($n = 3$) as described by Julien et al. (2019). Similarly, fresh sperm was also collected from males ($n = 3$) located at FWZ as a control for egg quality when conducting IVF with frozen sperm.

Ultrasonography was used to assess gravidity of female toads ($n = 3$) located at FWZ for hormone-induced ovulation and spawning. Ovarian development stages for PRCTs are shown in Figure 2. Only females with mature oocytes at an ultrasound grade 3 were administered the 0.4 µg/g BW GnRHa in order to induce

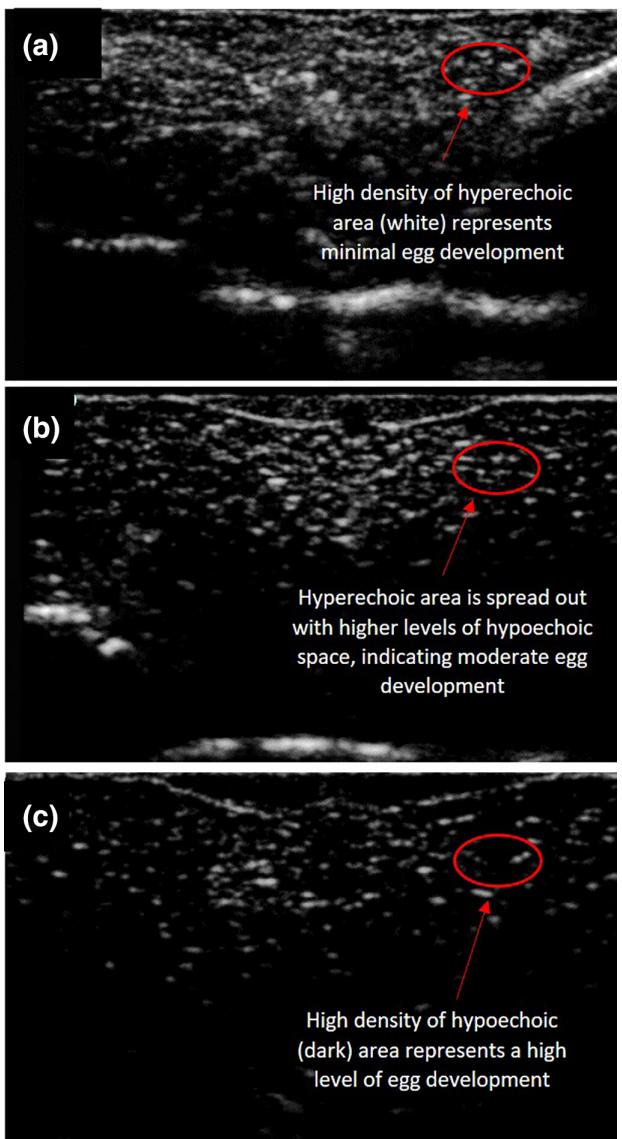


FIGURE 2 Representative ultrasound images of grades 1–3 of Puerto Rican crested toads. Fluid (or egg jelly) is indicated by hypoechoic areas and eggs by hyperechoic (white) areas. (a) Low levels of hypoechoic area shows that this female has minimal egg development; (b) mid-levels of hypoechoic area shows that this female has moderate egg development and could be administered a priming hormone dose prior to an ovulatory dose and IVF; (c) high levels of hypoechoic area shows that this female has fully developed eggs and can be administered an ovulatory dose for an IVF

ovulation and egg laying (Bronson & Vance, 2019; Graham, Langhorne, Vance, Willard, & Kouba, 2018).

2.3 | Sperm collection, analysis and cryopreservation

Spermic urine samples were collected from male toads at 1, 2, 3, 5, and 7 hr post-hormone administration by

holding the individual over a petri dish until urination occurred. Samples were immediately analyzed for sperm motility, quality, and concentration with an Olympus CX43 phase contrast microscope. Sperm motility was categorized as forward progressive motile (FPM, flagellar induced forward motion); nonprogressive motile (NPM, undulating flagellum without forward propulsion); and nonmotile (NM). Total motility was calculated as the sum of FPM and NPM sperm. Concentration was determined using a hemocytometer (Hausser Scientific #3200). Sperm was then stored in an ice slurry (~0 to 4°C) until all samples were collected and ready for the cryopreservation process or used as fresh controls on site at FWZ.

