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Dissertation

Big data as a source for tracking boar stud productivity and effect of extender centrifugation and sonication on sperm parameters of freeze-thaw boar semen

Camila Ribeiro Carvalho de Brito

Pelotas, 2019

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Dedicated to my Grandparents

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“Non, rien de rien/Non, je ne regrette rien/C'est payé, balayé, oublié/Je m'en fous du passé! […]
Aujourd'hui, ça commence avec toi!”
(Charles Dumont and Michel Vaucaire)

Resumo

BRITO, Camila Ribeiro Carvalho de. ***Big data* como fonte de rastreamento da produtividade de centrais de produção de sêmen e o efeito da centrifugação e sonicação do diluente de sêmen suíno sobre os parâmetros de congelamento-descongelamento**. 2019. 61f. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019

A grande expansão e transformação da produção de suínos durante o século XX deve ser atribuída principalmente ao uso de diversas tecnologias no manejo dos animais além da segmentação da produção em subáreas. Um dos setores que mais empregaram distintas tecnologias foi o da reprodução. Atualmente, reprodutores machos e fêmeas são mantidos em instalações separadas, sendo os primeiros alocados em centrais de produção de doses de sêmen. Tais centrais são dotadas de tecnologias que intensificam o uso de automação da coleta e produção de doses, que não seriam possíveis sem a obtenção informatizada de dados para a sua realização. Além disso, estudos em que são reportadas as características de manejo e produtividade são responsáveis por identificar tanto os pontos críticos quanto os pontos referências para a cadeia produtiva como um todo, o que é conhecido como 'benchmarking'. O primeiro trabalho demonstrou o perfil quantitativo e qualitativo de mais de vinte mil doses de sêmen produzidas durante um ano em onze centrais brasileiras em termos dos parâmetros espermáticos (concentração, motilidade, volume, coloração, etc.) e produtividade dos ejaculados coletados (p.ex., quantidade de doses produzidas) além das razões de descarte tanto dos ejaculados coletados quanto dos animais alojados nas granjas. Foi possível também compreender algumas características dos manejos adotados das granjas em termos das rotinas de trabalho adotadas (p.ex., dias de coleta, n° de funcionários, intervalo entre coletas, etc.). Por último, no segundo trabalho foi relatada uma inovação na metodologia de obtenção de diluente para o congelamento de sêmen suíno: a ultracentrifugação acompanhada ou não da sonicação. A ultracentrifugação do plasma de gema foi benéfica para os parâmetros de cinética espermática. Por outro lado, a aplicação da sonicação, com o objetivo de diminuir as partículas de LDL do diluente, isolada ou combinada com a ultracentrifugação foi deletéria a esses mesmos parâmetros. Portanto, o primeiro trabalho foi importante para determinar os pontos de referência da produção comercial de sêmen suíno em centrais brasileiras e o segundo forneceu uma alternativa viável e benéfica para o congelamento de doses de sêmen suíno.

Palavras-chave: *Big data*; Centrais de Inseminação Artificial; Criopreservação de sêmen; Diluentes; Suinocultura.

Abstract

BRITO, Camila Ribeiro Carvalho de. **Big data as a source for tracking boar stud productivity and effect of extender centrifugation and sonication on sperm parameters of freeze-thaw boar semen.** 2019. 61f. Dissertation (Master degree in Sciences) - Veterinary Post-Graduation Program, Veterinary College, Universidade Federal de Pelotas, Pelotas, 2019.

The great expansion and transformation of pig production during the 20th century must be attributed mainly to the use of different technologies in the management of animals in addition to the segmentation of production in sub-areas. One of the sectors that most used different technologies was reproduction. Currently, sires and dams are kept in separate facilities, with the first allocated into boar studs. Such plants are equipped with technologies that intensify the use of automation of the collection and production of doses, which would not be possible without obtaining computerized data for their realization. In addition, studies in which management and productivity characteristics are reported are responsible for identifying both critical points and reference points for the production chain as a whole, which is known as 'benchmarking'. The first study demonstrated the quantitative and qualitative profile of more than twenty thousand doses of semen produced during one year in eleven Brazilian boar studs in terms of sperm parameters (i.e., concentration, motility, volume, color, etc.) and productivity of the ejaculates collected (i.e., amount of doses produced) in addition to the reasons for discarding both the collected ejaculates and the animals housed in the farms. It was also possible to understand some characteristics of the management of the farms in terms of the work routines adopted (i.e., collection days, number of employees, interval between collections, etc.). Finally, in the second study, an innovation in the methodology for obtaining diluent for freezing swine semen was reported: ultracentrifugation, with or without sonication. The ultracentrifugation of the yolk plasma was beneficial for the parameters of sperm kinetics. On the other hand, the application of sonication, with the objective of reducing LDL particles in the diluent, isolated or combined with ultracentrifugation, was deleterious to these same parameters. Therefore, the first study was important to determine the reference points for the commercial production of swine semen in Brazilian plants and the second one provided a feasable and beneficial alternative for freezing boar semen doses.

Keywords: Big Data; Boar studs; Extenders; Pig Farming, Semen cryopreservation.

List of Figures

Article 1

List of Tables

Article 1

Article 2

List of Abbreviations and Acronyms

µL	Microliters
µM	Micromolar
ABPA	*Associação Brasileira de Proteina Animal*
ACSURS	*Associação de Criadores de Suínos do Rio Grande do Sul*
AI	Artificial Insemination
ALH	Amplitude of Lateral Head Displacement
Apr	April
Aug	August
BS	Boar Stud
BSA	Bovine Serum Albumin
BTS	Beltsville Thawing Solution
CAPES	*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*
CASA	Computer-assisted Sperm Analysis
CFDA	Carboxyfluorescein diacetate
cm	Centimeters
CNPq	*Conselho Nacional de Desenvolvimento Científico e Tecnológico*
Dec	December
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
Feb	February
FITC	Fluorescein Isothiocyanate
Fri	Friday
FURG	*Universidade Federal do Rio Grande*
G1	Group 1 (Total Motility lower than 70%)
G2	Group 2 (Total Motility ranging from 70% and 80%)
G3	Group 3 (Total Motility higher than 80%)

HCl	Chloridric Acid
Hz	Hertz
Jan	January
Jul	July
Jun	June
Kg	Kilograms
LDL	Low Density Lipoproteins
LIN	Linearity
LN	Liquid Nitrogen
Mar	March
mg	Milligrams
min	Minutes
mL	Milliliters
mm	Millimeters
mM	Millimolar
Mon	Monday
NaCl	Sodium Chloride
nm	Nanometers
Nov	November
Oct	October
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PM	Progressive Motility
Rh123	Rhodamine 123
ROS	Reactive Oxygen Species
Sat	Saturday
SEBRAE	*Serviço Brasileiro de Apoio às Micro e Pequenas Empresas*
Sep	September
STR	Straightness
Sun	Sunday
T1	Treatment 1 (Control – Egg-Yolk)
T2	Treatment 2 (Sonicated Egg-Yolk)
T3	Treatment 3 (Egg-Yolk Plasma)

T4	Treatment 4 (Sonicated Egg-Yolk Plasma)
Thu	Thursday
TM	Total Motility
TNE	Tris-NaCl-EDT Buffer
Tris	Tris(hydroxymethyl)aminomethane Buffer
Tue	Tuesday
UFPel	*Universidade Federal de Pelotas*
USA	United States of America
v/v	Volume Per Volume
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
Wed	Wednesday
WOB	Wobble

List of Symbols

%	Percent
±	Plus-Minus Sign
×	Multiplication Sign
=	Equal to
°C	Celsius Degree
$	Currency Sign
&	Ampersand (and)
<	Less than
>	Greater than
®	Registred Sign
G	Gravitational Force
™	Trade Mark Sign

Summary

1 Introduction

One of the most successful sectors in the modern agroindustry, pig farming experienced a radical shift in terms of modernization and appreciation of products (WOODS, 2012). In the beginning of 20th century, pork was considered as a “second class” meat, once the remnants of human food were given to pigs to be converted into meat (WOODS, 2012). The success of this industry, however, began when pigs were confined instead of reared in the open field (POND, 1983; WOODS, 2012). The confinement meant an effective control over all aspects of livestock production: breeding, feeding, and health (to some extent).

From the previous three aspects, breeding has gained great impulse especially after World War II, when there was a demand for standardization in carcass from industrial slaughter houses associated with the elevation of meat consumption during peace times (WOODS, 2012). Therefore, there was a need to select breeds, among the available ones, the most suitable for providing an adequate swine carcass: high meat percentage and low content of fat (FÁVERO et al., 2011). In Brazil, the efforts to reach these demands were implemented during the 1970s, when national breeders’ associations and the meat industry encouraged the adoption of foreign breeds to mate native ones, in order to improve the genetic material of national swine herd (FÁVERO e DE FIGUEIREDO, 2009).

It also dates from this time the implementation of boar studs in Brazil in two places: Estrela and Concórdia, respectively in the states of Rio Grande do Sul and Santa Catarina (CASTRO et al., 1996). Primarily, their role was to support breeders to adopt and spread Artificial Insemination (AI) technique in their farms. It enabled the reduction in the number of boars required to mate sows, rationalizing the use of the ejaculates, used previously in a single mate, to be extended and split into doses to inseminate more sows (BORTOLOZZO et al., 2008). Consequently, the genetic gain was facilitated because studs housed boars with higher genetic material and supplied sow farmers with their doses, establishing the multiplier farms (FÁVERO e DE FIGUEIREDO, 2009). Currently, boar studs are broadly widespread in Brazil and mainly located in regions where the pork production is well stablished (SEBRAE e

ABCS, 2016), albeit studies or official reports accounting the management characteristics of these farms are scarce.

Studies conducted in developed countries concerning management characteristics and yield in semen production, are commonly published in scientific journals, and offer invaluable information regarding the standards commonly adopted by farmers and they contain important data for a process called benchmarking. Benchmarking is defined as a reference parameter by which others are compared or measured (BENCHMARK, 2006). As such, the determination of standard parameters require huge amount of data, which can only be collected within farms. With respect to boar studs, these data may refer to sire performance (volume of semen collected, concentration of sperm cells, cell motility, etc.) or to the management implemented in the stud (collector, weekday, time for collection, etc.) (KNOX et al., 2008).

Until recently, data collected within farms were limited to internal management, rather than compose an overall diagnosis of the productivity in the pig chain (PIÑEIRO et al., 2019). However, with the sophistication of management applications, large databases were formed within those startups that developed these softwares. Thus, along the time, the amount of information assembled began to form immense clusters of data known today as ‘big data’, which can only be managed by a high scale analytics technology (WOLFERT et al., 2017).

