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Feasibility of semen collection in red-winged tinamou (*Rhynchotus rufescens*) by manual stimulation and sazonality implications

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ABSTRACT

This study aimed to report in detail, the technique and challenges of cloacal massage, to collect and evaluate semen from red-winged tinamou (*Rhynchotus rufescens*) keep in captivity, performed by only one technician. Sixty-four semen collection attempts, from 16 adult males, during breeding season and 16 attempts form these same 16 males in non-breeding season, were performed. Prior to collection, all animals were conditioned to cloacal massage for 6 weeks and the ejaculates were succeed with viable spermatozoa and then, evaluated for feces, urine and mucus contamination, volume, concentration, sperm vigor, motility, morphological defects and acrosome integrity. Semen collection success rate was 63% in breeding season and 2 (5%) samples were discarded by grade 5 contamination. Only 3 ejaculates from 16 tinamou were obtained in non-breeding season. Sperm concentration and acrosome integrity was higher ($p = 0.00$) in breeding season, and the percentage of total sperm morphological defects, were high in both in breeding and out breeding season. Overall, we concluded that the red-winged tinamou breeding season, is linked to photoperiod (spring and summer), and at this period time, semen can be obtained by cloacal massage collection satisfactorily, allowing its use in reproduction biotechnologies and sperm cryopreservation.

1. Introduction

The interest in breeding wildlife with commercial potential, such as tinamou (*Rhynchotus rufescens*), is a viable alternative for this species conservation with economic profitability. Knowledge on seminal characteristics and understanding of its reproductive biology [1] is essential.

The chosen semen collection technique varies by the research group experience. Different techniques, such as collection direct from the vas deferens [2], mannequin mate [1], artificial cloaca [3], cloacal massage [4], and electroejaculation [5]are described as methods of obtaining semen from poultry since 1930. Manual stimulation is a recommended method for wild birds and was described as a massage of the soft part of abdomen, beneath the pelvic bones [4]. As advantage, cloacal massage presents total control of the technician on the ejaculation moment and disadvantages, the need of conditioning the birds and samples contamination with urine and feces. To the authors' knowledge, in red-winged tinamou, cloacal massage technique for semen collection, wasn't previously described [4,6–8]. Thus, this study aimed to describe this technique in detail handled by only one operator, different from the published literature on domestic and wild birds. On the other hand, the semen collection allows seminal evaluation and helps the identification of subfertile and infertile individuals, allowing to classify male according sperm quality, for assisted reproduction programs [9]. Therefore, this study aimed to report in detail, the technique of semen collection by cloacal massage in red-winged tinamou (*Rhynchotus rufescens*).

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2. Material and methods

The tinamou used in this experiment were allocated at Fazenda Lageado (São Paulo State University, UNESP, Botucatu, Brazil) separated in boxes. Sixteen males (5 years–9 years-old) were used. The study was registered on Brazilian Biodiversity Authorization and Information System (Sistema de Autorização e Informação em Biodiversidade – SISBIO number 50367), conducted in accordance with ethical guidelines recommended by National Council for Control of Animal Experimentation and College of Animal Experimentation, and approved by the Institution's Animal Care and Experimentation Ethics Committee on protocol number 98/2016.

After collection by cloacal massage, semen was evaluated for volume (μL), concentration (x10^9 spermatozoa/mL), vigor (score 0 to 5), motility (%), morphological defects (minor and major defects %) and acrosome integrity (%). It was kept in a 37 °C in dry bath until performed all semen sample analysis. Ejaculate volume was determined by micropipette. Then semen was diluted in 0.9% saline solution in a proportion of 1: 50 (2 μL: 98 μL) and deposited in a lamina with a cover sheet, and evaluated to motility and vigor by a light microscope under 400× magnification. Acrosome integrity was evaluated using 10 μL of this dilution added to 10 μL of the POPE dye [10] and incubated for 70 s. Smear was prepared and evaluated under a light microscope. Were counted 100 cells and classified as intact acrosome (lilac stained) or not (pink/no stained) (Fig. 1) [11]. For sperm concentration, 1 μL of the ejaculate was diluted in 499 μL of buffered formol saline solution (1: 500) and counted in a Neubauer chamber. The remainder dilution was stored at 4 °C for morphology analysis under differential phase interference microscopy (1,000× magnification). The sperm morphological defects were classified as head, intermediate piece and tail defects.

