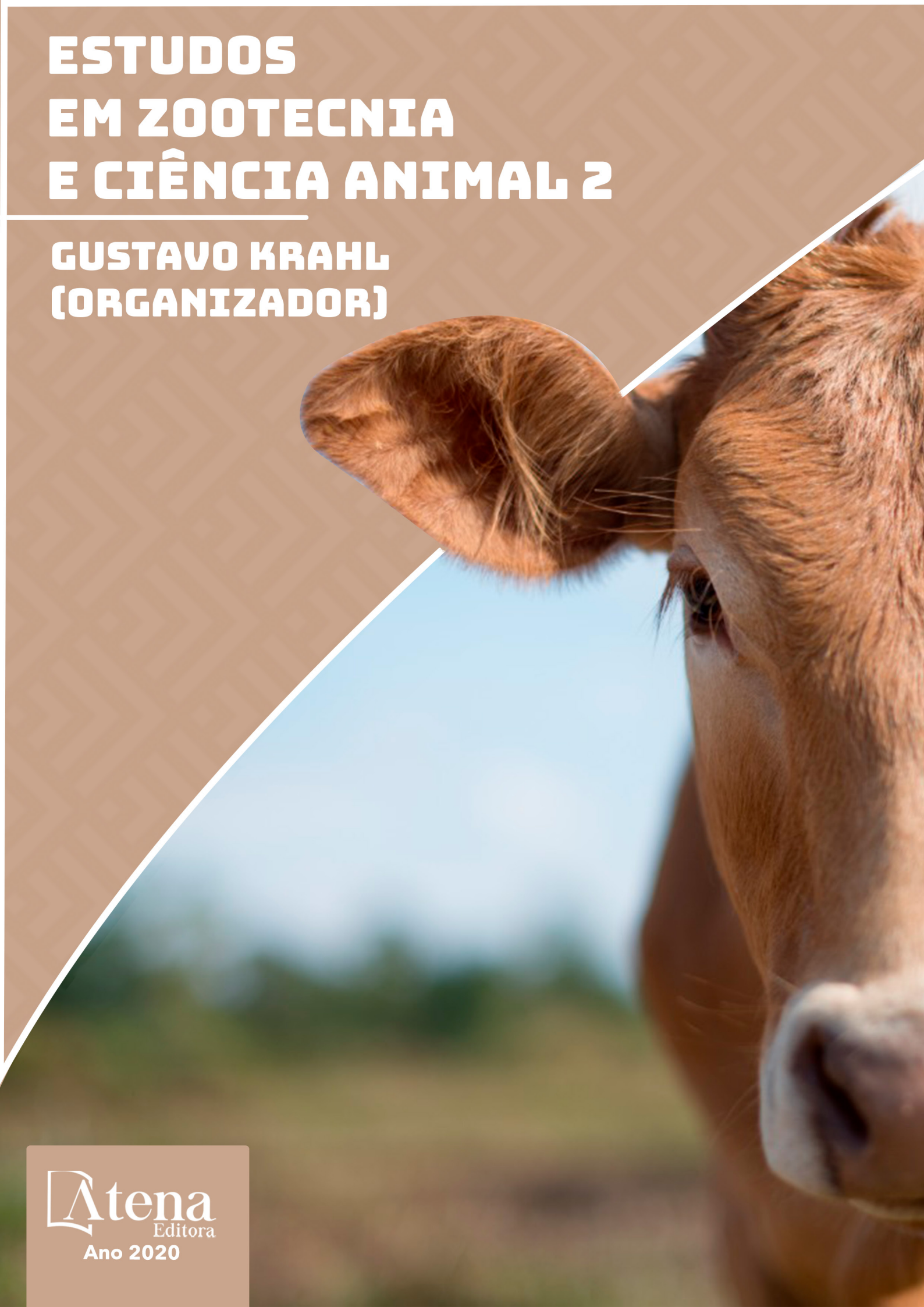
ESTUDOS EM ZOOTECNIA E CIÊNCIA ANIMAL 2

GUSTAVO KRAHL
(ORGANIZADOR)