After pre-freeze analysis, spermic urine was reanalyzed, pooled (if volume for one sample was <400 µL), separated into 100 µL aliquots, and mixed in a 1:1 ratio with 20% trehalose stock solution and either 20% N,N-dimethylformamide (DMFA) or 20% dimethyl sulfoxide (DMSO) for a final concentration of 10%. Once prepared, the 200 µL cryo-suspension was loaded into 0.25 mL freezing straws (Minitube International, Germany) and placed on a freezing rack situated 10 cm above a 5 cm depth of liquid nitrogen (LN₂) in a styrofoam freezing box (21 cm L × 16.5 cm W × 20.5 cm H). Straws were equilibrated in LN₂ vapor for 10 min with an initial freezing rate of -37.64°C/min, then plunged into LN₂, and transferred to a CX100 Vapor Cryogenic Shipper (Worthington Industries, AL). Cryopreserved sperm was transferred by air courier to the National Amphibian Genome Resource Bank at Mississippi State University, prior to the subsequent IVF trials at FWZ 1 year later.

2.4 | In-vitro fertilization

Of the 3 females chosen for IVF at FWZ, 1 female spontaneously laid overnight after hormone treatment, and the eggs could not be used. At approximately 12–24 hr post-hormone administration, females were gently squeezed to encourage egg expression as described in Bronson and Vance (2019). Once the females started spawning, approximately 100–200 eggs were expressed into separate petri dishes. Random straws from specific males were removed from the dry shipper and thawed by submerging in a 40°C water bath for 5 s. Once thawed, sperm were combined in a 1:10 ratio with distilled reactivation water (e.g., 10 µL cryo-suspension to 90 µL sterile embryo transfer water; Sigma #W1503, Kouba, Vance, Frommeyer, & Roth, 2003). Sperm was immediately analyzed for post-thaw motility and quality and placed on the eggs for IVF.

After 5 min of dry fertilization, the petri dishes were flooded with water and set aside. Embryos were monitored under a dissecting microscope at Gosner stages 3 (cleavage), 13 (neurula), 20 (hatched), and 46 (complete metamorphosis).

Once tadpoles reached Gosner stage 20, undeveloped eggs were removed, and tadpoles were relocated to 56-1 tanks with submersible plastic plants and kept separate according to genetic pairing structures. Water was continuously filtered and oxygenated by a raised 5 cm standpipe fed by a recirculating system. Water changes were done 3 times daily until all individuals fully metamorphosed. Water tank levels were decreased to approximately 8 cm once larvae developed front legs to prevent drowning and encourage metamorphosis transition to dry land. Tadpoles were fed Tetramin flake food, algae wafers, and spinach daily.

2.5 | Statistical analysis

We conducted a preliminary exploratory one-way analysis of variance (ANOVA) to determine if individuals had an effect on pre-freeze and post-thaw sperm motility. No significant differences were found for pre-freeze sperm motility ($F = 2.9$, $df = 5$, $p = .08$), so data were combined for analysis. There was a significant difference between males in post-thaw sperm motility ($F = 3.2$, $df = 5$, $p = .03$), so individual ID was included as a random effect in the analysis of post-thaw sperm to account for the variation. Comparisons between pre-freeze and post-thaw total motility were analyzed using a paired Wilcoxon Signed-Rank test. Post-thaw sperm motility differences between cryoprotectants (DMFA and DMSO) were analyzed using a Mann-Whitney test. We used a Generalized Additive Model for Location Scale and Shape (GAMLSS) test with a zero-inflated beta distribution to measure the effect of time of collection on pre-freeze motility, where time points were fixed effects and individual male was a random effect. Differences in development rates between sperm type (frozen-thawed vs. fresh) for cleaved eggs, neurulation, tadpole, and metamorphosis development following IVF were analyzed using Mann-Whitney tests. Neurulation, tadpole, and metamorphosis development rates were expressed as a percentage of cleaved embryos, not the number of eggs. We assessed conformity to model assumptions using a Shapiro-Wilk Test, visual inspection of residual plots, and Levene's Test for homogeneity in variance. Effect size was determined using Cohen's d . Statistical significance was set at .05, and all data was analyzed in RStudio with R version 4.0.2.

