Use of the ACP® and BTS extenders for cooling at 15°C white-lipped peccary (*Tayassu pecari*) semen[1]

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Knowledge about reproduction of white-lipped peccary is of great importance to assist with the conservation of this species and enable its rational use in captivity. This study aimed to evaluate the effect of ACP-103®, ACP-116® and BTS semen extenders on sperm viability during cooling of *Tayassu pecari* semen. Five ejaculates from four adult males were chilled. The animals were submitted to the protocols of sedation and anesthesia for semen collection by the electroejaculation method. After collection, the semen was macro- and microscopically assessed and diluted to reach 35×10^6 spermatozoa/mL in each of the three different extenders tested. The fresh-extended semen was packed in a BotuFLEX® thermal box to keep samples at 15°C for 24 hours. After cooling, the following semen parameters were analyzed: sperm motility, functional and structural integrity of sperm membranes, mitochondrial activity, chromatin condensation, and the thermoresistance test was performed. The parameters sperm motility, structural and functional integrity of sperm membranes, mitochondrial activity, and chromatin condensation were preserved after use of the extenders tested, and were similar to those of *in natura* semen ($p>0.05$). Curvilinear velocity (VCL) ($p<0.05$) was the only parameter with reduced values after cooling regardless of the extender used. The percentage of sperm with normal morphology was greater in samples cooled using the BTS extender ($p<0.05$). The ACP-103®, ACP-116® and BTS extenders can be used for the cooling and preservation of white-lipped peccary semen at 15°C for 24 hours.

INDEX TERMS: ACP® extenders, BTS extender, white-lipped peccary, *Tayassu pecari*, semen, coconut water, biobank, wild animals.

RESUMO.- [**Uso dos diluidores ACP® e BTS para refrigeração a 15°C de sêmen de queixada (*Tayassu pecari*).**] Para auxiliar na conservação da espécie e permitir o uso racional do queixada em cativeiro é de grande importância o conhecimento sobre a reprodução da espécie. Objetivou-se avaliar o efeito dos diluidores de sêmen ACP-103®, ACP-116® e BTS na viabilidade espermática durante a refrigeração do sêmen do *Tayassu pecari*. Foram refrigerados cinco ejaculados provenientes de quatro machos adultos. Os animais foram contidos com auxílio de puçá e submetidos ao protocolo de sedação e anestesia para realização da coleta de sêmen pelo método da eletroejaculação. Depois da coleta, o sêmen foi avaliado macro e microscopicamente e diluído para atingir 35×10^6 espermatozoides/mL em cada um dos três diferentes diluidores testados. O sêmen diluído foi acondicionado em caixa térmica BotuFLEX® para manter as amostras a 15°C por um período de 24 horas. Depois da refrigeração, os espermatozoides foram avaliados quanto aos parâmetros de movimento espermático, integridade funcional e estrutural das membranas espermáticas, atividade mitocondrial, condensação da cromatina e teste de termorresistência. Os diluidores testados preservaram as

características cinéticas, a integridade estrutural e funcional das membranas espermáticas, a atividade mitocondrial e a condensação da cromatina semelhante ao sêmen *in natura* (P>0,05). O único parâmetro que reduziu com o processo de refrigeração independente do diluidor utilizado foi a Velocidade Curvilínea (VCL) (P<0,05). Foi observado aumento do percentual de espermatozoides morfologicamente normais nas amostras refrigeradas em BTS (P<0,05). Os diluidores ACP-103®, ACP-116® e BTS podem refrigerar e conservar o sêmen de queixada a 15°C por 24 horas.

TERMOS DE INDEXAÇÃO: Diluidores ACP®, diluidor BTS, refrigeração, sêmen, queixada, *Tayassu pecari*, água de coco, biobanco, animais silvestres.

INTRODUCTION

White-lipped peccary (*Tayassu pecari*), a species commonly known as wild pig, is classified under the vulnerable category (VU) in the Red List of the International Union for the Conservation of Nature (IUCN) as a consequence of hunting and loss of habitat in its entire range of occurrence (Keuroghlian et al. 2013). This situation is more critical in some regions, such as in the significant remnants of Atlantic Forest in southeastern Brazil, where presence of peccaries was verified in only 31.4% of the area (SOS Mata Atlântica & INPE 2010). Owing to this sharp population decline, peccaries are considered as critically endangered in the regions of Atlantic Forest (Azevedo & Conforti 2008, Galetti et al. 2009). This species, however, plays a crucial role in the maintenance of forests through both dispersal and predation of seeds and seedlings (Keuroghlian & Eaton 2009, Altrichter et al. 2011). Because white-lipped peccary is a social ungulate that forms cohesive, large groups of hundreds of individuals (Fragoso 2004), they significantly influence biotic communities. For instance, a group of 400 peccaries represents the displacement of 12,000-20,000 kg of biomass, moving in synchrony, trampling the soil when consuming roots, seeds, and seedlings (Fragoso 2004, Beck 2006).

In contrast, this species is an important source of food for Neotropical indigenous populations and a source of income for rural populations (Fragoso 2004). In Brazil, commercial hunting is prohibited whereas subsistence hunting is simply ignored by the authorities, which has led to the disappearance of this and other native species (Nogueira & Nogueira Filho 2011). A captive breeding program could be one way to reverse the decline in white-lipped peccary population, enabling a more sustainable exploitation of the species in isolated communities that practice subsistence hunting (Figueira et al. 2003). In this context, studies addressing the behavior, nutrition and management of this species have been conducted (Nogueira Filho et al. 1999, 2014, Figueira et al. 2003, Nogueira et al. 2015, 2016), and although white-lipped peccary reproduce in captivity, there are reports that some males present low fertility (Sowls 1997).

When small, isolated populations are formed, considering linear social structure of white-lipped peccary, inbreeding can occur, reducing heterosis and compromising the reproductive efficiency and survival of the species (Biondo et al. 2011). The negative effect of inbreeding can be mitigated by the introduction of new animals or the use of fresh-extended or cooled and frozen semen from other breeding males and the exchange of biological material to increase the genetic variability and sustainability of these captive populations. The use of semen processing technologies may be an alternative for future use in assisted reproduction, such as in artificial insemination (AI) and *in vitro* fertilization of breeding females (Garcia et al. 2012, Souza et al. 2016, Campos et al. 2017).

Utilization of cooled semen is a possibility for the best use of ejaculates, facilitating the dissemination of genetic material in different regions (Silva et al. 2012), contributing to biodiversity conservation. However, semen needs to be processed into appropriate extenders prior to cooling to ensure sperm viability and cell survival during processing (Dziekońska et al. 2009). Nevertheless, spermatozoa are particularly sensitive to thermal shock, which induces changes in plasma membranes, resulting in reduction of their biological properties, mainly in the *Suidae* species (Johnson et al. 2000). Effect of thermal shock on spermatozoa depends on cooling rate, final storage temperature, and type of extender used (Paulenz et al. 2002). Because of this sensitivity to reduced temperature, swine semen has been extensively processed with dilution and storage at 15-20°C for 1-5 days (Johnson et al. 2000, Toniolli et al. 2010). In view of the general similarities with the domestic pig (*Sus scrofa*), the semen processing protocols used in white-lipped peccary were extrapolated from this species (Mercado et al. 2010). Recent studies conducted with collared peccary (*Pecari tajacu*) have demonstrated that semen cooled at 5°C and 17°C can remain viable for up to 36 h and 48 h, respectively (Garcia et al. 2012), however, no reports have been found in the literature on the behavior of cooled semen from white-lipped peccary.