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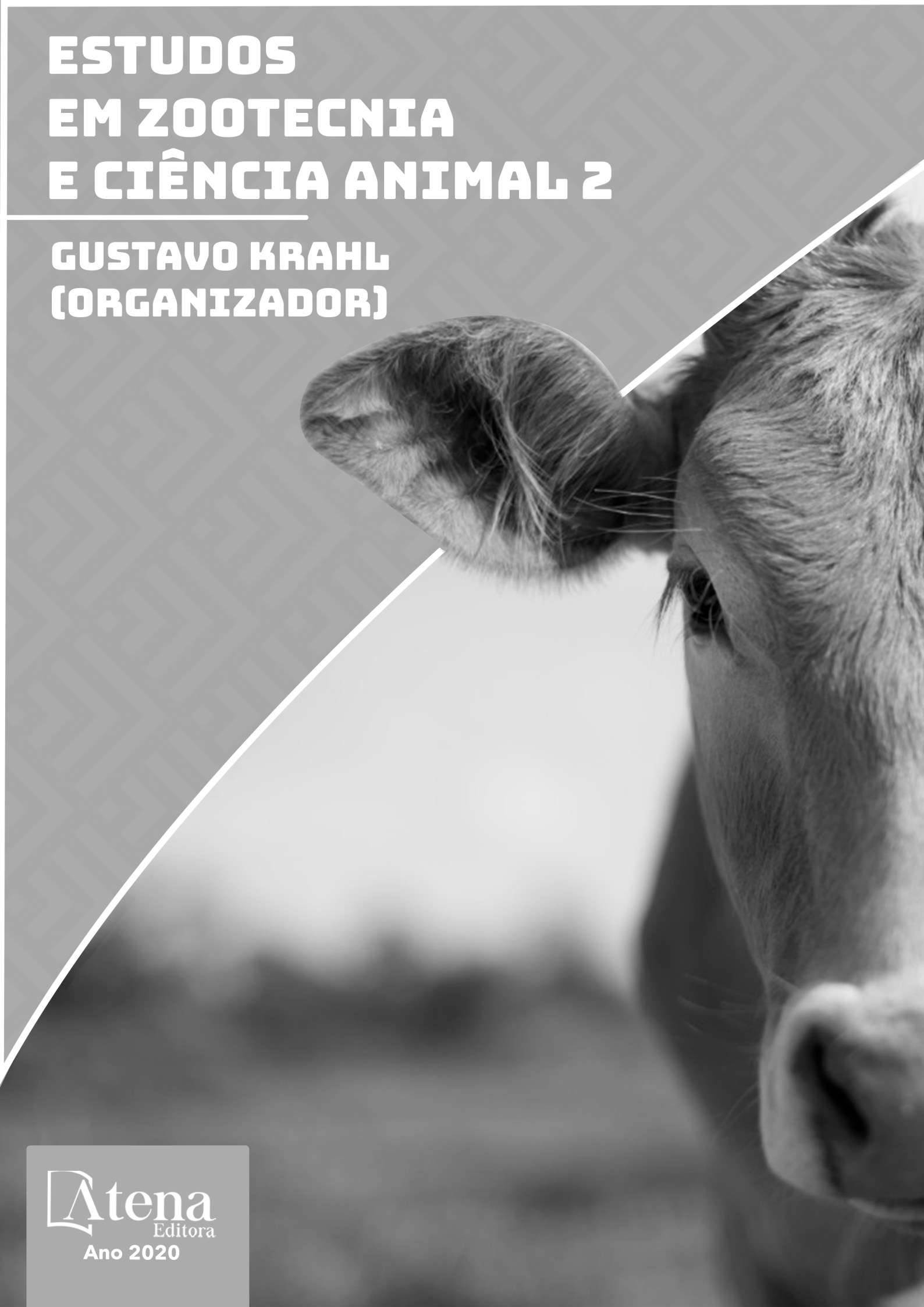

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APRESENTAÇÃO

As áreas da Zootecnia e Ciência animal englobam o setor agropecuário brasileiro, que por muitas vezes foi o responsável por dar a devida importância ao país na esfera global. Mas também deve-se destacar que este setor é o responsável pela produção de alimentos de origem animal e vegetal, geração de emprego e renda, tecnologias e ainda promove a conservação ambiental.

