EDGAR ANDRES DIAZ MIRANDA

PREDICTORS OF SUCCESS IN THE BREEDING SOUNDNESS EVALUATION AND INFERTILITY IN BULLS

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: José Domingos Guimarães

Co-adviser: Mariana Machado Neves

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APPROVED: August 26th, 2022

Assent:

EDGAR DIAZ

Edgar Andres Diaz Miranda
Author

José Domingos Guimarães
Adviser

DEDICATION

I dedicate this work to:
My parents: Edgar and Olga,
My brothers: David and Mateo,
My beloved wife: Kayla,
My family and friends.
My grandfather: Fredy in loving memory.

There are no words for all you have done for me. Even living thousands of miles away, I felt every day closer to you. Thank you for your guidance and support and for encouraging me to be better daily. Thanks to my wife for being my partner and supporting me, living abroad together and in the distance, keeping me focused, and challenging me every day. I will also thank all the friends and family I made in living abroad in Brazil and in the United States; lots of friends became brothers, and Brazil became home. I embraced your culture, and it made me a better person.

Thank you God, for all your blessings and for putting such amazing people around me.
Holy Mary intercedes for us.

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ABSTRACT

DIAZ MIRANDA, Edgar Andres, D.Sc., Universidade Federal de Viçosa, August, 2022. **Predictors of success in the breeding soundness evaluation and infertility in bulls**. Adviser: José Domingos Guimarães. Co-adviser: Mariana Machado Neves

Bull fertility is critical for the profitability and sustainability of the cattle industry. To date, the Breeding soundness evaluation (BSE) is the best methodology to estimate the reproductive potential of future bulls. However, the guidelines for the BSE differ between practitioners and societies, perhaps due to a lack in research in this area. Therefore, our first study hypothesized that by the implementation and use of strict BSE guidelines the number of approved Nellore bulls and their reproductive quality would increase overtime. Furthermore, our second study hypothesized that bulls born early in the calving season are more likely to be approved in their first BSE when compared to males calved later. To evaluate these two hypotheses, records of 20 years and 46,566 BSE were used in two separate analyses including a multivariate logistic regression to measure the effect of the year and farm, and to predict the probability of approval in Nellore young bulls at their first BSE according to their dams calving date. Bulls were classified as approved (satisfactory potential breeders and qualified for natural breeding service) and not approved (deferred and unsatisfactory potential breeders). Overall, there was no improvement in the percentage of approved bulls. However, there was an increase in the number of bulls approved in the first BSE and an increase in the percentage of bulls classified as satisfactory potential breeders (P-value <0.05), which means that the quality of the bulls improved over time. On the other hand, it was demonstrated that the calving date affected the probability of approval at the first BSE. Hence, bulls born on day 0 of the calving season had 1.26 more chances to be approved at the first BSE than bulls born 21 days later. In our studies, 53% of the bulls were not approved due to poor sperm quality, specifically abnormal sperm morphology. Bull ejaculates with the aplastic midpiece defect (AMD) have poor motility and fertility and it's related to have a putative genetic origin. Therefore, our third study aimed to understand the origin and factors contributing to the abnormal sperm production of the AMD in Dairy Gyr bulls. Six Dairy Gyr Bulls (three control bulls and three bulls with AMD) obtained from the same farm and management were castrated to conduct a sperm morphofunctional and proteomic study and evaluate the redox state of reproductive tissues. The morphological analysis demonstrated that the AMD is a consequence of defective spermiogenesis. Control bulls also produced a high percentage of AMD but have an effective mechanism of removal of

the defective sperm prior to epididymal transit. This mechanism generates mild oxidative stress in the testis and caput of the epididymis (measured as MDA and CAT; P-value <0.05); however, the redox state was reestablished in the corpus and cauda of the epididymis. Furthermore, qualitative and quantitative proteomic analysis showed that the sperm maturation of the bulls with the AMD should be deficient once they had differences in protein expression and a poor enrichment of critical pathways for sperm fertility. Finally, our last study hypothesized that binder of sperm (BSP) proteins could be used as a potential biomarker of defective sperm. Therefore, we characterized the binding properties and abundance of the BSP proteins after discontinuous gradient centrifugation and found that; in the supernatant fractions, there was an increase of BSP proteins abundance when compared to the sperm pellet fractions (P- value <0.05). In the sperm pellet fractions, BSP1 and BSP3 bound predominately to the acrosomal region of the sperm head, whereas BSP5 had a high affinity for the midpiece. Furthermore, high BSP proteins abundance was found to be correlated with acrosome and membrane damage induced by permeabilization. Therefore, the abundance of BSP proteins could be used as biomarker of defective sperm or sperm with compromise function. Our results allowed us to complement the knowledge on bull fertility, from the basic but functionally BSE to biomarkers for defective sperm specially on *Bos indicus* cattle.

Keywords: Bull fertility. Sperm morphology. BSPs. Biomarkers.

RESUMO

DIAZ MIRANDA, Edgar Andres, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Preditores de sucesso no exame andrológico e infertilidade em touros**. Orientador: José Domingos Guimarães. Coorientadora: Mariana Machado Neves.

A fertilidade dos reprodutores é fundamental para a lucratividade e sustentabilidade da indústria pecuária. Até o momento, o exame andrológico é a melhor metodologia para estimar o potencial reprodutivo de futuros reprodutores. No entanto, as diretrizes para o exame andrológico diferem entre profissionais e sociedades, talvez devido à falta de pesquisas nessa área. Portanto, nosso primeiro estudo avaliou se, seguindo rigidamente as diretrizes para o exame andrológico, o número de touros Nelore aprovados e sua qualidade reprodutiva aumentariam ao longo do tempo. O segundo estudo avaliou se os touros nascidos no início da estação de parição são mais propensos a serem aprovados no exame andrológico quando comparados aos touros nascidos mais tarde. Para avaliar essas duas hipóteses, foram utilizados registros de 20 anos e 46.566 laudos em duas análises separadas incluindo uma regressão logística multivariada para medir o efeito do ano e da fazenda, e para estimar a probabilidade de aprovação de acordo a data de nascimento na estação de parição em tourinhos Nelore no primeiro exame andrológico. Os touros foram classificados como aprovados (aptos para reprodução e aptos para monta natural) e não aprovados (inapto temporário e inaptos). No geral, não houve melhora no percentual de touros aprovados. No entanto, houve um aumento no número de touros aprovados no primeiro exame andrológico e um aumento na porcentagem de touros classificados como aptos para reprodução ($P < 0,05$), o que significa que a qualidade dos touros melhorou ao longo do tempo. Por outro lado, foi demonstrado que a data do nascimento na estação de parição afetou a probabilidade de aprovação no primeiro exame andrológico. Assim, touros nascidos no dia 0 da estação de parição tiveram 1,26 mais chances de serem aprovados no primeiro exame andrológico do que touros nascidos 21 dias depois. Em nossos estudos, 53% dos touros não são aprovados devido à má qualidade espermática, especificamente pela morfologia espermática. Ejaculados de touro com defeito de peça intermediária aplástica (PIA) têm baixa motilidade e fertilidade e podem ter origem genética. Portanto, nosso terceiro estudo teve como objetivo compreender a origem e os fatores que contribuem para a produção anormal de espermatozoides com PIA em touros Gir Leiteiro. Seis touros Gir Leiteiro (três touros controle e três touros com PIA) obtidos da mesma fazenda e manejo foram castrados para realizar um estudo morfofuncional e proteômico dos espermatozoides e avaliar o estresse oxidativo do trato

reprodutivo. A análise morfológica demonstrou que a PIA é consequência duma espermiogênese imperfeita, também demostrou que os touros controle produziram uma alta porcentagem de PIA, porém possuem um mecanismo de remoção dos espermatozoides defeituosos antes do trânsito epididimario. Esse mecanismo gera um estresse oxidativo leve no testículo e na cabeça do epidídimo (medido em MDA e CAT; $P < 0,05$); entretanto, o estresse é controlado no corpo e cauda do epidídimo. Além disso, a análise proteômica qualitativa e quantitativa mostrou que a maturação espermática dos touros com PIA deve ser deficiente, uma vez que apresentam diferenças na expressão de proteínas e baixa expressão de proteínas que participam em vias importantes para a fertilidade espermática. Finalmente, no nosso último estudo levantou se a hipótese de que as proteínas ligantes de espermatozoides (BSPs Binder of sperm proteins) poderiam ser usadas como um potencial biomarcador de espermatozoides defeituosos. Portanto, caracterizou se as propriedades de ligação e abundância das proteínas BSP após centrifugação em gradiente de densidade e verificou se que na fração sobrenadante, houve um aumento da abundância de proteínas BSP no espermatozoide quando comparadas à fração do pellet ($P < 0,05$). Nos espermatozoides obtidos dos pellets, BSP1 e BSP3 ligaram-se predominantemente no acrossoma, enquanto BSP5 apresentou alta afinidade pela peça intermediária. Além disso, a maior abundância das proteínas BSP foi correlacionada com danos no acrossoma e na membrana induzidos pela permeabilização do sêmen. Portanto, a abundância de proteínas BSP poderia ser utilizada como biomarcador de espermatozoides defeituosos ou com função comprometida. Nossos resultados nos permitiram complementar o conhecimento sobre fertilidade de touros, desde o exame andrológico até biomarcadores para espermatozoides defeituosos, especialmente em bovinos *Bos indicus*.

Palavras-chave: Fertilidade em touros. Morfologia espermática. BSPs. Biomarcadores.

SUMMARY

LITERATURE REVIEW

"Bull fertility: Breeding Soundness evaluation and sperm morphology analysis"

Meat consumption is projected to increase by 12% by 2029 compared to 2020 (OECD, 2020). Meeting this demand while remaining sustainable continues to be a challenge; the agriculture industry today strives to reduce the impact of greenhouse gases (global warming), biodiversity loss, water stress, and water pollution (Basso and Antle, 2020). Aligning with these goals, the cattle industry is dedicating considerable efforts to improving animal welfare, productivity, and efficiency (Brito et al., 2021). Fertility and bull fertility in particular, is critical for efficient cattle production, because bulls have a greater potential for spreading both reproductively favorable and deleterious gene variants through the herd than females. Nevertheless, bull fertility is often overlooked. Indeed, reproductive traits are considered economically more important than production traits in commercial operations (Trenkle and Willham, 1977). It is well known that using bulls with good semen quality can improve pregnancy rates by up to 6% (Wiltbank, 1983), and by improving only 1% from 86% calf crop, with 30 million beef cows in the US it would result in 200,000 more calves, or 340,000 fewer cows to obtain the same number of calves (Trenkle and Willham, 1977). Therefore, selection for bull fertility is key to improving the sustainability and profitability of the cattle industry.

In this context, determining a bull's fertility prior to using that bull for artificial insemination (AI) or natural breeding service (NS) is vital. However, to date, there is no precise procedure to estimate bull fertility, perhaps because bull fertility is a complex trait influenced by numerous factors. Therefore, the current best practice to assess the reproductive potential of a bull is the breeding soundness evaluation (BSE). The BSE consists of an evaluation of breeding behavior, a physical examination which includes an examination of the scrotum and its contents, the accessory sex glands, the penis, and the prepuce, as well as a semen evaluation.

Unfortunately, in the United States, only 27% of operations test their bull's semen, and only 16% performed scrotal measurements. Therefore, it is expected that a lower percentage of operations performed a complete BSE in their bulls (NAHMS, 2009), meaning that producers could be using bulls with high risks of subfertility or worse, infertility.

The use of BSE begun in the US in 1950, when the Rocky Mountain Society for the Study of Fertility in Bulls (RMSSFB) was formed with the goal of sharing, disseminating and standardizing the essential procedures for evaluation of bull fertility (Chenoweth et al., 2002). This exam is not just to diagnose infertility; it aims to determine the reproductive potential of the bulls and to estimate if a bull can be efficient during a breeding season. A bull which successfully passed the BSE should be able to have 60% of its cow herd pregnant at the first 21 days and have a 95% conception rate after 60 days of the breeding season in an optimal bull to female ratio (BFR) (Barth, 2018). However, as mentioned before, the BSE is not designed to predict the precise fertility of individual bulls.

Fertility variation is expected in the bulls approved by the BSE (Kastelic and Thundathil, 2014). This variation averages a 25% difference in pregnancy rates by AI in dairy cattle. However, this is entirely different than NS. In an AI breeding system, a low dose of cryopreserved semen is deposited in the uterine body of a cow. Although the intended outcome of AI and NS is the same (pregnancy and a live calf birth), differences in bull fertility are observed between the AI and NS. Differences in bull fertility may also be influenced by different breeding systems; indeed, many practitioners and societies have different guidelines for bull BSE dependent on their breeding system. This is further influenced by whether the system relies on NS, AI, or both. Thus, different bull BSE guidelines could represent another variation in the fertility of the bulls approved by the BSE. This means that more research is needed in this area. For instance, the Brazilian College of Animal Reproduction (CBRA) guidelines for the semen analyses stipulate ≥60% progressive motility; ≤ 30% total sperm

defects; ≤10% major sperm defects; ≤20% minor sperm defects; ≤5% major individual defects; ≤10% minor individual defects, whereas the Society for Theriogenology (SFT) thresholds for sperm motility and normal sperm morphology were established as 30% and 70%, respectively (CBRA, 2013; Koziol and Armstrong, 2018).