Coconut water-based extenders have been described to conserve semen from different domestic and wild species at low temperature, namely, the ACP-103® extender was reported cooling swine semen (Silva et al. 2015) and the ACP-116® extender cryopreserving collared peccary semen (Silva et al. 2012). These extenders are composed of standardized *in natura* coconut water and sugar, proteins, vitamins, amino acids, minerals, fat, and pH regulators (glycine and sodium citrate). Another extender widely used to cool swine semen is BTS (Beltsville Thawing Solution), which does not contain coconut water in its formulation, and is composed of sodium citrate, sodium bicarbonate, EDTA, potassium chloride, streptomycin, and glucose, and has been used in the cooling of semen of other species (Garcia et al. 2016, Zorinkimi et al. 2017).

Therefore, aiming to preserve the species for the possibility of facilitating dissemination of genetic material and due to lack of reports in the literature on the cooling of white-lipped peccary semen for use in assisted reproduction, the present study aimed to evaluate the effect of three different semen extenders (ACP-103®, ACP-116®, and BTS) on storage of semen of this species at 15°C and maintenance of sperm viability characteristics compatible with fertility.

MATERIALS AND METHODS

The experimental protocols applied in this study were approved by the Ethics Committee on Animal Use of the aforementioned Institution (CEUA-UESC) under protocol no. 031/16.

During the study period (March to June 2017), the adult, male white-lipped peccary presented an average wheight of 36kg. Animals from Experimental Captive Breeding Center of the State University of Santa Cruz (UESC), municipality of Ilhéus, Bahia state,

Brazil (14° 47' 47" S; 39°10' 20" W), were individually allocated in individual pens prior to semen collection. In these enclosures, the animals were fed a diet composed of corn mixture, soybean meal, and mineral supplements twice daily, providing 120 g/kg of crude protein and 14.5 MJ/kg of digestible energy, based on dry matter, following the recommendations by Nogueira-Filho et al. (2014), and water *ad libitum*.

At semen collection, the white-lipped peccary were restrained using a net and submitted to anesthetic protocol, with an association of acepromazine and ketamine, after feed fast of 12 h and water fast of 6 h. Drug administration was as follows: acepromazine (0.2 mg/kg, IM) and ketamine (5 mg/kg IM), as described by Kahwage et al. (2010), who performed similar procedures for collecting semen from collared peccary (*Tayassu tajacu*), a peccary of the same family (*Tayassuidade*) and with morphological and physiological characteristics similar to those of white-lipped peccary (Sowls 1997).

After anesthesia, 5 UI of oxytocin was administered in the saphenous vein 5 min before semen collection (Ungerfeld et al. 2016). Semen was collected using an electroejaculator (Eletrogen®) equipped with a probe with two linear electrodes connected to a 12 V source according to the protocol for collared peccary (Castelo et al. 2010).

After collection, the samples were immersed in dry bath at 37°C and the following macro- and microscopic parameters of *in natura* semen were assessed: volume, color, odor, appearance, pH, motility, spermatic concentration and morphology (CBRA 2013), as well as functional and structural integrity of the plasma membrane.

Volume of the ejaculates was measured using an adjustable volume micropipette. Spermatic concentration was evaluated by counting the spermatozoa in Neubauer chamber and pH was measured using urine strips (UROFITA®). For evaluation of sperm morphology, an aliquot of fresh semen of all ejaculates was fixed in 4% sodium citrate solution and 200 spermatozoa were analyzed under phase contrast microscopy (obj.1000x) in wet mount between slide and coverslip, and the spermatic defects were classified according to the region of the morphological change found.

Sperm Class Analyser® (SCA®; Microptics S.L, v.5.2, Barcelona, Spain) software was used for evaluation of the sperm motility parameters, according to the manufacturer's recommendations for swine spermatozoa, as follows: 25 images/sec with 25 Hz; particle size captured between 10 and 80μm/m^2; velocity of spermatozoa: static <10 μm/s, slow <25 μm/s, medium >25 μm/s and <45 μm/s, and rapid >45 μm/s, as described by Silva et al. (2012) when performing the same analysis in collared peccary semen, and according to Vieira et al. (unpublished data), who investigated the sperm morphometry of white-lipped peccary and reported that it was within the particle size captured by the swine setup. One 5 μL drop of semen was placed between slide and coverslip preheated on warming platen at 37°C, and an average of five different fields were digitized. The following parameters were assessed: total motility (TM); progressive motility (PM); linearity (LIN); straightness (STR): rapid, medium, slow and hyperactive in percentage (%); curvilinear velocity (VCL); straight-line velocity (VSL); average path velocity (VAP); wobble (WOB), expressed in micrometers per second (μm/s); amplitude of lateral head displacement (ALH), expressed in micrometers (μm); beat cross frequency (BCF), expressed in Hertz (Hz).

Assessment of the plasma membrane functional integrity was performed by the hyposmotic swelling test (HOST) by adding 10μL of semen to 90μL distilled water (0 mOsmol/L) followed by immersion in dry bath at 37°C for 40 min (Santos et al. 2013). After incubation, reading of percentage of HOST reactive sperm was performed under optical microscopy (Olympus® CX 21), using immersion objective (obj.1000x) in wet mount between slide and

coverslip stained with 3% rose bengal. A total of 200 spermatozoa were evaluated and classified as reactive or non-reactive to HOST based on the presence or absence of bended and/or coiled tails, respectively (Jeyendran et al. 1984). For calculation of the percentage of spermatozoa with functionally intact membrane, the formula described by Melo & Henry (1999) was used, in which the percentage of spermatozoa with tail defects (based on morphology evaluation) was subtracted from the percentage of spermatozoa reactive to HOST with bended and coiled tails.

Structural integrity of the plasma and acrosomal membranes was evaluated under fluorescence microscopy (Olympus® CX 31; obj.400x) after staining of spermatozoa with fluorochrome carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) according to the method of Harrison & Vickers (1990). CFDA staining was evaluated using a standard fluorescein filter set, whereas PI staining was assessed using a standard rhodamine filter set. Two hundred spermatozoa were analyzed per sample (Silva et al. 2006).

Five ejaculates from four different white-lipped peccary were used in the present study. Only ejaculates with minimum volume of 0.2 mL, minimum concentration of 35x10^6 spermatozoa/mL, and total motility >60% were used, which were considered compatible with the experimental objective. The ejaculates were analyzed *in natura* and divided into three fractions to be processed with three different extenders, namely, ACP-103® (ACP Biotecnologia, Fortaleza, CE, Brazil); ACP-116® (ACP Biotecnologia, Fortaleza/CE, Brazil), and Beltsville Thawing Solution (BTS)- Prolimax®BTSE (Vet life, Nova Odessa, SP, Brazil). Semen was diluted according to Barros et al. (2016) to show 35x10^6 spermatozoa/mL. The sperm motility parameters were assessed 5 min after completion of the dilution process (SCA®). The semen diluted using the ACP-103®, ACP-116® and BTS extenders was cooled in semen transport thermal box (BotuFLEX®, Botupharma Botucatu/SP, Brazil) soon after sperm motility analysis and verification of interaction between extender and semen. Final storage temperature of 15°C and cooling rate of 0.05°C/min were used. The samples remained chilled in BotuFLEX® thermal box for 24 h. After this period, the box was opened, the semen was thawed in dry bath at 37°C for 5 min, and the following sperm motility parameters were analyzed to verify the effect of the cooling process and the extenders tested on the behavior of the spermatozoa: sperm morphology, functional and structural integrity of the membranes, mitochondrial activity, and chromatin condensation.

