IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.1 Ser. No.86 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Short Paper

Characterization and seminal cryopreservation of three species of birds of prey

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10.22099/IJVR.2024.47943.6962

(Received 25 Jul 2023; revised version 23 Jan 2024; accepted 12 Feb 2024)

Abstract

Background: Assisted reproduction techniques in birds have contributed to many species' conservation and sustainable use. One of these techniques is semen cryopreservation, which is possible following the discovery of suitable cryoprotectants. **Aims:** This study aimed to characterize the fresh and post-thaw ejaculates of different species of birds of prey. **Methods:** The following species were included in the study: red-tailed hawk (*Buteo jamaicensis*) n=3, golden eagle (*Aquila chrysaetos*) n=3, and Harris's hawk (*Parabuteo unicinctus*) n=3. Twenty-five ejaculates were obtained for each species. The percentage of spermatozoa motility, viability, and morphology were evaluated. **Results:** Evident differences were observed among the ejaculates of the three species, particularly in sperm length and between the fresh and post-thaw parameters of the same species in which the motility reduced to approximately 40% after thawing. It was demonstrated that sperm cryopreservation of the studied species was possible using the same freezing protocol. **Conclusion:** This study showed that sperm characteristics could influence the parameters obtained during their *in vitro* conservation, both in the fresh and post-thaw states.

Key words: *Aquila chrysaetos*, *Buteo jamaicensis*, Falconiforms, *Parabuteo unicintus*, Spermatozoa

Introduction

Very little is known about the reproductive biology of raptors, both in the wild and captivity (Asano and Tajima, 2017). Approximately 10% are endangered (Dogliero *et al*., 2016). *Ex situ* reproduction using assisted reproduction techniques in birds is limited (Blanco *et al*., 2009); however, advancements in this area can contribute to these species' conservation and sustainable management (PREP, 2012). There is experience in semen collection and its preservation either in the fresh or cryopreserved state for subsequent use in artificial insemination, which has been successfully employed in over 40 species of birds under human care, including raptors, cranes, waterfowl, galliformes, and psittacids (Blanco *et al*., 2010; Long *et al*., 2014), and other species that interest (Nazeri *et al*., 2023).

The quality and characteristics of the ejaculate can be influenced by individual factors such as tolerance to handling (Umapathy *et al*., 2005). On the other hand, it is known that seminal characteristics vary among species; for example, sperm concentration is higher in domestic birds (Santiago *et al*., 2011) compared to wild species (Herrera *et al*., 2005; Umapathy *et al*., 2005), nevertheless, ejaculates in the latter have not been fully characterized. There are some differences between the ejaculates of domestic and wild birds, such as pH, which is more acidic in birds of prey due to their strictly carnivorous diet (Herbert *et al*., 2011). That is why it is necessary to carry out species-specific studies that allow us to understand the characteristics of semen in birds of prey to increase the success rate of assisted reproductive techniques. Furthermore, it is known that exposure to physical and chemical factors during the freezing process can modify the receptors involved in gamete recognition, interfering with the sperm membrane and its fertilizing capacity (Herrera *et al*., 2005). Therefore, the objective of this study was to characterize the sperm characteristics of the golden eagle (*Aquila chrysaetos*), red-tailed hawk (*Buteo jamaicensis*), and Harris's hawk (*Parabuteo*

unicinctus) in fresh and post-thaw conditions.

Materials and Methods

Three adult specimens of each species were included in the study: golden eagle (*Aquila chrysaetos*), red-tailed hawk (*Buteo jamaicensis*), and Harris's hawk (*Parabuteo unicinctus*). All species were under human care and assessed beforehand to be considered semen donors. The birds were housed in the central geographical area of Mexico. The handling of each specimen was carried out according to the Management Plan to comply with the conditions of animal care and welfare, authorized by the General Directorate of Wildlife (DGVS) of the Ministry of Environment and Natural Resources (SEMARNAT) Mexico; authorization was granted to each collection: Animal Kingdom Park: DGVS-ZOO-P-0074-03-MEX/América Football Club: DGVS-PIMVS-CR-IN-1847-CDMX/18/DILAJESH Aviary: DGVS-CR-IN-917-MEX/06.

