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Animal protein-free OptiXcell and shortened equilibration periods can replace egg yolk-based extender and slow cooling for rhinoceros semen cryopreservation

Jessye Wojtusik*, Monica A. Stoops, Terri L. Roth

Cincinnati Zoo and Botanical Garden, Center for Conservation and Research of Endangered Wildlife, 3400 Vine St., Cincinnati, OH, 45220, USA

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ABSTRACT

OptiXcell (OP) was tested as an animal protein-free alternative to an egg yolk-based extender for rhinoceros semen cryopreservation and shorter chilling/equilibration periods were evaluated. Semen was collected from three rhinoceros species: black (*Diceros bicornis*; n = 2), white (*Ceratotherium simum*; n = 2), and greater one-horned (GOH; *Rhinoceros unicornis*; n = 3). Controls were diluted with equine extender (EQ) or OP and equilibrated for 1 h. Treatments were diluted with extender and cooled for 15 min (fast: FEQ; FOP) or not cooled (immediate: IEQ; IOP), prior to cryopreservation. Motility decreased post-thaw (EQ: 50.7 ± 5.2%; OP: 52.9 ± 3.4%) from fresh (82.9 ± 2.9%), was higher in OP than IOP (38.6 ± 4.9%; $P \leq 0.05$) and decreased over time ($P \leq 0.05$). Post-thaw acrosomal integrity was lower in EQ, FEQ, and IEQ (56.9 ± 0.7; 56.6 ± 4.5; 54.9 ± 2.9%) than OP, FOP, IOP (71.8 ± 4.7; 71.9 ± 3.8; 69.9 ± 4.5%) and fresh (72.6 ± 1.4%; $P \leq 0.05$). Progression and viability were lower in EQ (2.8 ± 0.2; 61.9 ± 7.4%) and OP (3.1 ± 0.2; 53.4 ± 6.9%) than fresh (3.7 ± 0.2; 87.2 ± 1.3%), decreased over time ($P \leq 0.05$) but not different among treatments ($P > 0.05$). Morphology did not differ between fresh (75.0 ± 4.9% normal) and any treatment group (70.0–77.8%) or over time ($P > 0.05$). OptiXcell is comparable to egg yolk-based EQ when used for rhinoceros semen cryopreservation. Furthermore, chilling/equilibration can be reduced with little impact on sperm characteristics.

1. Introduction

Semen collection and cryopreservation are well established in three rhinoceros species: black rhinoceros (*Diceros bicornis*), white rhinoceros (*Ceratotherium simum*), and greater one-horned rhinoceros (GOH; *Rhinoceros unicornis*) [15–17,22,31,34,35,40]. As managed rhinoceros individuals and populations are often spatially segregated among institutions, assisted reproductive technologies (ARTs) can facilitate maintenance of genetic diversity [15,35]. However, few institutions have the equipment and/or expertise to collect, process, and store cryopreserved semen as per current protocols. Furthermore, in some cases, samples must be collected and processed in less than ideal field-type settings that often require flexibility in transport, storage, and processing techniques for optimal success. Modification of protocols to minimize necessary resources and add flexibility to strict protocols could facilitate semen collection procedures and encourage technique utilization.

Semen cryopreservation for rhinoceros species relies on the use of extenders which contain animal protein, such as egg yolk [16,17,35,40], to provide phospholipids to protect against cold shock and maintain sperm membrane integrity and therefore post-thaw motility and viability [7,28]. However, egg yolk is at risk of bacterial or viral contamination which may impact the fertilizing capacity of sperm [9,10,36]. Furthermore, egg yolk could act as a vector for bird-related diseases during transport, storage, or handling, raising concerns for regulatory agencies tasked with biosecurity [19,36]. Stricter regulations regarding semen transport due to extender components could hinder the application of ART for wildlife conservation. Egg yolk composition is variable dependent upon diet and health of the producing hens [11] and difficult to replicate with precision. Storage and transport of extenders containing egg yolk requires refrigeration. Furthermore, egg yolk can interfere with semen assessment, particularly computer-assisted sperm assessment (CASA), due to opaque composition and microscopic droplets within the extender [32]. To address the potential risk of microbial contamination and biosecurity concerns [19], several studies have investigated the efficacy of replacing egg yolk

* Corresponding author.
E-mail address: jessye.wojtusik@cincinnatizoo.org (J. Wojtusik).

