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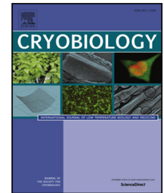

Cryopreservation and hormonal induction of spermic urine in a novel species: The smooth-sided toad (*Rhaebo guttatus*)

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Global amphibian declines have fueled an increased interest in amphibian assisted reproductive technologies. Within the genus *Rhaebo*, half of the species are experiencing decreasing population trends; however, insufficient information is available on many of these species' reproductive biology. Using the smooth-sided toad, *Rhaebo guttatus*, we present effective methods for collecting and cryopreserving an example of *Rhaebo* sperm. Specifically, our findings show that administering 10 IU/g body weight of hCG (human chorionic gonadotropin) yields the most motile and concentrated sperm and that cryopreserving spermic urine in a solution of 5% DMFA (N,N-Dimethylformamide) and 10% trehalose returns sperm with a 33 ± 3% average post-thaw motility. These findings may represent an important step forward in developing techniques that can be safely applied to other, more vulnerable species within the *Rhaebo* genus.

As amphibians continue to face global declines, assisted reproductive technologies (ART) play a critical role in combating biodiversity loss. These techniques have broken down many barriers pertaining to amphibian captive breeding, including asynchronous release of gametes, absence of breeding behavior, and low fertilization rates [5]. Recovery programs for multiple endangered anuran species, such as the dusky gopher frog (*Lithobates sevosus*), the Wyoming toad (*Anaxyrus baxteri*), and the Houston toad (*Anaxyrus houstonensis*) currently implement ART to facilitate captive breeding and achieve reintroduction goals. As such, the use of ART (e.g. exogenous hormone stimulations) to collect gametes for *in vitro* fertilization and cryostorage represent a worthy option to help sustain healthy captive populations in perpetuity.

However, these techniques are often taxon-specific, and gamete collection and cryopreservation protocols must be fine-tuned for novel groups [5]. To collect amphibian gametes, GnRH (gonadotropin-releasing hormone; des-Gly^{10}, D-Ala^{6}), hCG (human chorionic gonadotropin), or a cocktail of the two are most commonly used [3,7,10]. Reponses to hormone treatments differ widely across taxa, including time to peak sperm production, peak sperm concentration, and peak sperm motility [1,6], which highlight the need for adapting existing protocols to novel and understudied taxa. In doing so, we can increase the accuracy and effectiveness of methods used in captive breeding and enhance our understanding of amphibian reproductive physiology.

Cryopreservation protocols must also be tested and adapted accordingly for novel taxa. Sperm cells can vary in their cryosensitivities due to differences in cell morphology, cell membrane composition, and sperm concentration [2]. Fortunately, commonalities between taxonomically-related species exist. As such, cryopreservation studies are needed across diverse taxa so that the resulting knowledge and data from a new species or species branch may be applied to other related species that are in immediate need of these techniques.

Rhaebo is a genus of true toads (Family: Bufonidae) comprised of 13 species, all of which are native to Central and South America. This genus represents a neglected group in sperm collection and cryopreservation, as there are no published reports on either topic and even basic information of reproductive biology is absent for multiple species. Six species within the genus (*Rhaebo colomai, R. blombergi, R. haematiticus, R. hypomelas, R. caeruleostictus, and R. andinophrynoides*) exhibit decreasing population trends, due in part to commercial export, deforestation, and environmental contaminants [4]. Developing reproductive technologies for an example of this genus presents a great opportunity to gain knowledge on the fundamental biology of declining species that may be reliant on these conservation efforts in the future. Using *Rhaebo guttatus*, a widespread species with a secure population status, as a model organism for the genus, we seek to 1) characterize the effect of varying hormonal stimulations on sperm concentration and quality and 2) assess the freezability of *Rhaebo* sperm using a common cryoprotectant.

Adult, male *R. guttatus* (N = 5) were maintained indoors at 24 °C at

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the Memphis Zoo (Memphis, TN, U.S.A.). Individuals were housed in plastic enclosures (91 cm L × 61 cm D × 44 cm H) with a coconut shaving substrate, in groups of no more than two. Enclosures included multiple wooden hides and aged water. Individuals were fed crickets *ad libitum*. All experimental procedures were approved by the Memphis Zoo Animal Care and Use Committee (Approval 2018-7) and were conducted October–December 2018.

