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Assessment of ultra-rapid freezing as a simplified, field-friendly technique for semen cryopreservation in wild ocelots (*Leopardus pardalis*) and bobcats (*Lynx rufus*) in southern Texas

Ashley M. Reeves [a,b,*], William F. Swanson [c], Tyler A. Campbell [a], Michael E. Tewes [d], Amy Miller [c], Cary Springer [e], Debra L. Miller [b,f]

[a] East Foundation, San Antonio, TX 78216 USA
[b] Center for Wildlife Health and College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA
[c] Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Gardens, Cincinnati, OH 45220, USA
[d] Caesar Kleberg Wildlife Research Institute, Texas A&M University-Kingsville, Kingsville, TX 78363, USA
[e] Research Computing Support, University of Tennessee, Knoxville, TN 37996, USA
[f] One Health Initiative, University of Tennessee, Knoxville, TN 37996, USA

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ABSTRACT

Ocelot (*Leopardus pardalis*) populations in southern Texas have shown declines in genetic variability over time. Assisted reproductive technologies (ARTs[1]), such as semen cryopreservation and artificial insemination (AI[2]), can help improve species sustainability by preserving genetic diversity and connecting populations by transport of frozen gametes. Traditionally, felid semen has been cryopreserved by slow freezing in plastic straws (STRAW) for liquid nitrogen storage. However, another technique, ultra-rapid freezing (URF[3]), would decrease the time and effort needed to preserve samples and potentially facilitate cryopreservation in the field. The goal of this study was to compare the effectiveness of URF to traditional STRAW freezing with two wild felid species living in southern Texas. Semen was collected by urethral catheterization (UC[4]) and electroejaculation (EEJ[5]) and frozen using both cryopreservation methods from 12 free-ranging adult felids (n = 6 ocelots; n = 6 bobcats (*Lynx rufus*)). Post-thaw, sperm samples were assessed for progressive motility, acrosomal integrity, and heterologous in-vitro fertilization (IVF[6]) of domestic cat oocytes. For both species, sperm acrosomal integrity, percent progressive motility, and rate of forward progression declined ($p < 0.001$) over time, with no difference ($p > 0.05$) between cryopreservation methods. Frozen-thawed spermatozoa from both species fertilized mature domestic cat oocytes (range, 8.3 – 100 %), and oocyte cleavage percentage did not differ ($p > 0.05$) between cryopreservation techniques. Our initial results suggest that URF, in combination with UC, may allow wildlife veterinarians to routinely collect and bank semen samples from free-ranging cats for conservation purposes.

1. Introduction

Recent studies of wild ocelots living in southern Texas have documented loss of genetic variability in these small populations over time [1–3]. Genetic drift and inbreeding depression may be compromising ocelot population fitness and further reducing their viability [4–6]. Assisted reproductive technologies (ARTs), such as semen banking and artificial insemination (AI), may help to mitigate these declines in

* Correspondence to: 200 Concord Plaza Drive Suite 410, San Antonio, TX 78216, USA.
E-mail addresses: areeves@eastfoundation.net (A.M. Reeves), bill.swanson@cincinnatizoo.org (W.F. Swanson), tcampbell@eastfoundation.net (T.A. Campbell), michael.tewes@tamuk.edu (M.E. Tewes), Amy.Miller@cincinnatizoo.org (A. Miller), springer@utk.edu (C. Springer), dmille42@utk.edu (D.L. Miller).

[1] Assisted Reproductive Technologies.
[2] Artificial Insemination.
[3] Ultra- rapid Freezing.
[4] Urethral Catheterization.
[5] Electroejaculation.
[6] In-Vitro Fertilization.

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genetic diversity and its consequences in both wild and zoo-based cat populations. Specifically, ARTs can be used in felids to address behavioral and physical incompatibilities among breeding pairs, connect distant zoo-based populations by transporting reproductive materials such as semen and embryos, preserve genetic diversity within liquid nitrogen tanks, and link wild and human-managed populations without requiring removal of cats from the wild [7–10]. However, because species and individuals may differ in the capacity of their spermatozoa to survive cryopreservation, investigation of these techniques on a species-specific basis is often necessary [11,12].

Previous semen cryopreservation studies in ocelots and other felids have assessed sperm pelleting on indentations in dry ice [13–17] and straw freezing over liquid nitrogen vapor [14,18–21], but have shown limited pregnancy success when thawed samples were used in conjunction with AI. In an earlier study, one nulliparous female ocelot treated with exogenous gonadotropins and inseminated in utero with frozen-thawed spermatozoa cryopreserved by pelleting on dry ice, conceived and gave birth to a healthy kitten 78 days later [13]. However, it was suggested that increased total sperm numbers were necessary to compensate for freezing-induced acrosome damage in relation to freshly collected inseminates for AI procedures [13]. Compared with fresh ejaculates, frozen-thawed ocelot spermatozoa cryopreserved in straws exhibit similar values for progressive motility status but have decreased percentages of normal sperm morphology and intact acrosomes [22]. Cryopreservation findings in bobcats (*Lynx rufus*) are more limited, with just one previous report of semen cryopreservation, assessing TEST[7] (20 % egg yolk and 4 % glycerol) medium with a traditional straw freezing protocol [22–24]. This study showed that frozen-thawed bobcat sperm could fertilize in-vitro matured oocytes from domestic cats, with percentages of intact acrosomes and progressive sperm motility reduced post-thaw as seen with semen from other medium-sized cats [23]. To our knowledge, semen cryopreservation has never been reported previously with samples collected from free-ranging ocelots or bobcats in the wild.