A diversidade cultural observada no Brasil se estende à produção técnica e científica na área de zootecnia e ciência animal. A editora Atena, através da divulgação de trabalhos desta natureza, dá visualização nacional para pesquisadores que tem o papel fundamental de gerar conhecimento e desenvolver as mais diversas áreas voltadas a criação de animais, produção de alimentos e sustentabilidade. O desenvolvimento econômico, social e ambiental é um dos focos da comunidade científica que trabalha no setor agropecuário.

O e-book "Estudos em Zootecnia e Ciência Animal 2" traz trabalhos desenvolvidos em todo o Brasil, e contempla temas de importância regional e nacional. Os capítulos foram organizados e ordenados de acordo com as áreas predominantes. Os primeiros sete capítulos abordam temas relacionados a produção e conservação de forragem pela ensilagem, com foco na silagem de milho e de culturas alternativas. Os próximos cinco capítulos abordam a reprodução de bovinos machos e fêmeas, equinos e biotecnologias utilizadas. Na sequência, os cinco capítulos contemplam a avicultura de corte e postura, nos sistemas industrial e alternativo. Posteriormente, cinco trabalhos que abordam a bovinocultura leiteira e de corte. Também estão contemplados os com alguns capítulos com temas como a ovinocultura, avaliação sensorial e aceitabilidade de alimentos de origem animal e vegetal, piscicultura, entre outros assuntos com importância regional.

A organização deste e-book agradece a dedicação dos autores e instituições envolvidas pelo desenvolvimento dos trabalhos. Destaca-se que a socialização das informações aos leitores, faz parte do processo de geração de conhecimento e resulta na evolução sistemas produtivos. A troca de experiências materializada em trabalhos científicos, permite entregar ao leitor a informação com qualidade e confiabilidade.

Gustavo Krahl

SUMÁRIO

CAPÍTULO 10

SEMINAL PARAMETERS OF BRAZILIAN PONY STALLIONS IN FRESH AND COOLED SEMEN

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ABSTRACT: The objective of this study was to evaluate the effect of refrigeration and storage time (0, 12 and 24 hours) on the spermatic characteristics of Brazilian Pony stallions. The ejaculates of five stallions were collected in an artificial vagina, diluted (1:1) in BotuSêmen® and refrigerated in transport boxes for 24 hours. The seminal parameters were evaluated in fresh semen and at 0, 12 and 24 hours of refrigeration. The data were submitted to analysis of variance with mean comparison by the Duncan test at $P<0.05$. Sperm characteristics in fresh semen, with the exception of motility, varied between stallions and there was a significant effect of age on the gel volume. The refrigeration process affected the semen quality, causing a reduction in motility and viability and increased spermatic pathologies. Nevertheless, the semen characteristics were compatible with the equine species for both fresh and refrigerated semen. Therefore, the studied animals being considered

able to perform their reproductive activities in natural mating or artificial insemination with refrigerated semen.
KEYWORDS: Equine, semen refrigeration, sperm morphology.

PARÂMETROS SEMINAIS EM SÊMEM FRESCO E RESFRIADO DE GARANHÕES DE PONEIS BRASILEIROS

RESUMO: O objetivo deste estudo foi avaliar o efeito da refrigeração e do tempo de armazenamento (0, 12 e 24 horas) sobre as características espermáticas de garanhões da raça Pônei Brasileira. Os ejaculados de cinco garanhões foram coletados em vagina artificial, diluídos (1:1) em BotuSêmen® e refrigerados em caixas de transporte por 24 horas. Os parâmetros seminais foram avaliados no sêmen fresco e às 0, 12 e 24 horas de refrigeração. Os dados foram submetidos à análise de variância com comparação de médias pelo teste de Duncan em P <0,05. As características espermáticas no sêmen fresco, com exceção da motilidade, variaram entre os garanhões e houve um efeito significativo da idade sobre o volume do gel. O processo de refrigeração afetou a qualidade do sêmen, causando redução na motilidade e viabilidade e aumento das patologias espermáticas. Apesar disso, as características espermáticas foram compatíveis com a espécie equina tanto para o sêmen fresco quanto para o refrigerado. Portanto, os animais estudados são considerados aptos a realizar suas atividades reprodutivas em monta natural ou inseminação artificial com sêmen refrigerado.
PALAVRAS-CHAVE: Equino, refrigeração de sêmen, morfologia espermática