3 | RESULTS

No sperm was present in urine from any individual prior to giving hormones, and 9/10 responded to the treatment, with only two producing spermic urine at every time point (T1, T2, T3, T5, and T7). Variation in the number of males represented at each time point reflects missing urine collections. Occasionally, the males would urinate in the holding container prior to being restrained for collection.

Total sperm motility was high for the first four collection time points (T1–T5) and ranged between 61% (95% CI 29–93) and 70% (95% CI 56–84), yet began to decrease at T7 (41% [95% CI 14–68]; Figure 3a). We found a significant difference in sperm motility between T7 and all other time points ($p = .02$, $R^2 = 0.49$), but there was no difference in motility between T1 ($p = .06$, $R^2 = 0.49$), T2

($p = .63$, $R^2 = 0.49$), T3 ($p = .90$, $R^2 = 0.49$), and T5 ($p = .63$, $R^2 = 0.49$; Figure 3a). Average sperm FPM was 45% (SE 4), and average NPM was 35% (SE 4). Total sperm motility ranged from 34 to 93% and averaged 61% (SE 5) across all individuals. Average volume of spermic urine collected per male, across the entire collection period, was 2.2 mL (SE 0.3).

Of the nine males that produced spermic urine, samples from eight were selected for cryopreservation. A total of 93 straws were cryopreserved, with 68 frozen in DMFA, representing eight males, and 25 frozen in DMSO, representing six males (Table 2). Average pre-freeze motility of sperm frozen with DMFA and DMSO was 67% (SE 5) and 71% (SE 5), respectively. For post-thaw analysis, sperm were thawed from six males for the DMFA treatment and four males for the DMSO treatment. Average post-thaw sperm motility was 28% (95%

