

Short Communication

Semen Collection and Ejaculate characteristics of Black Cobra (*Naja naja karachiensis*)

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ABSTRACT

Black cobra has long been associated with human populations due to their size and continuously being captured for its body parts like liver, skin, fats and venom for use in traditional medicine. Pakistan Black cobra has been facing many threats like illegal hunting/poaching, habitat loss and climate change and need immediate measures for its conservation. Semen cryopreservation is one of the *ex-situ in vitro* conservation techniques to maintain genetic diversity and improve breeding success in animals. Hence, the present study was designed to standardize the semen collection methods and semen ejaculate characteristics *viz*; appearance, volume, concentration, sperm motility and sperm morphology. Semen was collected from seven mature males (121.9-144.7cm body length) during breeding season (April-June) by following caudal third massage method. Semen color was white, while most of the ejaculates collected from black cobra were found mixed with blood. Semen volume ranged from 10 μ L-55 μ L with spermatozoa concentration ranged from 2x10⁹-9x10⁹. Average sperm motility was recorded as 72.85% at the time of semen collection. Sperm shape was filiform with mean total length of 41.12 μ m. It is concluded that black cobra semen ejaculate exhibit high sperm concentration and motility, indicating its strong fertilization potential. These preliminary findings will support future studies to enhance husbandry practices, consequently contributing to the conservation of this species.

Article Information

Received 12 November 2024

Revised 25 September 2025

Accepted 10 October 2025

Available online 10 September 2026
(early access)

Authors' Contribution

AR had a primary role in writing and practical work, while the other authors contributed equally.

Key words

Caudal massage stimulation, Semen evaluation, Sperm motility, Sperm concentration, Sperm morphology

Black cobra (*Naja naja karachiensis*), a subspecies of *Naja naja oxiana*, is an important member of family elapidae distributed across southern regions of Pakistan (Asad *et al.*, 2012). This species is most important from an epidemiological perspective, since black cobra possess venom that has a potentially fatal neurotoxic effect on humans (Adler and Halliday, 2002). Therefore, black cobras are kept in captivity at National Institute of Health, Pakistan, to collect venom for antivenom production (Parveen *et al.*, 2017). Black cobra venom is utilized to treat various ailments, including arthritis, cancer, vision problems, leprosy, muscular pain, sexual weakness, and sciatica (Altaf *et al.*, 2020). The growing interest in snake venom, along with the increasing number of endangered species, highlights the impotence of captive propagation of

snakes to support conservation efforts (Vieira *et al.*, 2023).

Global status of black cobra is least concern on the (IUCN) red list (Coeti *et al.*, 2020) while in Pakistan, it faces substantial threats due to climate change, disease, habitat destruction, poaching for medicinal value, unsustainable collection for skin and meat (Chowdhury *et al.*, 2021). Considering the threats and persistent pressure on this group, the necessity and significance of black cobra conservation becomes clear. The survival of endangered snake species rely heavily on captive breeding (Oliveri *et al.*, 2018) and might be a viable activity in the conservation of black cobra. Semen cryopreservation as one of the *ex-situ in vitro* conservation technique in association with artificial insemination provide an effective method for preserving the genetic diversity of threatened species (Rakha *et al.*, 2016) and has been successfully applied in numerous species of snakes (Robests *et al.*, 2024; Mattson *et al.*, 2007; Quinn *et al.*, 1989). Understanding of basic seminal parameters (volume, motility, concentration and morphology) of a species help *ex situ* conservation programmers to successfully implement fertilization technique (Rakha *et al.*, 2015). Despite the importance of semen collection and evaluation towards artificial insemination, there are only few species of snakes for which basic semen parameters have been reported.

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0030-9923/2026/0001-0001 \$ 9.00/0



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In this study we describe an efficient method for semen collection and basic seminal parameters for black cobra. The findings from this study will contribute to establish a sizeable colony for venom extraction, study, and conservation of this species. Due to the lack of published reports, this study may serve as a starting point for the development of sperm cryopreservation protocols for black cobra.

Materials and methods

Ten mature male black cobra having mean body length of 133.46 cm, were used in this study. Every snake was given physical inspection to make sure it was healthy and each animal body weight was recorded. Snakes were kept in 4ft x 2.08ft x 2.08ft iron cages and provided with bowl of water and wooden bedding. Snakes were fed weekly based on their body length. For snake's ≤ 129.5 cm in length, 70ml of the prepared chicken mixture was administered by using a 60cc syringe connected to a feeding tube. After filling the snake's stomach, a gentle massage was performed from head to tail for 2 to 3 minutes to prevent vomiting. Cages were maintained with temperature around 26-28 °C, under normal photoperiod (light and dark cycle of 12 h) and humidity was kept between 50% to 60%.