Sperm mitochondrial activity was analyzed by 3,3'-diaminobenzidine (DAB) staining (Hrudka 1987) after incubation of 20μL of the sample in 20μL of DAB (1mg/mL of PBS) at 37°C for 60 min in the dark. After incubation, smears were fixed in 10% formalin for 10 min, washed in distilled water, and air dried under light protection. Two hundred cells were analyzed under phase contrast microscopy (Olympus® CX 31; obj.1000x). The cells were classified according to the amount of stain deposited on the mid-piece (MP). In class I, the spermatozoa presented the MP completely stained (all mitochondria active); in class II, the spermatozoa showed >50% of the MP stained (most mitochondria active); in class III, the spermatozoa had <50% of the MP stained (most mitochondria inactive); in class IV, the spermatozoa were not stained (all mitochondria inactive).

Spermatic chromatin condensation was evaluated using the metachromasia technique induced by toluidine blue (Naves et al. 2004). Smears were prepared with an aliquot of 10μL of the cooled sample, dried at room temperature, and fixed for 1 min in Carnoy's solution (3:1; 75mL 100% alcohol + 25mL acetic acid) and then in 70% alcohol for 3 min. Subsequently, the smears were hydrolyzed with 4N hydrochloric acid for 15 min, washed with distilled water, and

dried at room temperature. For smear staining, 20μL of 0.025% toluidine blue solution was deposited (0.00125g toluidine blue in 5mL McIlveine solution, at pH 4.0) between slide and coverslip, and 500 cells were analyzed under phase contrast microscopy (Olympus® CX 31; obj.1000x). Spermatozoa were classified as follows: with condensed chromatin (head region stained in light blue) and with fragmented chromatin (head region stained in dark blue or violet).

Sperm longevity after 24 h of cooling was assessed by the slow thermoresistance (STT) test after the samples were incubated at 37°C for 240 min. Sperm motility characteristics were evaluated using the SCA® software, as previously described.

For statistical analysis, a completely randomized block design was used, considering each individual as a block. All assumptions of normality and homoscedasticity were tested and found adequate. Results were submitted to analysis of variance (ANOVA). Comparison of the means of the sperm motility parameters between five ejaculates was performed by the Tukey test. Comparison of the means of the sperm motility parameters between the two ejaculates that showed resistance to cooling was conducted using the Scott-Knott test. The Friedman's non-parametric test was applied to compare the means of sperm morphology and structural and functional integrity of the membranes. The Tukey test was also used to compare the means of mitochondrial activity. Means were considered statistically different when $p<0.05$. All analyses were performed using the R Core Team (2016) software.

RESULTS

Macroscopic characteristics of *in natura* semen of five ejaculates from four white-lipped peccary evaluated were as follows: mean volume of 0.34 mL, white in color, *sui generis* odor, milky appearance, and pH values from 7.0 to 7.5. The microscopic characteristics of sperm motility are presented in Table 1. The mean concentration of spermatozoa in the ejaculate was 947.3×10^6/mL.

Dilution process of the five ejaculates using the ACP-103®, ACP-116® and BTS extenders did not influence the sperm motility parameters, except for VCL and VAP ($p<0.05$).

VCL and VAP were significantly reduced when fresh semen was diluted using the ACP-103® and ACP-116® extenders. The BTS extender preserved these parameters, which were similar to those of *in natura* semen (Table 1).

Table 2 shows the sperm motility parameters of *in natura* semen after dilution with the three extenders tested and after 24 h of cooling at 15°C. It is worth noting that only the semen of two peccaries showed resistance to the cooling process; the spermatozoa of the three samples from the two other animals submitted to cooling were dead after 24 h of storage at 15°C.

It was possible to observe that the percentage of spermatozoa with medium velocity increased after semen cooling using the ACP-103® and BTS extenders ($p<0.05$). VCL was reduced in the diluted and cooled samples ($p<0.05$). STR was increased in semen extended with ACP-116® and BTS ($p<0.05$). Increased percentage of morphologically normal spermatozoa was observed in the cooled samples using BTS ($p<0.05$), whereas decreased percentage of spermatozoa with distal cytoplasmic droplets (DCD) was observed in all chilled samples regardless of the extender used ($p<0.05$). Statistically significant increase in the percentage of spermatozoa with general tail defects (GTD) involving the mid piece, principal piece and end piece was observed in all cooled samples using the ACP extenders. It should be noted that there was a statistically significant reduction in the percentage of spermatozoa with most mitochondria inactive in the chilled samples using the BTS extender. It is worth emphasizing that the dilution and cooling process did not significantly affect the other parameters: sperm motility, structural and functional integrity of the membranes, mitochondrial activity, and chromatin condensation; although the predilection of white-lipped peccary sperm for the BTS extender is evidenced by analysis of the standard deviation of the means of the characteristics studied.

Figure 1 illustrates the sperm behavior of the ejaculates from two peccaries during the STT to evaluate sperm longevity using the SCA® software after 24 h of cooling. It was not possible to assess the data statistically, but it was observed that the spermatozoa of white-lipped peccary 1, despite showing sperm

Table 1. Sperm motility parameters of *in natura* and ACP-103®, ACP-116® and BTS fresh-extended semen of five ejaculates from four white-lipped peccary

Parameters	In natura	ACP-103®	ACP-116®	BTS
Total motility (%)	85.7± 9.3	72.7± 18.9	70.2± 17.7	84.3± 14.5
Progressive motility (%)	46.7 ±13.7	33.3± 19.9	32.8± 21.4	42.7± 17.8
Rapid (%)	48.8± 24.0	30.4 ± 23.9	28.3± 24.3	38.6 ± 22.4
Medium (%)	19.4± 7.8	19.2 ± 5.4	17.4± 5.7	24.1±4.1
Slow (%)	17.4± 10.6	23.0± 8.6	24.7± 10.2	21.4±9.5
Hyperactive (%)	5.2± 5.5	3.1± 3.7	2.2±1.9	3.8± 3.5
VCL (μm/s)	51.2± 13.8[a]	39.1 ±10.8[b]	38.8± 11.1[b]	43.6± 9.3[ab]
VSL (μm/s)	19.8 ± 6.1	13.4± 5.5	14.2± 7.8	14.6± 5.6
VAP (μm/s)	34.2± 12.8[a]	22.9± 8.4[b]	23.8± 11.3[b]	24.6± 8.4[ab]
LIN (%)	38.8± 8.1	33.8 ± 10.1	34.7± 10.2	32.7± 7.1
STR (%)	59.4± 8.6	57.4± 5.7	57.9± 5.1	58.2± 3.9
WOB (%)	65.4± 10.9	58.1 ± 11.9	59.1± 12.4	55.6± 8.7
ALH (μm)	2.3± 0.4	2.2± 0.3	2.2± 0.3	2.4± 0.1
BCF (Hz)	6.6± 0.9	6.2± 1.4	5.9± 1.3	6.0± 0.7

[a,b] Letters in superscript on the line indicate significant statistical difference (p<0.05), assessment of the dilution effect after 5min of addition of the extenders to in natura semen; VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 2. Parameters of sperm motility and morphology, functional and structural integrity of sperm membranes, mitochondrial activity, and chromatin condensation in semen *in natura* and ACP-103®, ACP-116® and BTS fresh-extended, and cold-stored for 24h at 15°C - data from two white-lipped peccary