Today, the operation of these large databases constitutes the basis for industrial automation. In the agribusiness sector, the automation is known as smart farming and is defined as the “application of modern information and communication technologies focused on a data-driven approach to deal with existing challenges and opportunities in agriculture” (HOSTE et al., 2017). The main purpose of applying such systems rely on the precision and standardization of processes that require the accuracy provided by collected data to output product quality and quantity as demanded from modern industry and the consumers’ needs (WOLFERT et al., 2017).

Despite the automation in boar studs is restricted to the semen collection technique and computer-assisted sperm analysis (CASA), these two important factors require the intermediation of specialized human labor that cannot, at least within a short time period, be substituted by robots. The intermediate steps between these two parts, such as semen extension and preparation of semen samples for CASA analysis, are still performed by a technician. Therefore, the full automation of this important part

of pig chain is still in process and, just as in other segments, will be fully accomplished as more data on other non-automated processes are continuously gathered.

1.1 Other technologies in AI

As previously reported, the AI was and still is a remarkable feat in not only swine reproduction but in all domestic animal's reproduction. However, different from other species, such as cows, the industrial AI in pigs is restricted to the use of cooled extended semen, rather than frozen (YESTE, 2016). The advantages of using frozen semen mainly relies on the undefined time of storage and transportation of doses (YESTE, 2015).

However, in the case of boars, the cryopreservation process leads to a loss of semen quality resulted from the technique itself, which makes its use on an industrial scale unfeasible for a species that produces a numerous offspring, unlike cows or mares. The most common alterations are the injuries caused to cell structures, such as the membrane, DNA, mitochondria and acrosome (YESTE, 2016). These injuries lead to loss of dose fertility for artificial insemination, when compared to the cooled semen (KNOX, 2015). Thereby, farmers invest in housing boars of high genetic merit for semen production and supply the market with high quality cooled extended semen doses (BORTOLOZZO et al., 2008). However, the cost to maintain these animals is high and the development of a high quality frozen semen doses would reduce these costs and improve the logistics in semen distribution (BORTOLOZZO et al., 2008). Moreover, cryopreservation of the doses would permit the storage for undetermined time, which is a drawback considering the current technology available (cooled semen) (YESTE, 2015).

2 Articles

2.1 Article 1

Management and sperm quality parameters in boar studs in three Brazilian states

C. R. C. de Brito, F. R. P. Bruhn, M. de Almeida, C. D. Corcini

To be submitted to the Journal *Ciência Rural*

Management and sperm quality parameters in boar studs in three Brazilian states

[Gerenciamento e parâmetros de qualidade espermática em centrais de inseminação de suínos em três estados brasileiros]

C. R. C. de Brito[1], F. R. P. Bruhn[2], M. de Almeida[3], C. D. Corcini[4]

Resumo

A centrais de inseminação de suínos representam um setor de destaque na suinocultura brasileira. Este estudo teve como objetivo avaliar os dados de produtividade, características de manejo e descarte de cachaços em 11 centrais. Em 2015, foram registrados 21.333 ejaculados ou tentativas de coleta de 983 cachaços e 86,1% deles foram considerados aprovados. Os demais ejaculados (13,9%) foram descartados principalmente por alterações morfológicas e baixa motilidade (32,1% e 21,4%, respectivamente). A produção mensal foi maior entre janeiro e abril (56,7% das coletas). Em média, cada central contava com 4,6 funcionários realizando grande parte das coletas durante a semana (86,2%), no geral às segundas e sextas-feiras (24,5% e 22,0%, respectivamente). O intervalo entre as coletas foi de 6,70 dias (± 3,25 dias). O volume médio de sêmen coletado, concentração, motilidade, temperatura de ejaculado e números de doses estimadas foram, nesta ordem: 311,16 mL (± 125,86 mL), 306,06 × 10^6

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células/mL (± 143,10 × 10^6 células/mL), 85,13% (± 17,67%), 35,47°C (± 1,01 °C) e 27,61 (± 8,84). O vigor espermático mais registrado foi ‘4’ (progressivo, em linha reta e rápido) em 70,1% dos ejaculados. Os aspectos visuais mais referidos foram Leitosos e Leitosos-Serosos (44,9% e 49,2%, respectivamente). Os machos foram descartados (n = 300) principalmente devido a: baixa viabilidade do sêmen (31,3%), melhoramento genético (22,7%) e idade (15,0%).

Palavras-chave: Suínos, centrais de inseminação, parâmetros espermáticos, benchmarking, inseminação artificial.

Abstract

Boar studs represent a remarkable sector in pig farming in Brazil. This study aimed to assess the productivity data, management characteristics, and boar culling in 11 boar studs. In 2015, 21,333 ejaculates or collection attempts from 983 boars were registered and 86.1% of them were considered approved. The remaining ejaculates (13.9%) were discarded primarily due to morphology alterations and low motility (32.1% and 21.4%, respectively). Monthly production was higher between January and April (56.7% collections). On average, each central had 4.6 employees performing great part of collections during weekdays (86.2%), overall Mondays and Fridays (24.5% and 22.0%, respectively). Interval between collections was 6.70 days (± 3.25 days). The average collected semen volume, concentration, motility, ejaculates temperature, and estimated dose numbers were, in this order: 311.16 mL (± 125.86 mL), 306.06 ×10^6 cells/mL (± 143,10 ×10^6 cells/mL), 85.13% (± 17.67%), 35.47°C (± 1.01°C), and 27.61 (± 8.84). Sperm vigor scored mostly ‘4’ (progressive, straight, and fast) in 70.1% of the ejaculates. The visual aspects most reported were Milky and Milky-Serous (44.9% and 49.2%,

respectively). Boars were culled (n=300) mainly due to: low semen viability (31.3%), genetic improvement (22.7%), and age (15.0%).

Keywords: Swine, boar studs, semen parameters, benchmarking, artificial insemination

Introduction

Despite of having a low consuming of pork (14.7 kg/ *per capita/* year consumption (ABPA, 2016)) when compared to European and Asian countries (15.8 and 34.6 kg/ *per capita*/ year, respectively (Source: FAOSTAT Database)), Brazil is one of the global leaders in the production of this protein. It encompasses the third largest herd of pigs (FAOSTAT, 2016) and is the fourth largest pork producer and exporter (ABPA, 2016). In a great measure, the success in the national production is due to the adoption of technologies in every part of the production chain. One of the largest adopted technologies by farmers, and very traditional, is the Artificial Insemination. According to data published in the report 'Mapping of Brazilian Pork Chain', this segment had an estimated revenue of 12.5 million dollars in 2015, considering marketed semen doses and AI supplies (SEBRAE & ABCS, 2016). During this period, 1.15 million semen doses were marketed at an average cost of R$ 20.50 / dose (US$ 6.52 / dose[5]).

Semen doses are produced within centers that house boars under two distinctive types of program: closed-like, in which the semen doses are employed in sows within the same farm, or open-like, in which the doses are marketed to different sow farms (BORTOLOZZO et al., 2008). In Brazil, the first Boar studs (BS) were set up in Estrela- RS and Concordia-SC municipalities (SCHEID, 1991). Since then, the number of BS had increased and up to this moment there is no official data accounting production in these centers. Consequently, few studies were dedicated to research their productivity aspects (CASTRO et al., 1996) and currently there are not reliable data concerning the practices and semen parameters of the doses they produce.

On the other hand, research groups in the United States and Canada have published numerous studies in which the practices on BS were surveyed (CRABO & DIAL, 1992; RUTTEN et al., 2000; SINGLETON, 2001; KNOX et al., 2008). Such studies shed light to

[5] Considering that the average currency in 2015 was 3.14 BRL/ 1.00 USD. Source: Brazilian Central Bank

some aspects in the pork chain that resulted in the identification of limitations in the production system that pointed out ways to improve the productivity further on (FOXCROFT et al., 2010a).

The primary objective of this study is, therefore, to report the productivity data from 11 BS in Brazil during the year 2015. Our secondary objective is to make this study the first reference on semen parameters produced in BS for benchmarking purposes, thus expecting to acknowledge a starting point for future improvements in this segment of the pork chain in Brazil.

Material and Methods

Production Databank

In this study, we retrieved data from a databank reporting semen productivity in 11 BS. This databank was fed and developed by a start-up that sources pig farms with a management software for the purpose of benchmarking. These BS were located in three Brazilian states: Santa Catarina, Goias and Mato Grosso do Sul. However, the geographical localization of each BS could not be determined due to anonymity policies. The database contained information related to semen parameters (motility, concentration, volume, vigor [score from 1 to 5], visual aspect, and temperature) and stud management (collection days, employee in charge of collection, collection interval for each boar, approbation or disapprobation and its reason). The number of doses produced was calculated based on concentration, volume, and motility data, considering each dose should have three billion sperm cells. The causes for boar culling in nine of the 11 BS were also assessed.

Statistical analysis

All variables were analyzed in terms of descriptive statistics, in which we displayed the average of the aforementioned parameters.

Results

During the year of 2015, 21,333 ejaculates or collection attempts of 983 boars were reported by 11 BS. From those, 18,373 (or 86.1%) were approved and 50,000 doses (9.5 thousand doses weekly) were produced and marketed, approximately. The remaining ejaculates (13.9%) were disapproved by the BS and in 1,583 (53.5%) of them, the causes were mainly: morphology alterations (32.1%), low motility (21.4%), and other (Fig. 1). Considering the year, there was decrease in the first two causes cited above between May and September, summing up only 431 disapproved ejaculates during this period (or 14.56% of the total disapproved). In the category 'other' (mentioned 681 times, 23.0% of total disapproved), the causes more cited were related to boar management in the BS, such as training or emptying (mentioned 667 times) and specific issues within the BS. Other categories listed were agglutination – 248 (8.4% of total); low concentration – 177 (6.0% of total); low volume – 49 (1.7 % of total); contaminations – 173 (5.8% of total); accidental loss – 18 (0.6% of total); impeditive boar behavior – 19 (0.6% of total); and low dose number – 12 (0.4% of total).

Regarding monthly production, high frequency of semen collections was concentrated between January and April (56.7% collections), with a marked decrease as of May (Fig. 2). On average, each BS employed 4.6 people to assist collections weekly.

In general, higher rate of collections occurred during weekdays (86.2%), being Mondays and Fridays (Fig. 3) the days with higher collection rates (24.5% and 22.0%, respectively). Intervals between collection for a given boar were on average 6.70 days (± 3.25 days), with higher average intervals in May (7.27 ± 3.33 days), June (7.13 ± 2.45 days), July (6.99 ± 3.65 days), August (6.93 ± 2.55 days), and September (6.97 ± 2.49 days).