Obtaining ejaculates

Twenty-five ejaculates per bird were obtained using the dorsoventral massage technique (Herrera-Barragán *et al*., 2021).

Basic semen evaluation

In each fresh sample, the ejaculated volume was directly determined in the pipette used for collection. The concentration was measured through optical microscopy using a Neubauer camera. Progressive mobility was determined in fresh and thawed samples by optical microscopy (×1000) to estimate the percentage of spermatozoa with vigorous movement. Sperm viability and morphology were evaluated in the same preparation using a vital staining method with eosin (1% eosin and 5% nigrosin). Two hundred sperm cells were evaluated per preparation under a phase contrast microscope. Morphology was assessed at a magnification of ×1000 (Herrera *et al*., 2005; Fischer *et al*., 2020).

Morphometric parameters

Sperm morphometry was determined in eosin-nigrosin-stained preparations using an Olympus BX51 microscope with Image-Pro 6.2 software (×1000), counting at least 1000 spermatozoa from each bird included in the study. The total length of each spermatozoon was measured, as well as the specific lengths of the head, neck, and tail (Fischer *et al*., 2020).

Sperm cryopreservation

Freezing and thawing were achieved by adapting two techniques described by Santiago *et al*. (2011). The semen was diluted in 50 µL with Beltsville Poultry Semen Extender (Sexton and Giesen, 1983) at 25°C supplemented with 7% dimethyl sulfoxide (DMSO) as a cryoprotectant. Aliquots of 30 µL were aspirated using a 0.25 ml straw, kept at 5°C for 10 min for stability, then frozen in liquid nitrogen vapor at -70°C for 10 min, and subsequently immersed directly in liquid nitrogen at -196°C for at least 30 days. The thawing of the straws was performed at 25°C for 30 s.

Statistical analysis

The analysis of the results was performed using the PAST software. The parameters of basic sperm evaluation were analyzed using the Wilcoxon test.

Results

Fresh sperm analysis

Variability in sperm concentration was observed among species, as has been previously reported in other raptor birds such as *F. mexicanus* (Boyd *et al*., 1917) and *B. jamaicensis* (Herrera *et al*., 2017).

The ejaculate volume of the three species also exhibited significant variability. *A. chrysaetos* (11.56 ± 2.14 µL) and *B. jamaicensis* (3 ± 1 µL) displayed the highest volume values, while *P. unicinctus* (2.68 ± 0.64 µL) exhibited a slightly lower volume.

The lowest value of sperm motility was observed in *P. unicinctus*, whereas in the other two species, motility was significantly higher ($P<0.0001$).

Regarding normal sperm morphology, only in *B. jamaicencis* were the parameters in fresh samples like those determined post-thawing ($P<0.05$). In *A. chrysaetos* and *P. unicinctus*, the parameters of normal morphology were lower in fresh samples than in post-thawed ones ($P<0.001$).

Sperm morphometry

The shape of the spermatozoa from the studied species corresponds to the general description known for birds: an elongated head, scarce cytoplasmic content, and a prominent flagellum (Fig. 1). When comparing the morphometric indicators of the spermatozoa from the three bird species (Table 1), a significant difference in the total length of the spermatozoa was observed ($P>0.001$).

Table 1: Morphometric parameters of spermatozoa in the three species studied

Length (µm)	*Aquila chrysaetus*	*Buteo jamaicencis*	*Parabuteo unicinctus*
Head	11.50±0.90	6.40±1.00	11.50±1.30
Neck	2.60±1.50	2.40±0.80	3.00±0.60
Flagellum	48.70±3.50	21.90±1.60	49.80±2.70
Total length	62.80±4.00	30.90±2.30	64.40±2.90

The sperm length of *B. jamaicensis* (30.90 ± 2.30 µm) was 50% shorter than that of *A. chrysaetos* (62.80 ± 4.00 µm) and *B. jamaicensis* (64.40 ± 2.90 µm). The differences in the neck length among the three species indicate that they are significant ($P<0.001$). The size of the flagellum undoubtedly makes the largest contribution to the total length of the sperm of all three species. The