Although the use of AI protocols has grown exponentially in the last decade, natural breeding service is still the predominant method for breeding in beef cattle production. Therefore, the assessment of the reproductive potential of bulls cannot be forgotten. The efficiency of herds can be improved by selecting bulls with better reproductive fittest and traits which strategically improve the herd via their offspring. The BSE should be objective and standardized. Furthermore, risk factors that affect bull fertility vary between breeds and are influenced by the environment. This literature review aims to discuss the importance and principal factors that affect or represent a risk to bull fertility evaluated by the BSE, with a special emphasis on the sperm morphology.

Genetic relationships with reproductive traits

Bull fertility is difficult to measured and has a low to moderate heritability (For review Butler et al., 2020). However, other traits such as the scrotal circumference (SC) have better estimates. Indeed, an immense gain in bull fertility has been made by selection based on SC, due to its high correlation with puberty, sexual maturity, and semen quality (CHANDLER et al 1985). Low SC is also one of the main reasons for bulls to fail a BSE. However, by selecting larger testis, the average SC has increased over time (Eriksson et al., 2012). Therefore, selection for this bull fertility marker can be done by following the guidelines of the BSE. Studies also have shown that bulls with larger SC can improve the conception rates in herds (Waldner et al., 2010). Furthermore, the SC has a negative and reproductively favorable genetic correlation with age of puberty in the female progeny (Vargas et al., 1998).

In this context, it is important to mention that cow and bull fertility are favorably genetically correlated; therefore, cow fertility could be genetically improved by indirect selection on bull fertility (Mackinnon et al., 1990). Indeed, a recent study showed that the best way to select for calving date was through the father side, instead of the mother side in order to increase the production of Brahman heifers (Earnhardt et al 2021). Calving date is the day within the calving season in which the heifer or cow calved (Cammack et al., 2009); it is one of the key reproductive traits in beef genetic programs because its high heritability and has an economic impact (Bourdon and Brinks, 1983).

It is well known that cows that calve early in the breeding season produce calves with higher weaning weights and pre-breeding heifer weights, which increases the chances of the heifers reaching precocious puberty and cyclicity, thus becoming pregnant and calving earlier (Patterson et al., 1992; MacGregor and Casey, 2000; Nafziger et al., 2021). Furthermore, it has been shown that early calving also increases the chances for bulls to be approved at their first BSE (Diaz Miranda et al., Manuscript in preparation). Therefore, selecting for calving date is a promising strategy to improve herd fertility.

Regarding semen quality, sperm motility is suggested to have low to moderate heritability, and the sperm defects have been reported to have a moderate heritability (Smith et al., 1989; Christmas et al., 2001; Garmyn et al., 2011). Semen quality has low heritability because is a polygenic trait and its highly influenced by the environment. Therefore, selection for semen quality is possible but slow, and a better strategy to improve these traits could be with genomic selection (Druet et al., 2009; Yin et al., 2019). To date, several candidate genes have been linked to bull fertility (Feugang et al., 2009; Blaschek et al., 2011; Li et al., 2012; Han and Peñagaricano, 2016), SC and early puberty (Fortes et al., 2013; Utsunomiya et al., 2014; Soares et al., 2017), sperm motility (Hering et al., 2014), and sperm concentration (Hering et al., 2014b). Recently, new polymorphisms have been associated with sperm

abnormalities (Sironen et al., 2010; Hiltpold et al., 2022; Nogueira et al., 2022). The implementation and validation of such markers will lead to faster genetic gain for bull fertility.

Risk factors that affect bull fertility

Multiple factors affect the ability of a bull to pass a BSE. The reasons for the failing the BSE includes small SC, unacceptable sperm morphology, low sperm motility, poor libido, and physical defects. Such reasons can be grouped as conditions that affect the desire or mating ability (*impotentia coeundi*) and/or the ability of spermatozoa to fertilize the oocyte (*impotentia generandi*) (Van Camp, 1997). These conditions are influenced by environmental factors including nutrition, age, herd management, weather, and genetic factors (Mathevon et al., 1998; Barth, 2013). The most common physical defects are poor body score condition, obesity, poor conformation of the musculoskeletal system and abnormalities of the eyes, feet, legs, the external genitalia and the accessory glands (Figure 1). However, most of these problems are easily diagnosed by herd managers, and these bulls frequently do not get a chance to be evaluated by veterinarians.

Figure 1. Common physical defects in bulls at the BSE. (A) Eye injuries (neoplasia), (B) Scrotal injuries (myiasis), musculoskeletal conditions ((C) feet injuries, (D) hygroma), preputial and umbilical conditions ((E) posthitis, (F) omphalitis, (G) phimosis).

On the other hand, semen quality is the primary reason for bull subfertility and infertility (Menon et al., 2011; Carson et al., 2014). Among the semen parameters evaluated by the BSE, sperm morphology is associated with conception rates (Fitzpatrick et al., 2002; Al-Makhzoomi et al. 2008). However, this sperm parameter is frequently neglected in the field even though there is a growing list of sperm defects considered to have a putative genetic origin, as well as new polymorphism related to such sperm defects (Chenoweth, 2005; Taylor et al., 2018). Due to the importance of this semen feature and the differences in the thresholds for an acceptable sperm morphology between societies and practitioners, an extended discussion will be dedicated to this topic in this review, including new approaches as sperm morphology/quality biomarkers.

Another critical test not routinely included in the BSE is the assessment of bull libido and mating ability. Libido is defined as the willingness and eagerness of a male animal to mount and attempt the service of a female. In contrast, mating behavior is the behavior of the male animal in the periods immediately before, during, and after service (Chenoweth et al., 1981). Assessing both behaviors is very important for the estimation of BFR. Non-selected bulls are estimated to have an optimal BFR of 1:24 to 1:30. However, this BFR does not represent a real challenge for sires with high libido. These high libido sires could increase that ratio and thus decrease the need for more bulls in an operation. It is worth mentioning that there are differences between breeds (Chenoweth et al., 1996). Libido tests employed for *Bos indicus* have been shown to be unsatisfactory in the identification of the different libido scores, because when it is done for longer periods of time, there is an increase of up to 30% of bulls approved in the test (Guimarães et al., 2011). Furthermore, another behavior to measure is the social ranking of bulls when a multiple-sire mating herds is used, because the social ranking within groups can influence their sexual activity (Petherick, 2005).

Abnormalities of the feet, legs, and back can detract from a bull's ability or desire to copulate (Van Camp, 1997; Wolfe, 2018), and it can also affect semen quality. A recent study showed that the odds of having a good semen quality were 4.4 times greater in not-lame bulls when compared with lame bulls (Boakari et al., 2022). A postmortem study of infertile bulls showed that 90% of them had joint lesions without symptoms of lameness, suggesting that physical defects should be considered as a contributing cause of reproductive failure in sires (Persson et al., 2007).

Production of an acceptable ejaculate depends on the normal development of the reproductive tract, including normal spermatogenesis, spermatozoa maturation and transport, normal accessory genital gland function, and nervous, musculoskeletal, and psychological factors (Foster et al., 2016). All major and minor congenital disorders are collectively called disorders of sexual development (DSD). There are several DSDs manifesting in the testis (For review McEntee, 2012). Careful examination of the gonads is required in the BSE of virgin bulls because any disorder in the testes directly affects bull fertility, as well as in the scrotum and spermatic cord, which can develop testicular degeneration affecting semen quality.

The testes should be symmetrical, mobile and without signs of pain upon palpation, the consistency must be fibro-elastic and may vary from very soft to very hard on a scale of 1 to 5. Any asymmetry, change in testicular conformation or consistency may indicate a pathological process (Roberts, 1986). Soft testes with low resilience are associated with high percentage of abnormal spermatozoa and low reproductive performance (Müller et al., 1992). Very hard testes can also be an indication that testicular degeneration is occurring from perhaps an inflammatory process (injury or infection) (Coulter et al., 1975). Among the testicular problems in young bulls, we can highlight testicular degeneration, hypoplasia, rotation, fibrosis, adhesion, cryptorchidism, orchitis and hydrocele (Figure 2). The use of ultrasonography is useful to

diagnose certain testicular problems, however, it does not necessarily reveal testis that have lost germinal epithelium (Barth and Kastelic, 2021) (Figure 3).

Figure 2. Common testicular problems in bulls at the BSE. (A) Hydrocele, (B) testicular fibrosis, (C) varicocoele, (D) orchitis.

The most common acquired condition that affects bull fertility is testicular degeneration, in which normal testicles undergo pathologic changes, eventually resulting eventually in small testicular size, abnormal function and poor sperm quality (usually <20% morphologically normal sperm) (Hopkins, 2007). It can be caused by several environmental factors, including heat stress and scrotal frostbite, by obesity when there is an excessive accumulation of scrotal fat, by fever, trauma or senility, in addition to pharmacologically induced (Barth and Kastelic, 2021). Testicular degeneration may be temporary or permanent; bulls with this condition should not be culled immediately because after removal of underlying cause, testis structure and function often recover. Such bulls should be reevaluated two months after diagnosis, the amount of time necessary for completing the cycle of the seminiferous epithelium and epididymal transit. However, advanced chronic testicular degeneration is manifested clinically and grossly as atrophy, mineralization, and fibrosis with permanent damage (Foster et al., 2016).

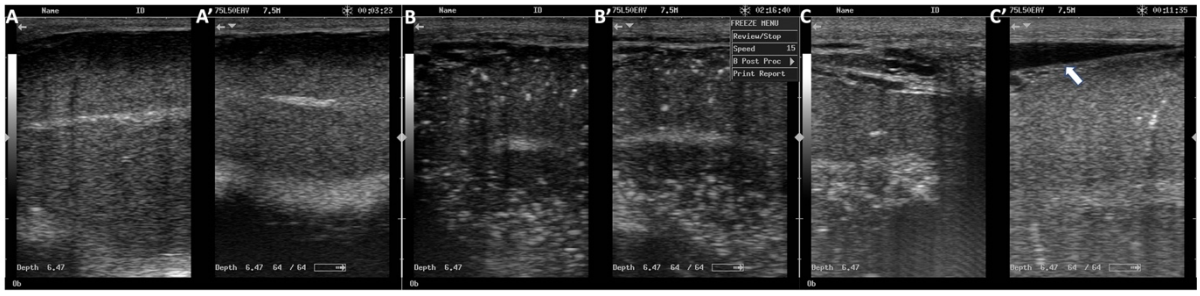

Figure 3. Ultrasonographic imaging of testicular problems at the BSE. (A) normal testis, (A') contralateral asymmetric testis, (B and B') fibrotic foci in testes, (C) Contralateral testis (C') to hydrocele (arrow).

The most common congenital condition that affect young bulls is testicular hypoplasia (TH), widely recognized as small testes. Studies report an incidence of 5 to 15% in Brazilian cattle herds (Vale Filho, 1988; Goiozo et al., 2003); it has been described in at least 12 breeds and appears to be more common in highly inbred cattle population (Steffen, 1997). The DSD is considered to be hereditary and caused by an autosomal recessive gene with incomplete penetrance that causes failures in migration and mitotic divisions of primordial germ cells (Venhoranta et al., 2013). Testicular hypoplasia can be unilateral or bilateral, and total or partial. In partial hypoplasia, some seminiferous tubules will have germ cells even in the affected testes. Bulls with unilateral or partial TH can be fertile and go unnoticed unless they are sexually challenged with a high density of females or serial semen collections. Testicular hypoplasia affects bull fertility by reducing sperm concentration, the number of fertile ejaculates and the percentage of spermatozoa with normal morphology, sometimes affecting sperm motility as well. Therefore, affected animals may be subfertile or infertile (Figure 4) (Hopkins, 2007).

