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Evaluation of Fresh and Thawed Semen of *Pecari tajacu* (Artiodactyla: Tayassuidae) in Yucatán Mexico

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Authors' contributions

This work was carried out in collaboration among all authors. All the authors collaborated in the execution, analysis and writing of this research work. They also approve the content of this manuscript.

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ABSTRACT

This research aimed to characterize the seminal properties of collared peccary *Pecari tajacu* before and after freezing, and to relate the morphometric measurements of the testes with sperm measurements before freezing. The study design was quasi-experimental in nature. The investigation was carried out at the Xmatkuil Wildlife Management and Conservation Unit and the Centennial Zoo in the state of Yucatan, Mexico, during the months of October to December 2001. Seventeen collared pecaries adult were electroejaculation from two captive populations. Macro and microscopic analyzes of fresh and thawed semen were carried out, also morphometric measurements of the testes, the data of sperm measurements were compared with Student´s t-test, a linear regression model was adjusted between spermatic and morphometric variables of the testis. Logistic regression was performed between seminal freezability and spermatic and morphometric variables. The ejaculate volume was 0.96 ± 0.98 ml, with 62.08 ± 19.10% sperm motility, concentration of 558.61 ± 537.04 x 10^6 spermatozoa / ml of fresh semen and vigor of 3.7 ± 0.58 on a scale of 1 to 5. On thawing the motility sperm was 35 ± 17.18% and 2.75 ± 0.62 vigor.

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The measurements of the sperm cell segments did not show a significant difference between the individuals of the two populations. Testicular width and sperm concentration were fitted to the logistic regression model with freezability (1 = yes, 0 = no). It is concluded that testicular width is a factor significantly associated with sperm motility and seminal freezability.

Keywords: Cryopreservation; collared peccary; reproductive management; semen; testicular size; Peccary tajacu.

1. INTRODUCTION

There is information on the characteristics of the semen of *Pecari tajacu* [1], as well as on its characteristics after freezing, the feasibility of cryopreserving the semen is also mentioned, tests have even been carried out with different diluents and cryopreservatives, which have demonstrated that they are efficient in maintaining the viability of frozen *P. tajacu* semen [2,3]. However, it is necessary to analyze the relationship between sperm viability versus freezing with sperm and testicular variables, information that may be valuable to identify the factors that are important to include them in the selection of semen donors in this species [4], in the same way that it has been reported in domestic mammals [5]. The objective of this work was to study the seminal characteristics of the Pecarí tajacu before and after thawing and relate them to some sperm and morphometric variables of the testes.

2. MATERIALS AND METHODS

2.1 Study Sites

The work was carried out in Zoo Centenario and Unit for the Management and Conservation of Wild Fauna (UMA) Xmatkuil in the state of Yucatán, Mexico. The climate of the region is warm subhumid Awo classification, with rains in summer, average annual temperature of 25.8ºC and annual relative humidity of 75 to 80% [6].

Seventeen *P. tajacu* males were used, eight of these confined in the UMA Xmatkuil and nine in the Centenario Zoo. All the specimens were adults between 3 and 5 years old and weights between 20 and 23 kg, kept in captivity from birth and fed, in the case of the UMA, with seasonal fruits and vegetables supplemented with pig feed with 9% crude protein, and in the case of zoo animals based on commercial cattle feed and a cut forage supplement (*Brosimum alicastrum*); there were no changes in diet during the study.

2.2 Testicular Morphometry and Collection of Seminal Samples

To measure testicles and electroejaculation, the animals were tranquilized with the intramuscular application of Ketamine hydrochloride at 10%, at a dose of 10 mg of ketamine per kg of live weight [7]. The specimens were fasted 12 hours prior to containment and were sedated, a blindfold was placed on their eyes, cotton plugs in their ears and a muzzle. Each testicle was measured for its width and length using a steel ruler graduated in mm and cm, also the testicular length and width of both testicles were measured. The semen collection took place from November to December 2007 and January 2008. For the semen collection, the preputial area was made asepsis with neutral soap and physiological saline solution and the foreskin hair was cut to facilitate semen collection. The probe used was specially designed for this species, the electrical stimulation was with a manual control electroejaculation from Standard Precision Electronics (Denver, Colorado). The electroejaculation protocol reported by [8] was used. In summary increasing electric shocks were applied according to the scale of the power source, each series of five shocks was for four seconds with an interval of two seconds, this procedure was repeated until until the ejaculated was obtained and an effective response to stimulation. Animals that did not respond to stimuli were not included in the study.