Semen traits analyzed (volume, concentration, motility, temperature, and vigor) exhibited great variability throughout the year (Tab. 1). In the surveyed period, the average collected semen volume was 311.16 (± 125.86) mL, with higher volume amounts obtained between April and August (mean 316.88, ± 99.19 mL). On the other hand, July, a month when the higher average volume amount was produced (332.16, ± 137.23 mL), exhibited lower average in ejaculate concentration among other months (278.34 ± 123.53 $\times 10^6$ cells/mL), when the annual average concentration was 306.06 $\times 10^6$ cells/mL (±143.10 $\times 10^6$ cells/mL). Regarding motility, the average value was 85.13% (± 17.67%), and June and October higher averages of this trait were reported. Average ejaculate temperature was 35.47°C (±1.01°C) and, likewise, lower average values were observed between June and October. The average of estimated dose number that each approved ejaculate produced was 27.61 (± 8.84), and in May it was recorded the higher average in estimated dose number by ejaculate, 30.29 (±8.92). With respect to vigor (CBRA, 2013), the higher score registered was '4' (progressive, straight, and fast) (70.1% year round), with fluctuations throughout the year, and from May to December this score was recorded the most (Fig. 4). Regarding the visual aspect, five options were listed: Aqueous, Milky, Dense-Milky, Milky-Serous, and Serous. The most mentioned were Milky and Milky-Serous both with 94.1% mentions (44.9% and 49.2%, respectively).

Lastly, the causes for boar culling were assessed in nine out of the 11 BS. During period analyzed, 300 boars were culled, in which 207 of them (69.0%) were discarded for three main causes: 94 for low semen viability (31.3%), 68 for genetic improvement (22.7%) and 45 for age (15.0%). The other reported causes were low demand (1.3%), disease or accident (1.7%), non-breeder boar (2.0%), locomotor impairments (3.7%), deterrent boar behavior for collection (5.0%), and other specific issues reported or non-reported by the BS (17.3%).

Discussion

Brazil has a continental dimension and the geographical, economical, and cultural characteristics are very distinctive throughout its regions. From the three analyzed states, two, Goias and Mato Grosso do Sul, are located in the same economic and cultural region that possess very similar climate traits, and are relatively new regions for pig farming. Santa Catarina, on the other hand, has the highest swine herd in the country (SEBRAE & ABCS, 2016) and is located in the southern region of Brazil, with cultural, economic and climate features very distinctive from the other two states, being a traditional state in pig farming. Although it was not possible to determine which BS belonged to which state, we believe that the great variations we observed in the data could be related to those differences.

The outcomes we observed may be explained by those climate and cultural contrasts. Interestingly, months in which higher demand for semen doses were observed (January to April), generally the average temperatures are high, and could have a negative impact in the comfort temperature for boars and, therefore, spoil the semen quality (WETTEMANN & BAZER, 1985). Despite the oscillations in the environmental temperature, the absolute amount of ejaculate discard rate remained stable throughout the year, which could indicate the usage of air conditioning in most studs. Motility, vigor, and concentration parameters had higher average values and the ejaculate temperature, on the contrary, exhibited lower average values from May to July, which is the period of the year when the average temperatures are generally low; therefore, resulting in a more comforting temperature for boars, influencing the improvement in semen parameters.

Regarding the techniques used in the studs, it was not possible to determine by which methods they employed to perform concentration and motility analyses, whether by an automated process, such as computer-assisted sperm analysis (CASA), or subjective measurements using an optical microscope, for instance. It is known that there are significant differences when analyses are performed by one or the other method (FOXCROFT et al.,

2010b; BROEKHUIJSE et al., 2011), affecting the outcomes in the parameters and the amount of doses produced.

Collections were performed mostly on Mondays and Fridays, probably due to the following reasons: (a) most Insemination Centers use Beltsville Thawing Solution (BTS) as semen extender, which has a lifespan of three days; therefore, sow farms would accommodate best their insemination days; and (b) there is a rationalization in the collection days, reducing the costs with labor payment and extra hours.

The remarkable fall in the approved ejaculates as of April to May were caused by a decrease in boars being collected (Fig. 2), possibly due to a shift in the type of artificial insemination performed in the studs, from cervical to intrauterine. In intrauterine insemination, the amount of sperm required for fertilization is approximately half or threefold lesser the required for traditional insemination (around 1 billion sperm per dose) (BORTOLOZZO et al., 2015). This allows the maximization of the doses produced with one ejaculate; therefore, less boars are required for semen production, consequently reducing housing and feeding costs, and increasing genetic gain in pig farming since genetically-superior boars stay longer in the studs.

One of the most cited causes for boar culling was the age; however, this data was not available in the collections databank, which prevented the determination on the average age of boars either being culled or collected. Likewise observed by KNOX et al. (2008), two of the most cited causes for culling in North America were genetic improvement and low semen quality, only differing with respect to age, more cited in this study. While in the study cited above the third most cited cause for boar culling regarded locomotive impairments (feet and legs). In this study, this cause was observed in only 3.7% of the cases reported. This could indicate improvements in detection and controlling feet and leg problems.

The results reported above could represent the reality of most Brazilian BS, although no official data is available to confirm this statement. We hope to obtain for future studies, a more

complete data of the BS from different states and from different years to perform a more complete analysis of the management, production parameters, and their general features and compare their performances.

Conclusion

This study evaluated productivity data and management characteristics of 11 BS in three Brazilian states and covered seminal parameters. Average motility of samples was 85.13%, volume 311.16mL, average concentration of 306.06 $\times 10^6$ sperm cells/mL, vigor score 4. Visual aspects most reported were Milky and Milky-Serous. Average ejaculate temperature was 35.47°C, and estimated dose number was 27.61. Each boar stud employed on average 4.6 people. January to April occurred most of semen collections (56.7%) and Mondays and Fridays concentrated most collections. Ejaculates were discarded mostly due to morphology alterations and low motility. Boars were culled due to: (1) low semen viability; (2) genetic improvement; and (3) age.

Acknowledgments

Our sincere thanks to Agriness, which provided the data we used in this paper and the boar studs that submitted their data to Agriness. We also want to thank the Agencies that financially supported our study: FAPERGS, CAPES, and CNPq. This work was conducted during a scholarship financed by CAPES – Brazilian Federal Agency for Support and Evaluation of Graduate Education within the Ministry of Education of Brazil.

References

ABPA. **Annual Report of the Brazilian Association of Animal Protein**. Brazilian Association of Animal Protein, p.131. 2016. Disponível em: <http://abpa-

br.com.br/storage/files/abpa_relatorio_anual_2016_ingles_web_versao_para_site_abpa_bloqueado.pdf>. Acesso em: 10 November 2017.

BORTOLOZZO, F., et al. New artificial insemination technologies for swine. **Reproduction in domestic animals**, v.50, p.80-84. 2015. Disponível em: <https://onlinelibrary.wiley.com/doi/full/10.1111/rda.12544>. Acesso em: 18 jan. 2018. doi: 10.1111/rda.12544.

BORTOLOZZO, F. P., et al. Inseminação Artificial em Suínos. In: P. B. D. Gonçalves, J. R. D. Figueiredo, *et al* (Ed.). **Biotécnicas Aplicadas à Reprodução Animal**. São Paulo: Editora Roca, 2008. Cap. 7, p.125-144

BROEKHUIJSE, M., et al. Additional value of computer assisted semen analysis (CASA) compared to conventional motility assessments in pig artificial insemination. **Theriogenology**, v.76, n.8, p.1473-1486. e1. 2011. Disponível em: <https://www.sciencedirect.com/science/article/abs/pii/S0093691X11003001?via%3Dihub>. Acesso em: 20 jan. 2018. doi: 10.1016/j.theriogenology.2011.05.040.

CASTRO, M. L. S. D., et al. Influence of the collection period on volume, motility and semen doses in swine. **Ciência Rural**, v.26, n.3, p.457-462. 1996. Disponível em: <https://www.scielo.br/scielo.php?script=sci_abstract&pid=S0103-84781996000300020&lng=en&nrm=iso>. Acesso em: 18 jan. 2018. doi: 10.1590/S0103-84781996000300020.

CBRA, C. B. D. R. A. **Manual para exame andrológico e avaliação de sêmen animal**. Belo Horizonte: Colégio Brasileiro de Reprodução Animal. 2013. 104 p.

CRABO, B. G.; G. D. DIAL. Artificial Insemination in Swine. **Veterinary Clinics of North America: Food Animal Practice**, v.8, n.3, p.533-544. 1992. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0749072015307027>. Acesso em: 18 jan. 2018. doi: 10.1016/S0749-0720(15)30702-7.

FAOSTAT. Live animals: Production of pigs. Rome: Food and Agriculture Organization of the United Nations 2016.

FOXCROFT, G., et al. Improving production efficiency in a competitive industry. **Proceedings of the Manitoba Swine Seminar', Winnipeg, Canada**, 2010a. p.81-98.

FOXCROFT, G. R., et al. Application of advanced AI technologies to improve the competitiveness of the pork industry. **Proceedings of the 21 st IPVS Congress, Vancouver, Canada-July**, 2010b. p.18-21.

KNOX, R., et al. An update on North American boar stud practices. **Theriogenology**, v.70, n.8, p.1202-1208. 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18656252>. Acesso em: 18 jan. 2018. doi: 10.1016/j.theriogenology.2008.06.036.

RUTTEN, S. C., et al. Boar stud production analysis. **Journal of Swine Health and Production**, v.8, n.1, p.11-14. 2000. Disponível em: <https://www.aasv.org/shap/issues/v8n1/v8n1p11.html>. Acesso em: 10 dez. 2018. doi.

SCHEID, I. Commercial swine artificial insemination in Brazil: development and current use. **International Conference on Boar Semen Preservation**. Berlin: Reproduction in domestic animals**:** 299-301 p. 1991.

SEBRAE; ABCS. **Mapping of Brazilian Pork Chain**. p.376. 2016. Disponível em: <http://www.abcs.org.br/images/-01_mapeamento_completo_bloq.pdf>. Acesso em: 9 September 2017.

SINGLETON, W. State of the art in artificial insemination of pigs in the United States. **Theriogenology**, v.56, n.8, p.1305-1310. 2001. Disponível em: <https://www.sciencedirect.com/science/article/abs/pii/S0093691X01006318>. Acesso em: 19 fev. 2018. doi: 10.1016/S0093-691X(01)00631-8.

WETTEMANN, R.; F. BAZER. Influence of environmental temperature on prolificacy of pigs. **Journal of Reproduction and Fertility. Supplement**, v.33, p.199-208. 1985. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/3910825/#:~:text=Exposure%20of%20male%20and%20female,result%20in%20reduced%20reproductive%20efficiency.&text=Decreased%20conception%20rates%20and%20reduced,0%20to%2016%20after%20mating.>. Acesso em: 19 fev. 2018. doi.