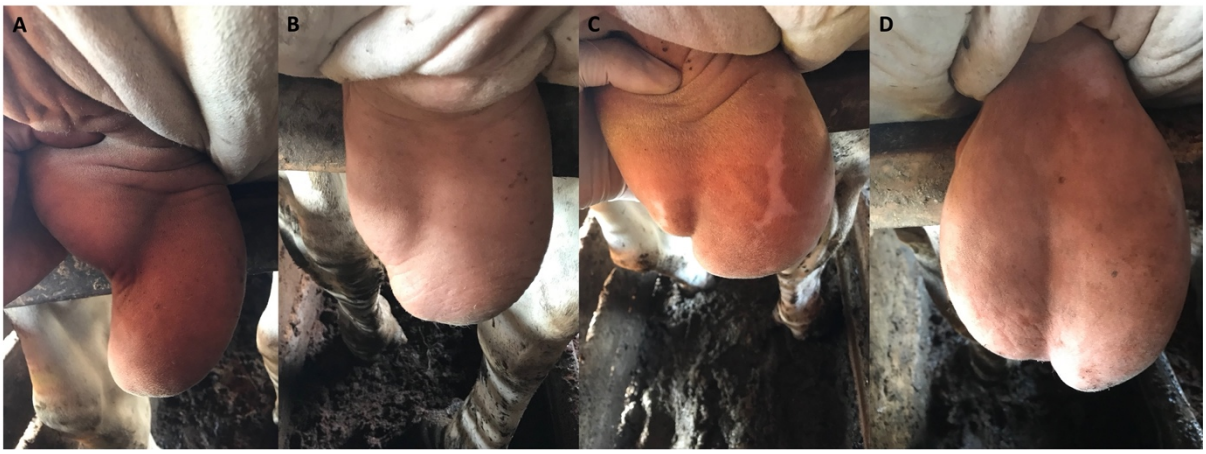

Figure 4. Different degrees of testicular asymmetry. (A) Testicular asymmetry due to a shortened spermatic cord (B) large, (C) medium and (D) small testicular asymmetry.

In the TH, degenerative lesions seem to occur more frequently in the dorsal portion of the testes. The confirmatory method is histological, and it is necessary to examine the tissue in at least three parts of each testicle (McEntee, 1990). The DSD is characterized by seminiferous tubules surrounded by a thickened and hyalinized basement membrane, lined with Sertoli cells devoid of the germinal epithelium; therefore, the tubule diameter is reduced. In the field, the SFT recommends culling bulls with SC measurements below 30 cm in post-pubertal *Bos taurus* bulls; this measurement is the suggested threshold for a bull to be diagnosed with testicular hypoplasia (Veeramachaneni et al., 1986; Hopkins, 2007), and with more than 25% of a size difference between testes (Chenoweth, 2015). Practitioners subjectively evaluate the difference; therefore, the CBRA recommends individual measures of the testes with a caliper. A difference of more than 1cm in the testicular width or length or above 10% of the testicular volume is diagnosed as testicular asymmetry, and the bull should be culled (CBRA, 2013). It is worth mentioning that the SC varies between breeds, especially among *Bos taurus* and *Bos indicus* bulls. Therefore, the diagnosis of small testes should be done very carefully.

Epididymal diseases are infrequent, especially genetic diseases such as segmental aplasia, because of intense natural and management selection pressure (Steffen, 1997).

However, epididymitis may be present after chronic seminal vesiculitis or orchitis (Figure 5). Furthermore, it could also be caused by pathogen-derived diseases such as brucellosis (Foster et al., 2016). Epididymitis causes infertility through poor motility, low percentage of morphologically normal sperm cells in the ejaculate, excessive white blood cells, or evidence of pus in the semen (Van camp, 1997).

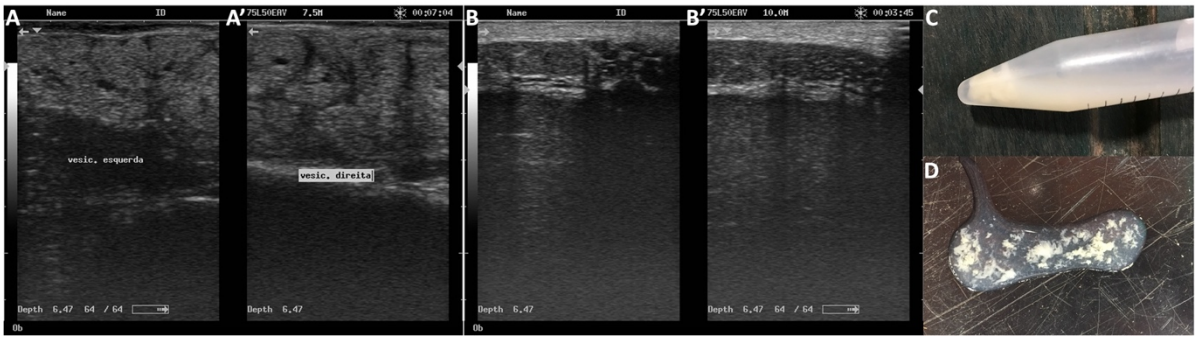

Figure 5. Ultrasonographic images of seminal vesiculitis, epididymitis and the presence of pus in the semen. (A) Normal vesicle and its counterpart with (A') seminal vesiculitis, (B) normal epididymal caput and corpus and its counterpart displaying (B') obstruction due to epididymitis. (C, D) presence of pus in the semen.

Among the accessory sex glands examination, seminal vesiculitis remain as one of the major reasons for culling bulls (Spitzer et al., 1988). It is diagnosed by rectal palpation, usually characterized by the detection of a loss of the typical lobulated architecture of one or both vesicular glands, an increase in glandular firmness and size, and in some cases the expression of pain on palpation of the glands. This disease has a prevalence of up to 10%, and close confinement and the feeding of high-energy diets appear to be associated with a higher prevalence of the disease (Cavalieri and Van Camp, 1997). Bacteria, viruses, chlamydia, fungi, and protozoa are the most common causes. Treatment includes using appropriate antibiotics; however, the outcome is often disappointing, and culling the affected bulls is probably the most cost-effective approach (Hopkins, 2007). Similar to epididymitis, semen from bulls with

seminal vesiculitis had poor sperm motility, excessive white blood cells, or evidence of pus in the semen (Hall and McEntee, 1981).

Common penile problems resulting in culling of bulls are fibropapillomas and the persistent penile frenulum (Bruner et al., 1995). Fibropapillomas in bulls are caused by bovine papillomavirus 1; they occur on the glans of the penis and are most common in young bulls. It could be noticed after mating when hemorrhages occur (Foster et al., 2016). Bulls should be culled because fibropapillomas are transmissible; if large, they may cause phimosis or paraphimosis, cause pain, and prevent copulation. On the other hand, a persistent penile frenulum is a congenital band of tissue extending from the median raphe of the prepuce to the ventral side of the penis near the glans (Van camp, 1997). Surgical correction is straightforward and fast. However, it is not suggested because a study showed that this condition could be transmissible as a recessive gene disorder (Elmore et al., 1978). There is less common congenital condition in bulls such as the micropenis, which is also related to be hereditary (Gilbert, 1989).

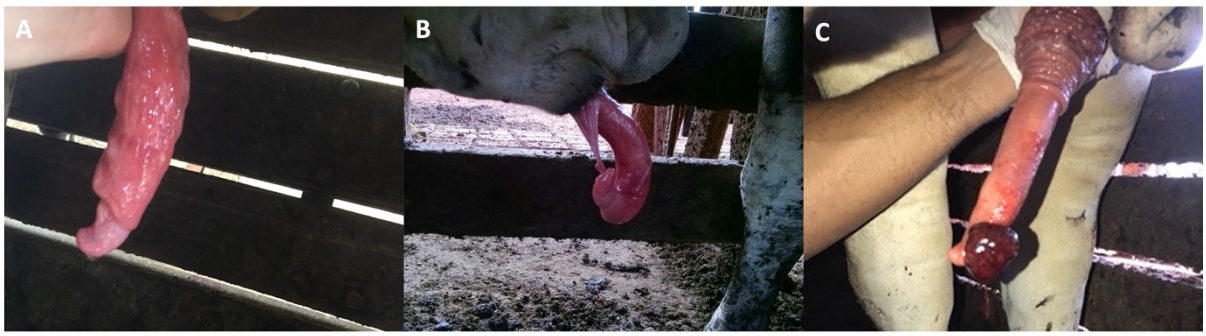

Figure 6. Common penile problems. (A) Prepubertal penile adherence, (B) persistent penile frenulum, and (C) penile fibropapilloma.

The risk of contracting venereal diseases by bulls is high, and some pathogens could survive cryopreservation and be transmissible via AI (Fore review: Givens and Marley, 2008; Thibier and Guerin, 2000). Careful attention should be given to health and management of bulls prior to breeding season (Engelken, 2008) in order to minimize the potential transmission of

these diseases into the herds. Most pathogens affect the health and semen quality of the bulls, and jeopardize the profitability of the producers by causing failure of cows to conceive and loss of pregnancies through abortion (BonDurant, 2005).

The risk factors affecting bull fertility vary according to the environment (Chacón et al., 1999; Higdon et al., 2000; Kennedy et al., 2002). Nevertheless, between 10 to 40% of the bulls fail the BSE (Barth, 2018; Bruner et al., 1995; Carrol et al., 1963; Carson and Wenzel, 1997; Elmore et al., 1975; Hoflack et al., 2006; Menegassi et al., 2012, Waldner et al., 2010), which makes the use of the BSE prior breeding season critical, especially in virgin bulls. For example, it was shown that using the BSE in Brazil can increase calf production by 31%; in addition, there is an increase of 13.8 additional calves during the lifespan of the bull and 24 kg more calves per year for cows (Menegassi et al., 2011).

Common sperm morphologic abnormalities in the bulls

The main purpose of examining sperm morphology is to determine the percentage and types of sperm abnormalities present in a semen sample, and to construct the sperm picture or spermiogram. The spermiogram is used to predict the fertility potential of the semen produced at the time of andrological examination or ejaculate collection, as well as to determine its etiopathology and treatment procedures when applicable (Barth, 2013). Among the possible causes, genetic, environmental, or a combination of both can be highlighted (Chenoweth, 2005). It is worth mentioning that the most common causes of abnormal spermatogenesis are environmental, including heat stress, pharmacological, and nutritional-toxicological (Barth and Oko, 1989).

The mammalian spermatozoon is divided into the acrosome, head (nucleus), connecting piece, midpiece, principal, and end piece (Figure 7). Abnormal sperm morphology has been classified over the years in different ways: into primary (testicular) or secondary (epididymal) defects according to the site of origin; into major and minors defects according to their

importance with fertility; into compensable or non-compensable according to their relationship of fertility with sperm number per dose at AI; into systematic or non-systematic according to the distribution in homogeneous or heterogeneous presentation of a sperm defect; and into head, midpiece and tail defects according to the location of the defect (Blom, 1950; Blom, 1973; Chemes and Rawe, 2003; Saacke, 2008; Söderquist et al., 1991).

Figure 7. Epifluorescence microscopy of bull sperm. Nuclear DNA was counterstained with DAPI, acrosome with PNA, and midpiece with ProteoStat aggresome probe.

Blom (1973) divided the common bull abnormal sperm defects into acrosome defects, underdeveloped, teratoid forms, coiled around the head, pouch formation, pyriform sperm, narrow at the base, abnormal color, abnormal contour, small abnormal heads, free abnormal heads, midpiece defects, strongly coiled tail, distal midpiece reflex, narrow heads, giant and short broad heads, free normal heads, abaxial tail implantation, proximal and distal cytoplasmatic droplet, bent tail, and coiled end piece. In addition, it mentioned other type of cells that can be found in the ejaculate such as medusa formation, spermatogenic, giant, desquamation, leucocytes, erythrocytes, bacteria, and pus cells. On the other hand, the SFT recognize 24 morphological abnormalities in bull semen.