1 I INTRODUCTION

The Brazilian Pony breed results from the crossing of several pony breeds, mainly from Shetland ponies of Scotland with Falabella ponies of Argentina, as well as some influence of animals from Paraguay and Uruguay who are descendants of ponies coming from Europe (NEVES et al., 2006). The importance of the Brazilian Pony in the national equine market is increasing due to the increase of its popularity and economic value. However, due to the reduced number of herds, the degree of inbreeding among this breed is high, being associated with a reduction in animal size which is attractive to the breeder, but on the other hand this may have adverse effects on other characteristics (ALVARENGA;PAPA, 2009), such as reproduction. Thus, breeding biotechnologies can make a significant contribution to the genetic improvement and diffusion of this breed.

Knowledge about the reproductive characteristics of these animals, and in particular on the breeding and management conditions in Northeast Brazil is scarce. Regarding the seminal characteristics of the breed, the few studies carried out in Brazil are those by Neves et al. (2006); Trentin et al. (2017) in Rio Grande do Sul, and Araújo and Araújo (2011) and Rua et al. (2013) in Rio de Janeiro. Much of the

available literature refers to pony breeds raised in other countries. For northeastern Brazil, studies with ponies have not yet been performed. Thus, it is necessary to obtain more knowledge on the characteristics of the breed in semiarid conditions for application of reproductive biotechnologies.

Semen refrigeration is very important to equine breeding, since in addition to maintaining the fertilizing capacity of the semen for longer periods of time, it also enables the transport of semen over long distances, facilitating dissemination of genetic material among breeders without the need to displace the breeding stock (CANISSO et al., 2008a). The refrigeration process preserves the longevity and the fecundating potential of the spermatozoa for up to 48 hours (CANISSO et al., 2008a; ALVARENGA; CARMO, 2016; VALE FILHO et al., 2011). In Brazil, the most commonly used equine semen refrigeration system is the passive, in which the temperature lowering is carried out in isothermal boxes near a cold source, being able to maintain the temperature between 4°C and 15°C (CANISSO et al., 2008a; FARRÁS et al., 2008; VIDAMENT et al., 2012; TRENTIN et al., 2017). However, better knowledge of this process is still needed to improve its efficiency since any damage to the sperm during collection; processing or storage can affect the fertilization process.

The objective of this study was to determine the seminal parameters from fresh semen, as well as to evaluate the effect of refrigeration and storage time (0, 12 and 24 hours) on the sperm characteristics of Brazilian Pony Stallions.

2 I MATERIALS AND METHODS

This study was carried out at a private property located in Santa Maria, Rio Grande do Norte, Brazil. Five Brazilian Pony stallions who were clinically healthy and in good nutritional condition were used (stallion 1 = 18 years old, stallion 2 = 10 years old, stallion 3 = 16 years old, stallion 4 = 6 years old and stallion 5 = 5 years old). All animals were kept under the same management conditions in individual stalls and fed native pasture, hay, concentrate, mineral salt and water *ad libitum*. Ten ejaculates were collected from each stallion in two periods of the year, between March 18 to April 15, and August 26 to September 20.

Semen was collected once a week with a Botucatu model artificial vagina (Botupharma, Botucatu-SP, Brazil) warmed up at 41-42°C and with the aid of an estrus female to stimulate the male. Immediately after the collection, the ejaculate was filtered on a nylon filter to remove the gel fraction and the volume of both fractions was determined. Then, the gel-free semen was kept in a water bath at 37°C and percentage of total motility and individual motility were evaluated.