Table List

Table 1. Average of semen parameters (volume, concentration, motility and temperature), interval between collections, and estimated dose number (considering a concentration of 3 billion sperm in each dose) obtained throughout the year and their standard deviation.

Month	Volume (mL)	Concentration (10^6 cells/mL)	Motility (%)	Temperature (°C)	Interval between collections (d)	Estimated Number of Doses
January	296.34 ±121.72	322.64 ±154.48	85.51 ±11.97	35.74 ±1.00	6.78 ±3.96	26.29 ±8.51
February	298.10 ±122.80	315.64 ±154.77	86.25 ±10.24	35.74 ±0.95	6.51 ±3.66	26.03 ±8.44
March	309.47 ±125.53	306.62 ±151.87	85.32 ±13.17	35.71 ±0.93	6.47 ±3.39	26.13 ±8.18
April	322.96 ±129.74	306.22 ±145.00	83.94 ±18.03	35.82 ±0.80	6.37 ±3.14	27.64 ±7.88
May	324.95 ±133.25	313.95 ±147.25	83.48 ±22.16	35.38 ±1.17	7.27 ±3.33	30.29 ±8.92
June	324.26 ±128.18	300.07 ±130.25	86.90 ±17.03	34.99 ±0.80	7.13 ±2.45	29.84 ±9.43
July	332.16 ±137.23	278.34 ±123.53	88.46 ±12.58	34.83 ±0.91	6.99 ±3.65	28.20 ±8.58
August	317.43 ±129.23	290.61 ±120.90	87.25 ±18.21	34.84 ±0.95	6.93 ±2.55	29.33 ±8.45
September	306.87 ±125.69	299.00 ±125.90	87.89 ±17.89	34.97 ±0.95	6.97 ±2.49	28.44 ±8.20
October	313.74 ±122.51	298.65 ±126.56	88.27 ±14.31	35.16 ±0.99	6.89 ±2.63	29.30 ±8.44
November	312.95 ±115.97	299.85 ±132.09	80.36 ±29.59	35.20 ±0.90	6.62 ±2.24	29.80±8.36
December	303.04 ±110.66	291.53 ±124.63	77.47 ±32.44	35.26 ±0.94	6.31 ±2.22	28.90 ±7.68
Average	311.16 ±125.86	306.06 ±143.10	85.13 ±17.67	35.47 ±1.01	6.70 ±3.25	27.61 ±8.48

Figure List

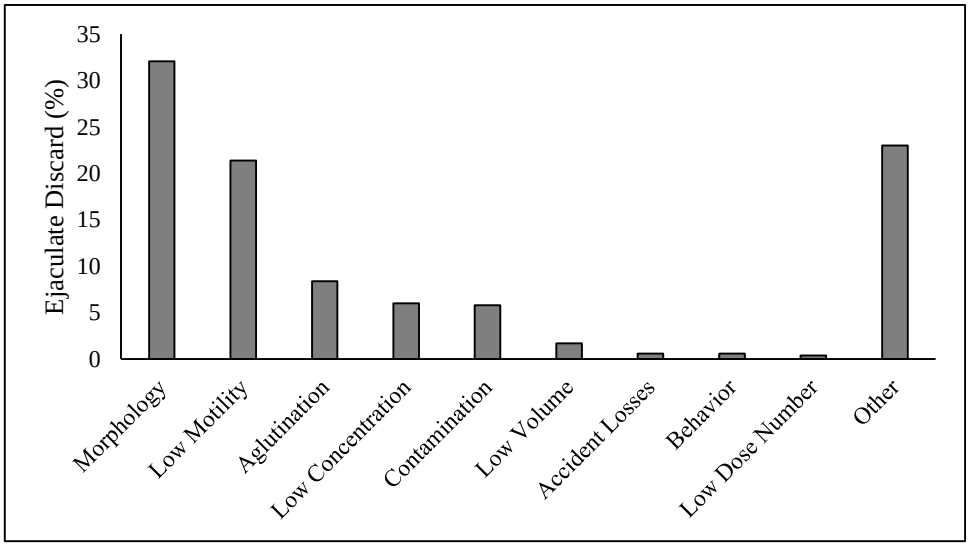

Figure 3. Frequency of ejaculates discarded according to discard reason.

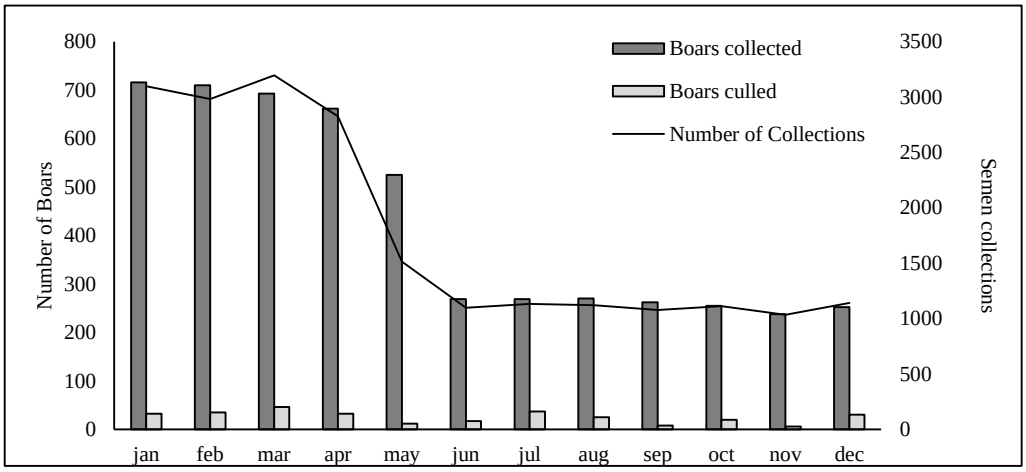

Figure 4. Number or boars being collected and culled and ejaculate collections throughout the year.

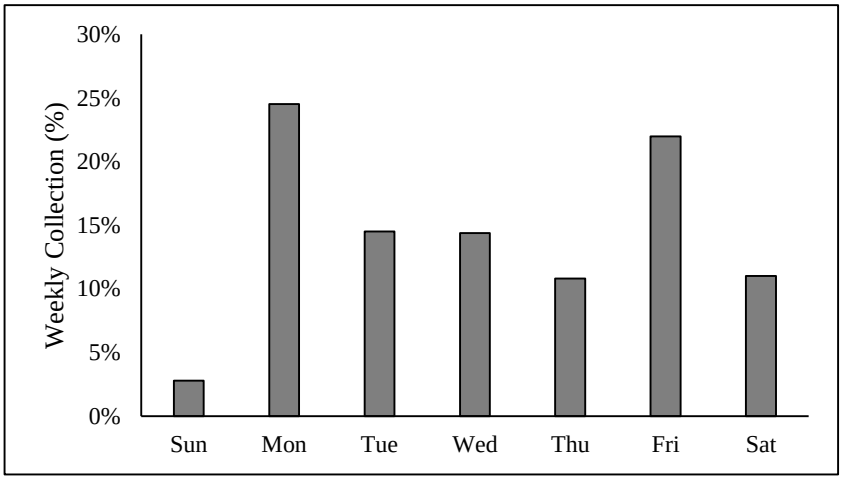

Figure 5. Frequency (%) of weekly semen collections.

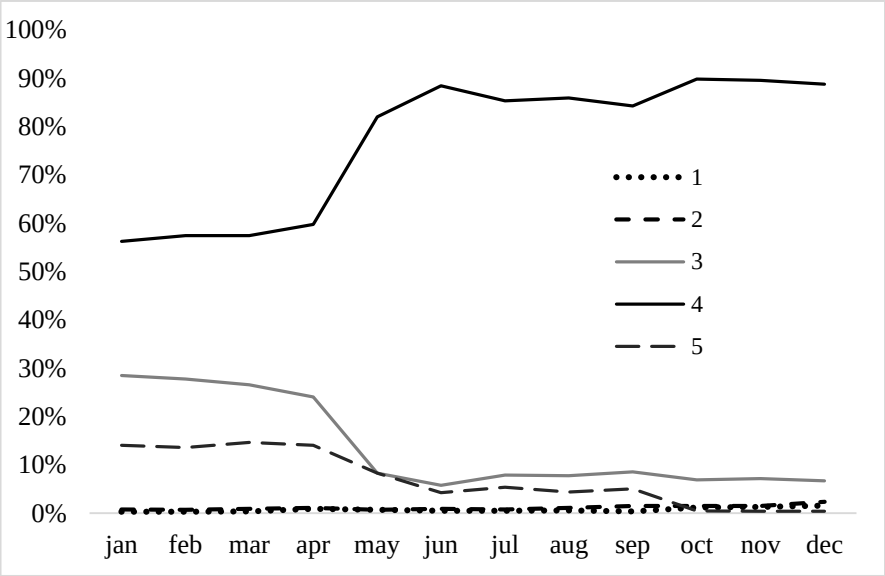

Figure 6. Frequency of vigor scores (1- exclusively oscillatory; 2 – slow; 3 – intermediate; 4 – progressive, straight, and fast; 5 – progressive, straight, and very fast) registered throughout the year. Source: brazilian college of animal reproduction – manual para exame andrológico e avaliação de sêmen animal (2013).

2.2 Article 2

Effect of high-speed centrifugation and sonication of extender on sperm parameters of freeze-thaw boar semen

Camila R. C. de Brito, Stela Mari M. Gheller, Izani B. Acosta, Andreia N. Anciuti, Norton Luiz S. Gatti, Edenara A. Silva, Nathalia W. Knabah, Antonio Sergio Varela Junior, Carine D. Corcini

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Effect of high-speed centrifugation and sonication of extender on sperm parameters of freeze-thaw boar semen

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Abstract

Researches with cryopreservation of boar semen still aim standardization of extenders and protocols to increase sperm viability post-thawing. Most common extenders are based on egg-yolk. Our objective in this study was to investigate if centrifugation and sonication of extender would improve kinematic and cell structure parameters post-thawing from boar ejaculates with distinct total motility pre-freezing. Twenty-seven semen doses were purchased from a commercial boar stud and processed for cryopreservation using egg-yolk lactose 11% (control) extender, processed using two different methods: high-speed centrifugation and sonication. Furthermore, semen samples were divided into three groups of motility profiles: < 70% (G1), > 70% and < 80% (G2), > 80% (G3). Then, they were submitted to freeze-thawing protocol and were assessed for kinematic and cell structural parameters. Samples in which extenders underwent centrifugation had better results in velocity parameters, meanwhile those that only sonication (T2) was performed had poorest results in this parameter. Moreover, G3 presented best results in the velocity parameters. However, only groups in which motility pre-freezing was lower (G1 and G2) had better results in preserving sperm cell membrane and mitochondria. Therefore, centrifugation of extender is beneficial for kinematic parameters of sperm cells and a low motility pre-freezing has positive effect in preserving the cell structure.