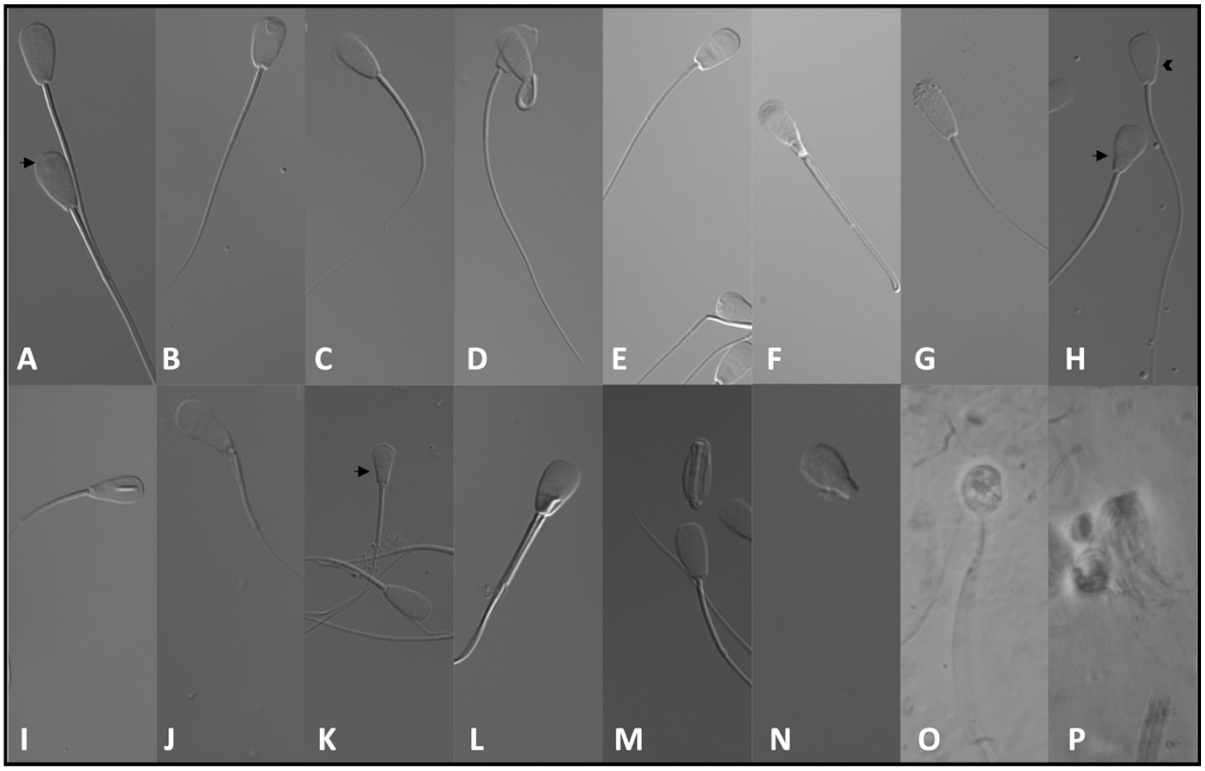

Figure 8. Examples of sperm head abnormalities and other sperm deviations. (A) Acrosome vacuoles, (B) flattening/inward knobbed, (C) swollen acrosome, (D) outward knobbed, (E) pale center, (F) narrow at the base, (G) tapered sperm head, (H) pyriform (arrow) and diadem (arrowhead), (I) nuclear crest, (J) abnormal contour, (K) microcephalic, (L) macrocephalic and multiple tails, (M) teratoid, (N) free abnormal head, (O) spermatogenic, (P) medusa formation.

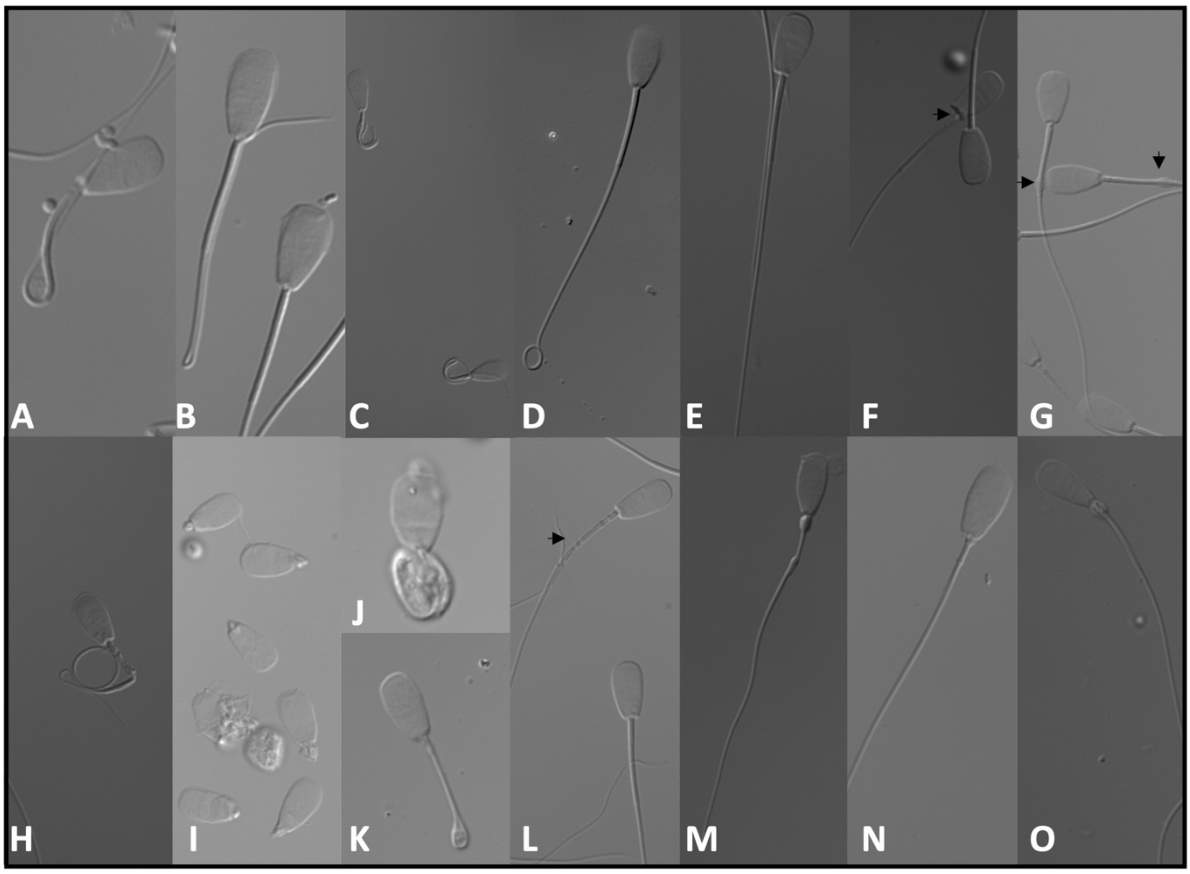

Figure 9. Sperm flagellum abnormalities. (A) Distal midpiece reflex, (B) bent tail, (C) strongly coiled sperm, (D) coiled end piece sperm, (E) abaxial tail, (F) abaxial tail with secondary implantation fossa, (G) distal droplets, (H) fractures, (I) stump tail, (J, K) Dag defect, (L) segmental aplasia of the mitochondrial sheath, (M, N) aplastic midpiece defect, and (O) proximal droplets.

The most common sperm acrosomal defect is the knobbed sperm defect. Is associated with sterility and subfertility in bulls. Donald and Hancock (1953) suggested that this defect was due to an autosomal sex-linked recessive mode of transmission in Friesian bulls, and genomic studies have found potential polymorphisms and locus related to knobbed sperm defect (Sironen et al., 2010; Nogueira et al., 2022). The knobbed defect can be described as a thickened acrosomal apex/ridge (outward) or an indented sperm apex (inward) (Thundathil et al., 2002); it is thought to be a result of the excess and folding of the acrosomal matrix over the

apex of the sperm head (Barth and Oko, 1989). The knobbed acrosome usually comprises two lobes bound by the outer acrosomal membrane and surrounded by the plasma membrane, the lobes can fuse, forming a cyst (Blom and Birch-Andersen, 1962; Cran and Dott, 1976). Bull ejaculates with high percentages of knobbed spermatozoa are rare (around 0.57%); however, is common to find knobbed spermatozoa in conjunction with other sperm defects (Barth, 1986). Knobbed sperm can also be found with other acrosomal defects including ruffled, swollen, and incomplete acrosomes (Saacke et al., 1968). It also appears that the outward knobbed have a major effect on bull fertility than the inward form (Andersson et al 1990; Meyer and Barth, 2001).

Knobbed spermatozoa were considered a compensable defect because of the reduced ability of the affected spermatozoa to bind the zona pellucida (Buttle and Hancock, 1965), presumably due to impaired plasma membrane function that predisposes them to premature capacitation and spontaneous acrosomal exocytosis (Thundathil et al 2002). However, in vitro studies have shown that oocytes penetrated by the apparently normal spermatozoa coexisting with the knobbed ones had a reduced cleavage rate and embryo development to the blastocyst stage, suggesting that this defect may be non-compensable (Thundathil et al., 2000). Furthermore, field studies showed a reduced fertility after timed AI of bulls with knobbed defect when compared to normal bulls (Nogueira et al., 2022).

Based on the sperm head shape, the pyriform, narrow at the base, and tapered sperm heads are the most common sperm defects. It is important to note that the nucleus, shaped by caudal manchette, histone-protamine swap and perinuclear theca deposition during spermatid elongation, primarily dictates the contour of the sperm head. The pyriform, pear-shaped head defect is characterized by a fully developed and round acrosomal region and a narrow equatorial and post-acrosomal region (Barth and Oko, 1989). On the other hand, in tapered heads, both acrosomal and post-acrosomal regions are narrow. When the predominant defect in the

ejaculate are the pyriform or tapered sperm heads, it is common to find both defects together; therefore, both head shape problems may have the same origin. Although a decision between normal and abnormal sperm head shape morphology may involve a degree of subjectivity, only spermatozoa with extreme narrowness of the post-acrosomal region are likely to have a reduced fertilizing potential (Barth et al., 1992). On the other hand, objective measures of the head shape morphology using Fourier harmonic amplitudes showed that it is possible to detect low fertility bulls with high accuracy. In addition, the most important measurement was not the harmonic amplitude means but the variances of the harmonic amplitudes (Parrish et al., 1998), meaning that variation in nuclear shape is more important than the mean sperm nuclear shape; even though bulls differ in the mean nuclear shape (Ostermeier et al., 2001b). Some differences observed in sperm nuclear shape may be explained by varying levels of chromatin stability (Ostermeier et al., 2001).

The development of abnormal nuclear shape could have both a genetic and an environmental origin/cause. However, the most common cause may be environmental; pyriform sperm is the pathognomonic sperm defect associated with heat stress or any disturbance with the thermoregulation of the testes (Shojaei Saadi et al., 2013). Abnormal head shape appears to have both a compensable and a non-compensable form because spermatozoa with severely misshapen heads do not reach the oocyte due to impaired hydrodynamics or because they are already dead at the time of sperm deposition. However, spermatozoa with subtle deviations in shape or normal shape but nuclear vacuoles can access the oocyte and probably compete for fertilization, generating low-quality embryos and becoming a non-compensable factor (Saacke et al., 1998). Lately, in vitro studies have confirmed that oocytes fertilized with pyriform spermatozoa produce fewer zygotes, with a lesser competence to cleave (Thundathil et al., 1999). Pale centers have been described as a narrowing of the sperm head in the post acrosomal sheath region and reported to decrease bull fertility. It was also suggested

that pale center spermatozoa usually appear in conjunction with vacuole abnormalities (Perry, 2021).

Nuclear vacuoles, pouch formation or diadem/crater defects are other common sperm abnormalities in bulls. These defects can be a transient phenomenon and the fertility could be normal following full or partial recovery (Larsen and Chenoweth, 1990). However, when presented in a high percentage, the fertility is severely affected, particularly due to embryonic mortality (Miller et al., 1982). In vitro studies have shown that the spermatozoa bearing nuclear vacuoles are defective in their zona binding (Thundathil et al., 1998), low fertilization rates and poor development of morulae and blastocysts (Esser et al., 2010). The hypothesis for the formation of nuclear craters in sperm heads implicates either the abnormal chromatin condensation or abnormal nuclear envelope assembly (nuclear invaginations). Even though nuclear vacuoles are generally caused by environmental conditions, hereditary origins must not be discarded. Among environmental conditions, nuclear vacuoles can be caused by elevated testicular temperature (Vogler et al., 1993) or frostbite (Heath and Ott, 1982), as well as dexamethasone treatment (Barth and Bowman, 1994). The incidence of bulls affected with nuclear vacuoles varies from 3 to 60% (Barth and Oko, 1989; Coulter et al., 1978).

Other head sperm defects in bulls include detached or decapitated spermatozoa (Settergren and Nicander, 1968; Blom and Birch-Andersen, 1970), macrocephaly or giant heads (Revay et al., 2009), rolled and crested heads (Blom, 1980; Cran et al., 1982), and teratogenic forms (Kopp et al., 2007). Teratogenic forms and macrocephaly head defects are generally caused by diploid spermatozoa and are very rare in cattle (Salisbury and Baker, 1966). The abnormal cells are frequently dead, and ejaculates with a high percentage of these defects generally do not improve; therefore, a genetic origin is suspected. The effect on fertility, as in most sperm abnormalities, depends on the number of cells affected, and it can vary from sterility to normal fertility (Barth and Oko, 1989).

Low numbers of detached heads are common in the semen of normal bulls. Higher percentages have been associated with testicular hypoplasia, testicular degeneration, lameness, nutrition, and sexual rest and idiopathic (Barth and Oko, 1989; Cooper and Peet, 1983; Williams, 1965; Holý and Barba, 1975; Wright, 1974). In addition, it can also be an artifact of the semen sample processing technique. However, studies have shown a rare congenital and hereditary sterilizing defect named decapitated sperm defect or disintegration of spermatozoa. The authors reported that the decapitated sperm defect occurred in the caput epididymis and affected 80 to 100% of the screened cells; generally, the tails remained motile. It was hypothesized that this defect was originated from a defective implantation foosa/groove and basal plate (Blom and Birch-Andersen, 1970).