A newer sperm cryopreservation approach, ultra-rapid freezing (URF), offers advantages of simplicity and minimal equipment needs over straw freezing, requiring only URF- specific medium and liquid nitrogen [25–27]. In domestic cats, sperm samples recovered by urethral catheterization and frozen by URF or conventional straw freezing showed no difference in post-thaw motility and acrosome status over time [27]. Preliminary in-vitro fertilization (IVF) results indicated that UC-URF spermatozoa could fertilize domestic cat oocytes in-vitro, and fertilization success with URF spermatozoa for all inseminated oocytes (30 %, 9/30) did not differ statistically from that observed with straw frozen samples (57 %, 17/30) [27]. Even if slightly compromised, URF semen may have adequate post-thaw quality for use with AI, depending on the specific technique used. With laparoscopic oviductal artificial insemination (LO-AI[8]), in which spermatozoa are deposited directly into the oviductal ampulla, sperm function and motility over time are not as critical as with intravaginal or intrauterine AI. High pregnancy rates (70–80 %) have been obtained in domestic cats using LO-AI with low sperm numbers (~ 1 million motile/oviduct) for insemination, including with semen frozen using standard straw cryopreservation methods [28–30]. Additionally, LO-AI has been used in domestic cats to produce kittens following insemination with frozen-thawed semen that were cryopreserved via URF [31].

As wild ocelot numbers continue to decline in southern Texas, semen collection and cryopreservation, could prove useful to store their genetic material for future use in conservation initiatives. While bobcat populations are not currently imperiled [32], they could one day face the same obstacles as many other feline species have over the last decade and may serve as a research model for closely related, threatened species, such as the Iberian lynx (*Lynx pardinus*) and the Eurasian lynx (*Lynx lynx*) [33,34]. Assessing reproductive techniques in bobcats while their populations remain stable could provide valuable data for future conservation efforts, if necessary. Accordingly, in this study, our objectives were to compare the effectiveness of URF to traditional straw freezing (STRAW) with semen collected from wild ocelots and bobcats in southern Texas, assessing post-thaw sperm motility and rate of forward progression, percentage of intact acrosomes, and heterologous in-vitro fertilization success in both species. If effective, this URF-based cryopreservation approach may provide scientists with a simple, rapidly applied field technique of sperm cryopreservation for conservation of free-ranging ocelots, bobcats and possibly other felid species.

2. Materials and methods

2.1. Animals

Twelve adult male wild cats (ocelot, n = 6; bobcat, n = 6) were used in this study. Wild felids were captured using modified Tomahawk traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) on private ranches in Willacy and Kenedy counties in southern Texas using previously described methods [35]. For heterologous IVF procedures, ovaries from domestic cats undergoing ovariohysterectomy for sterilization purposes were donated from local shelters. All animal use was approved by and in accordance with the policies of the Institutional Animal Care and Use Committees at Texas A & M University– Kingsville (TAMUK), Caesar Kleberg Wildlife Research Institute (CKWRI), the University of Tennessee- Knoxville and the Cincinnati Zoo's Center for Conservation and Research of Endangered Wildlife (CREW), with ocelot study permits provided by the United States Fish and Wildlife Service (USFWS).

2.2. Media

Feline-optimized culture medium (FOCM) was prepared from stock solutions as described by Herrick et al. [36]. Modifications to FOCM- for in-vitro maturation (IVM), IVF, and culture (FOCM-IVC) were made according to Herrick et al. [37]. All IVM, IVF and IVC media were equilibrated in 6 % CO_2 at 38.6 °C for 12–18 h before use. For straw freezing, soy-lecithin cryopreservation medium (with 4 % glycerol) was prepared from stock solutions as described by Vick et al. [24] and stored in a −40 °C freezer until needed. For URF, a variation of this cryopreservation medium containing Soy-lecithin with 0.2 M sucrose (but without glycerol) was prepared as described by Swanson [38] and stored in a −40 °C freezer.

2.3. Semen collection and cryopreservation

Male bobcats and ocelot were immobilized in the field and maintained at a light anesthetic plane for semen collection using anesthetic drug combinations, dosages, and sample collection procedures as previously described [35]. Semen samples were collected by urethral catheterization (UC) in both bobcats and ocelots and by electroejaculation (EEJ) in ocelots only [35]. Briefly, raw semen was measured for a total volume, an aliquot initially assessed for the presence or absence of spermatozoa using microscopy (100 ×), and for spermic samples, motility (percent progressively motile (PPM), 0–100 %) and rate of forward progressive motility (RFP, scale of 0–5) were evaluated microscopically (10 ×) [35]. A subset of raw semen (1–3 μl) was fixed in 0.3 % glutaraldehyde diluted in PBS (50 μl) for later sperm morphology assessment (35). Aliquots (4 μl each) were spread onto two microscope slides, dried at room temperature, and later stained with fluorescein isothiocyanate-peanut agglutinin (FITC-PNA, Sigma Aldrich Corporation), for fluorescent microscopy (100–200 sperm/sample) to determine percent of intact acrosomes (ACRO) [24,37].

Once processing was completed, the remaining volume of the UC sample was split into two aliquots and each diluted 1:5 in either URF

[7] Media containing TES and Tris buffers (TES + Tris= TEST).
[8] Laparoscopic Oviductal- Artificial Insemination.