Semen was collected during the mating season (April to July) (Khan, 2002) of this species. In order to prevent contamination during semen collection, cloaca was lavaged with 20ml physiological saline solution (1%). Semen collection procedure involved a team consisting of three people: One of the experienced snake handler placed a small plastic tube at the head of snake to make it immobilize. Another snake handler positioned the tail, and the third one perform caudal third massage. This massage was performed by rubbing the thumb over the ventral scales and gently pressing in a cranio caudal movement as described by Mengden *et al.* (1980). Following two to three minutes of constant pressure on the cloaca, small volumes of semen were released and collected using graduated disposable micropipette. All of the specimens went successfully through the procedure without showing any noticeable behavioral changes.

Following collection, volume and color of each ejaculate was assessed. Semen volume was measured by using micropipette. Fresh semen sample was divided in to three groups. First group was allocated for sperm motility measurement. One drop of semen sample diluted with PBS (1:5) and applied to a glass slide for motility measurement, cover slipped and observed under a phase contrast microscope at 400X. Second group was allocated for sperm concentration measurement. In order to evaluate sperm concentration, the already diluted semen sample was further diluted 1:5 in saline solution. Sperm cells were counted in a Neubauer hemacytometer using light

microscope at 400x. Sperm size was measured using digital image analysis software, Image j (Fig. 1). Images were captured at 400x under light microscope. In the software, scale was calibrated using a reference length in micrometer, which was determined from an image of a stage micrometer taken under the light microscope at 400x. Overall length of sperm, head length, tail length were measured. Tail length of sperm was calculated by deducting head length from sperm total length.

The data was presented as mean values of semen volume, sperm motility, sperm concentration and sperm size of black cobra.

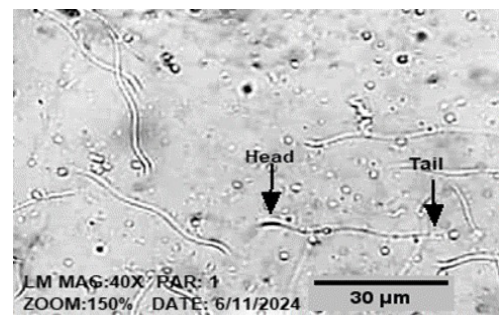


Fig. 1. Black cobra spermatozoan measured using digital image evaluation program image j split line section function. Tail length of sperm was calculated by deducting head length from sperm total length.

Table I. Body measurements and massage stimulation method mean outcomes in black cobra.

Parameters	Mean (Range)
Body weight (g)	584.38 (381.3-894)
Body length (cm)	133.46 (121.9-144.78)
Semen volume(μL)	36.42 (20-55)
Concentration(10^9 sperm/ml)	5×10^9 (2×10^9 - 9×10^9)
Motility (%)	72.85 (50-90)

Results and discussion

Semen was successfully collected from seven out of ten snakes after 2-5 min of caudal massage without eversion of hemipenes. This collection method did not require anesthesia because the specimen relax their cloacal muscles in response to technique, which facilitate successful ejaculation. Semen of black cobra had a liquid like consistency. The majority of ejaculates exhibited tan coloration with only few were white and blood tinged. Table I shows the data on motility semen volume and spermatozoa concentration in the samples collected from the black cobra. Sperms were filiform with slightly curved head and conical acrosome.

Sperm measurement data collected from black cobra is as follows:

Length of sperm: 41.12 ± 1.55 (37.74-43.92)

Length of head: 4.99 ± 0.58 (4.30-6.75)

Length of tail: 36.13 ± 1.56 (33.43-38.43)

Caudal massage stimulation (Zacariotti *et al.*, 2007) is a safe and fast method for semen collection and had been successfully applied in large snake species, lancehead pit vipers (Vieira *et al.*, 2023), *Pantherophis guttatus*, *Corallus hortulana*, *Sanzinia madagascariensis* (Oliveri *et al.*, 2018), golden lance head pit vipers (Silva *et al.*, 2015), red diamond rattlesnake (Zacariotti *et al.*, 2011), corn snake (Farhig *et al.*, 2007), *Elaphe gutatta* (Mattson *et al.*, 2007), *Boa constrictor occidentalis* (Tourmente *et al.*, 2006). This method is cost effective and less invasive in contrast to biopsy and electroejaculation which often yield immature samples. The present study demonstrates that massage method is an effective way by which urate free semen can be collected without any potential harm to individuals. Additionally, it eliminates the need for local anesthesia and eversion of hemipenes as it required in other snake species (Zacariotti *et al.*, 2007; Silva *et al.*, 2015).