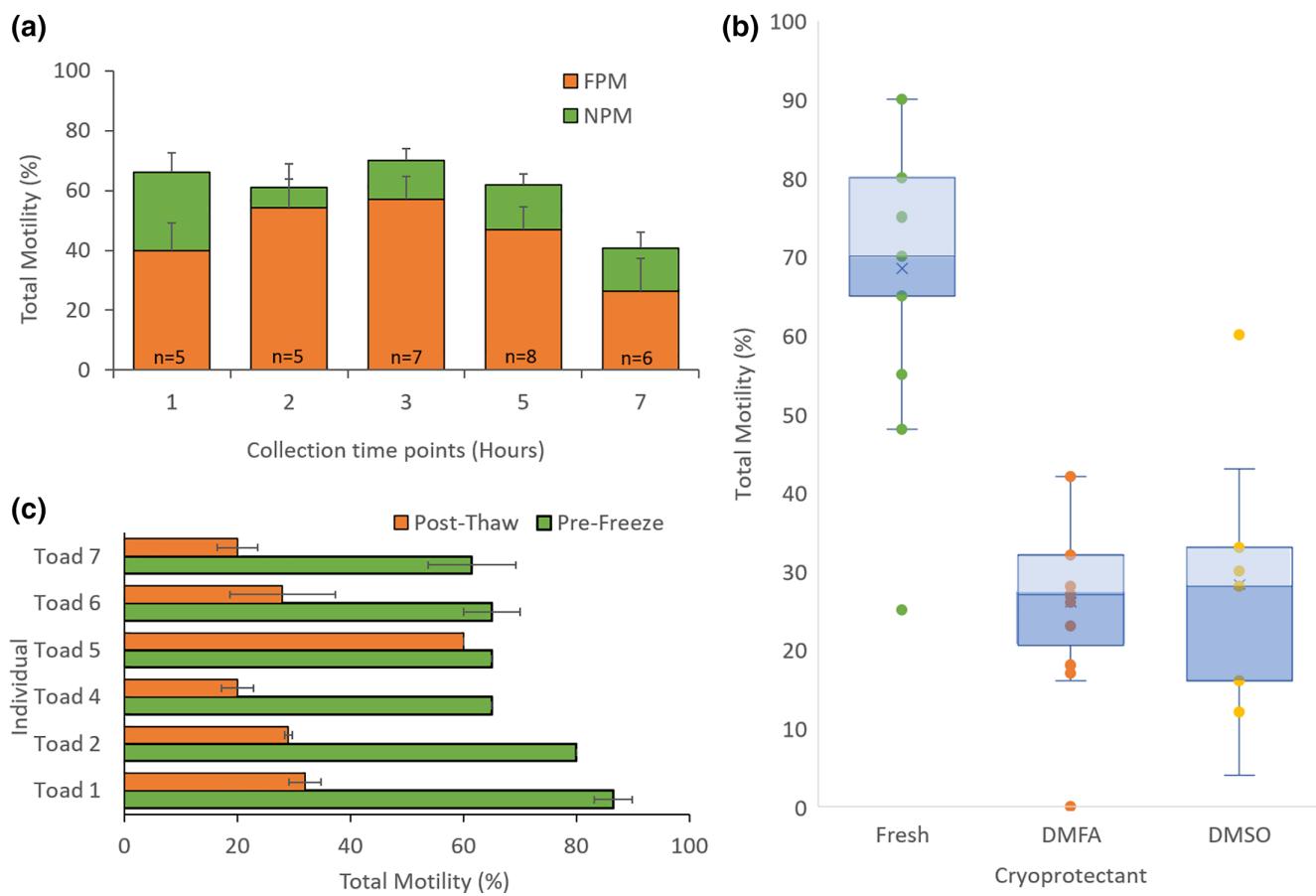


FIGURE 3 (a) Average sperm forward progressive motility (FPM) and nonprogressive motility (NPM) for male Puerto Rican crested toads ($n = 9$) at different collection time periods following hormone administration. Data are shown as mean \pm 95% CI with the number of individuals at each collection point indicated; variation in sample size per time point reflects missing urine collections. Different lowercase letters indicate significance of total motility between time points; (b) average pre-freeze and post-thaw motility for sperm frozen using either 10%DMFA or 10%DMSO as a cryoprotective agent (CPA); (c) total sperm motility for individual Puerto Rican crested toads before cryopreservation and after thawing. Due to no difference between CPA treatments, straws are averaged from both treatments for individual males. Straws from Toad 3 and 8 have not been thawed due to low sample numbers and remain in the Biobank for long-term genetic management. Data are shown as mean \pm 95% CI

TABLE 2 Motility and cryobanking summary separated by cryoprotectant and individual toad (studbook #)

CPA	ID#	Studbook#	Avg pooled pre-freeze motility (%) ^a	# of straws banked	# of straws thawed	Avg straw post-thaw motility (%) ^a	Avg relative post-thaw motility (%) ^a
10% DMFA	1	FWZ#210690	85 (SE 5)	19	5	30 (SE 4)	35 (SE 5)
	2	FWZ#210693	80 (SE 0)	7	1	27 (SE 0)	34 (SE 0)
	3	FWZ#210687	70	5	0	NA	NA
	4	FWZ#210689	65	10	3	20 (SE 3)	31 (SE 4)
	5	FWZ#210686	65	5	1	60	92 (SE 0)
	6	FWZ#210691	63 (SE 8)	12	5	25 (SE 8)	40 (SE 13)
	7	FWZ#210688	62 (SE 24)	5	2	26 (SE 3)	42 (SE 4)
	8	FWZ#210685	25	5	0	NA	NA
Total			67 (SE 5)	68	18	28 (SE 3)	37 (SE 4)
10% DMSO	1	FWZ#210690	80	4	4	35 (SE 4)	39 (SE 4)
	2	FWZ#210693	80	2	2	29 (SE 1)	36 (SE 1)
	3	FWZ#210687	70	5	0	NA	NA
	6	FWZ#210691	70	4	1	4 (SE 0)	6 (SE 0)
	5	FWZ#210686	65	4	0	NA	NA
	7	FWZ#210688	62 (SE 14)	6	2	14 (SE 2)	23 (SE 3)
	Total		71 (SE 5)	25	9	25 (SE 5)	37 (SE 8)
<i>p</i> -value ^b						<i>p</i> = .59	<i>p</i> = .24

^aData is shown as mean (SE).^b*p*-value shows significance between cryoprotectant treatments within a column.