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with vegan alternatives for a variety of species, with mixed outcomes [1,37,40]. OptiXcell (IMV Technologies U.S.A., Maple Grove, MN 55369, USA) is a commercially available animal protein-free, liposome-based extender that promotes sperm motility and viability in bulls [4], deer [33], and buffalo [5] and performed equally well to traditional extenders for bulls [21]. As OptiXcell does not contain animal products, risk of pathogenic contamination is minimal and there is negligible variability among batches. The extender is transparent, aiding assessment, and can be transported at room temperature (RT), facilitating its use in settings where refrigeration may not be an option.

The protective effect of liposome-based extenders, like OptiXcell, is reliant on phospholipids though the mechanism is not entirely understood. Liposomes, which are spherical vesicles made up of lipid bilayers, may deliver phospholipids to the sperm membrane or replace those lost during the cryopreservation process, providing stability and increasing chilling resistance [28,39,41]. The inclusion of liposomes to semen extenders improved post-thaw motility in stallions [39] and buffalo [18] and pregnancy rates of horses inseminated with sperm cryopreserved with liposomes were comparable to those achieved using other extenders [13].

Chilling of a semen sample prior to cryopreservation is a common technique intended to minimize damage associated with cold shock during spermatozoa transition from body temperature to a cryopreserved state [6]. Many protocols include an additional equilibration step during which a permeating cryoprotectant is added to allow the cryoprotectant time to enter the cell. Species-specific biophysical properties influence membrane permeability and interaction with cryoprotectants, which impact optimal cooling rates and cryosurvival [12,24]. Therefore, the timing of equilibration in relation to chilling and the necessity or benefit of including an equilibration step is often species dependent [14,27]. Standard rhinoceros semen cryopreservation protocols generally require a combined chilling and equilibration period of 1–2 h [16,23,25,34,40] though Hermes et al. limited equilibration to 45 min without detriment [17]. Glycerol, one of the commonly used cryoprotectants for rhinoceros semen cryopreservation [17,22,34,40], permeates cell membranes quickly [8] and employing a typical equilibration period of 1–2 h may be superfluous. A protocol with shortened equilibration time may add flexibility and ease to procedures, especially those conducted in the field, allowing samples to be frozen and stored quickly after collection.

This study investigated adjustments to rhinoceros semen cryopreservation methods that would obviate the need for an animal protein based medium and simplify current protocols without compromising sample value. The two specific goals were to 1) test OptiXcell as an animal protein-free alternative to egg yolk-based extenders for cryopreservation of rhinoceros semen and 2) evaluate the impact of shorter equilibration time periods during semen processing on sperm characteristics post-thaw.

2. Materials and methods

All chemicals were obtained from Sigma Aldrich (St. Louis, Missouri 63146, USA) unless otherwise noted.

2.1. Semen collection

All procedures were reviewed and approved by the Cincinnati Zoo and Botanical Garden's Animal Care and Use Committee (IACUC; protocol #14–120). A surgical plane of anesthesia was induced in seven adult male rhinoceros (two black rhinoceros, three GOH rhinoceros, two white rhinoceros; aged 10–26 years). Anesthetic drug choice and protocols were defined and administered at the discretion of veterinary staff at each of the participating institutions and therefore differed among individual rhinoceros. Various combinations of azaperone, butorphanol, detomidine, etorphine, flunixin, ketamine, midazolam, and/or medetomidine were selected, informed by personal experience of veterinary staff and established protocols [2,23,29,40]. Semen was collected via electroejaculation using rectal probes designed specifically for each species [31; Innovative Zoological Solutions, Cincinnati, OH 45205, USA) and an electroejaculator (Innovative Zoological Solutions; P-T electronics, Boring, OR 97009, USA). Stimuli (mean sum: 84.0 ± 7.3) of 2–9 V were administered over the course of 2–3 series with 5 min periods of rest between series. Fractions of semen were collected into whirlpak bags (Nasco, Fort Atkinson, WI 53538, USA) that were switched out intermittently to limit any possible urine contamination and stored in an insulated container until evaluation.