Keywords: Boar, cryopreservation, high-speed centrifugation, kinematic parameters, semen, sonication.

Introduction

Livestock production has achieved an intense use of techniques that have been able to preserve biological material with high genetic merit primarily by employing cryopreservation processes that store cells (such as the gametes) or complex tissues (i.e., ovaries) for undetermined time [3]. The cryopreservation of semen and its use in artificial insemination has been successful for the last seventy years [5] and, in some species, the commercial use of frozen semen is well stablished and largely adopted [23; 24]. The cryopreservation of boar semen, however, has its commercial use limited to conservation in germplasm banks, since fertility is reduced when compared to cooled or fresh semen [18; 20].

The poor freezability is attributed to intrinsic characteristics of the sperm cell in this species. The low content of cholesterol in the membranes and high levels of polyunsaturated fatty acids exposes the cell to lipoperoxidation and formation of reactive oxygen species (ROS), which in turn cause damages to the DNA [18]. Moreover, the formation of ice crystals, both inside and outside the cell, is an additional issue, considering that its formation lead to intracellular solutes to concentrate and destabilize cell structure, only decreased when cryoprotectants, such as egg-yolk (external or non-penetrating) or dimethylsulfoxide (internal or penetrating), are used in the extender [11; 30]. Currently, studies aim the development of the ideal composition of semen extender that would prevent the sperm cell to be injured during freezing-thawing process, especially harmful in swine spermatozoa [29].

Commonly, the extenders employed to freeze boar semen are based mainly on egg-yolk [29]. The egg-yolk has a high content of low density lipoproteins (LDL), which act in the sperm membrane by coating the cell [12]. Moreover, LDL is composed of fatty acids, especially phosphatidyl choline, that are thought to be incorporated into to the plasma membrane and protect the cell against cold shock during phase transition of the membrane [28]. However, egg-yolk also possess components that have detrimental effects on sperm survival, impacting on cell respiration thus decreasing its fertility [27]. Therefore, the extraction of egg-yolk LDL has been tested and employed with successful results in the preservation of semen of various species by using, among other steps, high-speed centrifugation [2; 12; 26].

Furthermore, some authors propose the addition of small molecules of fatty acids to the extender. The liposomes are the best example of these attempts, since they possess

a precise content of fatty acids forming a phospholipid bilayer [21; 22]. It is hypothesized that during incubation of sperm cells with liposomes in the extender, the outer membrane would fuse to the vesicles, increasing fatty acids content in the plasma membrane that would protect the cell against cold shock during freezing-thawing [10; 21]. Among the diverse methods by which these molecules and nanoemulsions are obtained is the high-power agitation generated by an ultrasound device. The energy generated by the intense vibration of the equipment into the biphasic liquid system causes the breakdown of the molecules dispersed in the solute into smaller droplets [1].

Therefore, this study aimed to test whether centrifugation and/or sonication of an egg-yolk-based extender would improve the quality of kinematic and structural sperm cell parameters post-thawed. Furthermore, we tested whether these two treatments would benefit quality parameters of semen doses that had different profiles of motility rates before freezing procedure, since they underwent 24 hours of shipment time before starting freezing processing.

Material and Methods

Characteristics of semen doses

To perform this study, semen doses were purchased from a commercial boar stud, located at the municipality of Estrela, distant from our laboratory facilities by 325 km. In this experiment, it was used semen doses from 27 different boars (n=27), from the same lineage, maintained in individual pens, and receiving the same feed. Doses were composed of ~80 mL of a fraction of the ejaculate (3 billion sperm cells) diluted in a commercial extender (Androstar®, Minitub, Germany). As semen doses purchased had distinct pre-freezing profiles of sperm total motility, they were divided into three groups: G1 – < 70%; G2 – > 70% and < 80%; and G3 – > 80%. To each of the previous groups, we tested four treatments on the cooling/freezing extenders, as follows.

Cooling and freezing extenders preparation

The cooling extender was prepared using fresh eggs obtained from a local grocery store. Yolks were separated from whites and placed onto paper towel to remove white and vitelline membrane debris. Then, they were mixed with a Lactose solution at 11%

concentration, with pH adjusted to 7.0. Lactose was purchased from Sigma-Aldrich (St. Louis, USA) and was diluted in deionized sterile water (Aquatec®, Caithec, Brazil). To extract yolk's plasma fraction we centrifuged three times the extender obtained in the previous step at × 10,000 g, for 45 min at 5°C, each time, always retrieving supernatant and discarding the pellet (Eppendorf Centrifuge 5804R, Germany) [2]. Lastly, we sonicated (Q55 sonicator, QSonica, USA) both extenders, centrifuged and non-centrifuged egg-yolk lactose, for 30 minutes at 50% amplitude, with a 3 mm probe tip diameter, with the extender in a tube immersed in an iced container to avoid rise in the temperature. By the end of this process, we obtained four cooling extenders: control egg-yolk (T1), egg-yolk sonicated (T2), egg-yolk's plasma (T3), and egg-yolk's plasma sonicated (T4).

The preparation of the freezing extender consisted of a mixture of 89.5% of the cooling extender (v/v) obtained in the previous step, 9% glycerol (v/v), and 1.5% detergent Equex-Paste (Minitub®, Germany) (v/v) [4]. Therefore, the straw contained sperm cells dispersed into 2/3 of cooling extender and 1/3 of freezing extender, making up 0.5 mL volume.

Semen doses manipulation and freezing procedure

Semen doses were shipped to our laboratory through postal service. When doses arrived, they were held for 90 min at 17°C. Then, doses were homogenized and 13.3 mL were distributed into four tubes of 15 mL capacity and centrifuged at 800 × g, for 10 min (centrifuge Baby FANEM, São Paulo, Brazil) [4]. Supernatant was discarded and the pellet formed in each tube, containing approximately 500 million sperm cells, was re-suspended in 334 µL of the cooling extender.

Samples were transferred to 1.5 mL tubes attached to floaters in a container with water and stored in a cooled box at 5°C for at least 90 min [4]. Afterwards, 166 µL of the freezing extender was added to the samples and the 500 µL of semen was loaded into the straws [4]. Thereafter, the straws were placed in a floater, distant from liquid nitrogen (LN) level by 5 cm, and left in contact with the nitrogen vapor for 10 min [4; 19]. Subsequently, straws were plunged into LN, stored in racks, and deposited in a LN vessel for at least 15 days before thawing.

Semen thawing and kinematic and cell structure analyses

In thawing process, straws were removed from the racks and thawed in water bath (~36.5°C) for 20 seconds. Afterwards, 50 µL of thawed semen was diluted into 450 µL of thawing medium: Bovine Serum Albumin (BSA) diluted into Beltsville Thawing Solution (BTS) (3 mg BSA/100 mL BTS). Thereafter, 3 µL of each sample obtained in the previous step was loaded into a counting chamber slide (Leja® Standard Count 4 Chamber Slide 20 micron, Luzernestraat, Netherlands), after incubation for 10 min at 36.5°C in a heated plate. Sperm cells were observed in an optical microscope attached to a computer-aided sperm analysis (CASA) system to assess kinematic parameters: total and progressive motilities (TM and PM, respectively), Average Path Velocity (VAP), Curvilinear Velocity (VCL), Straight Line Velocity (VSL), Linearity (LIN), Straightness (STR), Wobble (WOB), and Amplitude of Lateral Head Displacement (ALH).

Cell structure was assessed through the observation of semen samples conjugated with fluorescent probes, smeared into microscopy slides using a WU filter, with excitations of 450–490nm and emission of 516–617nm, by employing an epifluorescence microscope (Olympus BX 51, São Paulo, SP). Data were expressed as the percentage of whole cells (100 cells were counted for each assessment). For acrosome assessment, slides were dried, immersed in absolute ethyl alcohol for 5min, washed in PBS, and the fluorescent probe (FITC) was smeared on the slide surface [14]. They were subsequently washed in deionized water and drained in a dark room at 24ºC. In the remaining assessments, membrane integrity, mitochondria functionality, and DNA integrity, 20 µL sperm cells samples were conjugated with fluorescent-probes, incubated at 24°C for 5 minutes in a dark room, and smeared in a slide for observation in the microscope. Fluorescent probes used and classification of cell organelles are summarized on Table 1.

Statistical analysis

The statistical analysis was carried out in Statistix 10™ software. First, we tested normality of data by performing Shapiro-Wilk's test. Those variables that lacked normality the function Arcsine/100 was used in order to transform data. Then, data were compared using Analysis of Variance, first considering treatments applied to the extenders, then considering the groups formed according to motility profiles pre-freezing, and lastly the interaction between treatments and groups. Post-test used was LSD, with Interval Confidence of 95%.

Results

Prior to freezing, samples were categorized into total motility profiles: <70% (G1), ≥70% and <80% (G2), and ≥80% (G3). Means of TM and PM in each group were statistically different and were, respectively: G1 (n=36) – 58.7% (±1.39%) and 59.7% (±2.86%); G2 (n=44) – 76.1% (±0.40%) and 68.2% (±1.24%); G3 (n=28) – 84.5% (±0.60%) and 73.4% (±1.12%).

Post-thawing analysis of samples, when only the effect of treatments was compared, demonstrated that for the kinematic parameters VCL, VSL, ALH, and STR the treatment that had best result was T3, whereas T2 exhibited poorest results. The differences between other kinematic parameters were not statistically significant (P>0.05) (Table 2). When the results of the different groups formed according to pre-freezing total motility were compared, the G3 had the best results in the kinematic parameters STR and VSL. The other kinematic parameters did not differ statistically (P>0.05) (Table 3).

On the other hand, in the organelle integrity assessments no statistical differences were observed between treatments, despite groups formed according to pre-freezing total motility (Table 2). When only groups were considered, G1 and G2 had best results in preserving the membrane and mitochondria integrity, and G3 had poorest results (P<0.05) (Table 3).

When the effect of both variables, treatments and groups, were considered together the means did not differ statistically in both kinematic and cell integrity assessments (P>0.05).

Discussion

In this study, we tried to identify whether the centrifugation and sonication of the semen extender would have beneficial results in preserving boar sperm cells during freeze-thawing procedure. Moreover, since the doses obtained from a commercial boar stud took 24 hours to be processed after collection, we investigated whether these treatments would improve or at least contain the damages inflicted to the samples. In general, boar studs trade semen doses when total motility after ejaculation is higher than 70% [17]. Otherwise, the semen is discarded and if the low motility persists in other collections, the boar is likely to be culled [16]. For this study, we only obtained doses from high performance boars. However, since shipment time could not be shortened, we

had to test samples motility before processing to verify if total motility remained equal to or higher than 70%. This checkup showed that samples had different profiles of total motility, varying from 44% to 88%, and therefore we opted to categorize them in the aforementioned groups.