medium (soy-lecithin (SOY) with 0.2 M sucrose) or FOCM-HEPES[9] (for STRAW freezing). A sample aliquot (2.5 μl) was diluted (1:400) in water (to determine sperm concentration (CONC) using a hemocytometer method for each treatment (URFCONC, STRCONC). The diluted URF sample was allowed to equilibrate at ambient temperature (10–24°C) for five minutes and then cryopreserved using a micropipettor by individually depositing one ~ 20 μl drop directly into liquid nitrogen to create a frozen sperm pellet. This process was repeated for the entire volume, allowing each pellet to completely freeze (~ 30 s) before adding the next droplet. Frozen pellets were transferred into a labeled cryovial and stored in liquid nitrogen (for 1–6 months) until thawing for analysis. The diluted STRAW sample was centrifuged at 600 × *g* for 8 min and the resulting sperm pellet resuspended in straw-freezing medium (SOY with 4 % glycerol) to a CONC of 50×10^6 motile sperm/ml and loaded into 0.25 ml straws (30–100 μl/straw). Straws were heat sealed, transferred into a sealable plastic bag, submerged in room temperature water (100 ml) within a glass container, and cooled to 4 °C over a minimum of 2 h (maximum cooling time was 4 h). Straws were cooled initially in an electric, plug-in cooler during vehicular transport to the university laboratory and then transferred into a refrigerator for the final cooling period. Straws were then frozen using a modified two-step protocol [21,39]. Briefly, two metal racks were placed in a polystyrene foam container partially filled with liquid nitrogen (LN_2). Cooled straws were placed on the top rack (7.5 cm above the LN_2 surface) for one minute and then transferred to the bottom rack (2.5 cm above the LN_2 surface) for one minute before plunging directly into LN_2 for storage (1–6 months) until thawing for analysis.

Urine contamination occurred occasionally in both cat species during UC-based semen collection. If urine contamination was confirmed within the sample by low pH (< 8), a yellowish color, and/or sperm motility suggestive of osmotic shock under microscopic examination, the sample was immediately diluted with 1000 μl of FOCM-HEPES and centrifuged for 8 min at 600xg. The supernatant was removed, and the remaining sperm pellet diluted with 100–1000 μl FOCM-HEPES media, depending on estimated pellet volume. Sperm motility (PPM; 0–100 % and RFP; scale of 0–5, with 0 being non-motile and 5 being rapid forward progression) were evaluated microscopically. Two slides were made for later ACRO assessment using methods previously described [24], and an aliquot (3 μl) of each sample was fixed in 0.3 % glutaraldehyde diluted in PBS (50 μl) for later sperm morphology assessment [35]. At 5 min post dilution with FOCM-HEPES, the motility was re-assessed and if progressive motility remained at or above 30 %, the straw freezing protocol was completed.

For ocelots, one to three series of EEJ (2–5 V, 10 stimulations per series) were performed using techniques previously described [35]. For samples collected by EEJ (ocelots only), all series containing motile sperm were pooled and CONC was determined using a hemocytometer method. Total sperm per ejaculate (TSE) was calculated by multiplying VOL and CONC. If urine contamination occurred during an EEJ collection set, the sample was immediately diluted in FOCM-HEPES, centrifuged for 8 min at 600xg, and the supernatant removed. The sample was re-suspended with FOCM-HEPES and assessed for recovery of PPM and RFP for possible inclusion in the pooled sample. Aliquots (4 μl each) of the pooled sample were prepared as previously described to determine percent of intact ACRO [24]. The remaining diluted semen was centrifuged at 600xg for 8 min and the sperm pellet resuspended in straw-freezing medium (SOY with 4 % glycerol) to 50×10^6 motile sperm/ml. The extended semen sample was subsequently frozen using the same straw loading, cooling and freezing protocol as described above.[1, 2, 3, 4, 5, 6, 7, 8 and 9]

[9] Feline Optimized Culture Media- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2.4. Post-thaw analyses

Post-thaw sperm analyses were conducted using a standardized technique [40] with slight modification as detailed below. For heterologous IVF, domestic cat reproductive tracts were recovered immediately post-spay at a local spay-neuter clinic, stored in vials of chilled or room temperature PBS and transported to the laboratory within 1–3 h post-recovery. Ovaries were macerated in a petri dish containing FOCM-HEPES using a sterile scalpel blade to release oocytes. Recovered cumulus oocyte complexes (COCs) were graded for quality and only Grade 1 and Grade 2 oocytes were used for IVM [41] as shown in Fig. 1. For maturation, oocytes were rinsed through three pre-equilibrated microdrops of FOCM-IVM and then transferred into pre-equilibrated IVM drops (50 μl each; 10–15 COCs/drop) under mineral oil (Sigma-Aldrich Corporation). IVM dishes were transferred to a water-jacketed incubator (38.6 °C; 6 % CO_2 in air) and cultured for 24–26 h. For heterologous IVF, COCs exhibiting expanded layers of cumulus cells and an oocyte with uniformly dark cytoplasm [41] were randomly but equally divided between treatment groups (n = 10–20 COCs/treatment/cat). COCs were washed three times in FOCM-IVF and then placed in 95 μl microdrops of FOCM-IVF (10–15 oocytes per drop) under oil for pre-IVF equilibration (~ 1 h) in the incubator.

Sperm straws were thawed in the air for 10 s (sec) and then placed directly into a 38°C water bath for 30 s. The contents of each straw were emptied into a 1.5-ml microcentrifuge tube, slowly diluted with 300 μl FOCM-HEPES, and centrifuged at 300 × *g* for 8 min. The resulting sperm pellets were resuspended in 30 μl of pre-equilibrated FOCM-IVF and immediately evaluated for sperm motility (PPM, RFP (0 h)) and CONC. Sperm CONC was adjusted to 5–10 × 106 motile sperm/ml and aliquots were added to pre-equilibrated IVF microdrops (5 μl sperm into 95 μl drop, final sperm CONC ~ $2.5–5 \times 10^6$ motile sperm/ml) and motility microdrops (5 μl sperm into 20 μl drops, final sperm CONC $1–2 \times 10^6$ motile sperm/ml) under oil.

For URF pellet thawing, one sperm pellet was removed from the cryovial immersed within a LN_2 container, transferred into a warmed (38° C) glass test tube containing 100 μl FOCM-HEPES and gently mixed by hand for ~ 30 s. The sample was transferred to a microcentrifuge tube, slowly diluted with 200 μl FOCM-HEPES, and centrifuged at 300 × *g* for 8 min. The concentrated sperm pellet was resuspended in pre-equilibrated FOCM-IVF and processed for IVF as described above for straw frozen samples.