The variables were described in mean and standard deviation. Shapiro-Wilk test was used for variables normality and analyzed by *t*-test with significance level $p \leq 0.05$, using R software version 3.2.2., to compare the seminal parameters in non- and breeding season.

3. Results

The breeding season (September 2016 to February 2017) was identified by behavior, as egg laying, vocalization, mating and dorsal region pecking of males and females (dorsal areas of alopecia). Tinamou conditioning started in October 2016 by the contact of the technician and the birds (1st week). In the 2nd week, the birds were restrained for cloacal feather cut. By the 3rd week, cloacal and abdominal massages started (2 times/week). For semen collection, the technician seated, supported the bird on one of his legs. With the left hand, massage was performed on the tinamou back (caudally to the ribs and on the testicles) making light pressure against the bird's body to help restraint, and with the right hand, the abdominal region was stimulated between the pelvic bones to the cloaca until the phallus was slightly engorged (Fig. 2 - A). At this point, the head of the animal was trapped between the legs of the technician, leaving the bird upside down, for better visualization of the phallus and semen collection accuracy. Then continuous movements, with slight pressure, were made in the direction of the dorsal lips of the cloaca until ejaculation (Fig. 2 - B). The technician should not allow phallus exposure (Fig. 2 - C) until the ejaculation ends, as it has been noted that total exposure of this organ, inhibits ejaculation. Already in the 4th and 5th weeks it was possible to observe the elimination of mucous contents by the cloaca, containing spermatozoa, with rapid movements (head and tail), but attached to the mucus. Due to the failure of the collection, males were separated from the females and at the 6th week ejaculates were obtained with viable spermatozoa. The ejaculate was collected in heated 1.5 mL polypropylene microtubes with a flat surface cap (Fig. 2 – D) directly from the cloaca, without the need of micropipette or microcapillary. Mucus secretion, urine or feces can be eliminated after gentle cloacal massage and cleaning with a soft absorbent paper. The contamination of the sample was graded (score from 1 to 5) by direct observation [1].

During the breeding season, 64 attempts to collect semen were made (4 attempts per animal, once a week), with success rate of 63% (40/64). From 40 samples, only 2 (5%) were contaminated with feces and urine grade 5 and were discarded, 4 (10%) were grade 2 and 1 (2.5%) sample, grade 1.

Other collection attempt was made soon after breeding season ends (March 17, 2017). Only 3 (19%) ejaculates were obtained from 16 fowl and 1 sample had grade 2 contamination. Seminal parameters in breeding and non-breeding season were described in Table 1. None of the birds died after their manipulation (stress).

In breeding season, the morphological analysis showed 65% defects (80% tail, 14% intermediate piece and 6% head). Otherwise, in non-breeding season were observed 70% defects (6% tail, 7% intermediate piece and 87% head). The common sperm defects found were looped and bent head, round-headed, folded intermediate middle piece and strongly folded tail.

4. Discussion

Because they are wild animals, it is important a continuous contact between technician and birds, for adaptation and reducing seminal collection stress. Cloacal feather cutting facilitates the direct collection from the cloaca and together with the massages (cloacal and abdominal), make the fowl more tolerant and conditioned to the procedure.

This contact also aimed to observe individual behavior. We noted that some birds needed longer stimuli, while the mean time of massage for each animal was around 2 min until total ejaculation. Some males vocalized during stimuli, and these males ejaculated the greater seminal volume. It was possible to notice cloacal muscles movement (contraction). Some fowl were so conditioned, that start to present cloacal contractions already at the restraining. Also, we observed that the stimulus was correct, because the bird allows cloacal manipulation without struggle.

During the 4th and 5th week of conditioning, mucoid secretion was obtained with live spermatozoa. It's believed that this mucous secretion comes from secretory anal glands. The literature reports a

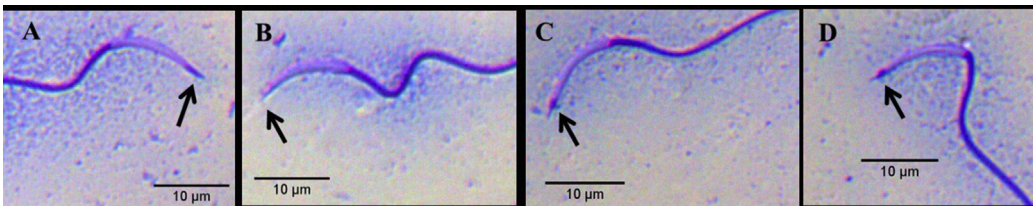

Fig. 1. Pope's coloration for Tinamous' acrossomal integrity. A. Intact acrossoma. B. Acrossoma not intact. C and D. Detached acrossome.