The most common sperm midpiece defects include distal midpieces reflexes (DMR), Dag defect, segmental aplasia of the mitochondrial sheath (SAMS), swelling midpiece, fracture, defibrillation, pseudodroplets, corkscrew defect, stump defect, and the aplastic midpiece defect (AMD). According to Barth and Oko. (1989), the most common midpiece defect is the DMR; however, they also classified simple bent tail as DMR. These defects should not be mixed, and the true DMR should not be confused with a simple bent tail because in the DMR, the midpiece is also included in the bend, and DMR is associated with the presence of the distal cytoplasmatic droplet (Perry, 2018). Thus, both conditions are classified as secondary, but the simple bent tail is a minor defect, whereas the DMR is a major defect (Blom, 1950; 1973). Some bulls are predisposed for the DMR defect, and spermatozoa with DMR display reverse motility and cannot penetrate the zona pellucida. However, the other normal spermatozoa in the DMR affected bulls can fertilize (Barth and Oko, 1989). Therefore, the DMR is classified as a compensable defect having a little negative effect on fertility until the frequency bypasses 30% in the ejaculate.

The Dag defect is described as folding and coiling of the midpiece and principal piece in conjunction with a misalignment or gaps in the midpiece and a high incidence of proximal cytoplasmatic droplets. Frequently, the midpiece is strongly coiled, folded, and split into the fibers. The defect was named Dag after the first carrier bull reported; that study also suggested that it was heritable because Dag and his full brother shared the same sperm defect (Blom, 1966). Later, pedigrees studies confirmed that the defect was due to the presence of an autosomal recessive factor (Koefoed-Johnsen et al., 1980; Sundararaman et al., 2014). It was also reported that spermatozoa with Dag defect also lack part of the axoneme, specifically one or two microtubule doublets with their corresponding outer dense fibers. Furthermore, it was suggested that the Dag defect had an epididymal origin because the sperm was morphologically normal in the testis, and the defect started appearing in the caput epididymis, probably after the spermatozoa gained motility (Koefoed-Johnsen and Pedersen, 1971). Regarding the genetic etiology, a deletion in the bovine QRICH2 was found responsible for low sperm count and immotile sperm with a morphological Dag-like defect (Hiltpold et al., 2022).

The SAMS is a defect characterized by gaps in the mitochondrial sheath, due to the missing mitochondria. Depending on the location and the size of the gaps, it could predispose spermatozoa to fractures and fibrillation, and spermatozoa with no mitochondrial sheath are usually dead (Zamboni, 1991). It is not uncommon to find small numbers of spermatozoa with this defect in ejaculate of fertile bulls; however, it is rare to find bulls with this sperm defect as the predominant feature of their ejaculate. This defect could have an environmental or genetic origin. It is the pathognomonic result of gossypol toxicity (by cottonseed intake) (Chenoweth et al., 2000), and it has been founded in viral diseases (Chenoweth and Burgess, 1972), in mice deprived of selenium (Wallace, 1983) and idiopathic (Hellmen et al., 1980; Lorton et al., 1983). The SAMS defect does not affect sperm motility or fertility (Barth and Oko, 1989). Likewise, Rocha et al. (2006) described a high fertility Charolais bull (semen used for repeat breeder

cows) with 24-36% of SAMS with no consequences for sperm motility or the conception rates of inseminated cows. This defect was characterized by mitochondrial aplasia near the connecting piece, mitochondrial segmental elongation and gaps, and thickening of the outer dense fibers at the apical region of the midpiece with loss of the cementing substance and development of plasma membrane extensions in the entire midpiece.

Relevant to SAMS, we studied a sterilizing sperm defect in Gir bulls named AMD, for aplastic midpiece defect. This defect is characterized by a lack or discontinuity of the mitochondrial sheath (e.g., gap, notch, filiform). The SAMS differs from the AMD in regard to the size and localization of the mitochondrial gaps. The gaps of the SAMS are minor and commonly localized in the proximal region of the midpiece. In contrast, the AMD-associated gaps are wider, generally discontinuing the mitochondrial sheath or causing a mitochondrial sheath absence (aplasia of the mitochondrial sheath); they are prevalently distal, located near the annulus. The structurally deficient mitochondrial sheath corrupt axoneme integrity, resulting in a wide variety of midpiece defects (Veeramachaneni, 2011). Therefore, defects such as the Dag, pseudodroplets, and stumps are frequently found in conjunction with the aplasia and/or SAMS. In addition, some spermatozoa display no midpiece at all and resemble the filiform spermatozoa (Savage and Isa, 1963). This defect was previously reported by Vale Filho et al. (1977) in 6 closely related bulls, one of them imported from India and two others being father and son; therefore, a putative genetic origin was suggested. The spermiograms showed an incidence of 12 to 100% of midpieces defects and sperm motility of 0 to 30%.

Our study showed that bulls with a high percentage of AMD had poor motility, poor viability (membrane integrity and functionality), and increased DNA damage when compared to bulls classified as satisfactory potential breeders (Diaz-Miranda et al., 2019). We hypothesized that the changes in the sperm parameters were due to oxidative stress and a reduction of the energy production capacity in conjunction with an alteration in the structural

composition of the sperm cell. Furthermore, we suggested that this defect was genetic. The incidence of midpiece defects in bulls is very low (Blom, 1959). In 48,592 Nellore bulls evaluated from our group, the incidence of bulls with a high percentage of midpiece defects was around 0.24%. However, in 282 Dairy Gir bulls evaluated, the incidence of this defect was 5.67%. Furthermore, this defect has affected several generations of Gir bulls, which definitively sustains the likelihood that this defect is heritable.

Nevertheless, further research should be done to estimate the effect of AMD on fertility, in addition to morphofunctional studies to verify if the defect is primary or secondary. Even though some midpiece defects have been suggested to originated in the epididymis, a similar defect in Swedish bulls has been shown to originate in the testis. The authors also observed a decreased percentage of midpiece defects after epididymal transit, suggesting that there was a removal of the abnormal spermatozoa (Hellmen et al., 1980). There is also a need to understanding the factors that influence an abnormal sperm midpiece structure, as well as the mode of inheritance and the genetic structure of the defect.

The corkscrew defect is a very rare sperm abnormality. This midpiece defect was named so because of the resemblance of the abnormal midpiece to a corkscrew; in the cases described, the bulls progressed from normal fertility to sterility, and frequently testicular degeneration (Blom, 1959). None of the spermatozoa affected had motility and most were dead. The mitochondrial sheath was characterized by an abnormal distribution of mitochondria over the midpiece accompanied with proximal droplets and sometimes swollen midpiece (Barth and Oko, 1989). In one study, this acquired defect was hypothesized to be a result of toxicity by nuclear fallout because they found a correlation of the number of cases with the periods of nuclear testing (Blom, 1978). Other rare abnormality in bulls is the “pseudodroplet” midpiece defect. However, when found in high percentages the fertility is low and it was suggested to

have a genetic origin (Blom, 1968). The defect can be described as a local thickening or swollen midpiece, denser than cytoplasmatic droplets (Chenoweth, 2005).

The stump defect, classified as a tail and a midpiece defect, is characterized by the sperm head and a short remnant of the tail or a droplet-like rounded structure attached to the implantation region of most of the "tailless" heads. Less than 1% of these cells keep a short tail rudiment (Vierula et al., 1983). The first known case in the United States was from an Ayrshire bull in 1985 (Arriola et al., 1985). The droplet-like structure is attached to the implantation groove and contains different types of vesicles and membranous debris, remnants of the capitulum, the segmented/striated columns, and/or a few dispersed round mitochondria (Blom and Birch-Andersen, 1980). Is not uncommon to see occasional stump spermatozoa in ejaculates; however, most of the time, this defect affects a majority of the cells and is often associated with other midpiece defects such as Dag defect, as well as with pyriform heads, which may suggest a disturbed caudal manchette function during spermatid elongation. Indeed, the first developmental abnormalities manifested themselves at the acrosome phase when the manchette was present. The common characteristic of abnormal spermatids with the stump defect is the failure of axoneme formation by the distal centriole and accumulations of disorganized mitochondria. Therefore, it was suggested that the origin of this defect was a dysfunction of the centriolar apparatus during spermiogenesis (Blom and Birch-Andersen, 1980). The etiology of this sterilizing defect in bulls was suggested to be hereditary. Accordingly, a frameshift mutation in ARMC3 gene was associated with the stump sperm defect in Swedish Red cattle (Pausch et al., 2016). In our study, some of the Gir bulls affected with AMD had the majority of their spermatozoa afflicted with this stump defect. Perhaps both AMD and stump sperm defect could be genetically linked or could be affected in the same gene by different mutations or a combination of mutations.

The abaxial tail attachment is no longer considered a bull sperm defect by the SFT and the Western Canadian Association of Bovine Practitioners (WCABP) (Koziol and Armstrong, 2018; Barth, 2013). The withdrawal of the abaxial tail sperm was based on insemination, superovulation and breeding trial using bulls with a high percentage of abaxial tails, in which the abaxial spermatozoa fertilized oocytes at a normal rate and were not associated with any increase in embryonic death. Therefore, it was concluded that the abaxial tail attachment has not a detrimental effect on bull fertility (Barth, 1989) and could be considered a normal variation of bovine sperm morphology, as well as is considered as such in other species (Dowsett et al., 1984; Hancock, 1956).

Cytoplasmatic droplets (CD) can be classified according to their localization as proximal or distal CD. The droplets are the remnants of cytoplasm after spermiogenesis, and the migration from proximal to distal midpiece is a step of the epididymal maturation. Finally, their release occurs prior to ejaculation in most mammals including bulls, when sperm become metabolically activated, and their motility increases. Notable exception is domestic boar in which CDs are mostly shed after ejaculation. Proximal droplets are classified as a major and primary defects while distal droplets are classified as minor, secondary defects (Blom, 1950; 1973). Although, the SFT excluded distal droplets from their list of defects (Koziol and Armstrong, 2018), proximal droplets are considered to be non-compensable due to reduced zona binding and early embryonic death in vitro (Saacke et al., 1995; Thundathil et al., 2001), affecting bull fertility (Nöthling and Arndt, 1995). Droplets are a pathognomonic defect associated with sexual immaturity in young bulls and testicular degeneration in old bulls. However, the percentage of droplets decreases when the bulls reach sexual maturity (Amann et al., 2000; Persson and Söderquist, 2005). In addition, most bulls experience a mild seasonal testicular degeneration with a decrease in sperm quality and an increase of proximal droplets (Erb et al., 1942). On the other hand, some bulls have a persistent production of abnormal

spermatozoa with cytoplasmatic droplets due to defective spermiogenesis (Barth and Oko, 1989).

Threshold for the percentage of normal spermatozoa or percentage of sperm abnormalities

Regarding the classifications of the defects, the compensable and non-compensable classification is widely known, and it has been helpful when evaluating bulls for semen cryopreservation and AI. Compensable defects are thought to impair events prior to sperm penetration into the oocyte membrane. On the other hand, non-compensable defects are believed to prevent events that occur after a spermatozoon enters the oocyte because the contribution of that spermatozoa cannot sustain fertilization and embryogenesis. There is also a positive association between embryonic quality with the number of accessory spermatozoa (11-20 desirable) (Amann and DeJarnette, 2012, Kastelic, 2013). However, it is uncertain whether a particular sperm defect is fully compensable or contributes to both types of outcomes (Amann and DeJarnette, 2012).

There is no clear baseline of an acceptable percentage of non-compensable or compensable defects and what would be the ideal insemination dose, which is more at the veterinarian's discretion since sperm/ejaculate conditions are individual and variable. The WCABP recommends limits of 20% of nuclear abnormalities (Pouch formation, vacuoles, chromatin defects, among others) or proximal droplets, and no more than 25% of acrosome or tail defects (Barth, 2013). However, the ACV in the bull reported for BSE suggested a tolerance threshold of 20% for the defects that they considered non-compensable (proximal droplets, pyriform heads, vacuoles, and teratologic sperm) and of 30% for compensable defects (midpiece and tail abnormalities, loose heads, and acrosome defects) (Fordyce et al., 2006). The approach differs in the North American AI centers, where farms are required to deliver data on semen fertility rates for individual sires, and sperm concentration is adjusted to have

adequate "fertility," consequently trying to decrease the sire-to-sire variation (Amann and DeJarnette, 2012). However, sperm concentration, morphology, and motility are not the only sperm parameters that affect fertility. Even with this adjustment, there is variation in the pregnancy rates per AI (Larson and Miller, 2000 Kastelic and Thundathil, 2008; Amann and DeJarnette, 2012). To date, in-vitro assessments of semen quality to predict the fertility potential of a semen sample only explains 50% to 60% of the variation among males (DeJarnette, 2005).