Two aliquots were then removed; one for evaluation of sperm concentration and another for evaluation of morphology. A smear by the vital staining method was also

prepared for later sperm viability evaluation.

After evaluating fresh semen, three milliliters of semen (3 ml) of each stallion were placed in a tube and diluted 1:1 with a milk-based medium (BotuSêmen®; Botupharma, Botucatu, SP, Brazil), which according to the manufacturer contains skimmed milk, glucose, preservatives, excipients and antibiotics.

After dilution (0 h), sperm motility (total and individual) were evaluated and then two aliquots of the diluted semen were taken which were placed in microtubes (1.5 ml) and then placed in two isothermal transport boxes (BotuFlex®; Botupharma, Botucatu-SP, Brazil) containing two blocks of ice, where they remained for 12 or 24 hours. The boxes were kept away from the light at room temperature for the entire storage period, and a digital refrigerator thermometer (Incoterm, -5+70°C) was placed in each them with only the bulb of the thermometer inside the box, avoiding contact with the cooling source. The temperature displayed on the thermometer was recorded at each evaluation.

Seminal evaluations were performed at the Laboratory of Cellular and Tissue Microscopy, Department of Morphology, at the Center for Biosciences, Federal University of Rio Grande do Norte (*UFRN*), located at the central Natal Campus - RN, and at the Laboratory of Andrology and Technology of Semen of the Agricultural Research Company of Rio Grande do Norte (*EMPARN*), situated in the municipality of Parnamirim - RN. The samples were evaluated for total motility, individual motility, concentration, viability and morphology. These evaluations were performed on fresh semen, immediately after dilution, and after 12 and 24 hours of refrigeration. Total motility evaluation was carried out subjectively. A small droplet of semen was placed on a glass slide (37°C) covered with a glass coverslip and then evaluated under light microscopy (400x) on a heating plate at 35-37°C, with three random fields/ areas being examined to estimate the mobile sperm percentage. Sperm individual motility was also subjectively evaluated according to the same procedure used for the sperm total motility evaluation, assigning scores ranging from zero to five (0, no movement; 5, “arrowing” spermatozoa with linear movements). The assessment has to take into account the type of movement observed for most spermatozoa and their velocity (Chemineau et al., 1991). To evaluate the sperm concentration, the semen was diluted to the ratio of 1:20 (10µl of raw semen in 200µl of distilled water) and then measured using a haemocytometer counting chamber under optical microscopy at 400x magnification. The percentage of live spermatozoa was determined using nigrosin-eosin stain. The smear was evaluated by optical microscopy (1000x, under immersion), counting 200 spermatozoa, which were classified as living (unstained head) or dead (totally or partially stained heads).

The sperm morphology evaluation was performed in semen samples fixed in formalin-saline solution and submitted to the wet preparation technique using a

phase contrast microscope at a magnification of 1000x under immersion. Cells were classified as normal and abnormal. Sperm morphologic abnormalities were classified as major defects (MAD) and minor defects (MID), according to Blom classification.

Data were submitted to analysis of variance (ANOVA) with a comparison of the means by the Duncan test at 95% confidence. Pearson's correlation analysis was also performed between gel volume and stallion age, as well as between motility and spermatic defects. The analyzes were performed with the aid of Statgraphics software (Statistical Graphics Corporation, Nottingham, UK).