The semen was collected in previously sterilized beakers and kept in a thermos at a temperature of 30 to 37°C. The container was placed next to the preputial orifice, so that when the penis was unsheathed it would remain inside the container. The semen samples were protected from sunlight at the time of collection.

2.3 Seminal Analysis

The volume was measured with a needleless insulin syringe and placed in a 5 ml test tube. From the fresh sample a drop was taken immediately to evaluate the motility and sperm

vigor by direct observation under the microscope at 30^0C, the motility was evaluated from a scale of 0 to 100%, the vigor from a scale of 1 to 5, where 1 is the minimum vigor and 5 is the maximum [9], Subsequently seminal samples were transported at temperature of 30 - 37^0C to the laboratory to be analyzed and evaluated 50 to 60 minutes after being collected, the concentration was measured with semen diluted (1:400) in physiological saline buffered with 1% formaldehyde and methylene blue by a Neubauer chamber at 400X. The amount of normal and abnormal sperm was measured by counting 100 cells in a smear stained with eosin-nigrosin, the result was expressed as a percentage. Morphometric measurements of the spermatozoa were carried out using 30 cells from smears of each seminal sample, sperm head lengths and width, neck length, sperm tail and total sperm length were measured under a micrometer microscope (Carl Zeiss cat. No. 454060). The criteria used to dilute and freeze the samples were the following: a) volume equal to or greater than 1 ml, b) initial motility equal to or greater than 50%, c) vigor equal to or greater than 3 within a scale of 1 to 5, and d) concentration equal to or greater than 100 x 10^6 sperm / ml.

The selected samples were diluted with triladyl between 25 to 28^0C adjusted to 100 x10^6 cells per ml. Subsequently they were placed in the refrigerator at 5^0C for 2 hours, and 0.25 ml straws were filled and sealed. Afterwards the straws were placed horizontally at 5 cm above the liquid nitrogen surface for 7 minutes, subsequently they were submerged in liquid nitrogen. The samples remained 72 h in liquid nitrogen before being analyzed. At the end of this time the straws were defrosted at 37^0C.

2.4 Statistical Analysis

A linear regression model was fitted to determine the relationship between some of the seminal variables that were significant (seminal volume, sperm concentration, initial sperm motility) and testicular or morphometric measurements of testicular length and width. A logistic regression model was also fitted between seminal variables and testicular morphometry with freezability, which corresponds to 1 = whether it freezes or not = 0, using the backward selection method. In our investigation the freezability assignment of the semen is in relation to the testicular and spermatic variables regardless of the four criteria for freezing the semen.

The measurements of the sperm segments, testicular length and width of both and each testicle of the animals from the UMA and Zoo were compared by means of the t-student test. Statgraphics 5.1 [10] was used to carry out the statistical analyzes. The significant effects were evaluated with a type I error of 5%.

3. RESULTS AND DISCUSSION

3.1 Seminal Characteristics

24 semen samples were obtained from 17 collared peccaries; three zoo animals ejaculated twice and six once; four UMA animals ejaculated twice and four once. Of these ejaculates, only 12 met the inclusion criteria for freezing and sperm analysis upon thawing. The results of the evaluations of all the fresh semen samples are presented in Table 1. The results of the evaluations of motility and sperm vigor before and after the freezing and thawing process are presented in Table 2. Table 3 presents the mean values of sperm morphometry per group and integrated measurements in the total populations analyzed, no significant differences were found between the sperm measurements of animals from the two facilities. The means of the length and width of the testicles of animals in the two study sites are presented in Table 4.

3.2 Statistical Evaluation

Fig. 1. shows the graph of the linear regression between the variables already described, where two outlayer points can be observed, however, most of the data approximates the fitted line. A linear regression model was fitted between the width of two testicles (total testicular width) and initial sperm motility, the regression model was initial motility = 15.3346 + 7.6689 total testicular width. The mean and standard deviation of the total testicular width was 6.089 ± 1.02 cm. The fitted logistic regression from the inclusion of the sperm and testicular variables is as follows:

$$P(CONG = 1) = \frac{e^z}{1 + e^z}$$

Where CONG is freezability (1 = yes, 0 = no), Z = -13.8069 + 2.55725 total testicular width - 0.00435621 sperm concentration. Percentage of deviation explained = 35.88. Tables 5 and 6 show respectively, the estimated parameters and the odds ratio, the analysis of variance, the adjustment test of the variables in the logistic

model, all the effects were significant. Both models are significant.