Spermatozoa and oocytes were co-incubated in IVF microdrops for 12–18 h. Oocytes then were transferred into microcentrifuge tubes containing FOCM-HEPES with 0.5 mg/ml hyaluronidase (Sigma-Aldrich Corporation) and vortexed vigorously to remove cumulus cells and loosely bound spermatozoa. Oocytes were then washed repeatedly before transferring into pre-equilibrated FOCM-IVC microdrops (95 μl each). At 48-h post-insemination, oocytes were evaluated microscopically for cleavage and developmental stage (Fig. 2) and then fixed separately as cleaving embryos or non-cleaving oocytes in FOCM-HEPES containing 1 % neutral-buffered formalin (Fisher Chemical, Fair Lawn, NJ, USA). Fixed oocytes and embryos were co-incubated with a Hoechst 33342 stain solution for 10 min (stain solution: 250 μl Hoechst 33342 added to 2.25 ml citrate buffer; 250 μl of Hoechst 33342:Citrate buffer mixture diluted 1:1 with FOCM) (Sigma-Aldrich) to determine oocyte nuclear maturation status or embryo developmental status: degenerate (D), germinal vesicle (GV), meiosis I (MI), meiosis II (MII), pronuclear (PN), number of blastomeres (BN), and number of accessory sperm (AS) bound to the zona pellucida of embryos (E) and mature oocytes (Fig. 2). Oocytes at the MII stage or pronuclear stage were classified as mature, and the presence of distinct blastomeres was considered indicative of fertilization. Fertilization percentage (FP) was calculated by dividing the total number of cleaved embryos by the total number of mature non-cleaved oocytes plus cleaved embryos and then multiplying by 100.

Motility dishes were maintained in the incubator for 24 h and

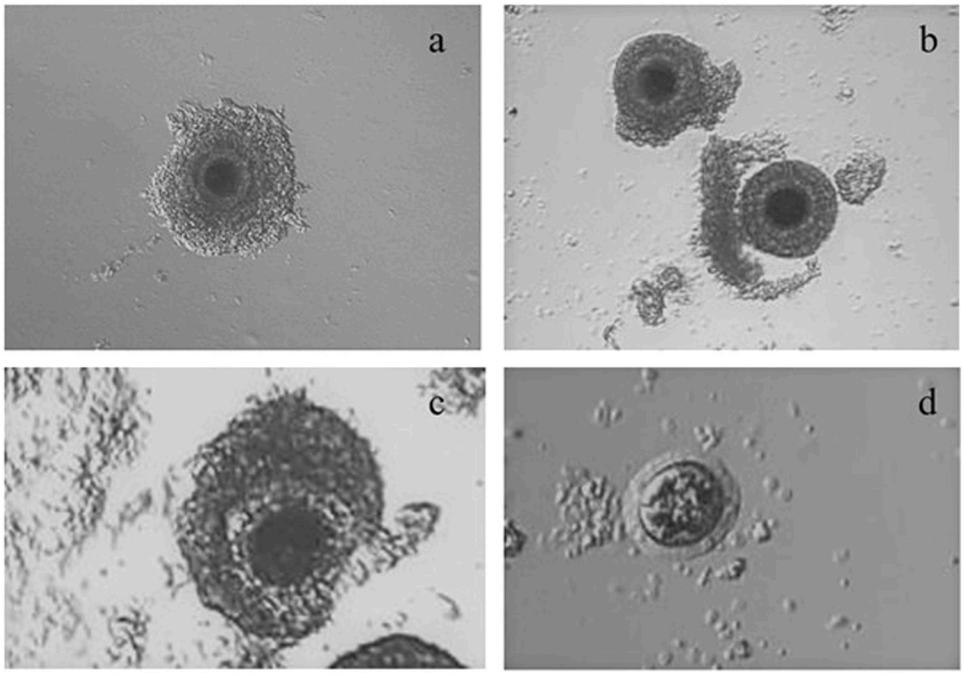

Fig. 1. Classification of cumulus oocyte complexes (COCs) utilized during heterologous IVF procedures with ocelot and bobcat sperm collected in southern Texas from 2019 to 2022. Grade 1 (a) = dark, uniform ooplasm with spherical eccentric nucleus, 5 + layers of tight cumulus cells surrounding the entire oocyte, complete intact zona pellucida; Grade 2 (b) = dark uniform ooplasm, lesser amounts of cumulus (< 5 layers) surrounding the oocyte, and intact zona pellucida; Grade 3 (c)= clear or clear patches in the ooplasm, very little cumulus, tear(s) in ZP; Grade 4 (d) = severe mosaic transparency in ooplasm, sparse complement of cumulus or denuded.