Exogenous hormonal induction was used to stimulate the production of spermic urine. Three hormone treatments were administered to test which yielded the most motile and concentrated sperm samples. Specifically, males received treatments of 1) 0.4 μg/g body weight (gbw, hereafter) of GnRH (Catalogue number: CG5, Sigma-Aldrich), 2) 7.5 IU/gbw hCG (Catalogue number: L4513, Sigma-Aldrich), and 3) 10 IU/gbw hCG. Hormone treatments were spaced every four weeks to eliminate the risk of receptor desensitization, receptor down-regulation, and sperm depletion [9]. Hormone dose selection was based on approximations of established protocols that are successful at promoting sperm production in bufonid and ranid species [3,6]. Injections were administered intraperitoneally using a 0.3 mL syringe and 29 gauge ½" needle. Immediately following injections, toads were placed in individual 5.7 L plastic boxes (36 cm × 20 cm × 12 cm) filled with 1 cm of aged water to promote urine production. Sample collection was facilitated by gently inserting plastic catheter tubing (0.86 mm inner diameter × 1.32 mm outer diameter, Scientific Commodities, Inc.) into the cloaca. Collections occurred at eight sequential time points as follows: 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 24 h post injection. A urine sample was also collected immediately prior to hormone injections (0 h) to ensure spermiation was the result of exogenous hormonal induction.

Each urine sample was assessed for the presence of sperm cells. Percent total motility and concentration were calculated for all samples containing sperm using an Olympus CX41 phase-contrast microscope at 400× magnification. Percent total motility was determined by counting all cells with flagellar movement within 100 cells. Sperm concentration was determined using a Neubauer-ruled chamber hemocytometer.

To evaluate the freezability of *R. guttatus* sperm, cells were cryopreserved in a solution of both penetrating and non-penetrating cryoprotective agents. Specifically, a stock solution of cryoprotectant was prepared using 10% DMFA (N,N-Dimethylformamide; Sigma-Aldrich) and 20% trehalose (Sigma-Aldrich) in distilled water. The solution was gradually mixed 1:1 with fresh spermic urine to create a sperm cryosuspension with a final concentration of 5% DMFA and 10% trehalose. Samples were then immediately loaded into 0.25 cc cryostraws (Reproduction Resources) and placed at 4 °C to equilibrate for 10 min. All samples were cryopreserved within 30 min of collection and were collected in the determined "peak" sperm production period (2–5 h post-injection) following a treatment of 7.5 IU/gbw hCG. Following equilibration, samples were placed 10 cm above liquid nitrogen (- 90 °C) for 10 min in an insulated polystyrene box, before being submerged into liquid nitrogen (- 196 °C). Cooling rates are estimated to be around 10 °C/min [8]. Samples were kept frozen for at least 24 h. Samples were thawed by a 10-s exposure to room temperature (23 °C), followed by a 10-s submergence in a water bath (40 °C), and then diluted 1:10 with distilled water. Total sperm motility was assessed following methods used for fresh sperm samples, as described above. Additionally, recovery rate (RR) for total motility and forward progressive motility was calculated as RR = (FV/IV) × 100, where IV = initial value of fresh samples and FV = final value after samples were cryopreserved and thawed.

To test if sperm total motility, forward progressive movement, and concentration differed among hormone treatments, statistical analyses were conducted using a beta-distributed generalized linear mixed model (R package 'glmmTMB'). Likelihood Ratio Tests were used to test the significance of hormone treatments, and *post hoc* Tukey's Honest Significant Difference tests determined differences in sperm metrics