The results obtained in the post-thawing analysis suggested that treatments, especially high-speed centrifugation (T3) but not sonication, improves sperm cell kinematic parameters and had no effect in preserving the cell integrity. However, it also showed that the higher the total motility, the poorer is the capacity to maintain sperm cell integrity, and no treatment was able to contain such damage.

It could be deduced from our results that during centrifugation process of T3 and T4 some constituents of egg-yolk that could be detrimental to sperm cells were pelleted, leaving only LDL molecules in the extender, that had good results in the kinematic parameters [2]. The supernatant was rich in LDL, which adheres to the membrane throughout freezing and thawing processes, coating the sperm cell [12]. When sonication was applied to the extender, it was expected that LDL would form microemulsions and/or nanoemulsions, to the size of liposomes or slightly larger [15]. In this study, the sonication demonstrated to be detrimental to extender performance in kinematic parameters, especially on T2, leading to the conclusion that the constituents of egg-yolk that are detrimental to sperm cells were decreased in size and had negative consequences to the kinematic parameters during the freeze-thaw routine [25]. However, in the T4 it was expected that sonication would decrease LDL size and make it more accessible to the cells to be coated, therefore enhancing both kinematic and structural parameters of sperm cells, which was not observed in our study. Others demonstrated that when liposomes were used on freezing extender on stallion sperm, kinematic parameters were inferior when compared to whole egg-yolk, corresponding partially with the results we obtained [21].

Looking closely at the cell integrity parameters, none of the treatments had an outstanding performance on preserving its integrity (Tables 2 and 3). They had similar results in preserving the membrane, acrosome, DNA, and mitochondria. However, when motility pre-freezing was taken into account, our results showed that the higher the motility pre-freezing the poorer is membrane and mitochondria integrities post-thawing. This may be due to the reactive oxygen species (ROS) production by sperm cells [31], not measured in our study. This byproduct of cell metabolism has the capacity of inducing sperm cell capacitation, and ultimately reduce their fertility by damaging through

oxidation organelles such as the plasma membrane, mitochondria, DNA, and acrosome [8]. This might explain the results we obtained on cell preservation in this study.

For cryopreservation process, especially in boar, general recommendation is to use ejaculates with high motility rate and extended as close to collection time as possible [29]. In our study, however, these two pre-requisites were missing, and doses had to be split into three groups, based on motility rate prior to freezing. Herein, the objective was to eliminate biases in post-thawing analyses. Surprisingly, except for specific samples and treatments used, even in the group with lowest motility ranks prior to freezing, the thawing process affected kinematic parameters less intensively than in the groups with higher motility pre-freezing. Moreover, it impacted less in cell structure integrity in those which motility was lower before freezing, than in the high motility samples.

Although motility is an adequate parameter to infer potential *in vitro* fertility, if the cell structure is compromised the general fertility is likely to be lower [13]. We demonstrated that the lower the motility pre-freezing, the better is cell structure conservation. However, since the doses we obtained took a long period from shipment to processing, we lacked to determine whether freshly extended semen would have different outcome in this parameter. Therefore, total motility itself is not the ultimate trait to predict poor/good freezability of boar sperm, and other parameters must be taken into account [29].

Conclusion

In summary, submitting cooling and freezing extenders to high-speed centrifugation, but not sonication, had best results in the kinematic parameters post-thawing. However, samples with lower motility sperm cells obtained better cell preservation than higher motility samples.

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References

[1] B. Abismail, J.P. Canselier, A.M. Wilhelm, H. Delmas, and C. Gourdon, Emulsification by ultrasound: drop size distribution and stability. Ultrasonics Sonochemistry 6 (1999) 75-83. 10.1016/S1350-4177(98)00027-3.

[2] L. Amirat, D. Tainturier, L. Jeanneau, C. Thorin, O. Gérard, J.L. Courtens, and M. Anton, Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. Theriogenology 61 (2004) 895-907. 10.1016/s0093-691x(03)00259-0.

[3] H.D. Blackburn, Biobanking Genetic Material for Agricultural Animal Species. Annu Rev Anim Biosci 6 (2018) 69-82. 10.1146/annurev-animal-030117-014603.

[4] P. Chanapiwat, K. Kaeoket, and P. Tummaruk, Cryopreservation of Boar Semen by Egg Yolk-Based Extenders Containing Lactose or Fructose is Better Than Sorbitol. Journal of Veterinary Medical Science 74 (2012) 351-354. 10.1292/jvms.11-0273.

[5] M.R. Curry, Cryopreservation of semen from domestic livestock. Rev Reprod 5 (2000) 46-52. 10.1530/revreprod/5.1.46.

[6] D. Evenson, L. Jost, D. Marshall, M. Zinaman, E. Clegg, K. Purvis, P. De Angelis, and O. Claussen, Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Human reproduction 14 (1999) 1039-1049. 10.1093/humrep/14.4.1039.

[7] D.L. Garner, C.A. Thomas, H.W. Joerg, J.M. DeJarnette, and C.E. Marshall, Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction 57 (1997) 1401-1406. 10.1095/biolreprod57.6.1401.

[8] J. Gosalvez, E. Tvrda, and A. Agarwal, Free radical and superoxide reactivity detection in semen quality assessment: past, present, and future. J Assist Reprod Genet 34 (2017) 697-707. 10.1007/s10815-017-0912-8.

[9] R. Harrison, and S.E. Vickers, Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. Journal of Reproduction and Fertility 88 (1990) 343-352. 10.1530/jrf.0.0880343.

[10] L. He, J.L. Bailey, and M.M. Buhr, Incorporating Lipids into Boar Sperm Decreases Chilling Sensitivity but Not Capacitation Potential. Biology of Reproduction (2001) 69–79.

[11] W.V. Holt, and L.M. Penfold, Fundamental and Practical Aspects of Semen Cryopreservation. in: P.J. Chenoweth, and S.P. Lorton, (Eds.), Animal andrology : theories and applications, CABI, Oxfordshire, UK, 2014, pp. 76-99.

[12] J.-H. Hu, Q.-W. Li, G. Li, X.-Y. Chen, H.-Y. Hai-Yang, S.-S. Zhang, and L.-Q. Wang, The cryoprotective effect on frozen-thawed boar semen of egg yolk low density lipoproteins. Asian-australasian journal of animal sciences 19 (2006) 486-494. 10.5713/ajas.2006.486.

[13] M. Jung, K. Rudiger, and M. Schulze, In Vitro Measures for Assessing Boar Semen Fertility. Reprod Domest Anim 50 Suppl 2 (2015) 20-4. 10.1111/rda.12533.

[14] A. Kawamoto, K. Ohashi, H. Kishikawa, L.Q. Zhu, C. Azuma, and Y. Murata, Two-color fluorescence staining of lectin and anti-CD46 antibody to assess acrosomal status. Fertil Steril 71 (1999) 497-501. 10.1016/s0015-0282(98)00507-x.

[15] S. Kentish, T. Wooster, M. Ashokkumar, S. Balachandran, R. Mawson, and L. Simons, The use of ultrasonics for nanoemulsion preparation. Innovative Food Science & Emerging Technologies 9 (2008) 170-175. 10.1016/j.ifset.2007.07.005.

[16] R. Knox, D. Levis, T. Safranski, and W. Singleton, An update on North American boar stud practices. Theriogenology 70 (2008) 1202-1208. 10.1016/j.theriogenology.2008.06.036.

[17] R.V. Knox, Artificial insemination in pigs today. Theriogenology 85 (2016) 83-93. https://doi.org/10.1016/j.theriogenology.2015.07.009.

[18] R.V. Knox, The Fertility of Frozen Boar Sperm When used for Artificial Insemination. Reprod Domest Anim 50 Suppl 2 (2015) 90-7. 10.1111/rda.12552.

[19] J. Li, J. Roca, C. Perez-Patino, I. Barranco, E.A. Martinez, H. Rodriguez-Martinez, and I. Parrilla, Is boar sperm freezability more intrinsically linked to spermatozoa than to the surrounding seminal plasma? Anim Reprod Sci (2018). 10.1016/j.anireprosci.2018.05.002.

[20] J.M. Morrell, and I. Mayer, Reproduction biotechnologies in germplasm banking of livestock species: a review. Zygote 25 (2017) 545-557. 10.1017/S0967199417000442.

[21] E. Pillet, C. Labbe, F. Batellier, G. Duchamp, V. Beaumal, M. Anton, S. Desherces, E. Schmitt, and M. Magistrini, Liposomes as an alternative to egg yolk in stallion freezing extender. Theriogenology 77 (2012) 268-79. 10.1016/j.theriogenology.2011.08.001.

[22] F. Puisieux, E. Fattal, M. Lahiani, J. Auger, P. Jouannet, P. Couvreur, and J. Delattre, Liposomes, an interesting tool to deliver a bioenergetic substrate (ATP). in vitro and in vivo studies. Journal of drug targeting 2 (1994) 443-8. 10.3109/10611869408996820.

[23] H. Rodriguez-Martinez, Sperm biotechnologies in domestic species: state of the art. Anim Reprod 10 (2013) 268-276.

[24] H. Sieme, and H. Oldenhof, Cryopreservation of semen from domestic livestock. Methods Mol Biol 1257 (2015) 277-287. 10.1007/978-1-4939-2193-5_10.

[25] J. Tosic, and A. Walton, Effect of egg-yolk and its constituents on the respiration and fertilizing capacity of spermatozoa. The Journal of Agricultural Science 37 (1947) 69-76. 10.1017/S0021859600013095.

[26] A.S. Varela Junior, C.D. Corcini, R.R. Ulguim, M.V. Alvarenga, I. Bianchi, M.N. Correa, T. Lucia, Jr., and J.C. Deschamps, Effect of low density lipoprotein on the quality of cryopreserved dog semen. Anim Reprod Sci 115 (2009) 323-7. 10.1016/j.anireprosci.2008.11.002.

[27] P. Watson, and I. Martin, The Influence of some Fractions of Egg Yolk on the Survival of Ram Spermatozoa at 5oC. Australian Journal of Biological Sciences 28 (1975) 145-152. https://doi.org/10.1071/BI9750145.

[28] P.F. Watson, Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of their Post-thawing Function. Reproduction, Fertility and Development 7 (1995) 871-891.

[29] M. Yeste, Recent Advances in Boar Sperm Cryopreservation: State of the Art and Current Perspectives. Reprod Domest Anim 50 Suppl 2 (2015) 71-9. 10.1111/rda.12569.

[30] M. Yeste, Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology 85 (2016) 47-64. 10.1016/j.theriogenology.2015.09.047.