Parameters	In natura	Fresh-extended semen			Cold-stored semen		
		ACP-103®	ACP-116®	BTS	ACP-103®	ACP-116®	BTS
MT (%)	91.0±11.3	92.5±5.4	87.9±3.9	96.3±0.8	93.5± 5.5	78.4± 24.3	97.0± 0.3
MP (%)	51.0±12.1	53.8±10.4	55.8±8.3	61.5±2.0	45.5± 16.6	34.8± 23.6	51.6±1.3
Rapid (%)	66.3±6.5	56.4±10.1	52.6±19.1	62.0±7.8	39.8±20.2	33.9± 25.4	46.5± 4.2
Medium (%)	16.1±6.8 [b]	22.2±6.7 [b]	19.6±10.0[b]	23.3±4.2[b]	31.5±2.8[a]	21.7± 7.4[b]	33.3± 0.3[a]
Slow (%)	8.7± 2.1	13.9±3.5	16.2±5.9	11.1±2.8	21.9±12.2	22.8± 8.4	17.2± 3.5
Hyperactive (%)	7.7± 8.1	5.4±5.6	3.6±1.8	6.7±3.7	4.6±5.3	3.5± 4.5	5.3± 5.3
VCL (μm/s)	63.7± 1.4[a]	50.9±0.6[b]	49.1±9.3[b]	53.0±2.7[b]	44.0±10.1[b]	42.5±11.3[b]	47.3± 1.4[b]
VSL (μm/s)	23.0± 2.6	18.0±4.1	22.5±4.5	20.8±0.0	14.4±6.1	14.0± 6.2	14.2±1.0
VAP (μm/s)	44.2± 9.5	31.0±4.6	35.6±6.4	33.9±0.3	25.8±8.4	25.5±8.8	25.7± 1.4
LIN (%)	36.0± 3.2	35.5±8.4	45.7±0.4	39.4±2.1	32.1±6.5	32.2± 6.2	30.2± 3.1
STR (%)	52.6± 5.6[b]	57.8±4.5[b]	63.1±1.3[a]	61.4±0.6[a]	55.1±5.4[b]	53.9± 6.0[b]	55.5± 0.9[b]
WOB (%)	69.1±13.4	61.0±9.7	72.5±0.9	64.1±2.7	57.9±6.0	59.4± 4.9	54.4± 4.7
ALH (μm)	2.7±0.7	2.5±0.1	2.1±0.1	2.6±0.1	2.4±0.2	2.3± 0.3	2.6± 0.1
BCF (Hz)	6.4±1.2	7.5±1.7	7.2±1.0	6.7±0.7	5.8±0.2	5.7± 0.1	6.1± 0.9
Normal (%)	45.0±38.1[b]	-	-	-	53.8±35.0[ab]	60.5±29.0[ab]	71.0±18.4[a]
PCD (%)	4.3±5.3	-	-	-	4.3±1.1	2.0±2.8	3.5±3.5
DCD (%)	32.8±26.5[a]	-	-	-	0.0±0.0[b]	6.0±8.5[ab]	0.0±0.0[b]
MPD (%)	5.8±6.7	-	-	-	4.0±5.7	0.0±0.0	2.5±3.5
GTD (%)	8.3±10.2[b]	-	-	-	32.3±28.6[a]	31.5±23.3[a]	23.0±18.4[ab]
Intact (%)	40.5±11.3	-	-	-	56.0±14.1	62.3±11.7	51.5±19.1
HOST-r (%)	51.0±22.6	-	-	-	46.5±24.7	56.5±19.1	60.0±8.5
DAB I (%)	-	-	-	-	90.8±5.3	91.5±6.4	97.3±1.8
DAB II (%)	-	-	-	-	0.0±0.0	1.0±0.7	0.5±0.7
DAB III (%)	-	-	-	-	2.8±1.1[a]	1.8±1.1[ab]	1.0±0.7[b]
DAB IV (%)	-	-	-	-	6.5±4.2	5.8±4.6	1.3± 1.8
DNA-c (%)	-	-	-	-	93.5±7.8	94.8±6.0	94.5± 6.4
DNA-f (%)	-	-	-	-	6.5±7.8	5.3±6.0	5.5±6.4

[a, b] Letters in superscript on the line indicate significant statistical difference (*p*<0.05), assessment of the dilution effect after 5min of addition of the extenders to *in natura* semen; (-) sperm motility parameters not assessed; VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, Normal = morphologically normal; Morphological changes in spermatozoa: PCD = proximal cytoplasmic droplets, DCD = distal cytoplasmic droplets, MPD = mid-piece defects, GTD = general tail defects, involving mid piece, principal piece and end piece; Intact = spermatozoa with integral structural membrane (CFDA+), HOST-r =spermatozoa reactive to the hyposmotic swelling test, with functionally intact plasma membrane, DAB I = all mitochondria active, DAB II = most mitochondria active, DAB III = most mitochondria inactive, DAB IV = all mitochondria inactive, DNA-c = condensed chromatin, DNA-f = fragmented chromatin.
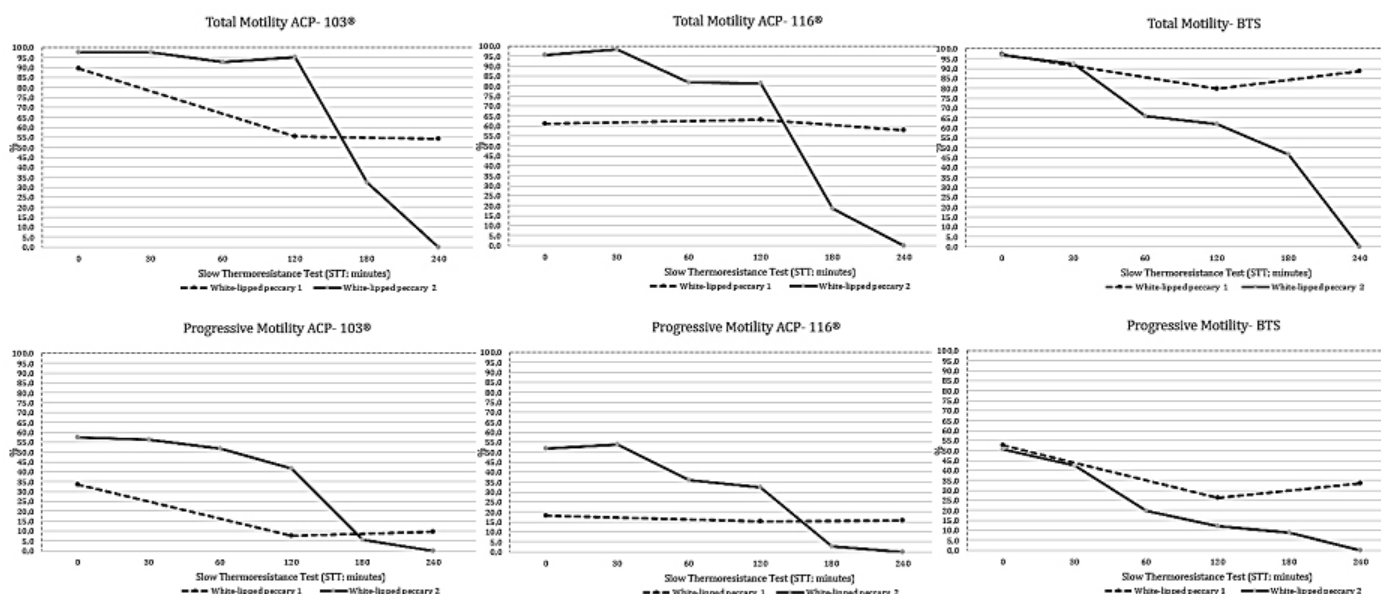

Fig.1. Total (TM) and progressive (PM) motility of the semen of two white-lipped peccary diluted using the ACP-103®, ACP-116® and BTS extenders and assessed with regards to sperm longevity for up to 240 min by the slow thermoresistance test (STT).

motility parameters slightly lower than those of white-lipped peccary 2, presented greater thermoresistance, since there was no significant variation in these parameters between the beginning and the end of the STT, as it was found in the second animal. Moreover, the spermatozoa showed motility for up to 240 min in the STT.