3 I RESULTS AND DISCUSSION

A significant difference ($P < 0.05$) was observed among stallions for most sperm characteristics observed in fresh semen. With the exception of total motility, the other parameters (semen volume, gel volume, concentration, individual motility, live sperm, and total defects) differed among the stallions (Table 1). According to Paccamonti et al. (1999), similarities in the percentage of motile sperm are expected since these values do not depend on the testicular mass, but rather on normal testicular parenchyma. Moreover, individual variation in seminal characteristics and fertility among stallions are considered normal and frequent in this species (DOWSETT; KNOTT, 1996; NEVES et al., 2006; CANISSO et al., 2008b; RUA et al., 2013). Factors such as breed, age among stallions of the same breed, ejaculation frequency, duration of excitation time, period of sexual rest, feeding, and handling (among others) have a significant influence on most seminal characteristics (DOWSETT;KNOT, 1996; CANISSO et al., 2008b). However, with the exception of volume, the other seminal parameters are within the limits recommended by the Brazilian College of Animal Reproduction for equine species (CBRA, 2013). We must take into consideration that the values established by the CBRA are for large-breed stallions, and because ponies are animals with smaller body size their testicular size is also smaller, which may interfere with sperm production. There was a positive correlation between the stallion age and gel volume ($r=0.69$, $P=0.004$), total motility ($r=0.32$; $P= 0.02$) and individual motility ($r=0.32$; $P=0.02$). The variation in the gel volume can be explained by almost 70% due to the variation in the age of the stallions; a fact evidenced by the high positive correlation coefficient observed between gel volume and age. The older animals (stallion 1 and 3) produced a larger volume of gel than the other animals. The influence of age is probably due to differences in daily sperm production and output, which in turn are related to immature spermatogenesis in younger animals and testicular degeneration in older animals (DOWSETT; KNOTT, 1996). Furthermore, factors such as breed differences and individual differences may also influence the variation in gel volume (DOWSETT; KNOTT, 1996; NEVES et al., 2006; CANISSO

et al., 2008b). This characteristic may be relevant for evaluating stallions for semen processing, considering that a large gel volume is difficult to separate and may interfere with semen quality for artificial insemination.

The percentage of live sperm differed significantly among stallions (Table 1). This difference probably occurred due to the fact that some stallions were in sexual inactivity before the beginning of the collections, which may have contributed to the increase in the dead spermatozoa percentage in the ejaculate. This characteristic is important in evaluating horse breeders, since there is a correlation between dead sperm percentage and pregnancy rate.

	STALLION				
Seminal Parameter	1	2	3	4	5
GFV (mL)	13.9±5.3^{ab}	15.5±6.3^{a}	10.9±4.8^{ab}	10.2±5.2^{b}	10.4±4.5^{ab}
Gel (mL)	12.9±8.7^{a}	0.0±0.0^{b}	11.9±5.1^{a}	2.3±1.5^{b}	7.6±6.6^{ab}
TM (%)	85.5±4.9	79.5±8.9	86.5±4.7	81.0±5.2	80.5±11.2
IM (0-5)	4.4±0.5^{ab}	4.0±0.5^{b}	4.6±0.5^{a}	4.3±0.5^{ab}	3.9±0.7^{b}
Live (%)	86.4±4.9^{a}	74.8±7.8^{b}	77.0±10.3^{b}	85.1±5.7^{a}	78.7±6.0^{b}
CONC (x 10^6/mL)	243.6±111.9^{ab}	225.7±85.9^{ab}	261.4±59.8^{a}	301.8±115.9^{a}	159.1±79.1^{b}
TSD (%)	37.6±11.9^{abc}	45.0±11.3^{a}	34.2±10.4^{bc}	39.5±10.6^{ab}	27.9±8.4^{c}
MAD (%)	31.2±10.9^{a}	32.7±11.8^{a}	25.8±10.7^{ab}	31.6±10.0^{a}	22.5±8.0^{b}
MID (%)	6.3±4.8^{c}	12.3±5.5^{ab}	8.4±3.0^{bc}	7.8±4.5^{bc}	5.5±3.2^{c}

Table 1. Seminal Parameter (mean ± sd) in fresh semen of Brazilian Pony stallions.

GFV, Gel-free volume; TM, total motility; IM, individual motility; CONC, sperm concentration; TSD, total sperm defects; MAD, major defects; MID, minor defects.

^{a,b,c} Different superscript letters within a same row denote significant difference ($P < .05$) by Duncan test.