The CBRA and the Scandinavian countries use the major and minor defect classification. This may be because the CBRA was heavily influenced by published work of Drs. N. Lagerlof, I. Settergreen, and E Blom (Vale Filho et al., 2010). The morphological analysis is focused on the types of defects rather than on % normal spermatozoa. All morphological abnormalities in the sperm cell are recorded, which means that more than one abnormality within a single sperm is considered in the evaluation, and both brightfield and phase contrast defects are evaluated in the analysis, with a minimum of 200 sperm counted. A uniquely applied threshold is the 5% of individual defects of the CBRA, which is based on the hypothesis that most sperm defects have genetic origin, thus aiming to limit the possibility of mass-dissemination of the heritable sperm defects.

The primary and secondary defect classification was widely used by the SFT until, in 2018, it was changed for the subcellular localization site of the defect. The change was because the primary or secondary classification only indicates the defect's suspected origin but does not necessarily correlate with the effect on fertility. There were also concerns regarding variation between practitioners because more than one collection is required to understand the etiology of some defects. In addition, some conditions may simultaneously affect both spermatogenesis and epididymal function, suggesting that primary and secondary defects can be equally important indicators of disturbance of reproductive function (Koziol and Armstrong, 2018).

There is no doubt that sperm morphology directly affects fertility (Foote, 1970; Wiltbank and Parish, 1986; Barth and Oko, 1989; Johnson, 1997; Steffen, 1997; Fitzpatrick et al., 2002; Chemes and Rawe, 2003; Chenoweth, 2005; Al-Makhzoomi et al., 2008; Freneau et al., 2010; Attia et al., 2016). However, a major question is whether the reference values reflect this characteristic and whether they are relevant both for both AI and NS, where billions of spermatozoa are deposited in the vagina during copulation. This concern is the reason why several societies and practitioners such as the Australian Cattle Veterinarian (ACV) and our laboratory approve bulls in the BSE only for NS (Chenoweth, 2015). Furthermore, little is known about fertility thresholds for individual sperm defects for NS, which is perhaps why the SFT does not recommend any threshold for specific defects.

Several studies have correlated fertility with the percentage of normal sperm (Larsen et al., 1990; Söderquist et al., 1991; Holroyd et al., 2002; Phillips et al., 2004; Al- Makhzoomi et al., 2008; Nagy et al., 2013). Overall, using bulls with 70% or more normal spermatozoa can improve the pregnancy rate in herds, whether in NS (Wiltbank and Parish, 1986; Fitzpatrick et al., 2002) or AI (Barth and Oko, 1989; Attia et al., 2016). Regarding the type or classification of the defects, Blom (1950) reported that fertile animals had a maximum of 10% of primary defects and animals with fertility problems had more than 15%. More recently, it was reported that major defects had a significant effect on the calving rate; if the proportion of major and minor defects increased by 10%, the calving rate decreased by 0.79 and 0.67 average units, respectively (Attia et al. al., 2016). Other studies have shown that sperm defects are associated with DNA damage, majors defects with 85% and minors with 18% (Enciso et al., 2011, Siddique et al., 2011; Nagy et al., 2013).

On the other hand, some studies have shown how individual defects affect bull fertility. For instance, high fertility bulls had up to 10% of spermatozoa with acrosome defects (Bacinoglu et al. 2008), as well as head defects (Al-Makhzoomi et al. 2008). Other studies

suggested that the incidence of 5 to 10% of any individual type of abnormal morphology would reduce fertility (Rollinson, 1951), including proximal cytoplasmic droplets (Johnson, 1997). Nevertheless, some studies have shown that certain sperm abnormalities did not affect bull fertility, such as the abaxial tail and distal cytoplasmatic droplets (Barth, 1989; Al-Makhzoomi et al., 2008).

The relevance of the sperm morphology analysis for bull fertility, is clear. However, there is no consensus about the correct thresholds for individual defects, even though a reduction in fertility is expected beyond 10% of major individual defects and/or 30% of total defects. Perhaps more research should be done aiming on sperm morphology standardization to have reliable results (Perry, 2021). However, selection against sperm abnormalities should be another focus because it is critical for improving bull fertility. Although there are no significant concerns about the genetic origin of sperm abnormalities from most societies, practitioners, or AI centers (For review Chenoweth, 2005), some studies have shown that it is possible to select against sperm abnormalities (Chandler et al., 1985). It is also the main reason for the strict threshold for individual defects in the sperm morphology analysis guidelines of the CBRA (CBRA, 2013). However, there is still a lack of knowledge about the inheritance mode of several sperm abnormalities and perhaps when or what is the limit to consider a bull as a carrier of a specific sperm defect. Therefore, further studies are necessary to understand the etiopathology of sperm defects and whether these abnormalities share mendelian inheritance or they are more complex polygenic traits.

Biomarkers of abnormal bull sperm morphology

It should be made clear that the sperm morphology is not the only parameter that affect bull fertility and sperm quality. A spermatozoon is a terminally differentiated, highly specialized and morphologically polarized cell, suspended in the complex seminal plasma at the time of ejaculation. The spermatozoon deliver more than just its DNA to the oocyte, it

carries other precious cargoes, which support sperm survival within female reproductive tract, fertilization and early embryo development. For instance, alterations in sperm chromatin (De Oliveira et al., 2013), miRNAs (Wu et al., 2020), metabolites in the seminal plasma (Velho et al., 2018), polymorphisms related to sperm function (Fernandez-Fuertes et al., 2017) or recessive haplotypes related to loss of function (VanRaden et al., 2011) have all been suggested to affect bull fertility.

Extensive research has been conducted in order to identify biomarkers of fertility in bulls (Peddinti et al., 2008; Taylor et al., 2018; Viana et al., 2018). The primary objective was to enhance the ability to accurately predict fertility and discover novel semen attributes associated with male fertility, as well as to develop methods to refine ejaculatesprocessed for AI, and to achieve better conception rates (Sutovsky et al., 2015). Although most of these approaches targeted low fertility bulls, the major reason for low fertility in bulls is abnormal sperm morphology. Paradoxically, defective spermatozoa can also look morphologically normal, and morphologically abnormal spermatozoa are not always dysfunctional (Kubo-Irie et al., 2004). Therefore, it is understandable that only a multi-trait or multifunctional analysis could offer objective and standardized procedures to predict male fertility.

Proteomic analyses have identified several proteins with increased abundance in low fertile bulls spermatozoa. For instance, the Outer Dense Fiber of sperm tails 2 (ODF2) and post-acrosomal assembly of sperm head protein (PAWP/WBP2NL) are more abundant in spermatozoa from low fertile bulls. The phenotypical characterization showed that high levels of PAWP might be indicator of macrocephalic spermatozoa, while high ODF content is mostly related to coiled tail/distal reflex, bent tail, and midpiece defects (Kaya et al 2021). The PAWP protein is localized exclusively in the post acrosomal sheath of the perinuclear theca and is suggested to participate in the sperm-induced oocyte activation process. Accordingly, another

study showed that a proper post acrosomal sheath localization and quantity of PAWP is correlated with field AI fertility and normal morphology in bulls (Kennedy et al., 2014).

Another negative biomarker is sperm ubiquitin because defective spermatozoa became ubiquitinated during epididymal transit (Sutovsky et al 2001). This post- translational modification of sperm surface proteins take place when a multiubiquitin chain binds to sperm proteins via covalent ligation. This was also corroborated by measuring the ubiquitin content of Percoll gradient separated immotile, dead, and defective sperm fractions. Thus, the measure of ubiquitin content in bull ejaculates is correlated with bull fertility (Odhiambo et al., 2014). In this context, increased levels of Platelet Activating Factor-Receptor (PAFr) correlated positively with sperm ubiquitin content, and it was suggested to be a potential biomarker for sperm quality. However, PAFr was also found in white blood cells, making its content in semen not a reliable measure for fertility. On the other hand, it could be an indicator of inflammation if WB are found in semen. Nonetheless, additional parameters have been suggested, such as DNA content to distinguish haploid spermatozoa from diploid WBC by flow cytometry, to eliminate the contamination and estimate the PAFr content in the sperm population without bias (Sutovsky et al., 2007).

The surface of the sperm plasma membrane is coated with a thick layer of glycans, and glycoproteins named the sperm glycocalyx, which influences sperm function (Xin et al., 2014). Lectins are natural binders of glycans, and a diverse family of carbohydrate-binding proteins found in all plants and animals. The composition of the glycans varies according to the different sperm membrane domains and, consequently, their binders. Therefore, Lectins have been used to determine specific sperm features such as acrosome integrity with PNA (Arachis hypogaea/peanut agglutinin) because they bind specific carbohydrate moieties of glycoproteins exclusively localized in the acrosome (Silva and Gadella, 2006). On the other hand, fluorescently conjugated LCA lectins have been used to identify abnormal spermatozoa by a

change in their binding properties. While in defective spermatozoa, fluorescently conjugated LCA binds to the entire sperm head and tail surface, in normal spermatozoa binds exclusively to acrosomal surfaces (Sutovsky et al., 2015).

Proteomic investigation of the sperm fractions containing defective sperm purified by nanoparticles covered in anti-ubiquitin and PNA showed an enrichment in the binder of sperm protein 5 (BSP5) (Odhiambo et al., 2014). In contrast, in another proteomic investigation, BSP1 was the most abundant protein in the immotile fraction isolated by percoll (D'Amours et al., 2010). Binder of sperm proteins (BSPs) are the major proteins found in seminal plasma and bind to spermatozoa upon ejaculation and may play a role in sperm capacitation and the formation of the sperm reservoir. There is no consensus regarding whether these proteins are beneficial or detrimental to bull fertility. For instance, BSP proteins were found to be upregulated in sperm and seminal plasma from high fertile bulls (Kasimanickam et la., 2019). On the other hand, there is also several studies suggesting a detrimental effect to bull fertility (Aslam et al., 2018; Roncoletta et al., 2006). This pivotal effect was described by Moura et al. (2006) who suggested that there is a quadratic effect, while lower amounts of BSPs may facilitate fertilization, higher amounts of BSPs may became detrimental to fertility.

Targeted proteomics of pyriform sperm induced by scrotal insulation showed a downregulation in cytoskeleton proteins and an upregulation in proteins involved in oxidative stress and protein folding and degradation. The authors also hypothesized that pyriform sperm was caused by a defect in sperm head cytoskeletal structures, due to a reduction in microfilament formation during spermatogenesis by PFDN5 (Shojaei Saadi et al., 2013).

As mentioned early, several pathways are altered in teratozoospermic males, including protein folding and degradation (Dias et al., 2019; Martínez-Heredia et al., 2008; Platts et al., 2007). Most of these proteins are heat shock proteins (HSP) which are molecular chaperones mediating protein folding and preventing protein aggregation. However, it is unclear why

aberrant chaperone expression may be a significant contributing factor to the defective sperm function in many cases of male infertility (For review Dun et al., 2012). Considering the role of HSPs in preventing protein misfolding during stress conditions, the increased expression could be associated with disturbed spermatogenesis by environmental effects or as a compensatory effect. Other proteins participating in these pathways are proteasomal proteins, which are in charge of degrading several proteins and organelles, as well as chromatin remodulation during spermatogenesis. Therefore, it seems likely that disturbed spermiogenesis can increase the expression of these proteins (Bracke et al., 2018).

Enormous efforts have been focused on building knowledge about bull fertility, and several biomarkers associated with abnormal morphology have been discovered. However, there is still a need to understand all the factors that affect sperm morphology. Perhaps with the rise of sequencing technologies and targeted proteomic approaches to understand specific phenotypes, the identification and validation of sperm morphology markers will accelerate, and abnormal sperm morphology could be removed as the main factor that detracts from bull fertility through the use of strategic breeding and genetic improvement programs.

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CHAPTER 1

Title: “Strict use of Breeding Soundness Evaluation (BSE) improves the reproductive quality of young Nellore bulls”.