Semen volumes obtained in *Naja naja* (121.9-144.7cm body size) ranged from 10-55 μ L, was significantly larger than that reported for *Bothrops insularis* of 1-6 μ L but quite similar to *Nerodia sipedon* of 10-30 μ L (Marques *et al.*, 2013; Schulte-Hostedde and Montgomerie, 2006). Variations in semen volume might be linked to differences in mating behavior of species. Both *Nerodia sipedon* and *Naja naja* exhibit male-male combat where several males compete to mate with single female. This form of competition favor males capable of producing large semen volume as it may offer a reproductive advantage (Shine and Richard 1994). On the other hand *B. insularis* lacks this combat matting pattern (Marques *et al.*, 2013), which could explain its lower semen volumes. Semen volume is also influenced by the technique used for collection. For instance Almeida-Santos *et al.* (2004) extracted up to 1000 μ L of semen from individual rattlesnakes (*Crotalus durissus*) by surgically removing ductus deference whereas Zacariotti *et al.* (2007) reported collecting only 3-70 μ L of semen from the same species using caudal massage stimulation Coeti *et al.* (2020). These differences highlights the importance of semen collection methods as they can significantly impact volume of semen retrieved. Vieira *et al.* (2023) reported semen volume ranging from 1.9-55.8 μ L in common lancehead, a species smaller in body size and weight than the black cobra. Conversely brazilian lancehead which has a similar body size and weight to black cobra exhibited lower semen volume ranging from 0.8 to 22.8 μ L (Vieira *et al.*, 2023). These findings suggest that there may not be a straightforward relationship between body size and semen volume.

Three semen color were noted: white, tan, and blood tinged. The majority of ejaculates were tan with only few were white. A blood tinged sample was most likely the

result of slight cloacal damage, either from plastic prob used during collection or from an underlying injury.

Sperm morphology of elapids had been poorly described. Black cobra sperm were filiform following general snake sperm morphology. Heads were slightly curved with short and conical acrosome. Sperm length ranged from 37.74 μ m micro meter to 43.92 micro meter with an average head length of 4.99 micro meter and an average tail length of 36.13 micrometer sperm length of black cobra is smaller than *N. rhombifer* which could be the result of interspecies variations and methods used for sperm measurement. Black cobra sperm length was measured by using light microscope while *N. rhombifer* sperm length was measured by using scanning electron microscope.

Average sperm motility of black cobra was recorded at 72.85%, which fall in the range of values reported for other snake species, garter snake, 70% (Quinn *et al.*, 1989); Brazilian rattlesnake, 64 % (Zacariotti *et al.*, 2007) Argentine boa constrictor, 63% (Tourmentea *et al.*, 2007) corn snake, 92.5% (Farhig *et al.*, 2007). Motility rates are influenced by maturity of snakes and season during semen collection. Silva *et al.* (2015) reported that during the mating period mature males had significantly higher sperm motility (85.7%) compared to immature snakes (62.5%). In this study semen was collected during the breeding season from both mature and immature males, which likely contributed to the overall motility rate of 72.85%. Taking in to account similar sperm motility parameters as recorded in this study Roberts *et al.* (2024) successfully produce snakelets through artificial insemination indicating that higher sperm motility is associated with increased insemination success rate.

The spermatozoal concentration (2×10^9 - 9×10^9 spermatozoa/ml) in the samples collected from the black cobra comparable to common lancehead pitvipers (0.42×10^9 - 8.1×10^9 spermatozoa/ml) but trended higher than Brazilian rattlesnake (0.94×10^9 - 2.23×10^9 spermatozoa/ml) (Vieira *et al.*, 2023; Zacariotti *et al.*, 2007). Despite the limited semen volume collected from black cobra, it was adequate for quality assessment, and sperm concentration was even higher than that obtained from larger volumes collected from Brazilian rattlesnake.

Conclusion

It is concluded that black cobra semen ejaculate exhibit high sperm concentration and motility, indicating its strong fertilization potential.

DECLARATIONS

Acknowledgement

The authors thanks snake handlers of National Institute of Health, Islamabad and Snakes handlers of Ayub National Park for providing assistance throughout.

Ethical statement

It is confirmed that the current study was conducted under the approval of Institutional Ethical Committee of PMAS-AAUR. Furthermore, no animal was harmed for semen collection.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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