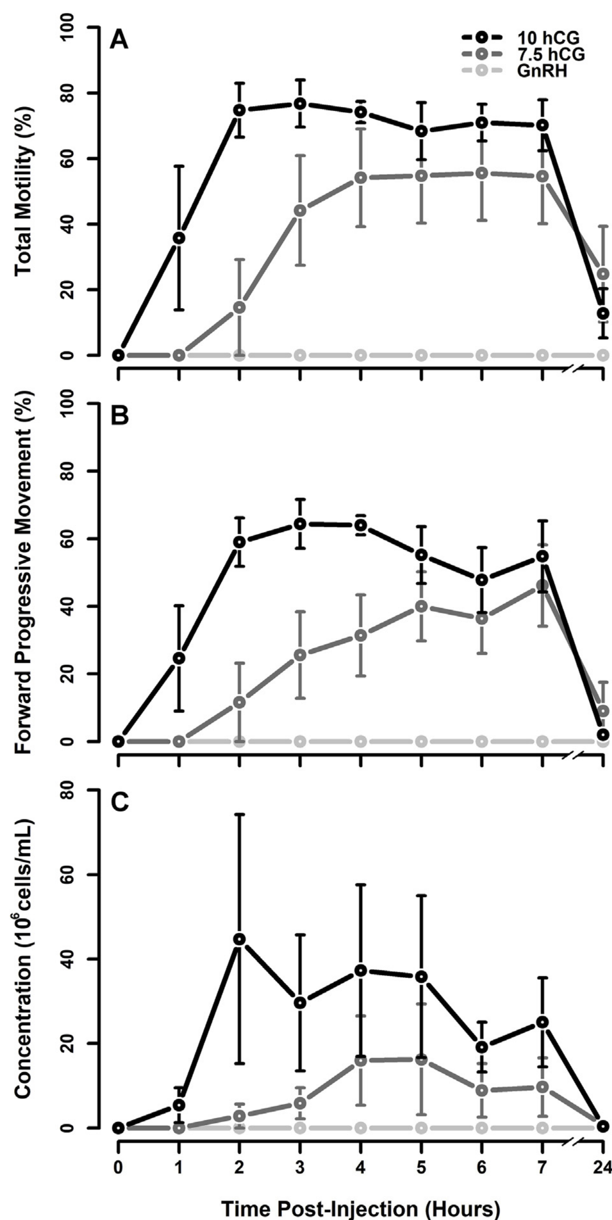

Fig. 1. *Rhaebo guttatus* (N = 5) total sperm motility (A), forward progressive sperm movement (B), and sperm concentration (C) following one of three hormone treatments: 1.) 10 IU/gbw hCG (black line), 2.) 7.5 IU/gbw hCG (dark gray line), or 3.) 0.4 μg/gbw GnRH (light gray line) over nine time points. Hour zero occurred prior to hormonal induction. Data are represented as mean ± standard error.

between hormone treatments (R package 'emmeans'). All statistical analyses were conducted in the R programming environment (v. 3.5.1) using a significance level of $\alpha = 0.05$.

R. guttatus sperm was successfully collected from all of the hormone treatments, although total motility, forward progressive movement, and concentration values varied greatly by individual, treatment, and time since injection (total motility: 0–92%; forward progressive movement: 0–84%; concentration: 0.005–150 × 10^6 cells/mL). No individuals produced sperm prior to hormonal induction (0 h, Fig. 1), indicating that exogenous hormones stimulated the production and release of sperm. Of the three treatments, 1/5 individuals responded to GnRH, 4/5 individuals responded to 7.5 IU/gbw hCG, and 5/5 individuals responded to 10 IU/gbw hCG. Hormone treatments elicited differences in all quantified sperm metrics: total motility ($p < 0.001$), forward progressive movement ($p < 0.001$), and concentration ($p < 0.001$)

(Fig. 1). Pairwise comparisons revealed significant differences between all three treatments in terms of total motility and forward progressive movement, with 10 IU/gbw hCG exceeding 7.5 IU/gbw hCG ($p = 0.015$ and $p = 0.016$, respectively) and GnRH ($p < 0.001$, both metrics) and 7.5 IU/gbw hCG exceeding GnRH ($p = 0.006$ and $p = 0.002$, respectively). 10 IU/gbw hCG elicited the release of significantly more concentrated sperm than 7.5 IU/gbw hCG ($p = 0.018$) and GnRH ($p < 0.001$), but no differences existed between the latter treatments ($p = 0.12$). Taken together, these results show that, of the three treatments, administering 10 IU/gbw hCG is the most effective method for collecting high quality sperm from *R. guttatus*. When using this method, between two and 5 h post-injection qualitatively appears to be the best time to collect highly concentrated, forward moving sperm. Interestingly, some individuals were found to still produce sperm up to 24 h post-injection.

Spermic urine samples from two males were successfully frozen in the cryoprotectant solution of 5% DMFA and 10% trehalose, with frozen-thawed sperm averaging a post-thaw total motility of 33 ± 3% and an average forward progressive movement of 16.5 ± 6.5%. Recovery rates were high and averaged 43.6 ± 2.5% for total motility and 27.5 ± 10.2% for forward progressive movement. These values are large enough to fertilize eggs through *in vitro* fertilization in other anuran species [e.g., Rana temporaria, 8]. However, whether these same values are sufficient to fertilize *R. guttatus* eggs or other eggs of this genus requires definitive investigation.

The collection of high quality sperm is a necessity for successful cryopreservation. Because amphibians vary considerably in their responses to exogenous hormones across taxonomic levels, investigations into effective hormone treatments must be completed in concert with cryo-strategies. Using *R. guttatus* as a model, we provide basal information on sperm collection and cryopreservation in a novel species and genus. We show that high concentration, motile sperm can be collected from *R. guttatus* via hormonally-induced spermic urine, and that viable cells can be recovered following cryopreservation. Additionally, the large body size of *R. guttatus* allows for a considerable amount of urine to be produced. Following hormonal induction, large volumes of urine (maximum urine volume = 15.53 mL) translate into large volumes of sperm (maximum sperm count = 1.67×10^9), which in turn provide a significant amount of sperm to be bio-banked and used in future ARTs. These findings are an important step forward in developing techniques that can be safely applied to vulnerable species within the *Rhaebo* genus. In particular, for species within *Rhaebo* whose main threats include commercial export, establishing a genome resource bank and developing assisted reproductive techniques could help create and facilitate captive breeding efforts to eliminate future take from the wild. The reproductive biology of *Rhaebo* has been largely overlooked and rarely studied, and as such, we present techniques that can be used as a “jumping off” point for future efforts.

Declaration of interest

None.

Role of funding source

The Institute of Museum and Library Services had no role in study design, in the collection, analysis, or interpretation of data, in the writing of the report, or in the decision to publish.

Conflicts of interest

We have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.05.007.

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