HIGHLIGHTS

- Records of 20 years and 46,566 BSE from a seedstock herd in Brazil were investigated
- About 14% of the bulls were not approved in the first BSE at two years old
- Semen quality (53%) & physical defects (47%) were the most common causes of failure
- Frequency of sperm defects did not improve over time but was controlled and low
- Testicular abnormalities decreased, & scrotal circumference increased over the years
- BSE use increased the percentage of bulls approved at first BSE in the herd over time

ABSTRACT

Breeding soundness evaluation (BSE) is the best methodology to estimate the fertility potential of future bulls and to perform an indirect selection for bull fertility. However, the outcome of the BSE is influenced by several factors, including genetics, environment, and the BSE guidelines. Therefore, we aim to characterize the reasons for failure in 46,566 BSE from ~2 years old Nellore (*Bos indicus*) bulls and investigate if the overall fertility of bulls improved over the years by using strict BSE guidelines. We also assessed the main reasons for BSE failure and determined if they decreased over time. In this context, we used a dataset of 44,776 BSE from young Nellore bulls born from 1998 to 2018 and investigated the effect of the year and farm with multivariate logistic regression. Bulls were classified as approved (satisfactory potential breeders and qualified for natural breeding service) and not approved (deferred and unsatisfactory potential breeders). The main reasons for failure at the BSE in Nellore bulls were poor semen quality (53.1%) and physical defects (46.9%), the main physical defect being testis abnormalities (19.7%). The overall percentage of bulls approved was 86.2%, with no improvement over the years of study. However, the percentage of approved bulls at the first BSE increased over the years. This increase was evident by a reduction in the difference between the percentage of the bulls approved vs the percentage of bulls approved at the first BSE. Furthermore, there was an increase in the percentage of bulls classified as satisfactory potential breeders in the BSE and a decrease in the percentage of bulls qualified only for natural breeding service (P-value <0.05). This indicates that the overall quality of the bulls improved over the years. To associate and identify the main sperm abnormalities, 3461 bulls were clustered. The most frequent defects were strongly coiled spermatozoa, proximal droplets, and acrosomal defects. Overall, there was no change in the frequency of bulls not approved by the sperm morphology over the years nor the frequency of the main sperm abnormalities. Nevertheless, the frequency of the defects remained very low, which implies that they were

controlled. Additionally, abnormalities in the testis decreased over the years in association with an increase in the average scrotal circumference (SC) of the herd and a decrease of bulls culled by low SC. In conclusion, this study demonstrates that there could be improvements in the reproductive quality of replacement bulls by implementing and using strict BSE guidelines.

Keywords: bull fertility, semen quality, scrotal circumference, sperm morphology.

1. Introduction

Bull fertility can be influenced by numerous factors that can affect its desire or ability to copulate (*impotentia coeundi*) or its ability to fertilize the ova (*impotentia generandi*) [1]. The causes of these conditions are acquired or congenital. Nonetheless, bull fertility is a key factor in the productivity and sustainability of the cattle industry [2]. Therefore, determining bull fertility is an important yet challenging task. There is no precision procedure to measure or estimate the real fertility of a bull before its use for breeding. Perhaps, because paternal fertility is a complex trait that involves several events; beginning with an early establishment of spermatogenesis (sexual precocity) and then producing spermatozoa capable of reaching and fertilizing the oocyte and contributing to embryo development, to the conceptus elongation, and the pregnancy establishment [3]. To date, the most accurate way to test bull fertility is by breeding a significant number of fertile females, which is expensive and time-consuming for routine use [4].

Therefore, the best practice to indirectly select for bull fertility is to conduct and follow strict guidelines of breeding soundness evaluation (BSE), which allows the detection of infertile and low fertile bulls. Nevertheless, it is estimated that only about 10% of the beef bulls in the US undergo a BSE [5]. Indeed, 20% to 40% of the bulls have been reported to have reduced fertility, with some bulls presenting infertility [6-7]. Bull fertility has been overlooked, and

improvements are necessary to increase efficiency in the livestock industry [8]. The BSE does not aim to predict the precise fertility of a bull. Instead, a bull approved in the BSE should be able to have 60% and 95% of the cows pregnant in the first 21 days and 60 days of the breeding season, respectively, in an optimal bull to female ratio [9-10]. Overall, the reason for the failure of the BSE includes low scrotal circumference (SC), unacceptable sperm morphology, low sperm motility, poor libido, and physical defects. Thus, the most common causes of failure in the BSE are associated with delayed puberty and sexual maturity, which are reflected in the SC and sperm morphology [11].

It is very important to highlight that the BSE guidelines differ between countries and practitioners. For example, the Society of Theriogenology (SFT) recommends 30% motility and 70% normal sperm thresholds to approve a bull submitted to BSE [10]. In contrast, other societies, such as the Brazilian College of Animal Reproduction (CBRA), use limits such as ≥ 60% motility in addition to the sperm morphology evaluation, which is based on the types of defects rather than on normal sperm [12]. Therefore, establishing thresholds for total, major and minor defects in addition to individual defects. In addition to that, some societies and practitioners classify bulls for natural breeding service (NS) when they consider that the quality of the semen is fair for NS, but not enough for artificial insemination (AI) [13]. Differences in these guidelines may suggest that more studies in the area are needed. Furthermore, large amounts of data are available from BSE of *Bos taurus* bulls, while on the *Bos indicus* side is very poor.

Another difference in BSE guidelines is the method to diagnose testicular hypoplasia (TH). TH is the most common congenital condition that affects bulls and is known as small testis. It is being described in at least 12 breeds, and studies in Brazil showed an incidence of 5-15% [14-16]. TH can be unilateral or bilateral and total or partial; depending on this, bulls can be subfertile or infertile. At the same time, the SFT recommends culling Bos taurus bulls

with < 30 cm of SC or more than 25% of a size difference between testes as a suspected diagnosis of TH [13,17,18]. The CBRA recommends individual measures of the bull testes with a caliper and differences of ≥ 10% of the testicular volume, to be diagnosed as testicular asymmetry [12]. Therefore, the guidelines can affect the percentage of bulls approved at the BSE [13,19,20].

The SC is the most used and important reproductive trait in bulls because it is fast, low-cost, and easily measured in the BSE. It is biologically important because it impacts puberty and sperm production [21]. For example, cows exposed to bulls with smaller SC were less likely to be diagnosed pregnant and had a longer interval from first bull exposure to calving when compared to cows exposed to bulls with larger SC [22]. Overall, bulls with small testis (<30 cm) have low conception rates [23]. Moreover, the SC has a medium to high heritability (h = 0.36 – 0.56) and differs between breeds and age at evaluation [8]. Sperm morphology is another important trait measured during the BSE. There is a growing list of sperm abnormalities considered to have a genetic origin, and these defects seem to have low heritability [24-25]. However, most heritability studies were conducted in bulls considered normal (70% of normal sperm). In contrast, the CBRA guidelines established that major individual sperm defects reaching more than 5% could have a potential genetic origin, and bulls with higher percentages are considered unsatisfactory potential breeders [12]. However, this baseline is more focused on genetic improvement rather than measuring the individual fertility of the bull. In fact, there are opportunities to study how sperm abnormalities change over time in *Bos indicus* bulls subjected to BSE.

In this context, empirical information suggests that after the implementation of the BSE in cattle operations, the percentage of approved bulls increases over time. However, this outcome is influenced by several factors, including the environment, genetics, BSE criteria, and bull age [13]. Therefore, we aimed to characterize the main reasons for failure the BSE from

~2 years old Nellore (*Bos indicus*) bulls and investigate if the overall fertility of bulls improved over the years by using strict BSE guidelines. We also assessed the main reasons for BSE failure and if they decrease over time. In addition, correlations between the testicular measurements and the semen quality were also investigated. Thus, our main hypothesis was that the strict use of the BSE would increase the number of approved Nellore bulls and their reproductive quality. This study will allow us to complement the knowledge on bull fertility, specifically on *Bos indicus* cattle.

2. Material and Methods

2.1. Bulls, location, and general management

This retrospective study used 46,566 BSE from *Bos indicus* Nellore bulls raised in grazing conditions and born between 1997 and 2018. Bulls were located on three farms in the states of Sao Paulo (SP), Mato Grosso do Sul (MS), and Bahia (BA), Brazil. Farm 1 (SP) is located at 20.6455° South, 50.2314° West, with annual average temperature of 22.0 °C and annual rainfall of 1,200 mm. Farm 2 (MS) is at 20.2947° South, 55.4454° West, with an annual average temperature of 23.3 °C and annual rainfall of 1,400 mm. Farm 3 (BA) is located at 13.3411° South, 44.6346° West, with an annual average temperature of 24.4 °C and annual rainfall of 924 mm.

In the three farms, the calves were kept with their dams in *Urochloa spp* pastures with water and mineral salt, and the weaning was performed at 7-8 months of age. After weaning, the calves were kept in pastures until 14-16 months, when they were confined on feedlots before the first BSE at 18-24 months. Starting in 2012, the first assessment of the BSE in the young bulls was performed around June or July, and a final assessment was conducted on the deferred bulls between August and September. Bulls classified as deferred or unsatisfactory potential breeders in the last assessment did not enter the breeding season and were destinated for

slaughter. Nevertheless, before 2012 the breeding season was not well established, and there were more than 2 BSE seasons. Therefore, some deferred bulls were evaluated more than twice.

2.2 Breeding soundness evaluation parameters

The BSE followed the standards determined by the Brazilian College of Animal Reproduction with some modifications [12]. First, a rigorous clinical andrological examination was conducted to determine the normality of testicular and epididymal function, as well as of the genital organs [26]. Further, data concerning scrotal circumference, testicular length, and width were also evaluated. Testicular width and length were obtained by using a caliper. The testicular volume was calculated with the equation proposed by [27]:

$$TV = \frac{4}{3}\pi\left(\frac{TL}{2}\right)\left(\frac{TW}{2}\right)\left(\frac{TW}{2}\right)$$

TV = testicular volume (cm^3); TL = testicular length (cm); and TW = testicular width (cm).

The semen was collected using electroejaculation and stored in prewarmed, graded, conical plastic tubes that were protected by a polystyrene cover from light, cold shock, and rapid temperature changes. Immediately after semen collection, mass motility, sperm motility, and sperm vigor were subjectively assessed using light microscopy. Sperm morphology was assessed by phase contrast microscopy of wet-mount semen sample fixed in isotonic formol saline [28]. Sperm morphology was evaluated by using major and minor defects classification [29].

Based on the BSE, bulls were classified as satisfactory, qualified for NS, deferred, and unsatisfactory potential breeders. A bull was classified as a satisfactory potential breeder when presenting a healthy and sound status, with adequate scrotal circumference (≥ 28 cm). Also, the bull must show sperm motility ≥ 50%; total sperm defects ≤ 30%; major sperm defects ≤ 10%; minor sperm defects ≤ 20%; major individual defects ≤ 5%; and minor individual defects ≤

10%. A bull was classified for NS when they presented the same sperm motility of ≥ 50%; total sperm defects ≤ 30%, but major sperm defects ≤ 20% and major individual defects between ≤ 10%. Moreover, a bull was classified as deferred when considered capable of improvement, which is pubertal bulls with SC ≥ 26 and/or poor sperm quality [30]. Finally, bulls were classified as unsatisfactory potential breeders when they were below one or more thresholds, and it is unlikely ever to improve their status. In addition, the unsatisfactory potential breeder classification included bulls with conditions considered genetics or irrevocable physical problems (including infectious disease) that would compromise breeding or fertility [13].

Starting on 2002, Bulls with SC > 26 and < 28 cm were classified as deferred bulls, and bulls with < 26 cm were classified as unsatisfactory potential breeders. Furthermore, bulls with testicular asymmetry (a difference of ≥ 1cm in width or length) were also classified as unsatisfactory potential breeders once they could potentially have testicular hypoplasia. From 1997 to 2002, the SC and testicular asymmetry thresholds were slightly different. That is, bulls with SC > 26 cm but reaching the sperm motility and morphology thresholds were classified as satisfactory potential breeders, and bulls with differences in the testicular width or length ≤ 1.5 cm were considered normal.

2.3 Dataset and Statistical analysis

The initial dataset included a total of 60,341 BSE. BSE from bulls with age > 30 months were excluded from the study (n = 5,425). Repeated BSE and bulls with misinformation were also excluded (n = 6,251). Data (n = 2,099) from the year 2009 was also excluded because of missing information. After editing, 46,566 BSE of young bulls born between 1997 and 2018 were used for the analysis of the main reasons for failure in the BSE. The Statistical Analysis System (SAS OnDemand) was used for data analysis [31].

In the following analysis, bulls evaluated on 1997 (n = 1,790 BSE) were not included because the BSE classification for bulls approved for NS was not established yet. Thus, a data

set including 44,776 bull BSE born between 1998 and 2018 was analyzed using PROC GLIMMIX to evaluate the effect of the birth year and the farm on the percentage of bulls approved and classified as satisfactory potential breeders at the BSE. The year 1998 and the farm SP were the references for comparison. In addition, the LS-means were compared by Tukey–Kramer test.

To objectively classify and associate the bulls according to their main sperm defect, a cluster analyses from the sperm morphology of 3,461 bulls not approved in the BSE was used. In addition, an analysis of frequency in the main abnormalities in the testis was evaluated over time. For research purposes, in this analysis, all bulls with testicular width or length differences of ≥ 1 cm were considered with testicular asymmetry, and bulls with < 28 cm of SC were considered low SC.

All the sperm abnormalities recorded from the 3,461 bulls were used in the cluster analysis using PROC FASTCLUS. The clustering method selected was the average linkage which bases clustering decisions on the average distance (linkage) between clusters. Subsequently, all the sperm abnormalities were included in a multivariate canonical analysis (PROC CANDISC) to find linear combinations of these quantitative variables that best summarized the differences among the clusters of the sperm morphology analysis.