2.2. Semen cryopreservation

Post-collection semen samples were evaluated on pre-warmed slides (37 °C) at 200x magnification for motility (% motile sperm) and progressive status (scale of 0–5; 5 = all motile sperm displaying rapid linear progression) using phase contrast optics. Five fields of view were evaluated per slide [23,40]. Sperm concentration was determined using a hemocytometer (American Optical, Buffalo, NY, 14215, USA). Only samples exhibiting greater than 60% motility and a concentration greater than 125×10^6/mL were included in this study.

For each rhinoceros, semen sample aliquots (500 μL) from the same ejaculate fraction were used for all controls and treatments. Traditional method control aliquots were diluted 1:1 with established equine semen extender (EQ; containing lactose (5.5% v/v), disodium EDTA (0.25% w/v), egg yolk (20% v/v), glucose (1.5% w/v), Equex STM (0.25% v/v; Nova Chemical, Moon Township, PA 15108, USA), 25 iu penicillin G mL^{-1}, 25 iu streptomycin mL^{-1}) and cooled in a water bath to 4–5 °C (~1.5 h), diluted 1:1 stepwise (25, 25, 50% v/v every 20 min) with extender containing 10% glycerol, and allowed to equilibrate at a final concentration of 5% glycerol for 1 h at 4 °C [22,34,40]. OptiXcell (containing carbohydrate, mineral salts, buffer, antioxidants, glycerol, phospholipids, water, and antibiotics: gentamicin, tylosin, linomycin, and specomycin) was prepared for use as per manufacturer's instructions (IMV Technologies). As OptiXcell contains glycerol (final concentration: 6.4%), the OptiXcell control sample (OP) was diluted 1:1 with RT OptiXcell and then chilled in a water bath at 4 °C for 1 h to mirror the cryoprotectant equilibration period of the EQ control sample. For the equilibration treatment groups, aliquots were diluted drop-wise, 1:1 with EQ + 10% glycerol or OptiXcell, accordingly, and equilibrated in a water bath at 4 °C for 15 min (fast; FEQ and FOP) or not subjected to an equilibration period (immediate; IEQ and IOP). Neither the fast nor immediate treatment groups were subjected to a chilling period prior to equilibration. All groups were loaded into 0.5 mL straws and lowered into a charged dry shipper (depth: 42 cm, capacity 3.6 L; Chart MVE Biomedical, Ball Ground, GA 301107) for 10 min before being plunged into liquid nitrogen (cooling rate: ~ −15 °C/min for 5 °C to −10 °C; ~ −40 °C/min for −10 °C to −60 °C) [30]. Straws (two straws/treatment/individual) were thawed for 10 s at RT then 37 °C for 20 s, maintained at RT protected from light and evaluated for sperm motility, viability, morphology, progressive status, and acrosomal integrity immediately (hour 0) and 1, 4, and 24 h post-thaw. For consistency, all assessments were conducted by the same individual and treatment group was withheld from assessor until assessments were complete.

2.3. Acrosomal integrity, morphological and viability assessment

Acrosomal integrity was assessed via staining with fluorescein-conjugated *Archis hypogeal* peanut agglutinin (FITC-PNA), as previously described [34,40] and examined using fluorescent microscopy at 400x (100 spermatozoa/straw). Acrosomes were considered 'intact' if there was staining of the apical ridge. If staining was incomplete, acrosomes were classified as 'damaged' or if staining was limited to the equatorial region or completely missing, as 'non-intact'. Morphological assessments were conducted under 400x magnification using phase contrast

optics (100 spermatozoa/straw), as previously described [22,34,40]. Morphological abnormalities were categorized as primary defects, those that impact the sperm head or midpiece, such as micro- or macro-cephaly and damaged midpieces or as secondary defects including bent midpieces or tails and proximal and distal droplets [40]. Spermatozoa absent of abnormalities were considered 'normal'. Viability was evaluated via staining with eosin-nigrosin live/dead exclusion stain (Jorgensen Laboratories, Inc., Loveland, CA 80538, USA) [23,40]. Slides were assessed under 400x brightfield magnification (200 spermatozoa/straw). Partial or full incorporation of the stain indicated the cell was non-viable.