DISCUSSION

Dilution process of the five ejaculates from white-lipped peccary using the ACP and BTS extenders, at the spermatic concentration used, did not negatively influence the motility characteristics of the spermatozoa. However, it was observed that the ACP extenders were not as efficient in preserving VCL and VAP as the BTS extender, which maintained these parameters with values similar to those found in *in natura* semen. The ability of the BTS extender to preserve VCL and VAP from spermatozoa after dilution of *in natura* semen may be associated with the medium constitution, which contains a small amount of potassium compared with that of ACP-based extenders. According to Alvarez & Storey (1982), this characteristic favors the Na/K pump, maintaining the intracellular concentration of this ion at physiological levels, fostering sperm motility. In contrast, reduction in sperm kinetics after use of ACP extenders had already been described by Rondon et al. (2008) when using this extender for the conservation of collared peccary (*Numida meleagris*) semen at 15°C for 24 h.

It was possible to verify that the semen of two white-lipped peccary was very resistant to the cooling process at 15°C for 24 h, because no effects of dilution with the tested extenders and of temperature decrease were verified on most of the parameters studied, namely, sperm motility, structural and functional integrity of membranes, mitochondrial activity, and chromatin condensation. The results of sperm quality after cooling found in the present research were higher than those reported by Garcia et al. (2012), who conserved the semen of collared peccary, a peccary of the same family of white-lipped peccary, which suggests that spermatozoa of the first may be slightly more resistant to metabolism reduction by cold than those of the latter.

Cooling of semen resulted in increased percentage of spermatozoa with medium velocity in the samples processed with the ACP-103® and BTS extenders compared with that *in natura*, diluted regardless of the medium used, and processed with the ACP-116® extender. In addition, VCL was reduced in all diluted samples and after cooling regardless of the extender used. The increase in medium velocity occurred because of reduction in the percentage of spermatozoa with rapid velocity and increase in the percentage of spermatozoa with slow velocity and, according to Campos et al. (2017), the rapid and medium velocities and the VCL are not kinetic parameters positively and significantly correlated with the capacity of spermatozoa to bind to oocytes or perivitelline membrane in *in vitro* binding tests. Nevertheless, the parameter of slow velocity presents statistically significant negative correlation with binding to perivitelline membrane (r=-80.5). It is worth mentioning that preservation of motility after 24 h of cooling is indicative of sperm quality, considering that these authors reported that this parameter is a good predictor of fertility in the perivitelline membrane binding assays in collared peccary semen.

STR was increased in the samples diluted with the ACP-116® and BTS extenders, although this parameter remained with the same percentage verified in *in natura* semen after the cooling process. Campos et al. (2017) also demonstrated that STR is a marker of sperm progression, and that it was positively correlated with the capacity of collared peccary sperm to interact with heterologous swine oocytes (r=66.5).

It is necessary that studies addressing the sperm motility parameters that influence sperm binding capacity be conducted with white-lipped peccary, because the success of fertilization is dependent on the meeting of gametes and, to this end, the spermatozoa must move along the genital system of the female and overcome anatomical and physiological barriers (Yaniz et al. 2014). Furthermore, knowledge of the correlations between the sperm viability and kinetics parameters and binding tests could provide relevant information about the *in vitro* fertilizing potential, enabling identification of which sperm quality parameters can effectively impair *in vivo* fertilization capacity (Campos et al. 2017).

There was significant increase in the percentage of morphologically normal spermatozoa after cooling with the BTS extenders and significant decrease in the percentage of cytoplasmic droplets after refrigeration with the ACP-103® and BTS extenders, because of the DCD were somehow released by the flagellum beat during semen processing. Leidl et al. (1999) and Saravia et al. (2007) reported that cytoplasmic droplets are not considered of great importance in swine, considering that they do not show significant correlation with change in fertility in this species. It is possible that this also occurs in peccaries.

Percentage of GTD, bending and coiling, increased in ACP-cooled samples, indicating that coconut water-based extender were not as effective in controlling the lesions caused by thermal shock. The extender tested were efficient in preserving more than 90% of the spermatozoa that showed all mitochondria active. Mitochondrial metabolism plays a crucial role in the regulation of several sperm functions (Flores et al. 2009), especially those associated with displacement and projection of the cell to the fertilization site (Halang et al. 1985, Ruiz-Pesini et al. 1998). According to Cummins et al. (1994), the effect of oxidation on mitochondrial activity and on the spermatic membrane can be considered the main causing factor of sperm motility reduction, thus evaluation of mitochondrial activity can be a good indicator of its integrity and functionality. However, it should be noted that the ACP-103® extender developed for swine resulted in increased percentage of spermatozoa with most mitochondria inactive.

In the present study, it was possible to demonstrate that the white-lipped peccary semen can be cooled at 15°C for 24 h using the ACP-103®, ACP-116® and BTS extenders. Nevertheless, it was also possible to observe that there was preference of the spermatozoa for the BTS extender, because the standard deviation of the means of the parameters sperm motility; percentage of rapid, medium, slow spermatozoa; VCL; VSL; VAP; LIN; STR; percentage of spermatozoa morphologically normal, functionally intact, and with all mitochondria active showed lower values compared with the results of samples processed using the ACP extenders. Coconut water-based extenders did not show a composition of substances capable of ensuring more homogeneous semen quality responses. This variation observed in the present research can also be attributed to the question of animal individuality in the semen

cooling process that has already been observed by other authors (Roca et al. 2006, Garcia et al. 2012). Garcia et al. (2012) reported significant difference in resistance of cooled spermatozoa stored at 17°C for up to 48 h between the collared peccary investigated and between the ejaculates of the same individual. It is important to note that the white-lipped peccary in this study did not undergo reproductive selection and nevertheless produced spermatozoa with potential to be chilled and stored for 24h with the possibility of use in assisted reproduction techniques such as AI.

Of the five ejaculates diluted and refrigerated, only two ejaculates from two white-lipped peccary remained with viable spermatozoa after 24 h of cooling. Three ejaculates from two white-lipped peccary, which were in sexual rest, presented spermatozoa with no resistance to the cooling process, since 100% of the cells were dead when the BotuFLEX® thermal box was opened. There are no reports in the literature on the behavior of white-lipped peccary chilled spermatozoa. Garcia et al. (2012) studied the spermatozoa of collared peccary and observed reduction in motility, vigor, integrity of the plasma membrane, and increase of total sperm defects compared with samples of *in natura* semen and cooled for 24 and 48h at 17°C, which evidences that collared peccary semen is less resistant to the cooling process than that of white-lipped peccary. It is also known that swine spermatozoa are very sensitive to temperature oscillations, because it is common the occurrence of thermal shock that causes irreversible lesions in cell structure, and loss of sperm motility, selective permeability and plasma membrane integrity, which can lead to cell death (Watson 1996). It is possible that the spermatozoa of white-lipped peccary that have not resisted the cooling process have suffered the effect of thermal shock, since it is known that spermatozoa stored for a long time in the tail of the epididymis are less resistant to semen manipulation and processing (Pellestor et al. 1994).

