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# Semen Collection and Evaluation in *Micrurus corallinus*

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***Abstract***.—**The Painted Coral Snake *Micrurus corallinus* is one of the Brazilian species kept in captivity to obtain venom for antivenom production. Difficulties in establishing a sizeable breeding colony make it necessary to find alternatives that increase the reproductive efficiency of captive individuals. Here, we tested a semen collection protocol and characterize the seminal parameters of captive *M. corallinus*. We collected semen during the mating season of the species (spring-summer) and were successful at every first attempt. Spermatozoa of *M. corallinus* are elongated and filiform, and the midpiece is the longest part. Sperm motility and progressive motility reached values of 80% and 3.6%, respectively, during the reproductive period of this species. Our results will allow further studies to improve husbandry, reproductive rates, and conservation of captive *M. corallinus*.**

*Key Words*.—reproduction; reproductive biotechniques; reptiles; sperm parameters

## Introduction

Reproductive biotechniques have been useful in implementing conservation projects for endangered species (Silva et al. 2015). We can also use these techniques to increase the reproductive rates of animals that rarely breed in captivity. One example is the Painted Coral Snake, *Micrurus corallinus* (Elapidae), a medium-sized venomous species that is widespread in Atlantic forest areas from northeastern to southern Brazil (Campbell and Lamar 2004; Marques and Sazima 2004; Nogueira et al. 2020). This species belongs to the group of coral snakes exhibiting black rings arranged in monads (BRM), with a black ring bordered by narrow white rings separated by red ones (Slowinski 1995). According to Marques et al. (2013), in the BRM group, gametogenesis is temporally dissociated, with spermatogenesis occurring in autumn and vitellogenesis in spring. Therefore, sperm need to be stored in the *ductus deferentia* until the mating season. Mating in *M. corallinus* occurs from spring to early summer (Marques 1996; Almeida-Santos et al. 2017), which means that males copulate with sperm that are stored in the *ductus deferentia*.

*Micrurus corallinus* has venom with a potentially lethal neurotoxic effect to humans (Gutiérrez et al. 2016). Therefore, individual *M. corallinus* have long been kept in captivity at the Butantan Institute in São Paulo, Brazil, to obtain venom for antivenom production (Mendes et al. 2018) and for development of immunotherapeutics (Castro et al. 2015; Tanaka et al. 2016). Although the species classification is Least Concern on the International Union for the Conservation of Nature (IUCN) Red List (Cacciali et al. 2019), the low encounter and capture rates of individual *M. corallinus* in the wild (Roze 1996) are worrisome and also make it difficult to establish a breeding colony with a substantial number of animals. Moreover, coral snakes produce little venom, and therefore, multiple extractions from several individuals are often required to obtain the necessary quantity of venom (Chacón et al. 2012; Mendes et al. 2018). Thus, finding strategies that increase the reproductive efficiency of captive snakes is crucial to establish a sizeable colony for venom extraction, study, and conservation of the species. Improved reproductive techniques will also reduce the necessity of removing more animals from nature. In this study, we tested a semen collection protocol with the aim of establishing a safe and non-invasive technique for *Micrurus* species that can be used for evaluating seminal parameters in captive and free-living coral snakes. Moreover, we assessed for the first time the status of sperm stored in the *ductus deferentia* of this species.

## Materials and Methods

***Animals and captive maintenance***.—We collected semen from seven clinically healthy adult males (snout-vent length > 390 mm) from different captivity locations and maintenance conditions. *Micrurus corallinus* specimens are difficult to find in nature and sensitive to excessive manipulation. The sampled individuals are held captive mainly to obtain venom for antivenom production. These factors restricted the number of animals we could evaluate.