2.4. Statistical analysis

Data from all three rhinoceros species were included as a set for statistical analysis [40]. Two straws per treatment per individual were assessed for all parameters to account for variability among straws; the mean value of the two straws was used for the analysis. To compare differences in post-thaw sperm characteristics between OP and EQ, data were subjected to paired samples *t*-test. To evaluate differences within the complete data set (all treatment groups), data were analyzed using repeated measures general linear model as it is the most appropriate test for interpreting these data, despite the violation of the assumption of normality due to small sample size. Sperm characteristics (motility, progression etc.) were subject to Mauchly's test which indicated none of the parameters violated the assumption of sphericity ($P > 0.05$). Analysis included post-hoc Bonferroni for pairwise comparisons. Data is presented as mean ± SEM and statistical significance was defined as $P \leq 0.05$. Statistical analyses were conducted using SPSS for Windows (Version 24; IBM Corporation, Armonk, NY 10504, USA).

3. Results

Fresh (undiluted, raw semen within 5 min of collection) ejaculate characteristics of black rhinoceros, GOH rhinoceros, and white rhinoceros collected via electroejaculation are presented in Table 1. Post-thaw motility (Fig. 1), progressive status, and viability were significantly decreased in all treatment groups compared to fresh ejaculate ($P \leq 0.05$). Morphology did not differ between fresh and post-thaw samples ($P > 0.05$). Most morphological abnormalities (~82%) were secondary defects. Acrosomal integrity decreased in EQ, FEQ, and IEQ post-thaw ($P \leq 0.05$) but not in OP, FOP, or IOP ($P > 0.05$) when compared to fresh.

3.1. EQ versus OP

Post-thaw motility (Fig. 1), progressive status, and morphology did not differ between EQ and OP treatment groups at any time point ($P > 0.05$). Viability did not differ between EQ and OP treatment groups at hours 0, 1, and 4 ($P > 0.05$), but was greater in the EQ group at 24 h post-thaw ($P \leq 0.05$). Acrosomal integrity was greater in the OP control treatment group than the EQ control group at 0, 1, and 24 h ($P \leq 0.05$) but was not different at 4 h ($P > 0.05$).

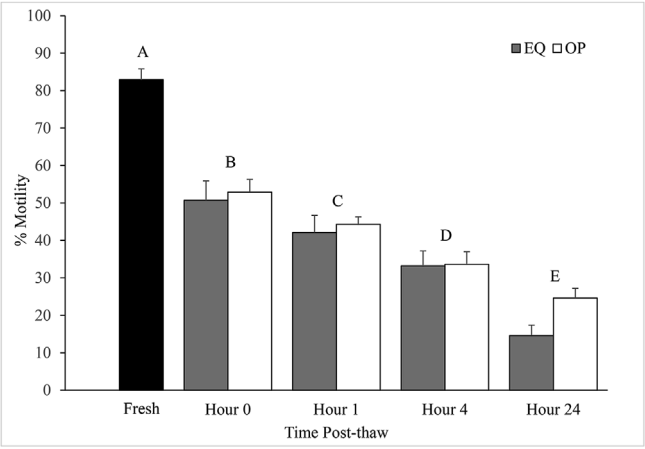

Fig. 1. Impact of semen extender treatment, egg yolk-based equine extender (EQ) versus animal protein-free OptiXcell (OP) on fresh rhinoceros sperm % motility and at 0, 1, 4, and 24 h post-thaw (n = 7). Data represents mean ± SEM. Different letters indicate statistical differences among each time point ($P \leq 0.05$). There were no significant differences between groups at each time point.

EQ and OP extender were impacted by time in similar patterns. Motility decreased at each time point ($P \leq 0.05$; Fig. 1). Viability and acrosomal integrity were lower at 4 and 24 h than 0 and 1 h ($P \leq 0.05$). Progressive status did not decrease until 24 h and morphology did not change over the course of any time points ($P > 0.05$).

3.2. Equilibration times

Post-thaw data for all equilibration treatment groups and corresponding extenders are summarized in Table 2. Morphology did not differ among treatment groups ($P > 0.05$) and did not change over time ($P > 0.05$). Progressive status did not differ among treatment groups ($P > 0.05$) but decreased at each time point post-thaw ($P \leq 0.05$). Motility was higher in OP than IOP ($P \leq 0.05$) but otherwise did not differ among treatment groups ($P > 0.05$) and decreased at each time point ($P \leq 0.05$). Acrosomal integrity was greater in all OP groups than all EQ groups and decreased at each time point ($P \leq 0.05$). Viability was greater in EQ than FOP and IOP ($P \leq 0.05$) but did not differ among all other treatments ($P > 0.05$) and decreased at each time point ($P \leq 0.05$).