Reduction in the quality of cooled spermatozoa is expected, because the process does not interrupt cellular metabolism, it only reduces the mechanical activity of the cells. Spermatozoa consume the nutrients available in the extracellular environment and excrete toxic products through constant production of substances reactive to the oxygen resulting from this metabolism (Johnson et al. 2000). In addition, sperm viability and cell osmotic resistance are also reduced by increasing the number of abnormal cells in semen (Johnson et al. 2000, Peña & Linde-Forsberg 2000). Additionally, it is known that spermatozoa undergo successive changes in the plasmatic membrane fluidity and funtionality during cooling (Holt 2000), resulting in reduced selective permeability, leading to calcium influx (Maxwell & Johnson 1997), as observed in samples diluted in ACP extenders (Garcia et al. 2012, Souza et al. 2016). Therefore, sperm stored for a long time in animals with long sexual rest may show greater sensitivity to the aforementioned factors.

Aiming to minimize the effects of thermal shock, a slow curve with a mean cooling rate of approximately 0.05°C/min and final storage temperature of 15°C in the BotuFLEX® thermal box was used to cool white-lipped peccary semen in this study. The cooling rate and storage container used were different from those described in the literature for cooling swine and collared peccary semen, namely, cooling rate of -0.38°C/min to decrease the temperature from 35°C

to 25°C and cooling rate of -0.11°C/min with temperature reduction from 24.9°C to 17°C, respectively (Nascimento et al. 1998, Garcia et al. 2012). The BotuFLEX® thermal box does not allow adjustment of the cooling rate, only selection of final storage temperature to 15°C or 5°C. In spite of that, it was observed that the semen of the two peccaries behaved quite well during cooling when the sperm viability data were compared with the findings by Garcia et al. (2012), who reported mean motility of approximately 22.5%, mean percentage of spermatozoa with intact membrane of 30.4%, and percentage of total defects of 39.4% after 24 h of cooling at 17°C in semen from collared peccary. Those authors used the cooling protocol for swine in collared peccary, and reported that the spermatozoa of the latter showed sensitivity to cold very similar to that of the first, which is considered one of the most sensitive to the cooling and freezing protocols compared with spermatozoa of other domestic species (Watson 2000) such as cattle, goats, and canines.

The low resistance of sperm to thermal shock observed in some species of wild and domestic mammals and between ejaculates of the same individual is associated with the chemical and molecular composition of the plasma and mitochondrial membranes, especially with the amount of fatty acids and phospholipids and of seminal plasma (Holt et al. 2005). Regarding swine spermatozoa, this sensitivity is closely associated with the cholesterol:phospholipid ratio (0.12) present in the plasma membrane, and it is lower than those observed in bulls (0.38) or rams (0.36). This characteristic predisposes spermatozoa to several modifications: changes in membrane fluidity and selective permeability, and it can lead to cell death when semen is chilled (Leeuw et al. 1991). No reports on the composition of semen plasma and plasma membrane of white-lipped peccary spermatozoa have been found in the literature.

During the STT, a sharp decline was observed in the sperm motility parameters of white-lipped peccary 2 - an individual that presented better parameters of sperm quality after cooling and had better viability results regardless of the extender tested. However, the spermatozoa of this animal did not show resistance at 37°C for up to 240 min, and somehow the cooling process contributed to reduction in sperm longevity. The sperms of white-lipped peccary 1 showed greater resistance in the STT and were longer lived regardless of the extenders used, although when conditioned with the BTS extender - a medium containing glucose, sodium citrate, sodium bicarbonate, potassium chloride, EDTA and streptomycin, there was no sharp decrease in the kinetic parameters of spermatozoa, as observed in samples diluted in ACP extenders. Indole-3-acetic acid, a molecule of the auxin group, a plant hormone present in coconut water and ACP extenders (Nunes & Salgueiro 1999), may somehow not have guaranteed sperm motility in white-lipped peccary for a long time, even though it is already known that ACP extenders are excellent for the sperm quality parameters of swine (Toniolli et al. 1996).

Sperms of the white-lipped peccary of the present research showed motility results after STT higher than those of swine in the study by Mendez et al. (2013), which suggests that they may have greater longevity in the female reproductive tract.

CONCLUSION

ACP-103®, ACP-116® and BTS extenders can be used for the cooling and preservation of white-lipped peccary semen at 15°C for 24h.

Conflict of interest statement.- The authors declare no competing interests.

REFERENCES

Altrichter M., Taber A., Beck H., Reyna-Hurtado R., Lizarraga L., Keuroghlian A. & Sanderson E.W. 2011. Range-wide declines of a key Neotropical ecosystem architect, the Near Threatened white-lipped peccary *Tayassu pecari*. Oryx 46(1):87-98. http://dx.doi.org/10.1017/S0030605311000421.

Alvarez J.G. & Storey B.T. 1982. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa: its effect on sperm motility. Biol. Reprod. 27(5):1102-1108. http://dx.doi.org/10.1095/biolreprod27.5.1102. PMid:6297627.

Azevedo F.C.C. & Conforti V.A. 2008. Decline of peccaries in a protected subtropical forest of Brazil: toward conservation issues. Mammalia 72(2):82-88. http://dx.doi.org/10.1515/MAMM.2008.027.

Barros T.B., Toniolli L.S., Guimarães D.B., Freitas E.N., Nunes T.G.P. & Toniolli R. 2016. Curvas de resfriamento do sêmen do varrão diluído em ACP®103 adicionado de gema de ovo em concentração fixa. Ciênc. Anim. Bras. 17(4):540-549. http://dx.doi.org/10.1590/1089-6891v17i417645.

Beck H. 2006. A review of peccary-palm interactions and their ecological ramifications across the Neotropics. J. Mammal. 87(3):519-530. http://dx.doi.org/10.1644/05-MAMM-A-174R1.1.

Biondo C., Keuroghlian A., Gongora J. & Miyaki C.Y. 2011. Population genetic structure and dispersal in the white-lipped peccaries (*Tayassu pecari*) from the Brazilian Pantanal. J. Mammal. 92(2):267-274. http://dx.doi.org/10.1644/10-MAMM-A-174.1.

Campos L.B., Peixoto G.C.X., Silva A.M., Souza A.L.P., Castelo T.S., Maia K.M., Pereira A.F. & Silva A.R. 2017. Estimating the binding ability of collared peccary (*Pecari tajacu* Linnaeus, 1758) sperm using heterologous substrates. Theriogenology 92:57-62. http://dx.doi.org/10.1016/j.theriogenology.2017.01.008. PMid:28237343.

Castelo T.S., Bezerra F.S., Lima G.L., Alves H.M., Oliveira I.R., Santos E.A., Peixoto G.C. & Silva A.R. 2010. Effect of centrifugation and sugar supplementation on the semen cryopreservation of captive collared peccaries (*Tayassu tajacu*). Cryobiology 61(3):275-279. http://dx.doi.org/10.1016/j.cryobiol.2010.09.005. PMid:20858475.

CBRA 2013. Manual para exame andrológico e avaliação de sêmen animal. 3ª ed. Colégio Brasileiro de Reprodução Animal, Belo Horizonte. 104p.

Cummins J.M., Jequier A.M. & Kan R. 1994. Molecular biology of the human male infertility: links with aging, mitochondrial genetics and oxidative stress. Mol. Reprod. Dev. 37(3):345-362. http://dx.doi.org/10.1002/mrd.1080370314. PMid:8185940.

Dziekońska A., Fraser L. & Strzeżek J. 2009. Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. J. Anim. Feed Sci. 18(4):638-649. http://dx.doi.org/10.22358/jafs/66438/2009.

Figueira M.L.O.A., Carrer C.R.O. & Silva Neto P.B. 2003. Ganho de peso e evolução do rebanho de queixadas selvagens em sistemas de criação semi-extensivo e extensivo, em reserva de Cerrado. Revta Bras. Zootec. 32(1):191-199. http://dx.doi.org/10.1590/S1516-35982003000100024.