Fig. 2. Handling and restraint Tinamous (*Rhynchotus rufescens*) for semen collection. A. Dorsal stimuli and abdominal massage. B. Cloacal massage, with phallus exposure (arrow). C. Tinamous (*Rhynchotus rufescens*) phallus exposure (arrow) during semen collection. D. Microtube with tinamous' (*Rhynchotus rufescens*) ejaculates.

Table 1
Semen parameters (mean ± standard deviation) from 16 red-winged tinamous (*Rhynchotus rufescens*) collected by manual stimulation in breeding and non-breeding season.

Semen parameter	Breeding season (n = 38)	Non-breeding season (n = 3)	p value*
Volume (μL)	58.4 ± 44.5	55.5 ± 13.0	0.42
Concentration (x10^9 sperm/mL)	1.62 ± 0.73	0.72 ± 0.05	0.00
Motility (%)	73.0 ± 14.0	75.0 ± 7.0	0.37
Vigor (0–5)	3.7 ± 1.0	3.5 ± 0.7	0.42
Acrosome integrity	92.0 ± 3.2	79.0 ± 2.2	0.01
Total sperm morphological defects (%)	70.0 ± 13.0	75.0 ± 16.0	0.28

*significance level $p \leq 0.05$.

waxy, greasy or mucous material in ratites, some large raptors, and a few passerines. Even the presence of mucoid secretion has not been reported in many other species, secretory anal glands have been found in 72 species in 42 families [12]. It is unclear if it was vestigial spermatozoa due to previous copulation, due to the lack of technician experience or the lack of tinamou familiarity to the procedure. At this point, it was decided to separate the male from female, and apparently it had a positive influence, because at the 6th week, viable spermatozoa were acquired. The cloacal mucoid gland function is unclear, however, this secretion compromises seminal quality [6].

Seminal color is a good index of sample contamination with urine and feces. The color without contamination ranges from opaque white, white transparent to yellowish. In contaminated samples, the color range from white/green/brownish to white/yellow/green. Because it is a very subjective analysis, the score (1–5) will depend on the experience of the observer. Samples with contamination 4 and 5° were discarded because semen analysis showed ejaculate of poor quality, which make it unfeasible to biotechnologies applicability. Urine contamination leaves the semen pH acidic and reduces sperm viability, which could be bypassed by washing it with neutral extender, but its benefits, is

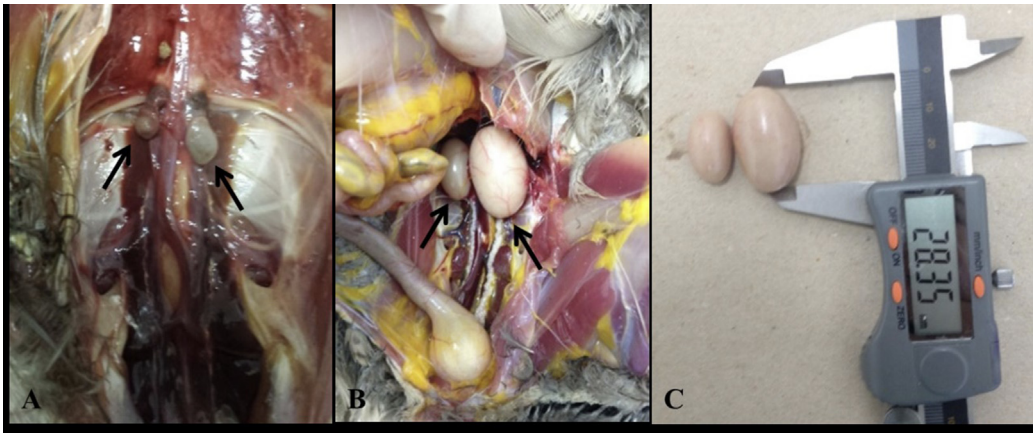

Fig. 3. Tinamous (*Rhynchotus rufescens*) testicle. A. Non-breeding season. B/C. In breeding season/measure in mm.

under discussion [13]. In endangered species this shall be used as attempt to help its preservation.

As this study shows, semen concentration was higher in breeding than non-breeding season. It was already predictable due the changes in the libido, testicular size, LH and testosterone levels during non-breeding season [14].