TABLE 3 Fertilization, embryo, larvae, and metamorph development results following IVF with either fresh or frozen-thawed Puerto Rican crested toad sperm

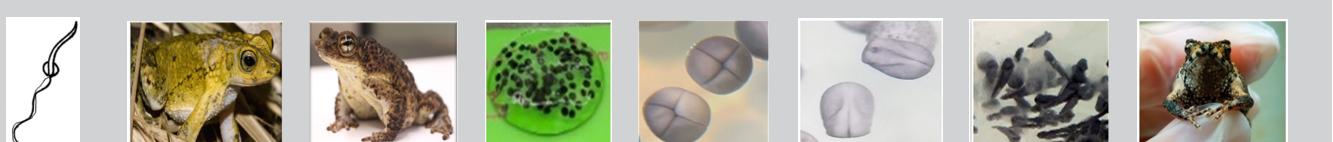
Sperm	No. males	No. females	Eggs	Cleaved (%) ^a	Neurula (%) ^b	Hatched (%) ^b	Metamorph (%) ^b
Frozen	5	2	6,981	306 (4)	150 (49)	55 (18)	46 (15)
Fresh	3	2	2,691	525 (20)	400 (76)	215 (41)	140 (27)
<i>p</i> -value				<i>p</i> = .44	<i>p</i> = .70	<i>p</i> = .82	<i>p</i> = .94
Cohen's <i>d</i>				<i>d</i> = 0.63	<i>d</i> = 0.24	<i>d</i> = 0.22	<i>d</i> = 0.09

^aDevelopment rate shown as percentage of eggs fertilized.^bDevelopment rate shown as percentage of cleaved embryos.

CI 22–35) for sperm frozen with DMFA and 25% (95% CI 16–35) for sperm frozen with DMSO (Figure 3b). The two cryoprotectants yielded similar total sperm motility post-thaw ($p = .59$, $d = 0.75$).

Post-thaw sperm data were pooled across CPAs for analysis. Fresh sperm had a significantly higher ($p = .03$, $d = 2.24$) total motility (mean = 71%, 95% CI 62–79) than post-thaw sperm (mean = 27%, 95% CI 22–32), yet post-

thaw sperm still maintained over one-third of the total motility that was initially frozen (Figure 3c). Fresh sperm motility ranged from 48 to 90% with no significant difference found in total motility between individuals. Post-thaw sperm motility ranged from 0 to 60%. We did find a significant difference in post-thaw sperm motility between Toad 5 (mean = 60%) and Toad 4 (mean = 50%, 95% CI 15–26, $p = .04$, $R^2 = 0.47$), Toad 6 (mean = 28%, 95% CI



9–46, $p = .04$, $R^2 = 0.47$), and Toad 7 (mean = 20%, 95% CI 13–27, $p = .03$, $R^2 = 0.47$), but not the other individuals (Figure 3c). This variation could be caused by the low sample size of straw replicates.

For the IVF experiments, 9,672 eggs were used between cryopreserved and fresh sperm. Eggs fertilized with frozen–thawed sperm and fresh sperm produced a total of 306 (4%) and 525 (19%) cleaved embryos, respectively (Table 3). There was no significant difference found between frozen–thawed (mean = 5%, 95% CI 1–10) or fresh sperm (mean = 17%, 95% CI 1–33) on the rate of cleavage ($p = .44$, $d = 0.63$). A high rate of neurulation was found from cleaved eggs using frozen–thawed sperm (49%) and fresh sperm (76%), suggesting that fertilized embryos have a high probability of advancing through development. Ultimately, IVFs using frozen–thawed sperm produced 46 toadlets, compared to 140 toadlets produced by fresh sperm. This data shows that frozen sperm was able to produce at least one-third the number of metamorphs as fresh sperm (Table 3). Overall, we also did not find any significant differences in neurulation (frozen: mean = 63%, 95% CI 33–92; fresh: mean = 51%, 95% CI 7–94, $p = .70$, $d = 0.24$), hatching (frozen: mean = 23%, 95% CI 7–39; fresh: mean = 32%, 95% CI –4 to 68, $p = .82$, $d = 0.22$), or metamorphosis (frozen: mean = 16%, 95% CI 1–32; fresh: mean = 15%, 95% CI 2–28; $p = .94$, $d = 0.09$) rates for tadpoles derived from fresh vs. frozen sperm (Table 3).