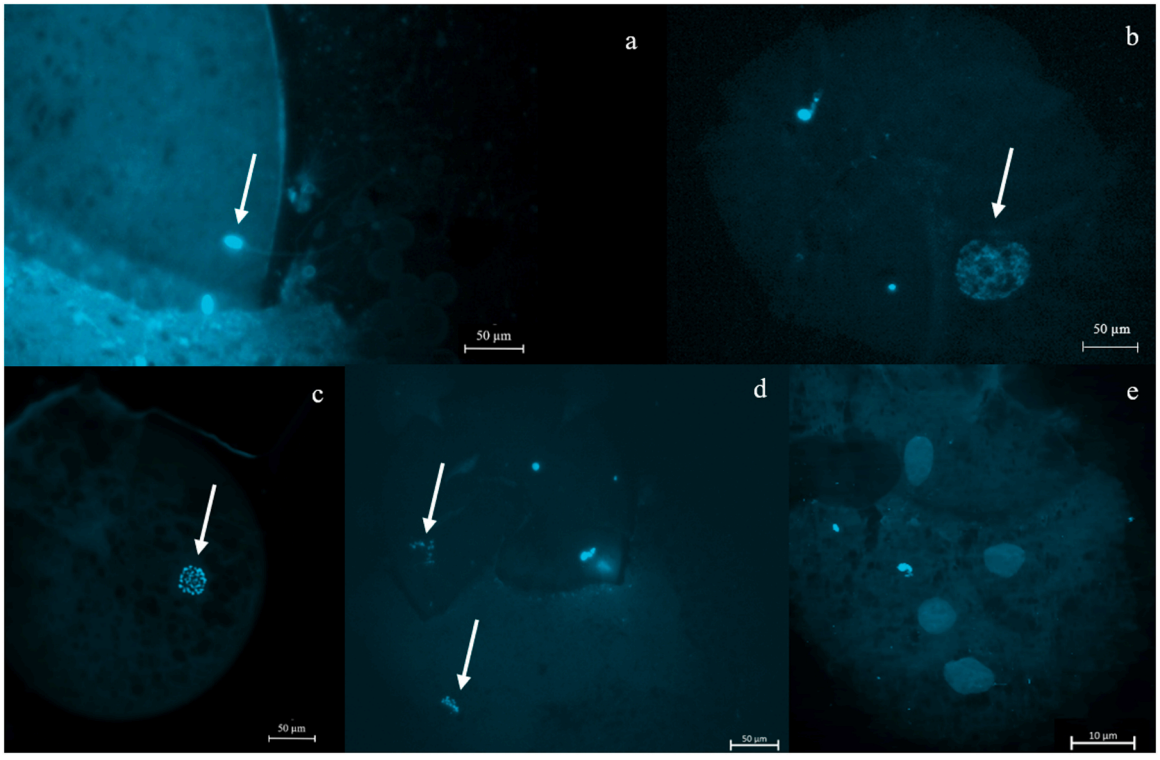

Fig. 2. Classification of oocytes and embryos from heterologous IVF procedures with ocelot and bobcat sperm collected in southern Texas from 2019 to 2022. (a) Sperm binding to the zona pellucida. (b) Germinal Vesicle (GV). (c) Meiosis I cell (MI). (d) Meiosis II cell (MII). (e) 4-cell Embryo (E). White arrows signify important cell structure for each cell type; *, denotes 4 nuclei of the 4-cell embryo.

aliquots (2.5 µl) assessed at 1, 3, 6, and 24 h of incubation for sperm motility (PPM, RFP). Sperm aliquots (4 µl each) were also used to assess percentage of intact ACRO post-thaw at 0 h and 6 h of culture. Acrosome slides were prepared and stained as previously described. Acrosome

status was classified as intact, partially intact or not intact and assessed using fluorescence (excitation 465–495/emission > 515) on a Zeiss Axioscope Fluorescent microscope equipped with AxioCam ERc5s. Oocytes and embryos stained with Hoechst were visualized using fluorescence (excitation 375 ± 28 nm; emission > 435 nm). Images were obtained and processed using Zen 3.1 (blue edition; Carl Zeiss Microscopy GmbH, Germany).

2.5. Statistical analyses

Normality was assessed using the Shapiro-Wilk test at a 95% confidence interval. If a normal distribution was reported, the mean values ± SEM were reported in the results. If there was a non-normal distribution, the median (minimum-maximum) values were reported in the results. The effect of treatment, time, and treatment by time on post-thaw parameters was assessed using mixed model analysis of variance (ANOVA) with animal as a random factor. Descriptive analysis of the number of sperm bound to mature oocytes per animal was reported as mean values ± SEM. All analyses were performed in SPSS 28 with an $\alpha = 0.05$.

3. Results

During three field seasons (from 2019 to 2022), semen was collected from 12 adult felids (n = 6 ocelots; n = 6 bobcats). Mean (± SEM) and/or median (minimum- maximum) values for pre-freeze seminal traits in ocelots and bobcats assessed in this study are presented in Table 1. For bobcats (B) and ocelots (O), there was a significant effect of post-thaw time on acrosome integrity (B, $p < 0.001$; O, $p < 0.001$), percent motility (B, $p < 0.001$; O, $p < 0.001$), and forward progressive motility (B, $p < 0.001$; O, $p < 0.001$) (Fig. 3) with all parameters declining over time. There was no significant effect of treatment (URF vs STRAW) or treatment by time for either species. Table 2 shows the mean fertilization percentage at 48 h post-insemination for both treatments (URF vs STRAW) and the mean (± SEM) of number of sperm bound to mature oocytes. For ocelots, there was only one male for which both freezing techniques were applied after urethral catheterization, with the STRAW sample showing a greater fertilization percentage (61.5%; 8/13 mature oocytes) than the URF sample (29.4 %, 5/17 mature oocytes). For bobcats and ocelots, fertilization percentages were similar (Bobcat, $p = 0.66$; Ocelot, $p = 0.133$) between the two cryopreservation techniques, although small sample size could account for this lack of significance.

4. Discussion

In this study, we have conducted the first detailed assessment of the feasibility for semen collection and cryopreservation from free-ranging wild ocelots and bobcats. Our study design incorporated the same semen collection methods, cryomedia and cryopreservation techniques previously used for semen banking of ocelots and bobcats in human-managed populations, but with our specific study design adapted for field usage. Although further research is needed across cat species, our initial results suggest that URF, particularly in combination with UC, may be of value as a rapid, field-friendly approach for collection and banking of semen from wild ocelots, bobcats and other felid species living in situ.