Table 1
Fresh ejaculate characteristics for black rhinoceros (n = 2), GOH rhinoceros (n = 3), and white rhinoceros (n = 2) collected via electroejaculation.

	Black	GOH	White		
	Rhinoceros	Rhinoceros	Rhinoceros	Overall	
Characteristics	Mean	Mean	Mean	Mean ± SEM	Range
Seminal volume (mL)	55	28.6	13.5	31.8 ± 8.7	7.9–73.5
Sperm motility (%)	85	80	85	82.9 ± 2.9	70–90
Progressive status (0–5)	3.8	3.8	3.5	3.7 ± 0.2	3.0–4.5
Total concentration (x10^6/mL)	275	545	837.5	383.0 ± 109.2	135.0–837.5
Total count (x10^9)	13	9.7	7.5	10.1 ± 3.0	1.4–20.2
Sperm viability (%)	89.3	85	88.5	87.2 ± 1.3	83.0–93.0
Intact acrosomes (%)	73.5	73.9	69.9	72.6 ± 1.4	68.0–77.2
Normal morphology (%)	64.5	78.7	80	75.0 ± 4.9	58.0–89.0
Seminal pH	8.2	8.4	8.5	8.4 ± 0.1	7.7–8.7

Table 2
Post-thaw (hour 0) rhinoceros sperm characteristics of egg yolk-based equine (EQ) and OptiXcell (OP) extenders and equilibration times (n = 7).

	Motility	Progression	Acrosomes	Viability	Morphology
	(% motile)	(0–5 scale)	(% intact)	(% alive)	(% normal)
EQ	50.7 ± 5.2^{AB}	2.8 ± 0.2^{A}	56.9 ± 0.7^{A}	61.9 ± 5.6^{A}	70.0 ± 6.4^{A}
FEQ	48.6 ± 5.9^{AB}	2.9 ± 0.2^{A}	56.6 ± 4.5^{A}	55.9 ± 3.8^{A}	77.1 ± 4.2^{A}
IEQ	50.7 ± 3.0^{AB}	3.0 ± 0.2^{A}	54.9 ± 2.9^{A}	54.8 ± 2.1^{A}	77.8 ± 4.6^{A}
OP	52.9 ± 3.4^{A}	3.1 ± 0.2^{A}	71.8 ± 4.7^{B}	53.4 ± 5.2^{A}	74.2 ± 5.8^{A}
FOP	42.1 ± 2.6^{AB}	3.1 ± 0.1^{A}	71.9 ± 3.8^{B}	38.2 ± 2.3^{A}	75.1 ± 6.3^{A}
IOP	38.6 ± 4.7^{B}	3.0 ± 0.2^{A}	69.9 ± 4.5^{B}	39.1 ± 3.9^{A}	75.1 ± 4.9^{A}

F = fast equilibration (15 min), I = immediate equilibration (0 min). Data presented as mean ± SEM. Different letters indicate statistical differences within each characteristic measure ($P \leq 0.05$).

4. Discussion

This study demonstrated that animal protein-free OptiXcell is comparable to traditional egg yolk-based extender for protecting rhinoceros sperm through cryopreservation and subsequent thawing. Rhinoceros semen samples frozen in the presence of OptiXcell displayed similar post-thaw characteristics to those cryopreserved in a traditional egg yolk-based equine extender (EQ). Motility, progressive status, and morphology did not differ between the two types of extender at any measured post-thaw time point. These results are consistent with results in bulls [21] which demonstrated that OptiXcell performed equally well to traditional egg yolk-based extenders. At 24 h post-thaw, viability was greater in the EQ treatment group, demonstrating greater longevity, though the importance of this finding is likely limited as semen is not generally thawed so far in advance of a procedure and once the sample is deposited into a female, the extender is diluted by fluid within the female reproductive tract. Acrosomal integrity was greater in OP treatment groups in all but one time point, possibly due to the greater glycerol content in OptiXcell (6.4% versus 5.0% in EQ) and perhaps indicating greater ability to maintain fertilizing capacity. Increased glycerol content may also account for decreased longevity in OP samples due to glycerol's cytotoxic nature. Hermes et al. limited the cytotoxic effect of glycerol by reducing its concentration and including methylformamide [17]. As OptiXcell contains glycerol, it is not possible to reduce the concentration without diluting the other components as well, but it may be worth investigating the addition of methylformamide to OptiXcell. Dilution in OptiXcell was not detrimental to non-return rate in dairy cows inseminated with frozen-thawed semen [21] and fertility rates were improved in bulls [4] and buffalo [5] when semen was frozen with OptiXcell, therefore it appears that OptiXcell does not hinder fertilizing capacity of sperm. However, this has yet to be tested in rhinoceros species.