[31] Z. Zhu, T. Kawai, T. Umehara, S.A.M. Hoque, W. Zeng, and M. Shimada, Negative effects of ROS generated during linear sperm motility on gene expression and ATP generation in boar sperm mitochondria. Free Radic Biol Med 141 (2019) 159-171. 10.1016/j.freeradbiomed.2019.06.018.

Table List

Table 1. Classification of sperm cells according to plasma membrane integrity, mitochondria functionality, acrosome integrity, and DNA integrity by fluorescence emission, in terms of intact cells.

Assessment	Fluorescent probe and sample preparation	Fluorescence emission and classification	References
Membrane integrity	Carboxyfluorescein diacetate (CFDA – 20µM), Propidium Iodide (PI – 7.3µM), and formaldehyde (1.7 µM) diluted in sodium citrate buffer.	Intact - Green fluorescence Injured - Red fluorescence	Harrison and Vickers [9]
Mitochondria integrity	Rhodamine 123 (Rh123 – 13mM) and Propidium Iodide (PI).	Functional - Mid-piece with intense green fluorescence Non-functional - Mid-piece with few or with no green fluorescence	Garner *et al.* [7]
Acrosomal integrity	Propidium iodide (PI - 20µL) and *Arachis hypogaea*-Fit-C Lectin Conjugate (20µL – 20mg/mL).	Intact - Green fluorescence and half-moon shape Injured - No green fluorescence and/or abnormal shape	Kawamoto *et al.* [14]
DNA integrity	10µL of TNE (0.01M Tris-HCl, 0.15M NaCl, 0.001M EDTA, pH 7.2); 100µL of Triton (1x); and 50µL of Acridine Orange (2mg/mL in deionized water)	Normal (double-strand intact) - Green fluorescence Denaturated - Red or yellow fluorescence	Evenson *et al.* [6]

Table 2. Effect of the different treatments on kinematic parameters and organelles integrity post-thawing, regardless pre-freezing motility. Data are expressed as means (±SEM).

Kinematic Parameters	T1	T2	T3	T4	P-value
TM (%)	28.0 ±2.25	22.4 ±2.23	25.3 ±2.35	27.8 ±2.43	>0.05
PM (%)	19.7 ±1.95	15.1 ±1.77	17.0 ±1.82	18.7 ±1.96	>0.05
VAP (µm/s)	39.9 ± 1.28	36.5 ±1.02	40.7 ±1.45	39.4 ±1.46	>0.05
VCL (µm/s)	77.5 ±2.84^{A}	70.9 ±1.60^{B}	79.1 ±3.24^{A}	75.9 ±2.84^{AB}	**<0.05**
VSL (µm/s)	30.4 ±1.07^{AB}	28.0 ±0.99^{B}	32.3 ±1.25^{A}	30.9 ±1.32^{AB}	**<0.05**
[a]STR	75.7 ±0.81^{B}	75.8 ±1.18^{B}	78.8 ±0.88^{A}	77.5 ±0.71^{AB}	**<0.05**
[a]LIN	39.7 ±1.17	39.0 ±0.97	41.9 ±2.22	40.3 ±0.98	>0.05
[a]WOB	52.0 ±1.44	50.9 ±0.63	52.3 ±2.04	51.7 ±1.01	>0.05
[a]ALH (µm)	215.3 ±5.43^{A}	194.1 ±6.05^{B}	208.7 ±6.70^{A}	200.9 ±5.01^{AB}	**<0.05**
BCF (Hz)	31.8 ±0.94	31.5 ±0.71	33.4 ±1.08	32.6 ±1.12	>0.05
Organelles Integrity (%)					
Membrane	42.5 ±4.05	41.2 ±3.89	46.4 ±3.66	39.5 ±4.05	>0.05
Mitochondria	34.8 ±3.48	37.4 ±3.09	37.9 ±3.31	33.8 ±3.21	>0.05
DNA	75.0 ±4.72	77.4 ±4.41	78.0 ±4.50	74.4 ±4.92	>0.05
Acrosome	54.7 ±3.13	58.9 ±3.16	48.9 ±2.86	49.2 ±3.45	>0.05

TM – Total Motility; **PM** – Progressive Motility; **VAP** – Average Path Velocity; **VCL** – Curvilinear Velocity; **VSL** – Straight Line Velocity; **LIN** – Linearity; **STR** – Straightness; **WOB** – Wobble; **ALH** – Amplitude of Lateral Head Displacement; **BCF** – Beat-Cross Frequency; **G1** – Low Motility (<70%); **G2** – Medium/Standard Motility (>70% and <80%); **G3** – High Motility (>80%); **T1** - Control Egg-Yolk; **T2** – Egg-Yolk Sonicated; **T3** – Egg-Yolk's Plasma; **T4** – Egg-Yolk's Plasma Sonicated. Distinct uppercase letters indicate statistical differences within rows (P<0.05).

[a] Data are expressed as $\times 10^{-2}$.

Table 3. Kinematic parameters and organelles integrity when samples were divided in groups according to pre-freezing motility and without considering treatments applied to extenders. Data are expressed as means (±SEM).

Kinematic Parameters	G1	G2	G3	P-value
TM (%)	25,3 ±1,99	26,1 ±1,81	26,2 ±2,42	>0.05
PM (%)	17,0 ±1,69	17,5 ±1,40	18,6 ±1,96	>0.05
VAP (µm/s)	39,0 ±1,10	37,9 ±0,86	41,3 ±1,62	>0.05
VCL (µm/s)	75,7 ±2,05	74,2 ±1,80	78,5 ±3,60	>0.05
VSL (µm/s)	30,0 ±1,02^{AB}	29,2 ±0,76^{B}	32,8 ±1,37^{A}	**<0.05**
[a]STR	76,1 ±0,94^{B}	76,3 ±0,54^{B}	79,1 ±0,94^{A}	**<0.05**
[a]LIN	39,3 ±0,93	39,2 ±0,76	43,0 ±2,13	>0.05
[a]WOB	50,9 ±0,67	51,1 ±0,92	53,8 ±2,02	>0.05
[a]ALH (µm)	203,2 ±5,83	203,8 ±4,10	208,3 ±6,02	>0.05
BCF (Hz)	32,5 ±0,63	31,9 ±0,67	32,8 ±1,35	>0.05
Organelles Integrity (%)				
Membrane	47,0 ±3,90^{A}	46,2 ±2,86^{A}	31,8 ±2,81^{B}	**<0.05**
Mitochondria	39,0 ±3,40^{A}	38,2 ±2,51^{A}	29,5 ±2,20^{B}	**<0.05**
DNA	83,6 ±3,81	73,5 ±3,65	72,5 ±4,29	>0.05
Acrosome	50,6 ±3,00	52,1 ±2,32	57,3 ±3,16	>0.05

TM – Total Motility; **PM** – Progressive Motility; **VAP** – Average Path Velocity; **VCL** – Curvilinear Velocity; **VSL** – Straight Line Velocity; **LIN** – Linearity; **STR** – Straightness; **WOB** – Wobble; **ALH** – Amplitude of Lateral Head Displacement; **BCF** – Beat-Cross Frequency; **G1** – Low Motility (<70%); **G2** – Medium/Standard Motility (>70% and <80%); **G3** – High Motility (>80%); **T1 -** Control Egg-Yolk; **T2** – Egg-Yolk Sonicated; **T3** – Egg-Yolk's Plasma; **T4** – Egg-Yolk's Plasma Sonicated. Distinct uppercase letters indicate statistical differences within rows (P<0.05).

[a] Data are expressed as $\times 10^{-2}$.

3 Final Considerations

For such an organized industry, few aspects still need improvements, and certainly, one liming factor is the development of a successful protocol and the right supplies that would efficiently preserve gametes using cryopreservation. Due to biological intrinsic factors, freezing boar semen is unavailable for commercial AI since performance in fertilizing eggs is below the performance of cooled and fresh semen Currently, worldwide research groups aim to develop protocols, techniques, and the right combination of substances in extenders to give the best results. Therefore, the achievement of an effective technique will represent a paradigm shift for the Pig Industry.

Meanwhile, the alternatives for the production of semen for insemination are linked exclusively to boar rearing for this purpose in boar studs. Although it corresponds to a productive sector of the chain that has been present for more than forty years in Brazil, little is known about the productivity of these plants in the country. Therefore, it is believed that the advances in reproduction research, as well as in collecting data and automation, will lead to the improvement in swine industry, consolidating it as one of the greatest markets in agroindustry in the future.

References

ABISMAIL, B.; CANSELIER, J. P.; WILHELM, A. M.; DELMAS, H.; GOURDON, C. Emulsification by ultrasound: drop size distribution and stability. **Ultrasonics Sonochemistry,** v.6, n.1-2, p.75-83, 1999.

ABPA. **Annual Report of the Brazilian Association of Animal Protein**. Brazilian Association of Animal Protein, 2016. 131 p. Available at: <http://abpa-br.com.br/storage/files/abpa_relatorio_anual_2016_ingles_web_versao_para_site_abpa_bloqueado.pdf>. Accessed: 10 November 2017.

AMIRAT, L.; TAINTURIER, D.; JEANNEAU, L.; THORIN, C.; GÉRARD, O.; COURTENS, J. L.; ANTON, M. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. **Theriogenology,** v.61, n.5, p.895-907, 2004.

BENCHMARK. In: SOANES, C. and STEVENSON, A. (ed.). **Oxford Dictionary of English**. London: Oxford University Press, 2006. p. e63852.

BLACKBURN, H. D. Biobanking Genetic Material for Agricultural Animal Species. **Annual review of animal biosciences,** v.6, p.69-82, 2018.

BORTOLOZZO, F.; MENEGAT, M.; MELLAGI, A.; BERNARDI, M.; WENTZ, I. New artificial insemination technologies for swine. **Reproduction in domestic animals,** v.50, p.80-84, 2015.

BORTOLOZZO, F. P.; BERNARDI, M. L.; BENNEMANN, P. E.; WENTZ, I. Inseminação Artificial em Suínos. In: GONÇALVES, P. B. D.;FIGUEIREDO, J. R. D.*, et al* (ed.). **Biotécnicas Aplicadas à Reprodução Animal**. São Paulo: Editora Roca, 2008. p.125-144.

BROEKHUIJSE, M.; ŠOŠTARIĆ, E.; FEITSMA, H.; GADELLA, B. Additional value of computer assisted semen analysis (CASA) compared to conventional motility assessments in pig artificial insemination. **Theriogenology,** v.76, n.8, p.1473-1486. e1471, 2011.

CASTRO, M. L. S. D.; DESCHAMPS, J. C.; MEINKE, W.; SIEWEDT, F.; CARDELINO, R. A. Influence of the collection period on volume, motility and semen doses in swine. **Ciência Rural,** v.26, n.3, p.457-462, 1996.