We sampled three individuals housed in the Vital Brazil Institute (Rio de Janeiro, Brazil) and kept them

individually in enclosures formed by two boxes (with a transparent polycarbonate base, 200 × 230 × 300 mm) with water *ad libitum*. The snakes were fed a diet prepared with animal protein (bovine liver and chicken eggs) and mineral-vitamin supplement (Hemolitan®, Vetnil, Brazil). We also sampled two individuals housed in the Biological Museum of the Butantan Institute (São Paulo, Brazil) and kept individually in terrariums (700 × 500 × 400 mm) with ventilated lids and water *ad libitum*. Weekly, these individuals were fed snakes or amphisbaenids (20–25% of their body mass) provided by the Animal Reception Section or the Biological Museum. Finally, we also sampled two newly captured individuals that were temporarily housed in the Animal Reception Section of the Butantan Institute. In this case, we collected the semen within 24 h of arrival at the institute.

***Semen collection and evaluation***.—We conducted the semen collections between 2016 and 2018, always between November and March (austral spring and summer), which corresponds to the mating season of the species (Marques 1996; Almeida-Santos et al. 2017). We restrained the snakes using a transparent plastic tube. Next, we massaged the ventral portion of each snake towards the cloaca for 1-3 min (Mengden 1980) and collected the semen directly from the urogenital papilla using 75 mm long × 1 mm internal diameter microhematocrit capillary tubes (Precision® glass line, Shandong Harmowell Trade Corporation, Shandong, China). We collected and evaluated the semen of each individual once.

We measured semen volume and evaluated semen appearance and color. Next, we divided the semen samples into two groups. The first sample was diluted in Ham's F10 medium (dilution 1:100 µL) to evaluate sperm motility and progressive motility immediately after collection. The second sample was fixed in 10% saline formaldehyde solution to evaluate sperm concentration and morphology. We assessed sperm motility and progressive motility using an Eclipse E100 optical microscope (Nikon Corporation, Tokyo, Japan) at ×100 magnification. For the motility analysis, we placed a drop of diluted semen onto a glass slide, cover slipped samples, estimated the percentage of progressively motile sperm cells by the mean of five different microscopic fields (Fahrig et al. 2007). We graded progressive motility on a 0–5-point scale, where 0 = no motility; 1 = moving but without forward progress; 2 = moderate side-to-side movement, forward progress in spurts; 3 = slow forward progress; 4 = moderate forward progress; and 5 = rapid forward progress (Mattson et al. 2007; Silva et al. 2015). To avoid observer bias, the same researcher evaluated all samples under the microscope. We examined sperm concentration in a Neubauer hematimetric chamber using a Leica DM 400B optical microscope (Leica Microsystems, Wetzlar, Germany) at ×400 magnification. To assess sperm morphology, we first imaged the cells of each semen sample using a phase-contrast microscope (at ×1,000 magnification). We then measured sperm head length, midpiece length, tail length, and total length of 70 sperm using ImageJ, version 1.52a (Schneider et al. 2012).

***Statistical analysis***.—Data were normal (Shapiro–Wilk test) and variances were homogeneous (Levene's test). We used Pearson's Correlation Coefficient ($\alpha$ = 0.05) to test whether male morphological traits (body mass and snout-vent length) and semen parameters (semen volume and sperm concentration) were correlated. We used the program Statistica, version 7 (StatSoft Inc., Tulsa, Oklahoma, USA) to perform all the statistical analyses.

## Results

In all sampled individuals, semen was successfully collected in the first attempt. We observed no contamination of semen samples with feces or urate (Fig. 1). The semen of *M. corallinus* was white and thick (Fig. 1). The males presented different measures

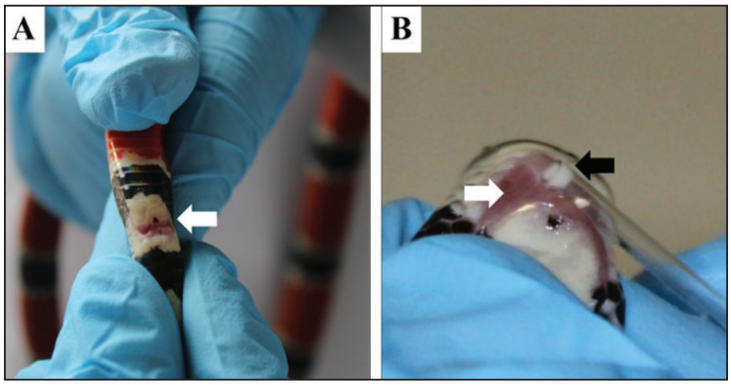


**Figure 1.** Semen collection from a captive male Painted Coral Snake (*Micrurus corallinus*). (A) Massage of the ventral portion of the abdomen of the snake. (B) Semen collection using a microhematocrit capillary. The white arrow points to the cloaca and the black arrow points to semen.