Table 2: Basic sperm evaluation of the three species studied

Sperm count	*Aquila chrysaetuos*		*Buteo jamaicensis*		*Parabuteo unicinctus*	
Ejaculated volume (µL)	11.56±2.14		3.00±1.00		2.68±0.64	
Sperm concentration ($\times 10^6$/ml)	3.94.00±388.00		2.06±0.25		1.60±0.15	
Sperm quality	Fresh	Thawed	Fresh	Thawed	Fresh	Thawed
Sperm viability (%)	73.91±6.19[a]	56.26±7.94[b]	91.20±3.68[a]	77.92±4.99[b]	84.63±9.50[a]	69.09±8.83[b]
Sperm motility (%)	74.39±7.61[a]	47.39±7.36[b]	75.60±9.38[a]	40.00±8.66[b]	64.90±10.64[a]	35.45±5.09[b]
Sperm abnormalities (%)	2.69±1.29[a]	9.86±2.28[b]	3.64±1.77[a]	3.68±1.54[a]	3.86±2.64[a]	11.59±4.75[b]

Different labels in the same parameter (fresh/frozen) and same species indicate P<0.001 (Wilcoxon test)

longest flagellum corresponds to the sperm of *P. unicinctus*, while the shortest flagellum corresponds to the sperm of *B. jamaicencis* (Table 2).

The length of spermatozoa is greater in the ejaculates of flock-living birds such as *P. unicinctus*, compared to monogamous birds (Blas *et al*., 2011), such as *B. jamaicensis* (Dogliero *et al*., 2015) and *A. chrysaetos* (Villaverde-Morcillo *et al*., 2015).

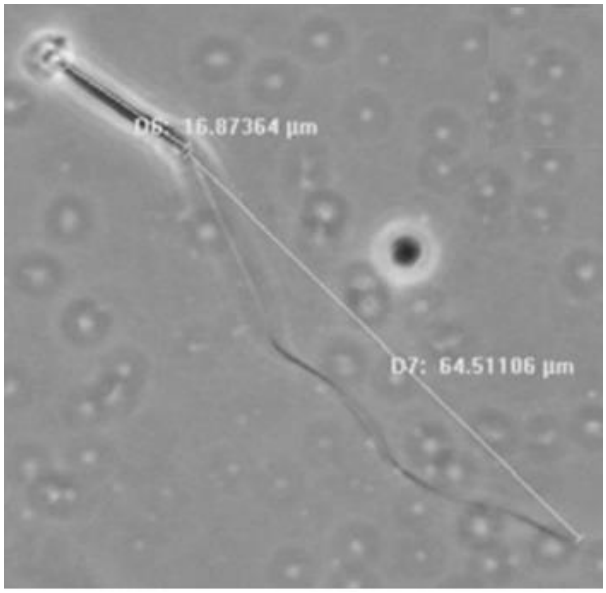

Fig. 1: The normal morphology for raptor spermatozoa is an elongated head, low cytoplasmic content, and prominent flagellum (source: own work)

Post-thawing indicators

In the three species studied, sperm motility and viability were approximately 40% lower (P<0.001) in the post-thawed samples compared to fresh ejaculates (Table 2).

Discussion

The ejaculate volume of *B. jamaicensis* and *P. unicinctus* were similar, in contrast to the considerably higher volume observed in *A. chrysaetos*. This disparity may be attributed to the species' body weight, with the former weighing approximately 1.5 kg, whereas *A. chrysaetos* can weigh up to 4.0 kg. It is widely recognized that a strong correlation exists between this parameter and the size and weight of birds (Etches, 1996). It is important to highlight that sperm viability in *A. chrysaetos* exhibited a significant decrease (P<0.0001) in the collection month, alluawith the highest sperm viability observed in March. This finding aligns with the research of Blanco *et al*. (2000), who reported varying sperm viability percentages ranging from 50.8% to 93.2% in raptor species such as the golden eagle (*Aquila chrysaetos*), booted eagle (*Hieraaetus fasciatus*), Spanish imperial eagle (*Aquila adalberti*), and peregrine falcon (*Falco peregrinus*). The motility parameter was higher, they never reached the values of other species, such as *Gallus gallus*, which can reach up to 90% sperm motility (Herrera *et al*., 2005; Froman, 2013).