Semen volume collected was greater than others studies [5,10], however concentration, motility, vigor, morphological defects and acrosome integrity were similar in breeding season. Although the last author [15] used the same tinamous' group that we use in this study, we believe that there is a difference in the volume of ejaculate, due to the type and intensity of the stimulus used at the time of collection, and because the technique that they use was cloacal digital manipulation, and we performed abdominal and cloacal massage, followed by cloacal digital manipulation.

Because of the semen high concentration, it was necessary a dilution (1:50) for immediately motility and vigor evaluation. According to Cavalcante [16], the red-winged tinamous ejaculate dilution with 0.9% NaCl, a commercial turkey extender, and other two extenders, for fresh analysis, did not differ. The differences were found after 24 and 48 h of refrigeration. 0.9% saline solution (380 mOsm) is considered an isotonic solution, since the red-winged tinamous semen osmolarity is around 300 mOsm [16].

Reproductive features vary in the birds genera in the same order and there is differences in the seasonality expression [17]. In this study, the seasonality of tinamou in São Paulo (latitude: 22° 53′ 09″ S; longitude: 48° 26′ 42″ W; altitude: 804 m) state in 2016/17 was elucidated by behavioral patterns and semen analysis. Too, by the necropsy of some males tinamou, we could notice the difference of the testicle size in breeding and no-breeding season (Fig. 3). There were scarce studies about sperm parameters of the red-winged tinamou (*Rhynchotus rufescens*), and this study contributed to understand some their reproductive characteristics and behavior. The difficult of tinamous' semen parameters standardization is due to the lack of collection experience, seasonality, inbreeding and fowl training. Those facts interfere directly in the ejaculate quality. Consequently, semen biotechnology development and breeding program success is hampered.

Percentage of morphological defects found in breeding season in this study was very high compared with a study in other tinamous' species [15], that found around 30%. In breeding season, folded tail was the main morphological defect and in non-breeding season was those associated to the spermatozoa head, which are related with the spermatogenesis. Sperm morphological defects in mammals, can be related to aging [18]. Red-winged tinamou reaches its sexual maturity around ten months [19], and to our knowledge, there are no scientific records about their lifespan. It's believed that tinamou species can live up to 10 years [20]. This life expectancy might increase in captivity. Previous study conducted by Góes et al. [11], that studied these same fowls, two years before (3–4 years age), showed similar results (60%). By this, we believe that these features could be related with high endogamy degree, as they are being raised in captivity since 1987, without the introduction of new fowls, as it was previous studied in other animal species, endogamy coefficient showed a strong inverse relationship with ejaculate quality [21]. In our knowledge there are scarce studies mentioning tinamous morphological spermatozoa defects [15] and considering the role sperm morphology plays in fertility, further studies about morphological defects are essentials.

Acrossomal integrity has higher indices in breeding season than non-breeding season. It's an indication, together with low concentration, that the sperm capacity to fertilize an egg, is righer in breeding season.

This paper describes, to our knowledge, for the first time, the successful cloacal massage, to collect and evaluate semen from red-winged tinamous, performed by only one technician. Overall, we concluded the red-winged tinamou breeding season, which is linked to photoperiod (spring and summer), occurs from September to March in the south hemisphere. Semen collection by cloacal massage can be performed by only one technician. The red-winged tinamou are easily adaptable to handling, and semen collection can be facilitated by the experience and perception of the technician. The absence of females in male donors of semen group, for a few days, before the collection, increases the libido and the sperm quality. Feces and urine contamination occurs due the lack of bird adaptation to technique, technician experience, and the semen contaminated should be discarded, but can be used in endangered species. Semen characteristics, excluding morphological defects, allow to use the samples in reproduction biotechnologies as sperm cryopreservation in commercial breeding and to species conservation.

5. Conclusion

Cloacal massage performed by one person is possible in red-windged tinamou, but its seminal quality is strong related to breeding season.

Conflict of interest

None of the authors of this paper has any financial or personal relationship with any other person or organisation that might

inappropriately influence or bias the content of the paper.

Author contribution

All authors made a substantial contribution to the execution of this study. Paranzini, Ferreira, Correia, J. Silva and Veiga designed the study. Paranzini, conducted seminal collection with França, K. Silva and Correia support. Paranzini, Ferreira and Camargo were responsible for seminal analysis. França and Correia were responsible for management, handling, separating and feeding, all animals including weekend and holidays. Paranzini with Ferreira helps, drafted the paper. All authors contributed to the manuscript revisions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2017.10.032.

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