Flores E., Fernández-Novell J.M., Peña A. & Rodríguez-Gil J.E. 2009. The degree of resistance to freezing thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72(6):784-797. http://dx.doi.org/10.1016/j.theriogenology.2009.05.013. PMid:19604570.

Fragoso J.M.V. 2004. A long-term study of white-lipped pecacary (*Tayassu pecari*) population fluctuations in northern Amazonia, p.286-296. In: Silvius K.M., Bodmer R.E. & Fragoso J.M.V. (Eds), People in Nature: wildlife conservation in south and central America, Columbia University Press, New York. http://dx.doi.org/10.7312/silv12782-018.

Galetti M., Giacomini H.C., Bueno R.S., Bernardo C.S.S., Marques R.M., Bovendorp R.S., Steffler C.E., Rubim P., Gobbo S.K., Donatti C.I., Begotti R.A., Meirelles F., Nobre R.A., Chiarello A.G. & Peres C.A. 2009. Priority areas for the conservation of Atlantic forest large mammals. Biol. Conserv. 142(6):1229-1241. http://dx.doi.org/10.1016/j.biocon.2009.01.023.

Garcia A.R., Kahwage P.R., Guimarães D.A.A. & Ohashi O.M. 2012. Chilled semen of captive collared peccaries (*Pecari tajacu*): effects of preservation at 17°C on semen quality. J. Agr. Sci. Techn. 2:921-929.

Garcia R.R.F., Vasconcelos A.C.N., Povh J.A., Oberst E.R., Eloy L.R. & Streit Junior D.P. 2016. Different extenders solutions for tambaqui semen cooling. Pesq. Agropec. Bras. 51(6):780-784. http://dx.doi.org/10.1590/S0100-204X2016000600011.

Halang W., Bohnensack R. & Kunz W. 1985. Interdependence of mitochondrial ATP production and extramitochondrial ATP utilization in intact spermatozoa. Biochim. Biophys. Acta 808(2):316-322. http://dx.doi.org/10.1016/0005-2728(85)90014-3. PMid:3848331.

Harrison R.A.P. & Vickers S.E. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J. Reprod. Fertil. 88(1):343-352. http://dx.doi.org/10.1530/jrf.0.0880343. PMid:1690300.

Holt W.V. 2000. Basic aspects of frozen storage of semen. Anim. Reprod. Sci. 62(1/3):3-22. http://dx.doi.org/10.1016/S0378-4320(00)00152-4. PMid:10924818.

Holt W.V., Medrano A., Thurston L.M. & Watson P.F. 2005. The significance of cooling rates and animal variability for boar sperm cryopreservation: Insights from the cryomicroscope. Theriogenology 63(2):370-382. http://dx.doi.org/10.1016/j.theriogenology.2004.09.018. PMid:15626405.

Hrudka F. 1987. Cytochemical and ultracytochemical demonstration of cytochrome C oxidase in spermatozoa and dynamics of its changes accompanying ageing or induced by stress. Int. J. Androl. 10(6):809-828. http://dx.doi.org/10.1111/j.1365-2605.1987.tb00385.x. PMid:2828243.

Jeyendran R.S., Van Der Ven H.H., Perez-Pelaez M., Crabo B.G. & Zaneveld L.J. 1984. Development of an assay to assess functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70(1):219-228. http://dx.doi.org/10.1530/jrf.0.0700219. PMid:6694140.

Johnson L.A., Weitze K.F., Fiser P. & Maxwell W.M.C. 2000. Storage of boar semen. Anim. Reprod. Sci. 62(1/3):143-172. http://dx.doi.org/10.1016/S0378-4320(00)00157-3. PMid:10924823.

Kahwage P., Garcia A.R., Guimarães D.A.A., Ohashi M.O., Luz-Ramos R.S., Dias H.L.T., Albuquerque N.I. & Bartha M.M.P. 2010. Biometria testicular, eletroejaculação e características seminais de caititus, *Tayassu tajacu* Linnaeus, 1758 (Mammalia, Artiodactyla, Tayassuidae) mantidos em cativeiro na Amazônia Oriental. Acta Amazonica 40(4):771-778. http://dx.doi.org/10.1590/S0044-59672010000400019.

Keuroghlian A. & Eaton D.P. 2009. Removal of palm fruits and ecosystem engineering in palm stands by white-lipped peccaries (*Tayassu pecari*) and other frugivores in an isolated Atlantic Forest fragmente. Biodiversity

Conservation 18(7):1733-1750. http://dx.doi.org/10.1007/s10531-008-9554-6.

Keuroghlian A., Desbiez A., Reyna-Hurtado R., Altrichter M., Beck H., Taber A. & Fragoso J.M.V. 2013. *Tayassu pecari*. IUCN Red List of Threatened Species. Avaliable at <http://www.iucnredlist.org/details/41778/0> Accessed on Jul. 12, 2018.

Leeuw A.M., Den Daas J.H.G. & Woelders H. 1991. The fix vital stain method: simultaneous determination of viability and acrosomal status of bovine spermatozoa. J. Androl. 12(2):112-118. PMid:1711023.

Leidl W., Stolla R., Schefels W. & Schad C. 1999. Morphology of sperm: description, classification and assessment. Part 2. Boar (*Sus scrofa* f. domestica). Software distributed by Minitub of America Inc., Version 7.

Maxwell W.M.C. & Johnson L.A. 1997. Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology 48(2):209-219. http://dx.doi.org/10.1016/S0093-691X(97)84068-X. PMid:16728120.

Melo M.I.V. & Henry M. 1999. Hypoosmotic test for the evaluation of equine semen. Arq. Bras. Med. Vet. Zootec. 51(4):71-78.

Mendez M.F.B., Zangeronimo M.G., Rocha L.G., Faria B.G., Pereira B.A., Fernandes C.D., Chaves B.R., Murgas L.D. & Sousa R.V. 2013. Effect of the addition of IGF-I and vitamin E to stored boar semen. Animal, Cambridge 7(5):793-798. http://dx.doi.org/10.1017/S1751731112002285. PMid:23211508.

Mercado E., Rodríguez A., Gómez E. & Sanz E. 2010. Cryopreservation of Iberian pig spermatozoa, comparison of different freezing extenders based on post-thaw sperm quality. Anim. Reprod. Sci. 118(1):54-61. http://dx.doi.org/10.1016/j.anireprosci.2009.06.006. PMid:19586729.

Nascimento E.F., Vale Filho V.R., Silva Filho J.M. & Nascimento J.K.F. 1998. Efeito da taxa de resfriamento sobre a motilidade, sobrevivência e morfologia espermática de varrões. Arq. Bras. Med. Vet. Zootec. 50(6):691-694.

Naves C.S., Beletti M.E., Duarte M.B., Vieira R.C., Diniz E.G. & Jacomini J.O. 2004. Avaliação da cromatina espermática em equinos com azul de toluidina e "acridine orange". Biosci. J. 20(3):117-124.

Nogueira-Filho S.L.G., Nogueira S.S.C. & Sato T.A. 1999. Estrutura social de pecaris (Mammalia, Tayassuidae) em cativeiro. Revta Etologia 1(2):89-98.

Nogueira-Filho S.L.G., Borges R.M., Mendes A. & Dias C.T.S. 2014. Nitrogen requirements of white-lipped peccary (Mammalia, Tayassuidae). Zoo Biol. 33(4):320-326. http://dx.doi.org/10.1002/zoo.21141. PMid:24958733.