Although post-thaw seminal traits did not differ significantly between the two cryopreservation methods in ocelots, straw freezing tended to produce superior sperm parameters compared to URF, regardless of the semen collection technique (UC vs EEJ). In ocelots, only one sample collected by UC was cryopreserved using both freezing methods, limiting the direct comparison of IVF success with thawed semen. In addition, semen collected by EEJ was not cryopreserved using the URF method, precluding direct comparisons of URF with straw freezing. Our assessment of the interaction of semen collection method with freezing method may have been affected by the exclusion of three urine-contaminated, non-viable UC samples. In felids, UC has been suggested to reduce urine contamination risks relative to EEJ, but ocelots show a high propensity for urination with either collection method [35]. With EEJ, the negative impact of contamination with highly acidic, hyperosmotic urine is muted due to concurrent dilution with alkaline seminal fluids secreted during collection. In contrast, UC-collected samples contain minimal seminal fluid and may be exposed to urine for 10–15 min in the urethra before catheter recovery, producing highly detrimental effects on viability (i.e., motility, acrosomal, and/or morphological status). Because one goal of this study was to bank semen from the critically endangered southern Texas ocelot population, EEJ was incorporated into the study design during the 2021–2022 trapping season to maximize the opportunity for recovery and cryopreservation of their invaluable semen samples. For EEJ samples that were contaminated with urine, salvaging methods (i.e., immediate dilution with culture medium and centrifugation) allowed recovery of some viable sperm for cryopreservation, although subtle damage possibly occurred. Despite EEJ-based collection being more difficult to conduct in a field setting, this method, used in conjunction with straw freezing, remains the most consistent approach for obtaining high numbers of good quality spermatozoa with adequate post-thaw viability for in-vitro fertilization success in ocelots. However, our findings suggest that if UC samples can be recovered without urine contamination, they also may be effectively frozen by the traditional straw method, with possibly superior results compared to urine-damaged EEJ samples.

In contrast to ocelots, bobcat semen samples frozen by URF had consistently greater post-thaw seminal values as compared to straw samples, but fertilization percentages with IVF were similar between the two cryopreservation methods. A previous study of zoo-housed bobcats examined post-thaw traits in bobcat semen collected by EEJ and frozen in straws using TEST (egg yolk based) cryopreservation medium [23]. Findings showed that cryopreserved bobcat sperm could fertilize domestic cat oocytes with a relatively high (46 %) cleavage rate [23]. The heterologous fertilization percentage using this egg yolk-based cryopreservation medium was similar to that reported in semen freezing studies in other small-sized wild felid species [40]. However, compared to egg yolk-based media, cryomedium containing soy lecithin is often preferable, since non-animal-based proteins avoid the greater risk of bacterial contamination, and reduce regulatory barriers for international transport and use. Based on our initial results, additional studies in bobcats should include assessment of EEJ and straw freezing using the SOY-based cryomedium and further investigation of EEJ combined with URF. In our study, EEJ was not included as a collection technique with bobcats due to their comparatively robust conservation status and lack of urgency to develop reproductive technologies to improve bobcat population sustainability under human care or in the wild. In previous

Table 1
Mean (± SEM) values for pre-freeze seminal traits in ocelot and bobcat samples processed for cryopreservation in southern Texas from 2019 to 2022.

	Ocelot UC (n = 3)	Ocelot EEJ (n = 4)	Bobcat UC (n = 6)
VOL	113.5 (± 34.5)	468 (200–922)*	30.9 (14–60)*
CONC	415.2 (± 120.3)	29.5 (0–97)*	48.25 (5.5–122.5)*
TSE	79.25 (23.3–269.5)*	53.55 (0–152.5)*	2.28 (0.137–36.8)*
TMS	55.12 (14–80.85)*	42.85 (0–137.3)*	0.195 (0.003–14.7)*
ACRO	52 (± 10.1)	73.3 (± 5.0)	45.83 (± 7.7)
MORPH	40.3 (± 11)	58.1 (± 12.8)	23.08 (± 3.86)
PPM	50 (± 15.3)	83.8 (± 2.4)	21.3 (± 7.3)
RFP	3.3 (± 0.44)	3.9 (± 0.4)	3.25 (± 0.25)

UC, urethral catheterization collection technique; EEJ, electroejaculation collection technique; VOL, semen volume (μl); CONC, sperm concentration ($\times 10^6$/ml); TSE, total sperm per ejaculate ($\times 10^6$); TMS, total motile sperm ($\times 10^6$); ACRO, intact acrosome status (%); MORPH, normal sperm morphology (%); PPM, percent progressively motile (%); RFP, rate of forward progression (0–5); SEM, Standard Error of the Mean.

* Values reported are median values (min-max) due to a non-normal distribution.