Sperm morphology also differed between stallions and between the type of semen (fresh x refrigerated) (Table 2). The percentage of sperm pathologies was high (~37%) based on the parameters recommended for equine by the Brazilian College of Animal Reproduction - CBRA (2013). However, values above 30% are frequently observed in this specie without interfere with the fertility of the stallion (ARAÚJO; ARAÚJO, 2011; VALE FILHO et al., 2011). According to Vale Filho et al. (2011) the majority of sperm morphology studies of stallions show values of 50 to 60% of normal spermatozoa in the ejaculate; they also add that there are no studies establishing tolerable levels of certain sperm-related fertility defects in the stallion, making it difficult to use criteria such as major and minor defects for this species. Probably, the high rate of pathologies presented by some animals is also due to the low genetic variability among individuals (ALVARENGA; PAPA, 2009; ARAÚJO; ARAÚJO, 2011). All these factors may have contributed to the high percentage of

sperm morphologic abnormalities observed in the fresh semen of the animals in this study.

Seminal Parameter	Fresh semen	Cooling time (hr)			*P* value
		0	12	24	
TM (%)	82.6±7.71[a]	77.2±10.26[a]	60.5±11.53[b]	53.0±12.41[c]	< 0.0001
IM (0-5)	4.24±0.59[a]	4.08±0.72[a]	3.68±0.77[b]	3.44±0.73[b]	< 0.0001
Live (%)	80.4±8.31[a]	80.4±7.66[a]	59.4±16.44[b]	51.8±19.33[c]	< 0.0001
TSD (%)	36.83±11.64[b]	38.53±10.51[b]	46.88±13.60[a]	39.79±9.54[b]	0.0001
MAD (%)	28.75±10.73[b]	31.68±10.74[ab]	36.31±13.83[a]	27.43±8.28[b]	0.0004
MID (%)	8.09±4.74[bc]	6.85±5.44[c]	10.57±7.91[ab]	12.36±5.44[a]	< 0.0001
ACROD	8.07±0.78	7.55±7.04	8.31±12.8	7.26±0.78	0.4429
CT	5.41±0.99[c]	9.0± 8.7 [bc]	15.89±10.8[ab]	11.07±1.03 [a]	0.0002
BT	3.68±2.75[b]	3.67±3.2[b]	7.56±7.2[a]	9.24±5.69[a]	< 0.0001

Table 2. Sperm parameters (mean ± sd) of Brazilian pony semen evaluated after collection (fresh semen) and after cooling at 5-8°C for 24 hours (n=50).

TM, total motility; IM, individual motility; TSD, total sperm defects; MAD, major defects; MID, minor defects, ACROD, acrosome defects; CT, coiled tails; BT, bent tails.

[a,b,c] Different superscript letters within a same row denote significant difference (P < .05) by Duncan test.

The refrigeration process affected all sperm characteristics evaluated, causing decreases in motility, as well as damage to the plasma membrane (sperm viability) and sperm morphology when compared to fresh semen; particularly, after 12 hours of refrigeration (Table 2). The sperm possibly suffered thermal shock during the cooling of the ambient temperature to the storage temperature of approximately 5°C, resulting in tail coiling which led to a decrease in motility, damage to the plasma membrane and consequently reduction in viability (WATSON, 2000; NUNES et al., 2006; OLIVEIRA et al., 2013).

The deleterious effect of refrigeration on the structure and function of equine spermatozoa has been reported in both pony stallions (VIDAMENT et al., 2012; OLIVEIRA et al., 2013; DEICHSEL et al., 2016; TRENTIN et al., 2017) and in large breeds (MAIA, 2010; FREITAS-DELL'AQUA et al., 2013; FLOREZ-RODRIGUEZ et al., 2014). In our study, the first 12 hours of refrigeration appeared to be the critical point for inducing injury during storage at 5°C, as this was when the seminal quality began to deteriorate. This result is in agreement with the expected result, since spermatic quality decreases over time, independent of storage conditions, due to cellular aging and accumulation of metabolites (MAIA, 2010; OLIVEIRA et al., 2017). For this reason, use of refrigerated semen has limited time, usually 24 to 48 hours. According to Alvarenga and Carmo (2016), the refrigeration time should not exceed

24 hours so that there is no impairment in the fertility indexes.