Traditional methods of rhinoceros semen cryopreservation involve slow and lengthy equilibration periods for both chilling and cryoprotectant exposure [16,23,25,34,40], but data from this study indicate these periods are not always necessary. Although equilibration is touted as benefitting sperm by avoiding cold shock and allowing permeation by the cryoprotectant [6], it is not similarly required by all species [8,20,27]. Reduction of the combined chilling and equilibration period to 15 min (fast) and 0 min (immediate) had no impact on post-thaw characteristics of treatment groups extended with the traditional EQ extender. This pattern did not hold true for samples diluted with OptiXcell; though progressive status, acrosomal integrity and morphology were not impacted by the shortened equilibration, motility was decreased in OptiXcell samples frozen without an equilibration period (IOP). However, ~15–20% more acrosomes were intact in all OptiXcell samples which might compensate for the reduction in motility. Furthermore, though not statistically significant herein, viability tended to decrease in both fast and immediate treatment groups, and with additional samples, data would likely reflect significance.

The discrepancy in cryosurvival following reduced or eliminated equilibration between extender types in this study is surprising because both extenders contain glycerol. It is possible the reduced chilling/equilibration period did not allow enough time for the liposomes within OptiXcell to fuse with the sperm membrane limiting their protective effect or perhaps, slowed the permeation of glycerol. Regardless, a chilling period prior to equilibration is not necessary when using either extender for rhinoceros cryopreservation, reducing total processing time by 1 h or more. The response to shortened equilibration periods is not universal among taxa [20,27]. Pradiee et al. successfully shortened equilibration to 15 min when processing epididymal sperm from Iberian Ibex without detriment [26]. However, a follow up study encompassing additional species and ejaculated sperm revealed a species-dependent response to shortened equilibration periods and demonstrated a difference in response for ejaculated sperm suggesting composition of the sperm membrane also influences response [27]. The timing of glycerol addition relative to chilling may also impact post-thaw measures, with results varying among taxa with no discernable pattern. Stallion sperm benefitted with increased motility and fertility when glycerol was added at RT [38] whereas timing did not impact brown bear sperm characteristics [3]. In contrast, domestic cat sperm benefitted from glycerol being added after the sample was chilled [37]. As OptiXcell contains glycerol, it is not possible to adjust when glycerol is added to the sample in relation to chilling.

All post-thaw characteristics, excluding acrosomal integrity in OptiXcell diluted samples and morphology for all treatment groups, declined as compared to fresh ejaculate values. Despite the use of semen extenders and cryoprotectants, cryopreservation and subsequent thawing of rhinoceros semen results in a typical loss of motility of 15–30% often accompanied by a decrease in progressive status, viability, and acrosomal integrity [15–17,23,25,34,40]. Future studies should investigate methods for further reducing such losses to ensure the use of samples of the highest quality for assisted reproductive technologies.

Results of this study indicate that two simple changes to the traditional rhinoceros semen cryopreservation protocol can be adopted without compromising post-thaw sperm characteristics. First, the animal protein-free extender, OptiXcell is comparable to egg yolk-based equine extender in preserving rhinoceros sperm characteristics following cryopreservation. Additionally, glycerol equilibration can be eliminated for samples diluted with EQ extender and limited to ~1 h for those diluted with OptiXcell, greatly reducing the amount of time needed to process samples prior to cryopreservation. Such modifications simplify rhinoceros semen cryopreservation, which may broaden its adoption and implementation by others, an important step in the effort to collect genetic material from valuable individuals, bolster genome resource banks, and ensure the genetic health of future managed populations.

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Conflicts of interest

None.

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