Toadlets produced from this study were head-started at FWZ and released to the wild at 4.5 months of age. Fourteen toadlets produced from frozen–thawed sperm and 92 toadlets produced from fresh sperm were released in Puerto Rico by USFWS in November 2019. As of February 2021, 24 adult toads produced from frozen–thawed sperm and 21 adult toads from fresh sperm are still living after 1.5 years in the captive assurance colony. These individuals have been retained for genetic management and future breeding efforts. One of the males produced by cryopreserved sperm has now produced another F2 generation of offspring with 5,085 tadpoles released, indicating these animals are reproductively viable.

4 | DISCUSSION

In this study, we report the first production of endangered PRCT offspring using frozen–thawed sperm collected and cryopreserved through the use of exogenous hormone therapy from live animals. Furthermore, this is one of the first reports to link in-situ and ex-situ amphibian populations using assisted reproductive technologies to enhance genetic management and population stability. In total, these reproductive techniques led to the production

of 46 offspring, 14 of which were released back into Puerto Rico. These released individuals may breed naturally with other PRCTs in the wild, increasing population numbers as well as genetic variation of this metapopulation. The importance of these results toward conservation management cannot be understated, especially for threatened amphibian species in the wild, which are constantly under demographic and environmental stochasticity. For instance, the karst formation where the males for this study were collected collapsed following a series of earthquakes in 2020, leading to the status of the PRCTs in that area being unknown for approximately a year. Fortunately, USFWS were able to locate PRCTs in early 2021, indicating that they are still able to use the karst formation regardless of the collapse (Pacheco, personal communication). Nevertheless, events like these could potentially eradicate the few remaining populations on the island, which for the PRCT, could be the end of the species if not mitigated.

If population numbers of wild and captive PRCTs decline, the likelihood for inbreeding, loss of fitness, and loss of genetic variation increases (Frankham, 2008; Frankham et al., 2010; Robert, 2009). In order to reverse these effects, three strategies can be applied to transfer genetics from the wild to captive colonies (Table 1). The first two strategies require the removal of adults or tadpoles from the wild to be transferred to the assurance colonies. However, the northern wild PRCT is now believed extirpated (D. Barber, personal communication) and environmental threats to the southern wild population are increasing, meaning these two strategies may not be successful long-term. The third strategy, on the other hand, applies exogenous hormone therapy and cryopreservation to collect and transfer gametes from wild individuals to the captive collection (Table 1). These gametes can then be used in reproductive efforts to increase population numbers and potentially introduce new genetic variation into the assurance population, while allowing reproductively viable individuals to remain in the wild. This strategy has been applied to mammals and has led to the successful production of genetically diverse offspring (Wildt, Rall, Critser, Monfort, & Seal, 1997), and it has now been successfully applied to amphibians as well.

This study focused primarily on the third strategy (Table 1), which employs a coordinated series of assisted reproductive technologies for introducing new genetic lineages into PRCT populations, thus maintaining, if not increasing, current levels of genetic diversity. The original PRCT founders were collected mostly from a northern population site that is now believed to be extirpated, as well as a few from the southern population. For this study, males were collected from a recently discovered southern group that is not planned for population

augmentation and, according to preliminary genetic analysis, has significant genetic variability from the neighboring southern source population (D. Williams, unpublished data). Furthermore, past and current reintroductions of captive-bred PRCTs occur over six geographically separate locales across Puerto Rico, negating the possibility of genetic integration between this newly discovered “source group” and other established populations (Barber, personal communication, 2021). The genetic variation between the recently discovered southern population (where our males were collected from) and its neighbor, along with the lack of additional individuals integrated from release efforts or dispersal, indicates that the wild males used in this study serve as a potential unrepresented founder base in the captive collection of PRCTs, possibly expanding the current gene pool of the captive and subsequent reintroduced populations following release. We recognize that genetic analysis of the populations are needed to confirm this supposition; however, it is highly likely given the time since last importation from this site in 2014, absence of augmentation from the captive population, low dispersal between isolated populations, and underrepresentation in the captive collection. Therefore, it is quite probable that through the application of biobanking and IVF (third strategy, Table 1) we increased the gene variation of captive populations by adding eight new founders to the current genetic structure. Although the importance is not that new founders were secured for the population, but that the methods themselves could revolutionize the management of rare amphibians.