The electroejaculation technique was used in P. tajacu because, according to [11], it is necessary to obtain semen from wild animals, it is also the best method to obtain semen from valuable animals, at risk of extinction or from the zoo [12,13]. The level of success achieved in this experiment is high, because there were no lesions in the animals, and we were also able to repeat the procedure in some specimens, which led to obtaining two ejaculates in seven peccaries, fasting the animals subjected to 12 hours previously, avoided the risk of death due to bronchoaspiration, which usually occurs when fasting is not applied [14], the intramuscular administration of the dissociative sedative Ketamine prior to the application of the electroejaculator, allowed the handling with maximum safety for the technicians and for the submitted animal testing.

Of 24 samples obtained, only 12 (50%) were suitable for the freezability test, because some were contaminated with urine (n = 4), in others they had a very low volume that was not enough for processing, they did not present sufficient vigor or sperm concentration values.

The appearance and color of the semen varied between opalescent, watery and milky, as well as white and yellowish white, these data agree with samples of semen rich in sperm concentration according to [15]. The volume of ejaculates is less than that reported by [9] and [16], whose values are 2.9 ± 2.29 ml and 2 ± 0.2 ml respectively; but what was reported by Kahwage et al. [15] was 0.81 ± 0.86 ml, similar to what was found in our research, a reasonable explanation for low semen volumes, due to the low secretory capacity of the prostatic and bulbourethral glands of this captive population with respect to other locations from America.

Table 1. Values of five variables (mean ± standard deviation) of 24 fresh semen samples from *Pecari tajacu*, obtained by electroejaculation in Yucatán, México

Variable	Results
Volume (ml)	0.96 ± 0.98
Motility (%)	62.08 ± 19.10
Concentration (sperm/ml)	558.61 ± 537.04 x 10^6
Vigor	3.7 ± 0.58
Abnormal sperm (%)	39.84 ± 22.27

Vigor is rated from 1 (minimum value) to 5 (maximum value)

Table 2. Motility and sperm vigor (mean ± standard deviation) of 12 samples selected with the expected inclusion criteria, of *Pecari tajacu* semen before and after freezing

Variable	Fresh	Thawed
Motility (%)	67 ± 13.06	35 ± 17.18
Sperm Vigor	3.21 ± 0.69	2.75 ± 0.62

Vigor is rated from 1 (minimum) to 5 (maximum).

Table 3. Measurements of the sperm cells of two captive populations of *Pecari tajacu*, there were no significant differences in the variables between samples of both populations

Variable	UMA	Zoo Centenario	Integrated measurements
Head length	5.6 ± 0.5 µm	5.3 ± 0.6 µm	5.5 ± 0.5 µm
Head width	3.7 ± 0.6 µm	3.5 ± 0.6 µm	3.6 ± 0.6 µm
Neck length	1.0 ± 0.3 µm	1.0 ± 0.5 µm	1.0 ± 0.4 µm
Tail length	42.1 ± 4.7 µm	43.0 ± 3.9 µm	42.5 ± 4.3 µm
Total length	48.8 ± 7.9 µm	49.4 ± 6.4 µm	49.1 ± 4.4 µm

Table 4. Mean values and standard deviation of the testicular morphometry of 17 collared peccaries, there were no significant differences between the measurements of both testicles

Testicle	Length (cm)	Width (cm)
Right	4.458 ± 1.25	3.052 ± 0.97
Left	4.358 ±0.80	3.03 ± 0.28

Table 5. Estimated parameters of the logistic regression (Maximum likelihood) between the independent variables total testicular width, sperm concentration and the freezability dependent variable

Parameter	Estimators	Standard error	Odds ratio
Constant	-13.8069	8.45689	
Testicular coefficient	2.55725	1.46223	12.9003
Sperm concentration coefficient	0.00435621	0.00319724	0.995653

Freezability 1=yes, 0 = no

Table 6. Analysis of variance of the adjusted logistic regression model. Independent variables are total testicular width and sperm concentration, dependet is freezability