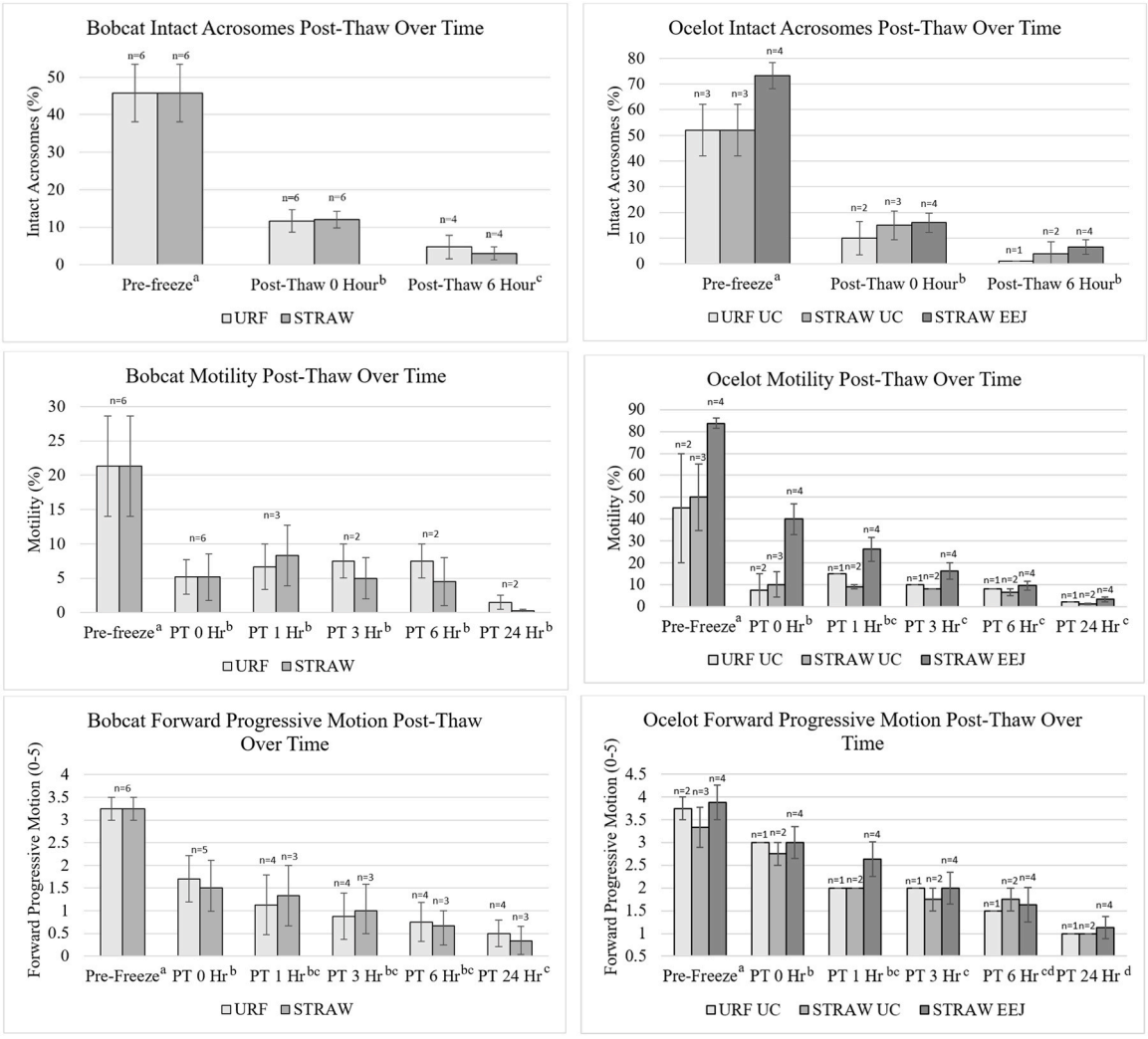

Fig. 3. Percentage of intact acrosomes, percent sperm progressive motility, and forward progressive motion over time for bobcats and ocelots in southern Texas from 2019 to 2022 comparing two semen cryopreservation techniques. URF: ultra-rapid freezing; STRAW: straw freezing; UC: urethral catheterization collection method; EEJ: electroejaculation collection method; PT: post-thaw; Hr: hours. Standard error bars are represented. Lower case superscripts denote differences ($p < 0.05$) among time points. Treatment (URF vs STRAW) did not differ and therefore, time points were assessed collectively.

studies, bobcats showed seasonal differences in semen quality with higher concentration and sperm per ejaculate [35] and IVF success [23] in spring (March-April). In our current study, all bobcats were collected and successfully cryopreserved in March indicating seasonal changes may have affected our ability to successfully cryopreserve semen and produce heterologous embryos. We were unable to compare across seasons given our limited sample size and the concentration of our samples to a single month.

Our findings also suggest that the efficacy of semen cryopreservation methods in wild felids would benefit from further refinement before wider field application. In ocelots and bobcats, both URF and STRAW freezing resulted in significant declines in sperm quality traits (i.e., acrosome integrity, sperm progressive motility, rate of forward progression) over time - from pre-freeze to immediately post-thaw to 6 h post-thaw. Sperm motility and acrosomal integrity are essential for fertilization [42–45] but damage observed following cryopreservation in other studies have been associated with decreased oocyte penetration and IVF success [17,44,46]. With natural breeding and uterine AI, spermatozoa are normally stored in-vivo within the isthmus of the oviduct [47], requiring a period of incubation for capacitation [48], and then are released close to the time of ovulation to complete fertilization [42,49]. Our findings of pronounced time-dependent reductions in post-thaw sperm motility traits and acrosome status suggest that fertilization success would be compromised using these cryopreserved sperm samples with standard uterine AI procedures.

However, with LO-AI in felids, semen typically is inseminated into the oviductal ampulla after ovulation induction. Accordingly, the total number of motile spermatozoa may be less critical for fertilization since the sample is deposited in such close proximity to mature oocytes and sperm storage is presumably not a factor. For LO-AI with frozen-thawed semen, the rate of forward progression and percent of intact acrosomes would still be critically important to ensure penetration of the cumulus cell masses and zona pellucidae to complete fertilization.

The decreased quality of frozen-thawed semen in wild felids is consistent with observations in domestic cats, with reports of up to 50 % loss of acrosomal integrity [19] and 30 % loss of progressive motility [44]. Across species, reasons for sperm quality decline with cryopreservation may be related to ice crystal formation rupturing cellular membranes during rapid cooling [50], damage to the internal mitochondrial structure [51], and premature acrosomal reaction and capacitation shortening the sperm life span and reducing fertility [45]. Although we have documented similar post-thaw reductions in acrosomal integrity and motility in ocelots and bobcats, investigation of other sperm parameters using CASA systems or alternative in-vitro assays, such as homologous zona pellucida (ZP) adhesion and/or homologous or heterologous (intraspecific) ZP penetration, could provide

Table 2
Cleavage percentage of domestic cat oocytes (mature only) at 48 h post-insemination with ocelot and bobcat cat sperm cryopreserved by ultra-rapid freezing or straw freezing in southern Texas from 2019 to 2022.