According to the current ratio of effective populations size to breeding census size ($N_e/N = 0.09$), there is a high level of inconsistency present in the overall breeding success among adult individuals. Moreover, a majority of adults do not contribute the genetic material that is expected by current population sizes to the next generation, producing a skew of founder representation in offspring (Sánchez-Montes, Wang, Ariño, Vizmanos, & Martínez-Solano, 2017), as seen in the founder genome equivalent (FGE; Table 1). There are 48 founders in the captive population that could conceivably contribute to genetic variation, yet the FGE shows that only 16.5 are actively contributing to the population's diversity (Table 1). While the current mean inbreeding (0.027) and kinship (0.03) values of the PRCT captive population are low, this unequal representation in founder stock can lead to an increase in both rates, creating an unsustainable population level. If we rely on the first strategy (Table 1) to maintain genetic variation and reduce the levels of relatedness and inbreeding, up to 10 new founders will need to be added every four years. However, in our application of the third strategy, sperm

collected from 8 males generated 4 genetically unique cohorts and 46 new individuals. Furthermore, one reproductively viable male adult was produced from cryopreserved sperm utilizing a captive female that was at risk due to a condition called brown skin disease (Crawshaw et al., 2014), highlighting this technology for recovering gene diversity that is about to be lost from an animal that is no longer able to breed naturally due to disease. As of June 2021, this first-generation adult male produced 5,095 offspring through a hormone-induced natural breeding event. Of these offspring, 5,085 were released back into the wild and 10 were kept to augment the captive population (Barber, personal communication, 2021). Through this most recent breeding event, the genetic lineage of the deceased female has been preserved and will continue to be passed on to generations in both captive and reintroduced groups. Even though not all individuals produced through this study survived to adult hood, 2.5–12 times more offspring were produced and introduced into the captive population in a quarter of the time. Thus, linkage of in-situ and ex-situ populations through the use ART has introduced more genetic variation to the captive population, which can subsequently be used in future efforts to bolster the gene diversity within wild populations.

Noninvasive methods of sperm collection from live animals are becoming more common for conservation management of threatened amphibians with low reproductive output (Julien et al., 2019; Langhorne et al., 2021), although sperm from testes macerates would still be useful for sick or deceased individuals (Upton et al., 2018). The main drawback of collecting sperm from live animals is that sperm are metabolically activated when released to the environment (Kouba et al., 2003), resulting in a short lifespan along with the need to be immediately cooled to temperatures near 4°C to maintain motility and viability (Arregui et al., 2021; Kouba & Vance, 2009; Langhorne et al., 2021). In addition, during the cryopreservation process sperm become deactivated from the high osmolality of the cryoprotective agents and after thawing become reactivated by dilution to a low osmotic environment for IVF. These shifts in sperm activation lead to a loss in sperm motility, especially forward progression, at each step of the freeze-thaw process. Such extreme shifts in sperm activation are not experienced by sperm from testes macerates, in which sperm remain immotile in the physiological osmolality of the testes (280 mOsm), the freezing medium, and up until the time of fertilization (Kouba et al., 2003). In the current study on PRCTs, sperm cryopreservation from live individuals was highly successful, achieving 33% recovered sperm motility post-thaw, high fertilization capacity, and the development of metamorphs and adult offspring, even after the extreme fluctuations in osmolality

and the freezing process. Two other studies from our lab have shown that post-thaw sperm motility can be achieved following cryopreservation in wild male Boreal toads (*Anaxyrus boreas boreas*) (Langhorne, 2016) and male Chiricahua leopard frogs (*Lithobates chiricahuensis*) (unpublished data), which was then used to successfully fertilize eggs from captive females to produce adult animals and tadpoles, respectively. Hence, the current study contributes to the growing number of species demonstrating that linking ex-situ and in-situ populations is feasible and could be integrated enabling larger genetic management plans for these two populations.