**Table 1.** Body measurements and seminal parameters of seven captive Painted Coral Snakes (*Micrurus corallinus*). The abbreviation SD = standard deviation.

| Parameter | Mean | SD | Range |
|---|---|---|---|
| Snout-vent length (mm) | 488.1 | 6.6 | 392–565 |
| Body mass (g) | 32.7 | 10.1 | 19.2–51.0 |
| Semen volume (µL) | 4.4 | 2.1 | 3.0–8.4 |
| Sperm motility (%) | 85 | 5 | 80–90 |
| Sperm progressive motility (0–5 scale) | 3.6 | 0.5 | 3–4 |
| Sperm concentration ($\times 10^9$ spermatozoa/mL) | 1.3 | 0.6 | 0.3–1.9 |

of morphological traits, and the smallest male collected was a snout-vent length of 392 mm (Table 1). Seminal parameters showed individual variation; however, the minimal percentage of motility detected was 80% (Table 1). Despite the variations, we found no significant correlations between male morphological traits and seminal parameters ($P$ = 0.132–0.679). The spermatozoa of *M. corallinus* were elongated, filiform, and the tip of their heads were slightly curved (Fig. 2). Mean total length of sperm was 103.73 ± (standard deviation) 1.35 µm. The midpiece was the longest part of the sperm (64.18 ± 1.16 µm), followed by the tail (29.05 ± 0.68 µm) and the head (10.50 ± 0.08 µm).

## Discussion

The efficiency of semen collection techniques in snakes has improved in recent decades. Mengden et al. (1980) adopted a successful technique of massaging the posterior abdomen of snakes, which reduced sample contamination. Further studies replicated their technique in other snake species (Tourmente et al. 2006; Fahrig et al. 2007; Mattson et al. 2007; Oliveri et al. 2018). Zacariotti et al. (2007) and Silva et al. (2015) used ventral massage coupled with anesthesia of the cloacal region, resulting in a safe and effective collection of semen from the South American Rattlesnake (*Crotalus durissus*) and the Golden Lancehead Pitviper (*Bothrops insularis*), respectively. Semen collection in *M. corallinus* was fast, effective, and minimally invasive. Moreover, it required no use of local anesthesia, as in other snake species (e.g., *Crotalus durissus*: Zacariotti et al. 2007; *Bothrops insularis*: Silva et al. 2015). Considering the similar seminal parameters found from different maintenance conditions (captive versus free-living animals), this technique may be replicable to other *Micrurus*.

The spermatozoa of *M. corallinus* have three regions: head, midpiece, and tail, which is similar to that described in other reptiles (Oliver et al. 1996). The sperm morphology of elapid snakes has been poorly studied (Oliver et al. 1996; Gribbins et al. 2016) and for *Micrurus*, sperm morphology has been described only in *M. fulvius* (Austin 1965). Therefore, this is the first paper describing the sperm morphology of *M. corallinus*.