Nogueira S.S.C. & Nogueira-Filho S.L.G. 2011. Wildlife farming: an alternative to unsustainable hunting and deforestation in Neotropical forests? Biodiversity Conservation 20(7):1385-1397. http://dx.doi.org/10.1007/s10531-011-0047-7.

Nogueira S.S.C., Macêdo J.F., Sant'Anna A.C., Nogueira-Filho S.L.G. & Costa M.J.R.P. 2015. Assessment of temperament traits of white-lipped (*Tayassu pecari*) and collared peccaries (*Pecari tajacu*) during handling in a farmed environment. Revta Anim. Welfare 24(3):291-298. http://dx.doi.org/10.7120/09627286.24.3.291.

Nogueira S.S., Caselli C.B., Costa T.S., Moura L.N. & Nogueira-Filho S.L.G. 2016. The Role of Grunt Calls in the Social Dominance Hierarchy of the White-Lipped Peccary (Mammalia, Tayassuidae). Plos One 11(7):e0158665. http://dx.doi.org/10.1371/journal.pone.0158665. PMid:27409797.

Nunes J.F. & Salgueiro C.C.M. 1999. Utilização da água de coco como diluidor do sêmen de caprinos e ovinos. Revta Bras. Saúde. Prod. Anim. 1(1):17-26.

Paulenz H., Söderquist L., Pérez-Pé R. & Berg K.A. 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology 57(2):823-836. http://dx.doi.org/10.1016/S0093-691X(01)00683-5. PMid:11991386.

Pellestor F., Girardet A. & Andreo B. 1994. Effect of long abstinence periods on human sperm quality. Int. J. Fertil. Menopausal Studies 39(5):278-282. PMid:7820161.

Peña A. & Linde-Forsberg C. 2000. Effects of Equex, one or two step dilution and two freezing thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54(6):859-875. http://dx.doi.org/10.1016/S0093-691X(00)00397-6. PMid:11097040.

R Core Team 2016. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Roca J., Hernández M., Carvajal G., Vázquez J.M. & Martínez E.A. 2006. Factors influencing boar sperm cryosurvival. J. Anim. Sci. 84(10):2692-2699. http://dx.doi.org/10.2527/jas.2006-094. PMid:16971570.

Rondon R.M.M., Rondon F.C.M., Nunes J.F., Alencar A.A., Sousa F.M. & Carvalho M.A.M. 2008. Uso da água de coco em pó (ACP®) em diferentes temperaturas como diluente de espermatozóides de capote (*Numida meleagris*). Revta Bras. Saúde. Prod. Anim. 9(4):848-854.

Ruiz-Pesini E., Diez C., Lapeña A.C., Pérez-Martos A., Montoya J., Alvarez E., Arenas J. & López-Pérez M.J. 1998. Correlation of sperm motility with mitocondrial enzymatic activities. Clin. Chem. 44(8):1616-1620. PMid:9702947.

Santos E.A.A., Sousa P.C., Peixoto G.C.X., Simão B.R., Oliveira M.F. & Silva A.R. 2013. Establishing the hypoosmotic swelling test for sperm analysis in collared peccaries (*Pecari tajacu*). Arq. Bras. Med. Vet. Zootec. 65(4):1257-1260. <http://dx.doi.org/10.1590/S0102-09352013000400044>

Saravia F., Núñez-Martínez I., Morán J.M., Soler C., Muriel A., Rodríguez-Martínez H. & Peña F.J. 2007. Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry are not related to chromatin integrity. Theriogenology 68(2):196-203. <http://dx.doi.org/10.1016/j.theriogenology.2007.04.052> <PMid:17555808>

Silva A.R., Cardoso R.C.S., Silva L.D.M., Chirinéa V.H., Lopes M.D. & Souza F.F. 2006. Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. Theriogenology 66(2):456-462. <http://dx.doi.org/10.1016/j.theriogenology.2005.12.009> <PMid:16427120>

Silva C.G., Cunha E.R., Blume G.R., Malaquias J.V., Bào S.N. & Martins C.F. 2015. Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose. Cryobiology 70(2):90-94. <http://dx.doi.org/10.1016/j.cryobiol.2015.01.001> <PMid:25595634>

Silva M.A., Peixoto G.C., Lima G.L., Bezerra J.A., Campos L.B., Paiva A.L., Paula V.V. & Silva A.R. 2012. Cryopreservation of collared peccaries (*Tayassu tajacu*) semen using a powdered coconut water (ACP-116c) based extender plus various concentrations of egg yolk and glycerol. Theriogenology 78(3):605-611. <http://dx.doi.org/10.1016/j.theriogenology.2012.03.006> <PMid:22538005>

SOS Mata Atlântica & INPE 2010. Atlas dos remanescentes florestais da mata atlântica, período 2008-2010, relatório parcial. Relatório Técnico, Instituto de Pesquisas Espaciais. 120p. Available at <http://www.inpe.br/noticias/arquivos/pdf/atlasrelatoriofinal.pdf> Accessed on Jul. 12, 2018.

Souza A.L.P., Lima G.L., Peixoto G.C.X., Silva A.M., Oliveira M.F. & Silva A.R. 2016. Use of Aloe vera-based extender for chilling and freezing collared peccary (*Pecari tajacu*) semen. Theriogenology 85(8):1432-1438. <http://dx.doi.org/10.1016/j.theriogenology.2016.01.007> <PMid:26830302>

Sowls L.K. 1997. Javelinas and other peccaries: their biology, management, and use. 20th ed. Texas A&M University Press, College Station, Texas, p.325.

Toniolli R., Bussière J., Courot M., Magistrini M. & Combarnous Y. 1996. Effect of indole-3-acetic acid (plant auxin) on the preservation at 15 degrees C of boar semen for artificial insemination. Reprod. Nutr. Dev. 36(5):503-511. <http://dx.doi.org/10.1051/rnd:19960506> <PMid:8987102>

Toniolli R., Toniollo G.H., Franceschini P.H. & Morato F.M.A.C. 2010. Use of powder coconut water as extender (ACP-103®) for boar semen longer preservation: in vitro and in vivo evaluations. Braz. J. Vet. Res. Anim. Sci. 62(5):1072-1079.

Ungerfeld R., Abril-Sánchez S., Toledano-Díaz A., Beracochea F., Castaño C., Giriboni J. & Santiago-Moreno J. 2016. Oxytocin administration before

sperm collection by transrectal ultrasonic-guided massage of the accessory sex glands in mouflons and bucks. Anim. Reprod. Sci. 173:13-17. <http://dx.doi.org/10.1016/j.anireprosci.2016.08.002> <PMid:27530370>

Watson P.F. 1996. Cooling of spermatozoa and fertilizing capacity. Reprod. Domest. Anim. 31(1):135-140. <http://dx.doi.org/10.1111/j.1439-0531.1995.tb00016.x>

Watson P.F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61:481-492. <http://dx.doi.org/10.1016/S0378-4320(00)00099-3> <PMid:10844218>

Yániz J.L., Carretero T., Recreo P., Arceiz E. & Santolaria P. 2014. Three-dimensional architecture of the ovine oviductal mucosa. Anat. Histol. Embryol. 43(5):331-340. <http://dx.doi.org/10.1111/ahe.12078> <PMid:23848134>

Zorinkimi A., Ahmed F.A. & Lalrintluanga K. 2017. Effect of different extenders on the quality of mongrel dog semen preserved at 5°C on the basis of hypo-osmotic sperm swelling test (HOSST). Int. J. Curr. Microbiol. Appl. Sci. 6(12):961-964. <http://dx.doi.org/10.20546/ijcmas.2017.612.106>