CBRA, C. B. D. R. A. **Manual para exame andrológico e avaliação de sêmen animal**. 3. ed. Belo Horizonte: Colégio Brasileiro de Reprodução Animal, 2013. 104 p.

CHANAPIWAT, P.; KAEOKET, K.; TUMMARUK, P. Cryopreservation of Boar Semen by Egg Yolk-Based Extenders Containing Lactose or Fructose is Better Than Sorbitol. **Journal of Veterinary Medical Science,** v.74, n.3, p.351-354, 2012.

CRABO, B. G.; DIAL, G. D. Artificial Insemination in Swine. **Veterinary Clinics of North America: Food Animal Practice,** v.8, n.3, p.533-544, 1992.

CURRY, M. R. Cryopreservation of semen from domestic livestock. **Reviews of reproduction,** v.5, n.1, p.46-52, 2000.

EVENSON, D.; JOST, L.; MARSHALL, D.; ZINAMAN, M.; CLEGG, E.; PURVIS, K.; DE ANGELIS, P.; CLAUSSEN, O. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. **Human reproduction,** v.14, n.4, p.1039-1049, 1999.

FAOSTAT. Live animals: Production of pigs. **Food and Agriculture Organization of the United Nations**, Rome, 2016. Available at: <http://www.fao.org/faostat/en/#data/QA/visualize>. Accessed: 22 April 2018.

FÁVERO, J. A.; DE FIGUEIREDO, E. A. P. Evolução do melhoramento genético de suínos no Brasil. **Ceres,** v.56, n.4, 2009.

FÁVERO, J. A.; DE FIGUEIREDO, E. A. P.; IRGANG, R.; COSTA, C. N.; SARALEGUI, W. H. L. Evolução da genética: do porco tipo banha ao suíno light. In: SOUZA, J. C. P. V. B.;TALAMINI, D. J. D.*, et al* (ed.). **Sonho, desafio e tecnologia: 35 anos de contribuições da Embrapa Suínos e Aves**: Embrapa Suínos e Aves, 2011. cap. 4, p.105-136.

FOXCROFT, G.; PATTERSON, J.; DYCK, M. Improving production efficiency in a competitive industry. In: Proceedings of the Manitoba Swine Seminar', Winnipeg, Canada, 2010, **Anais do Proceedings of the Manitoba Swine Seminar', Winnipeg, Canada**. 2010. p.81-98.

FOXCROFT, G. R.; PATTERSON, J.; CAMERON, A.; DYCK, M. K. Application of advanced AI technologies to improve the competitiveness of the pork industry. In: Proceedings of the 21st International Pig Veterinary Society Congress, Vancouver, Canada, 2010, **Anais do Proceedings of the 21st International Pig Veterinary Society Congress, Vancouver, Canada**. 2010. p.25.

GARNER, D. L.; THOMAS, C. A.; JOERG, H. W.; DEJARNETTE, J. M.; MARSHALL, C. E. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. **Biology of reproduction,** v.57, n.6, p.1401-1406, 1997.

GOSALVEZ, J.; TVRDA, E.; AGARWAL, A. Free radical and superoxide reactivity detection in semen quality assessment: past, present, and future. **Journal of Assisted Reproduction and Genetics,** v.34, n.6, p.697-707, 2017.

HARRISON, R. A.; VICKERS, S. E. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. **Journal of reproduction and fertility,** v.88, n.1, p.343-352, 1990.

HE, L.; BAILEY, J. L.; BUHR, M. M. Incorporating Lipids into Boar Sperm Decreases Chilling Sensitivity but Not Capacitation Potential. **Biology of reproduction**, n.64, p.69–79, 2001.

HOLT, W. V.; PENFOLD, L. M. Fundamental and Practical Aspects of Semen Cryopreservation. In: CHENOWETH, P. J. and LORTON, S. P. (ed.). **Animal andrology : theories and applications**. Oxfordshire, UK: CABI, 2014. cap. 5, p.76-99.

HOSTE, R.; SUH, H.; KORTSTEE, H. **Smart farming in pig production and greenhouse horticulture**. ed. Wageningen Economic Research, 2017. p. ISBN 9463432183.

HU, J.-H.; LI, Q.-W.; LI, G.; CHEN, X.-Y.; HAI-YANG, H.-Y.; ZHANG, S.-S.; WANG, L.-Q. The cryoprotective effect on frozen-thawed boar semen of egg yolk low density lipoproteins. **Asian-australasian journal of animal sciences,** v.19, n.4, p.486-494, 2006.

JUNG, M.; RUDIGER, K.; SCHULZE, M. In Vitro Measures for Assessing Boar Semen Fertility. **Reproduction in domestic animals,** v.50 Suppl 2, p.20-24, 2015.

KAWAMOTO, A.; OHASHI, K.; KISHIKAWA, H.; ZHU, L. Q.; AZUMA, C.; MURATA, Y. Two-color fluorescence staining of lectin and anti-CD46 antibody to assess acrosomal status. **Fertility and sterility,** v.71, n.3, p.497-501, 1999.

KENTISH, S.; WOOSTER, T.; ASHOKKUMAR, M.; BALACHANDRAN, S.; MAWSON, R.; SIMONS, L. The use of ultrasonics for nanoemulsion preparation. **Innovative Food Science & Emerging Technologies,** v.9, n.2, p.170-175, 2008.

KNOX, R.; LEVIS, D.; SAFRANSKI, T.; SINGLETON, W. An update on North American boar stud practices. **Theriogenology,** v.70, n.8, p.1202-1208, 2008.

KNOX, R. V. The Fertility of Frozen Boar Sperm When used for Artificial Insemination. **Reproduction in domestic animals,** v.50 Suppl 2, p.90-97, 2015.

_____. Artificial insemination in pigs today. **Theriogenology,** v.85, n.1, p.83-93, 2016.

LI, J.; ROCA, J.; PEREZ-PATINO, C.; BARRANCO, I.; MARTINEZ, E. A.; RODRIGUEZ-MARTINEZ, H.; PARRILLA, I. Is boar sperm freezability more intrinsically linked to spermatozoa than to the surrounding seminal plasma? **Animal Reproduction Science**, 2018.

MORRELL, J. M.; MAYER, I. Reproduction biotechnologies in germplasm banking of livestock species: a review. **Zygote,** v.25, n.5, p.545-557, 2017.

PILLET, E.; LABBE, C.; BATELLIER, F.; DUCHAMP, G.; BEAUMAL, V.; ANTON, M.; DESHERCES, S.; SCHMITT, E.; MAGISTRINI, M. Liposomes as an alternative to egg yolk in stallion freezing extender. **Theriogenology,** v.77, n.2, p.268-279, 2012.

PIÑEIRO, C.; MORALES, J.; RODRIGUEZ, M.; APARICIO, M.; MANZANILLA, E. G.; KOKETSU, Y. Big (pig) data and the internet of the swine things: a new paradigm in the industry. **Animal Frontiers,** v.9, n.2, p.6-15, 2019.

POND, W. G. Modern pork production. **Scientific American,** v.248, n.5, p.96-103, 1983.

PUISIEUX, F.; FATTAL, E.; LAHIANI, M.; AUGER, J.; JOUANNET, P.; COUVREUR, P.; DELATTRE, J. Liposomes, an interesting tool to deliver a bioenergetic substrate (ATP). in vitro and in vivo studies. **Journal of drug targeting,** v.2, n.5, p.443-448, 1994.

RODRIGUEZ-MARTINEZ, H. Sperm biotechnologies in domestic species: state of the art. **Animal Reproduction,** v.10, n.3, p.268-276, 2013.

RUTTEN, S. C.; MORRISON, R. B.; REICKS, D. Boar stud production analysis. **Journal of Swine Health and Production,** v.8, n.1, p.11-14, 2000.

SCHEID, I. Commercial swine artificial insemination in Brazil: development and current use. In: International Conference on Boar Semen Preservation, 1991, Berlin. **Anais do International Conference on Boar Semen Preservation**. Reproduction in domestic animals, 1991. p.299-301.

SEBRAE; ABCS. **Mapping of Brazilian Pork Chain**. ABCS, 2016. 184 p. Available at: <http://www.esalq.usp.br/biblioteca/pdf/Pork_Chain_in_Brazil_Cadeia_Suinos_no_Brasil_2016_Fava_Neves_et_al.pdf>. Accessed: 15 January 2019.

SIEME, H.; OLDENHOF, H. Cryopreservation of semen from domestic livestock. **Methods in Molecular Biology,** v.1257, p.277-287, 2015.

SINGLETON, W. State of the art in artificial insemination of pigs in the United States. **Theriogenology,** v.56, n.8, p.1305-1310, 2001.

TOSIC, J.; WALTON, A. Effect of egg-yolk and its constituents on the respiration and fertilizing capacity of spermatozoa. **The Journal of Agricultural Science,** v.37, n.1, p.69-76, 1947.

VARELA JUNIOR, A. S.; CORCINI, C. D.; ULGUIM, R. R.; ALVARENGA, M. V.; BIANCHI, I.; CORREA, M. N.; LUCIA, T., JR.; DESCHAMPS, J. C. Effect of low density lipoprotein on the quality of cryopreserved dog semen. **Animal Reproduction Science,** v.115, n.1-4, p.323-327, 2009.

WATSON, P.; MARTIN, I. The Influence of some Fractions of Egg Yolk on the Survival of Ram Spermatozoa at 5oC. **Australian Journal of Biological Sciences,** v.28, n.2, p.145-152, 1975.

WATSON, P. F. Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of their Post-thawing Function. **Reproduction, Fertility and Development,** v.7, n.4, p.871-891, 1995.

WETTEMANN, R.; BAZER, F. Influence of environmental temperature on prolificacy of pigs. **Journal of Reproduction and Fertility. Supplement,** v.33, p.199-208, 1985.

WOLFERT, S.; GE, L.; VERDOUW, C.; BOGAARDT, M.-J. Big data in smart farming– a review. **Agricultural Systems,** v.153, p.69-80, 2017.

WOODS, A. Rethinking the history of modern agriculture: British pig production, c.1910-65. **20 Century British History,** v.23, n.2, p.165-191, 2012.

YESTE, M. Recent Advances in Boar Sperm Cryopreservation: State of the Art and Current Perspectives. **Reproduction in domestic animals,** v.50 Suppl 2, p.71-79, 2015.

_____. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. **Theriogenology,** v.85, n.1, p.47-64, 2016.

ZHU, Z.; KAWAI, T.; UMEHARA, T.; HOQUE, S. A. M.; ZENG, W.; SHIMADA, M. Negative effects of ROS generated during linear sperm motility on gene expression and ATP generation in boar sperm mitochondria. **Free Radical Biology & Medicine,** v.141, p.159-171, 2019.