	UC-URF	UC-STRAW	EEJ STRAW
Ocelots	n = 1	n = 2	n = 4
1	29.4 (17; SB 1.88 ± 0.36)	61.5 (13; SB 2.38 ± 0.86)	
2		61.5 (13; SB 2.15 ± 0.48)	
2			31.3 (16; SB 1.56 ± 0.38)
4			33.3 (12; SB 0.92 ± 0.34)
5			15.4 (13; 0.78 ± 0.26)
6			27.3 (11; SB 1.0 ± 0.18)
Bobcats	n = 3	n = 2	
7	100 (8; SB 1.25 ± 0.25)		
8	45.5 (11; SB 0.91 ± 0.83)	54.5 (11; SB 1.27 ± 0.43)	
9	8.3 (12; SB 0.17 ± 0.11))	16.7 (18; SB 0.56 ± 0.26)	

Data are reported as the percent of mature oocytes that cleaved (total mature oocytes per treatment). Collection methods: UC = urethral catheterization, EEJ = electroejaculation; Cryopreservation methods: URF = ultra-rapid freezing, STRAW = straw freezing. SB = sperm bound to zona pellucida for mature oocytes (mean ± SEM).

further information about sperm capacitation, zona binding and acrosomal function [45,52]. In this study, a higher number of accessory sperm were bound to the ZP for samples frozen by straw cryopreservation in both cat species. Although fertilization percentages were similar for the two freezing methods in most cases, the slightly higher fertilization observed with straw samples may be a consequence of greater acrosomal damage resulting from URF, with fewer acrosomes remaining intact post-thaw over time.

Although our study confirmed that frozen-thawed ocelot and bobcat sperm can fertilize domestic cat oocytes in-vitro, similar outcomes are not guaranteed in-vivo when thawed samples are used for AI in conspecific individuals. Multiple studies have investigated IVF success using domestic cat oocytes to test functionality of frozen-thawed sperm samples from non-domestic feline species, showing no apparent barriers to cross-species fertilization [7,21,37,53–55], and allowing heterologous IVF to provide initial insights into fertilization capabilities among all felids. Because domestic cat oocytes are more readily available from spay clinics, collection of conspecific oocytes from wild felids can be avoided with its multitude of challenges (i.e., small populations, endangered intractable species, invasive oocyte recovery procedures, wastage of valuable genetic resources, etc.). However, heterologous IVF does require use of CO_2 incubators and complex media formulations to create a suitable environment for cross-species gamete support and embryo development, presumably approximating the in-vivo environment found within the oviducts of both domestic and wild felids. While our fertilization success using frozen-thawed semen with heterologous IVF in ocelots and bobcats was encouraging, absolute confirmation of post-thaw sperm function with both semen cryopreservation methods would require demonstration of fertilization and conception in-vivo following AI procedures. Although the URF method is more easily performed in the field and results in similar post-thaw parameters as seen with straw freezing, efficacy for offspring production following LO-AI has been shown to date only in two domestic cats [31]. In contrast, AI with straw-frozen semen in domestic cats and zoo-housed ocelots has produced multiple pregnancies in both species [13,15,28–30] over the past 20 years. Further studies with URF semen are warranted to assess AI success across cat species but, in the interim, wildlife veterinarians might consider applying this field-friendly method, in combination with UC, for opportunistic semen banking of wild felids. Our findings suggest, at a minimum, that those frozen samples will have value for use with IVF and could enable production of endangered felids following embryo transfer procedures.

5. Conclusions

Semen collection by urethral catheterization and subsequent cryopreservation by ultra-rapid freezing may be of value as a rapid, field-friendly approach to collection and banking of semen samples from wild felids. However, further refinement of cryopreservation methods in wild felids would be necessary before wider field application as both freezing techniques resulted in significant declines in sperm quality and function. These declines have been associated with decreased sperm functionality (oocyte penetration and fertilization) in other studies further indicating the need for refinement to utilize these samples in insemination procedures with successful outcomes. In ocelots, while urethral catheterization did result in successful semen collection, electroejaculation was superior when urine contamination was present most likely due to concurrent dilution with alkaline seminal fluids secreted during collection. Despite EEJ being more difficult to perform in the field, this method of collection combined with straw freezing remains our most consistent approach in ocelots. In contrast, collection by UC and cryopreservation by URF and straw freezing showed similar fertilization percentages in bobcats. The efficacy of EEJ collection in bobcats would need to be further explored. Exploration of semen parameters by CASA systems or alternative in-vitro assays could provide further information about functional parameters of semen samples. Furthermore, while our fertilization success in-vitro with domestic cat oocytes was promising, confirmation of sperm function with both semen cryopreservation methods would require demonstration of fertilization and conception in-vivo following AI procedures. Although several AI offspring have been produced in zoo-housed ocelots using frozen semen over the last few decades, no pregnancies have been documented following AI using semen samples from free-ranging individuals. At present, UC collection and URF cryopreservation may be most relevant for field-friendly semen banking of wild felids for future use in IVF and embryo transfer procedures.

CRediT authorship contribution statement

Debra L Miller: Writing – review & editing, Supervision, Project administration, Investigation. **Ashley M Reeves:** Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **William F Swanson:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Tyler A Campbell:** Writing – review & editing, Resources, Funding acquisition. **Michael E Tewes:** Supervision, Resources, Project administration. **Amy Miller:** Writing – review & editing, Supervision, Methodology. **Cary Springer:** Writing – review & editing, Formal analysis.

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Competing Interests

None to declare.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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