However, if we compare the mobility parameters determined in this study for *A. chrysaetos* to those reported by Villaverde-Morcillo *et al*. (2015), they were superior. The parameters determined in the three species studied can be considered low, or this difference may be because sperm mobility is often overestimated in highly concentrated ejaculates such as in *G. gallus* (Etches, 1996). On the other hand, factors such as the health of the specimens and the "contamination" of the ejaculate with feces and urates in individuals who have not undergone prior training or genetic selection must be considered for breeding males (Bailey and Lierz, 2017). The proportion of spermatozoa with morphological abnormalities, ranging from 2.69% to 3.86% that was like other reports for ejaculates of birds used for zootechnical purposes (Etches, 1996). The main abnormalities observed were bent and swollen heads, bent and angled necks, as well as bent or coiled tails. The length of spermatozoa only coincides with the greater length of spermatozoa in *P. unicinctus* compared to the other two species.

Sperm morphometry contributes to predicting fertilizing capacity (Villaverde-Morcillo *et al*., 2017). In birds, sperm length is related to the morphological characteristics of the sperm storage tubules in females. It should be noted that the sperm storage cells are present in smaller proportions when the homologous sperm is longer (Briskie and Montgomeri, 1992; Sasanami *et al*., 2013). In our results, it was evident that the spermatozoa of *B. jamaicensis* were the smallest in size among the three species studied. This could be related to their longer sperm production time, as sperm can be found for up to four months. This extended sperm production period helps increase males' fertilization capacity during the breeding season. This is because *B. jamaicensis* is a migratory and monogamous species that only forms pairs during the mating season, therefore requiring greater

effectiveness to achieve offspring. Sperm viability in the three species was determined to be between 56.26% and 91.2%, which falls within the range of 50.8% and 93.2% reported by Blanco *et al*. (2000), for various raptor species such as the golden eagle (*Aquila chrysaetos*), Bonelli's eagle (*Hieraaetus fasciatus*), Spanish imperial eagle (*Aquila adalberti*), and peregrine falcon (*Falco peregrinus*) when similar thawing protocols were used as in our study. Sperm viability decreased by approximately 20% post-thawing. In this study, using 7% DMSO, the percentage of motility in *P. unicinctus* was like that reported by the mentioned authors using 6% and 8% in *Falco sparverius*. These values were approximately 10% lower than what we observed in *A. chrysaetos* and *B. jamaicensis*. The percentages of viability and sperm motility in post-thaw semen of the golden eagle (*A. chrysaetos*), Bonelli's eagle (*H. fasciatus*), Spanish imperial eagle (*A. adalberti*), and peregrine falcon (*F. peregrinus*) depend on the freezing technique and cryoprotectants used (Asano and Tajima, 2017; Kuzlu and Taskin, 2017; Cardoso *et al*., 2020). In *F. sparverius*, there is evidence of the effects of different concentrations of DMSO in the diluent used to freeze semen samples on sperm motility and semen fertility after thawing. The authors used concentrations ranging from 4% to 10% DMSO and reported infertile eggs when using 4% and a significant decrease in motility with 10% compared to 8% and 6% (Gee *et al*., 1993).

Basic sperm characteristics were determined in fresh and post-thawed samples, revealing differences between species in the parameters assessed in fresh and post-thawed conditions. This highlights the need to develop appropriate sperm cryopreservation protocols for each species in *ex situ* conservation programs. Sperm characteristics can have an impact on parameters during *in vitro* preservation.

Acknowledgements

We express our gratitude to Dr. J. A. Herrera-Barragán for his significant contributions to avian reproduction research. His passion and dedication continue to inspire us.

To CONAHCYT for the scholarship awarded to I. Rodríguez-Montiel.

Conflict of interest

All authors declare that there is no conflict of interest of any kind.

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