The volume of semen obtained depends on factors such as collection technique, species, and reproductive timing. Protocols involving euthanasia and then excision, stretching, and washing of the *ductus deferentia* certainly will provide a higher semen volume. For example, Almeida-Santos et al. (2004) obtained approximately 1000 µL of semen from individual rattlesnakes (*Crotalus durissus*), while techniques based on the mechanical compression of the *ductus*, like ventral massages, provide a lower volume when performed in the reproductive season on the same species (3–7 µL; Zacariotti et al. 2007). The semen volume obtained in *M. corallinus* (4.15 ± 2.13 µL; this work) was lower than that obtained in the Corn Snake (*Pantherophis guttatus*; 10 µL; Fahrig et al. 2007) but similar to that obtained for the Northern Common Boa (*Boa imperator*; 3.62 µL; Meza-Manriquez et al. 2015) using the same collection technique. The semen

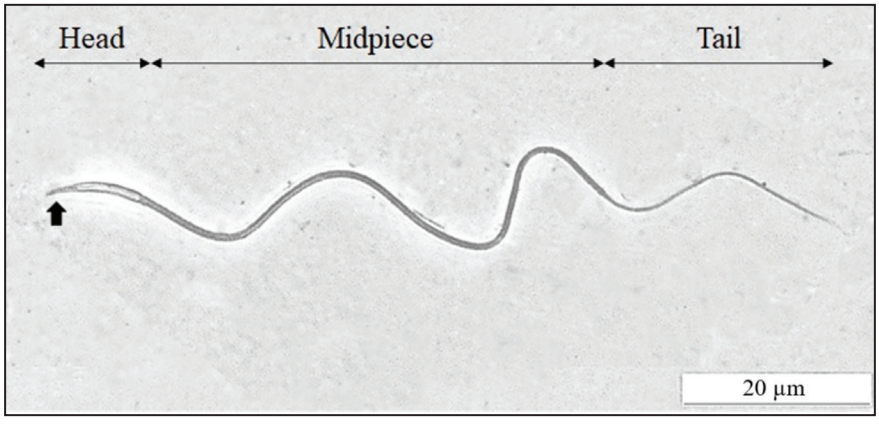


**Figure 2.** A spermatozoon of the Painted Coral Snake (*Micrurus corallinus*) observed in a phase-contrast microscope with black arrow indicating the acrosome.

concentration of *M. corallinus* that we found ($1.3 \times 10^9$ spermatozoa/mL), however, was higher than that of *P. guttatus* ($0.8 \times 10^9$ spermatozoa/mL; Fahrig et al. 2007) and for *B. imperator* ($0.001 \times 10^9$ spermatozoa/mL; Meza-Manriquez et al. 2015). The small volume of semen collected from *M. corallinus* was sufficient to evaluate its quality, and semen concentration was even higher than that obtained in semen collection of other snakes using euthanasia: $0.041 \times 10^9$ spermatozoa/mL for *C. durissus* (Almeida-Santos et al. 2004) and $0.00047 \times 10^9$ spermatozoa/mL for the Zanjani Viper (*Vipera albicornuta*; Moshiri et al. 2014).

The sperm motility of 80% and progressive sperm motility of 3.6 in *M. corallinus* semen is similar to reports in the Green Ground Snake (*Erythrolamprus poecilogyrus*; 80%; Silva et al. 2017) and higher than *C. durissus* (63.8% and 2.9%; Zacariotti et al. 2007). Considering similar parameters of motility (63–75.7%) and progressive sperm motility (3.5–4), Mattson et al. (2007) succeeded in artificial insemination of *P. guttatus* and suggested that insemination success rates are proportional to higher concentrations and motilities. Thus, the semen parameters observed in captive *M. corallinus* indicate a potential for fertilization. Furthermore, our results confirm the viability of semen stored in the *ductus deferentia* during the mating season, corroborating results obtained from preserved animals (Almeida-Santos et al. 2006; Marques et al. 2013).

We obtained semen in every first collection attempt, which shows that the technique works in coral snakes. We also demonstrated that the *Micrurus corallinus* semen has high sperm concentration and motility in the reproductive season. The adoption of effective techniques to collect and characterize semen parameters are fundamental for the planning and applicability of other reproductive biotechniques such as artificial insemination, cooling, and cryopreservation (Durrant 2009). Thus, the protocol described in this work has great potential to be applied in future studies on semen evaluation, morphology, and reproductive management in this snake genus and will allow further studies to improve husbandry and reproductive rates of captive *M. corallinus* and, consequently, contribute to coral snake conservation.

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