Source	Deviation	Degrees of freedom	P value
Model	8.87377	2	0.0118
Residuals	15.8568	15	0.3916
Total (corr.)	24.7306	17	

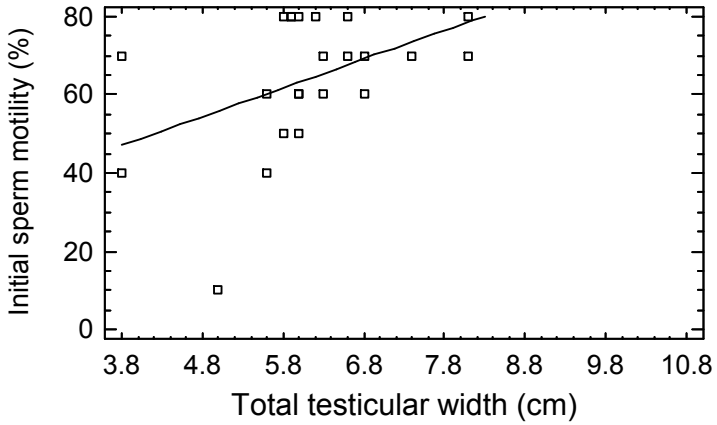

Fig. 1. Linear regression between the width of the two testicles and initial sperm motility. The adjusted regression model is Initial motility = 15.3346 + 7.6689 total testicular width, R^2 = 0.167

The mean sperm concentration in this study is higher than that reported by [9], whose mean value was 87.0 ± 53.1 x 10^6 sperm / ml, [16] reported values of 371 ± 30 x 10^6 sperm / ml, and [15] report a concentration of 137.44 ± 153 x 10^6 sperm / ml. Sperm concentration depends on the volume of seminal fluid secreted by the prostate and bulbourethral glands, therefore if a sperm quantity is suspended in a lower volume of seminal plasma, then the sperm concentration increases, or the opposite generates a lower concentration, therefore low volumes of seminal fluid generated by the peccaries in this research lead to higher sperm concentrations than reported by the aforementioned authors [17]. Report that other factors that can generate variations in sperm concentration are the measurement methods, either by CASA, sperm density meter, spectrophotometer, as well as ejaculate volume, age of the reproductive male, genetic line, interval between extractions, time of year and the interactions between these factors.

The motility of fresh semen obtained in this work is higher than those reported by [9] and [15], which were 48.7 ± 31.5 and 52.66 ± 28.79%, respectively. It is important to note that the

variation in motility values in the works reported as the present one suggests that there are various factors that affect the motile behavior of sperm; it is not possible at this time to explain what factors involved in this result, some them are for example mice fed excess fat alters sperm quality: increased DNA fragmentation, decreased motility, problems in spermatic capacitation and lower rates of fertilization [18] or the interaction with climatic factors, in pigs it has been shown that heat stress at temperatures around 42°C for six hours, induces a decrease in sperm motility by up to 32% [19], It is necessary to formulate experiments that identify the relevant sources of variation that define this sperm behavior, since this factor is important to evaluate the results of the tests before and after sperm freezing.

The value of sperm vigor reported by [9] is 1 ± 0.0 to 4.5 ± 0.6, [15] report values of 2.2 ± 0.8 on the scale of 1 to 5, so the values obtained in this study for fresh semen were within the range found by these authors. This variable also needs to be investigated in terms of the origin of the variation between populations or to define techniques to reduce imprecision, sperm vigor is characterized according with the intensity of sperm movements, sperm motility is essential for sperm reach the uterine environment and the fertilization site, being considered the most important criterion in the evaluation of sperm cells before and after cryopreservation [20]. Numerous seminal evaluations of sperm motility and vigor in livestock and wildlife use subjective techniques, through evaluation by eye [1,2]. The alternative to reduce measurement imprecision is through the use of dynamic evaluation methods such as the Computerized analysis method [21], which allows obtaining more precise data on sperm motility, velocity and morphology. Madrigal-Valverde M et al. [22] reported that with method Computer-assisted semen (CASA) it is possible to identify subpopulations of sperm with different motility characteristics and sperm measurements. Sperm motility and vigor values after freezing and thawing of the 12 selected samples is observed to decrease, this means sperm damage and loss of viable cells. Although damage to cell integrity was not measured in this work, it is possible to deduce the aforementioned, since in other reports there is a direct relationship between sperm damage and decreased motility or vigor due to alterations in the mitochondria of the middle part of the body sperm, however motility is not directly related to fertilizing activity [23].