The minor limitations of this study are the small sample size of wild males we located, number of straws we thawed vs. held back for management purposes, and number of females used for IVF. When working with wild animals, the number of individuals that can be collected depends on being able to locate, capture, and properly house the animals for the short duration needed for sperm collection. Moreover, the number of straws that can be frozen depends on if the male produces spermatogenic urine in a usable volume of sufficient concentration. Given the importance of these wild genetics to the future population structure, a limited number of straws could be thawed for IVF without impacting the reserve holdings. Importantly, since increasing genetic variability among the PRCT populations was one of the main purposes of this study, specific individuals must be matched up based on the population studbook and pedigree. If a female already has a high mean kinship value, another female with a lower value will be selected for breeding. By introducing new wild male genes into the captive population, the level of relatedness will decrease, and additional females can be used in reproductive efforts. As more females are used, the number of offspring produced increases, resulting in more individuals that can be introduced to the wild or held to bolster captive populations. As these studies progress and gametes from more wild individuals are collected, the genetic variation and stability of PRCT populations can increase exponentially.

Here, we show how ART for amphibians can be used to link in-situ and ex-situ populations of endangered species, especially where “at risk” captive populations exist and long-term sustainability is a challenge without the emigration of wild individuals. Just as hormone administration has been shown to be species- or genera-specific (Clulow et al., 2018; Kouba et al., 2009), the protocols described here for cryopreservation and IVFs may be species-specific as well, indicating the importance of expanding this applied work to other threatened and endangered species. Furthermore, solidifying a cryopreservation protocol that optimizes post-thaw sperm motility and IVF success produces not only an increase in

offspring numbers, but also introduces new genetics that can then be held in captive colonies or released to the wild. However, as mentioned earlier, there are numerous challenges that come with cryopreservation in the field. These include shipping supplies, sometimes internationally, that are fragile; obtaining LN₂ in remote locations to use for the cryopreservation process; organizing experts and volunteers to locate and capture animals; and acquiring all necessary permits and permissions for working with threatened species, to name a few. Overcoming these challenges and expanding this work to other species would help strengthen the overall body of knowledge of sperm cryopreservation, creating a more holistic approach to amphibian conservation management.

Captive assurance colonies play an integral part in the conservation and management of threatened and endangered amphibian species. Maintaining a high level of gene diversity in these colonies is imperative to the recovery of wild populations, which are experiencing drastic declines in population numbers. Cryopreservation and biobanking of male gametes to link in-situ and ex-situ groups of threatened and endangered species has been a recommended conservation strategy for decades, yet never successfully implemented in amphibians. In this study, we explored the feasibility of linking in-situ and ex-situ PRCT populations by transporting sperm from the wild into captive colonies for managed genetic crossings using IVF. We show that the use of exogenous hormone treatment is a viable and nonlethal way to collect sperm from individuals; sperm can be cryopreserved, transported, and recovered for coordinated IVF breeding efforts to produce viable second-generation offspring; and metamorphosed toadlets originating from frozen-thawed sperm can be incorporated into both captive and wild populations, potentially increasing the overall genetic variation. As amphibian populations decline, developing new conservation strategies and linking in-situ and ex-situ populations will continue to grow in importance for conservation and genetic management of captive and wild populations.

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CONFLICT OF INTEREST

The author and co-authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Isabella Burger—data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, review and editing. Allison R. Julien—conceptualization, data curation, investigation, methodology, supervision, validation, visualization, writing—review & editing. Andrew J. Kouba—conceptualization, supervision, funding acquisition, resources, project administration, writing—review and editing. Diane Barber—conceptualization, investigation, methodology, project administration, resources, supervision, writing—review and editing. Kristen R. Counsell—investigation. Carlos Pancheco—resources, project administration. Jessi Krebs—resources, project administration. Carrie K. Kouba—conceptualization, supervision, project administration, funding acquisition, resources, data curation, investigation, methodology, validation, writing—review and editing.

DATA AVAILABILITY STATEMENT

The data supporting the study's findings can be accessed by contacting the corresponding author.

ETHICS STATEMENT

The authors are not aware of any ethical issues regarding this work. All data collected was approved by the Institutional Animal Care and Use Committee at the Fort Worth Zoo as well as the Fort Worth Zoo—United States Fish and Wildlife Service permits, as stated in Section 2 of the manuscript.

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