Total and individual motility progressively decreased over the course of storage, which was confirmed in other studies with milk-based diluents or equivalent (MAIA, 2010; VIDAMENT et al., 2012; DEICHSEL et al., 2016; OLIVEIRA et al., 2017; TRENTI et al., 2017). On the other hand, Farrás et al. (2008) using the BotuSêmen diluent and Botutainer transport system (in fiberglass) did not observe any impairment in motility and sperm viability in equine semen stored at 5°C for 12 or 24 hours in relation to fresh semen. However, in our study, a Botuflex transport box (styrofoam) was used, which did not keep the temperature constant during the 24 hours and reaching a maximum of 8.6°C at that time. The proper refrigeration rate was probably not reached because the volume to be cooled was lower than the one indicated by the manufacturer (100 mL), thus interfering with the cooling rate of the device. However, there was no major damage in semen quality despite this variation in the refrigeration temperature, reaching 24 hours with 53% of motility and 51% of viable cells. Freitas-Dell'aqua et al. (2013) demonstrated that the semen quality is maintained in both storage at 5°C and at 15°C for transport lasting up to 24 hours.

Although a significant difference in motility occurred after 12 hours (Table 2), it was observed that there was already a large reduction in this parameter shortly after the dilution. A similar behavior was observed in the study by Santos et al. (2015) using the Botu-semen diluent at a dilution rate of 1: 1. Possibly, there has been an interaction between the seminal plasma and the diluent components, resulting in decreased motility in relation to fresh semen (BRINSKO et al., 2000; CARVER;BALL, 2002). In addition, there is a difference between stallions regarding the resistance to the cooling process. In our study, stallions 2 and 4 presented a degradation rate in motility higher than 40% at 24 hours, being considered "poor coolers" according to Brinsko et al. (2000). Thus, spermatozoa of these two animals were possibly sensitive to refrigeration, impacting the mean values of motility and other sperm parameters obtained in this study.

Sperm viability showed a progressive decline over the refrigeration time, mainly after 12 hours of storage (Table 2), as reported in previous studies (FLOREZ-RODRIGUEZ et al., 2014; DEICHSEL et al., 2016; OLIVEIRA et al., 2017). However, the percentage of viable spermatozoa (intact plasma membrane) after 24 hours of storage was 51%. Although there was a reduction in relation to fresh semen, our result was higher than that obtained by Trenti et al. (2017) in semen of pony stallions diluted 1:1 with milk-based diluent and stored at 5°C. The difference between the diluents used and among the evaluation techniques (our study used vital staining, and the study by Trenti et al. (2017) used the hypoosmotic swelling test) may have contributed to the variation between the results in the two studies. According to Florez-Rodriguez et al. (2014), the longevity of the sperm depends on the diluent

composition.

No significant increase in morphologic abnormalities was observed after 24 hours of storage at 5°C in comparison to fresh semen (Table2). However, there was a significant increase in spermatic defects, MAD and MID at 12 hours, mainly in the increase in the percentage of bent tail and coiled tails. This tail alteration may have been induced by a cold shock, as reported in other studies with equine semen refrigeration (NUNES et al., 2006; CANISSO et al., 2008b; FLOREZ-RODRIGUES et al., 2014). According to the CBRA (2013), sperm abnormalities for equine refrigerated semen should not exceed 40%. Thus, the results obtained in this study are at the threshold considered desirable for the species (42.84%).

The integrity of the acrosomal membrane was not affected by the refrigeration process, contrary to that observed by Vidament et al. (2012) in which the percentage of intact acrosome significantly reduced after 22 hours of storage at 4-7°C or 8-10°C. The evaluation technique used (wet chamber) may not have been effective in accurately detecting this type of alteration, as Vidament et al. (2012) used the epi-fluorescence technique with FITC-PSA.

4 I CONCLUSIONS

1-There are differences between stallions in sperm characteristics in the fresh semen and some these characteristics, like gel volume, are influenced by stallion age.

2-The refrigeration process affect the spermatic quality after 12 hours of storage, however without great damage since the seminal parameters remained above the reference value recommended by the Brazilian College of Animal Reproduction for equine specie.

3- The values found in this study can serve as a basis for establishing the proper seminal patterns for small size horses, considering that the prediction based on the seminal parameters obtained for large-sized stallions may not be adequate for evaluating pony breeds.

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