The amount of abnormal sperm is similar to that reported by [24] who indicate values between 27 to 67% and higher than that reported by [2] who mention 3 to 25%. The wide variability between reports of sperm abnormalities has multi-factorial causes, from nutritional management, even factors external to the animal such as hyperthermic conditions, due to the fact that the testes are sensitive to this type of changes, this happens in all mammals [18].

The integrated measurements of length, width and total length of the spermatozoa of *P. tajacu* in this investigation are lower than that reported by [25], whose mean values are 6.34 ± 0.018 (µm) for head length, 4.2 ± 0.019 (µm) wide, 50.68 ± 0.121 (µm) total length.

The testicular measurements are similar to those reported by [26] which mentions values of 4.4 cm in length and diameter, while [8] reports 5.2 cm in length, 3.0 in width and 3.8 in diameter for the right testicle and 5.3 cm, 2.9 and 4.0 respectively, for the left testicle, these being the highest values, while the lowest values were 4.1cm, 3.5 and 2.8 for the right testicle and 4.5 cm, 3.3 and 2.9 for the left. Sonner JB et al. [27] present values of testicular sizes of 20 peccaries whose means are 4.36 cm long, 2.74 wide and 2.33 in diameter in the right testicles and 4.19, 2.68 and 2.34 respectively for the left ones.

Kahwage PR et al. [15] report that there are positive correlations with values between 0.609 to 0.952 between length and width between testicles ($p < 0.001$), and correlations between 0.63 to 0.681 between body weight and length and width of testicles ($p < 0.001$), they conclude that these correlation values are intermediate, although highly significant, therefore there is little body variation because the animals are adults and have stability in body development [28] report that there is no significant correlation between ejaculate volume, sperm concentration and quantity of spermatozoa in the semen with testicular volume, scrotal circumference and area of seminiferous tubules, the values range from 0.01 to -0.16; but [29] report that in pigs there are low correlations ($r = 0.24$) between paired testicular diameter and total average sperm, [28] mention that there are similarities in testicular morphophysiology in mammals, therefore comparisons between these species are valid. These same authors do not precisely explain the factors that determine the variability between testicular morphometry and sperm characteristics, they mention some such as age,

sexual maturity, nutritional status, endocrine balance, season of the year, even the efficiency of electroejaculation that limits obtaining good quality of semen or the separation between the sexes that eliminates copulation and generates greater sperm storage in testicles.

This information is important, but the trait to consider for the selection of reproductive male is the relationship between sperm traits and testicular morphometry, which in practical terms would allow the selection to be made easily and quickly for the cryopreservation of useful semen doses.

The logistic model shows that freezability (CONG) is related to sperm concentration and testicular width, with a relatively low percentage of deviation explained by the model, which is due to the small sample size as mentioned by [30] or the variability of these traits between animals of the same population as mentioned by [28]. However, the p-value of residuals is greater than 0.39, which means that the loss of fit without being explained is not significant. The likelihood ratio test with p-value for both predictor variables less than 0.05, shows that the data adequately fit the logistic model [31]. The probability calculation from the odds ratio values to assign freezability considering testicular width is 0.928 and the probability per concentration is 0.4989, which indicates that testicular width is a more efficient predictive variable than sperm concentration.

The adjustment by linear regression between sperm motility and testicular width shows that the model is significant, both models have in common the predictor variable testicular width. These regression results are to be expected, since the greater the amount of spermatogenic testicular tissue, the greater the testicular width or diameter and also the greater production of sperm cells, as occurs in domestic species for this type of relationship [32,33], according to the aforementioned, it is possible to propose the hypothesis that one of the criteria for selecting male peccaries that could be donors of seminal samples to be frozen, may come from males that present relatively high testicular diameters or widths, within the mentioned range in this study.

4. CONCLUSION

The resistance of sperm cells to the process of freezing and thawing in liquid nitrogen, makes it possible to obtain around 30% motility of thawed cells. There is a low but significant linear relationship between initial sperm motility and testicular width; there is also a significant logistic relationship between the freezability allocation of the semen sample with testicular width and sperm concentration. It is possible to propose in this species that testicular width is a variable to consider when selecting semen to cryopreserve in liquid N.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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