Correlations among scrotal circumference, testicular volume, sperm motility, and sperm defects were evaluated by Pearson`s correlation coefficient (CORR Procedure).

3. Results

The percentage of bulls classified as satisfactory, qualified for NS, deferred, and unsatisfactory potential breeders were 78.7%, 7.5%, 6.7%, and 7.1%, respectively. Table 1 shows the descriptive statistics of the parameters evaluated in the bulls in the BSE. The main reasons young Nellore bulls fail in the BSE are shown in Fig.1a. and they represent 13.8% of

the bull population. The reasons were also separated according to the BSE bull classification. That is, unsatisfactory potential breeder and deferred (Figs. 1b and 1c). Thus, the overall frequency of undesirable semen quality and abnormalities in the testis, accessory glands, penis, prepuce, feet and legs, and epididymis in the whole bull population was 7.3%, 2.7%, 1.3%, 1.1%, 0.1%, 0.8%, and 0.1%, respectively. In addition, 0.4% of the bulls were not approved due to voluntary culling. In summary, bulls were not approved due to the semen quality (53.1%) and physical defects (46.9%). On the other hand, semen quality represented 17.3% and physical defects 82.7% of the bulls classified as unsatisfactory potential breeders. Furthermore, the bulls were classified as deferred mainly because of poor semen quality (90.3%), low SC (5.3%), and adherence in the penis (4.4%). Therefore, the main two reasons for failure at the BSE were abnormal sperm morphology and abnormalities in the testis.

The percentage of young Nellore bulls approved by the BSE was 86.2%. In contrast, the percentage of approved bulls at the first BSE was 74.2%. Fig. 2. shows the significant differences in the overall percentage of approved bulls during the years of the study, and there was no improvement in the percentage of bulls approved over time. However, the percentage of approved bulls at the first BSE increased throughout the years. This increase is evident in the difference between the number of bulls approved at the BSE and the bulls approved in their first BSE in Fig. 2. Furthermore, there was an increase in the percentage of bulls classified as satisfactory potential breeders in the BSE over time (Fig. 3; P-value <0.05). Suppl. Tables 1 and 2. shows the results of the multivariate logistic regression and the odds ratio estimates according to the farm and year on the percentage of Nellore bulls approved and the percentage of bulls classified as satisfactory potential breeders at the BSE, respectively. Suppl. Fig. 1. demonstrates that farms influenced the percentage of bulls approved in the BSE.

Subsequently, we assessed the main two reasons for culling bulls, which were abnormal sperm morphology and abnormalities in the testis. Fig. 4. shows a heatmap with the mean value

of each sperm abnormality by cluster. Each cluster was denominated by the main sperm abnormality, which is the sperm abnormality with a higher mean value. However, there was sperm abnormalities association (i.e., cluster one had an association between proximal droplets and strongly coiled tail sperm). The main sperm abnormalities in the culled bulls were strongly coiled spermatozoa (clusters 3 and 13), proximal droplets (clusters 8, 11, and 12), and acrosomal defects (clusters 4 and 6). On the other hand, cluster 10 was the bulls with random defect combinations or a small number of bulls with the same sperm picture. Overall, there was not a change in the frequency of bulls culled by sperm abnormalities over time (Suppl. Fig. 2a). After identifying the high-frequency sperm abnormalities, we evaluated them by year, and there was also no tendency to decrease the frequency of the main sperm abnormalities (Suppl. Fig. 2b, c, d).

Regarding the abnormalities in the testis, the main reasons for culling bulls were testicular asymmetry and low SC. There was a decrease in the percentage of bulls culled due to abnormalities in the testis over time. An increase in the bulls SC was also evidenced during the years of the study. This was also accompanied by a decrease in the percentage of bulls that were culled due to low SC over the years. However, there were no significant differences in the frequency of bulls culled because of testicular asymmetry across the years (Fig. 5). A low correlation between the testicular measurements and the sperm morphology in the approved bulls was founded (Table 2). Despite being low, the greatest correlation between the testicular measurements and the sperm morphology in the not approved bulls was negative with proximal droplets. Total, major, and midpiece defects were moderately correlated with sperm motility.

4. Discussion

Breeding soundness evaluations is still the main procedure to select bull for their reproductive potential regardless of its limitation [32]. The BSE should be used routinely or at

least prior to the use of bulls for natural mating or for sperm cryopreservation. As mentioned before, the guideline of the BSE varies depending on the countries, societies, and practitioners, and there is no consensus on several parameters evaluated in the bulls, such as sperm motility and morphology [33]. Therefore, variation in a procedure established in the early 1950s probably means that more studies are required. Regarding the CBRA guidelines, some thresholds are mainly focused on genetic improvement rather than the true fertility of the bull in the field.

In this context, the hypothesis of our study was supported since we showed that by the implementation and strict use of the BSE following the CBRA guideline, the reproductive potential of the bulls improved. Even though there was not an increase in the percentage of bulls approved through the years, it was very clear that more bulls were approved in their first BSE, which could be explained by the bulls reaching puberty and sexual maturity early. In addition, there was an increase in the percentage of satisfactory potential breeders and a decrease in the bulls qualified for NS. These findings illustrate that there was an improvement in the overall semen quality of the bull herd since the NS classification is used for bulls with a fair semen quality for NS but not enough for AI.

According to the literature, 10% to 40% of bulls fail the BSE [9,34-40]. Therefore, the percentage of failure in this study (13.8%) could be considered normal or low. Comparisons with other studies may be difficult once there are differences in the BSE guidelines, breed, genetics, age at the evaluation, and environment. Indeed, as in other studies, we found that environmental effects, such as the farm influenced the percentage of approved bulls in the BSE; even though the bulls in this study had the same genetic background [19,20,41,42]. However, this high percentage of bulls approved in the BSE can be because this herd has been selected for fertility and sexual precocity for over 40 years. This fact could also explain that there were no changes in the overall percentage of approved bulls.

Overall, the main reasons for failure in the BSE were poor semen quality and physical defects being 7.3% and 6.5%, respectively. These percentages are also low compared to the literature [19,20,41]. However, it agrees with most of the literature suggesting that poor semen quality is responsible for ~50% of bull failure at the BSE [20,36,39]. On the other hand, it is worth mentioning that producers easily notice physical defects, and most bulls with physical defects do not reach the BSE season. Especially on farms with breeding genetic programs, such as the one in this study, which have SC measurement at yearling; therefore, most of the testicular problems are likely to be diagnosed early. Even so, by breaking down the categories of bulls that did not pass the BSE, abnormal sperm morphology and abnormalities in the testis were the main reasons for failure.

Although the environment is the factor that more influences sperm abnormalities, the breed, and genetics affect their frequency and incidence [24,43]. Therefore, the bulls were clustered according to their sperm morphology to determine whether the main sperm abnormalities of the herd were decreasing or increasing after strict use of the BSE guidelines. This approach would eliminate subjectivity because, to date, little is known about the percentage of defects to consider a bull as a carrier. Interestingly, strongly coiled tails were the most frequent sperm abnormality in the not approved bulls. Indeed, a short tail defect was reported to be potentially genetic in this herd [44]. The second most frequent defect was proximal droplets, which may be associated with delayed sexual maturity and is a major reason for yearling bulls to fail the BSE [45]. Regarding the acrosome defects, there is potential evidence suggesting a genetic defect, especially when the defect remains for several ejaculates with no other sperm abnormality [46]. However, there was not a decrease in the frequencies of a bull being culled exclusively by the sperm morphology nor the three most frequent sperm abnormalities.

These results could be explained by the fact that sperm abnormalities were already low frequent in the herd. To test such hypothesis, studies should be carried out in herds of unselected bulls. However, it demonstrates that, by selection, sperm abnormalities remained low for several years. In this sense, if the goal is to eliminate sperm abnormalities from herds, genomic approaches may be key to achieving it, since there is evidence of the potential genetic origin of sperm defects [25]. This hypothesis may also explain why there were no changes in the frequency of bulls culled by testicular asymmetry. The strict measure of the testes is used to control and/or eliminate TH from the herds due to the hereditary background and the high frequency reported in some *Bos indicus* breeds [16,47]. On the other hand, the abnormalities in the testis decreased over the time of this study and that outcome is associated with the reduction of bulls culled by low SC, which is explained by the increase of the SC in the bull herd.

The SC is a trait that responds to selection. Indeed, by selecting larger SC, the SC increased over time [48]. Low to moderate favorable correlations were found in the SC, testicular volume, or motility with sperm quality in the approved bulls, which agrees with most of the literature [49]. However, a moderate negative correlation between the SC and the proximal droplets was found in the non-approved bulls. Proximal droplets are common defects in puberal bulls, which may suggest that selection to larger SC and sexual precocity could decrease the percentage of proximal droplets in that population of bulls. This is not surprising as selection for SC is widely used because it correlates with early puberty and sperm production [21,50]. In addition, this study confirms the correlations between sperm motility and midpiece defects, total defects, and major defects in bulls that were not approved [51].

Most semen traits are considered low to moderate heritability [52-53]. Therefore, improvements in bull fertility can be obtained through selection [13]. This was proved in this study and encourages the producers and veterinaries to implement and follow the BSE guidelines. Although there was no decrease in the frequencies of bulls being culled by specific

sperm abnormalities, these frequencies were very low, and the overall semen quality in the herd improved, with bulls reaching early sexual puberty and maturity and fewer bulls qualified for NS. Another advantage could be that using rigorous guidelines, it may decrease the variation in the fertility reported in bulls approved in the BSE even though this variability in fertility may not be totally related to the parameters evaluated in the BSE. An ultimate goal in BSE research is the standardization of the guidelines [54]. This could help with the international trading of genetics, including bulls and straws, to avoid differences in the regulations and policies of different countries [55]. However, in the field, these guidelines should only be a reference. The interpretation of any values should be biological and not mathematical [56]. The outcome of the BSE is individual and mainly derived from the technical criteria of the veterinarian.

5. Conclusion

In conclusion, there was a clear improvement over time in the reproductive quality of Nellore bulls in a Brazilian seedstock herd by the strict use of the BSE. This was reflected in more bulls approved as satisfactory potential breeders and fewer bulls approved for NS. Furthermore, there was an increase in the percentage of approved bulls in the first BSE and a decrease in the percentage of bulls with low SC suggesting that the bulls are reaching puberty and subsequently sexual maturity early. Nonetheless, there was no improvement in the overall percentage of approved bulls over the years or in the frequency of sperm abnormalities in the herd. However, these defects remained low, suggesting that there were controlled in the herd. Therefore, this study demonstrates that there could be improvements in the reproductive quality of replacement bulls by implementing and using strict BSE guidelines.

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TABLES

Table 1. Descriptive statistics of the testicular measurements and semen traits from young Nellore bulls in the breeding soundness evaluation (BSE).

Variable	n	Mean (SD)	Median	Minimum	Maximum
Age (months)	46566	22.0 (1.7)	21.8	17.9	30
Scrotal Circumference (cm)	46239	33.2 (2.6)	33	22.5	48.4
Volume (cm^3)	43879	318.6 (89.3)	311.3	32.5	1369.3
Motility (%)	43951	70.1 (12.1)	70	0	95
Total (%)	43713	17.4 (11.3)	15	0	168
Major[a] (%)	43713	12.6 (9.8)	10.5	0	146
Minor[a] (%)	43713	4.8 (4.1)	4	0	75

[a]Major and minor sperm defect classification by [29].

Table 2. Correlations coefficient of the scrotal circumference, testicular volume, and sperm motility with the sperm abnormalities in the approved and not approved Nellore bulls.

Defects	Approved[a]			Not approved[b]		
	Scrotal Circumference	Volume	Motility	Scrotal Circumference	Volume	Motility
Acrosome	-0.01*	-0.02**	-0.06**	0.02^{ns}	-0.03*	0.02^{ns}
Head	-0.04**	-0.04**	-0.07**	-0.04*	-0.05*	-0.19**
Midpiece	-0.01^{ns}	0.03**	0.00^{ns}	0.02^{ns}	0.05*	-0.42**
Tail	-0.03**	-0.02**	-0.09**	0.09**	0.08**	-0.16**
Proximal	-0.05**	-0.08**	-0.07**	-0.18**	-0.13**	-0.12**
Distal	-0.03**	-0.03**	-0.09**	0.02^{ns}	0.08**	0.13**
Major[c]	-0.07**	-0.06**	-0.12**	-0.08**	-0.06*	-0.34**
Minor[c]	-0.02**	-0.04**	-0.10**	0.03*	0.06**	0.00^{ns}
TOTAL	-0.07**	-0.07**	-0.15**	-0.07**	-0.03*	-0.34**

[a]Bulls approved include the satisfactory potential breeders and qualified for NS and [b]Not approved includes the deferred and unsatisfactory potential breeder bulls. [c]Major and minor sperm defect classification by [29] (*P-value <0.05, **P-value <0.0001).

FIGURES

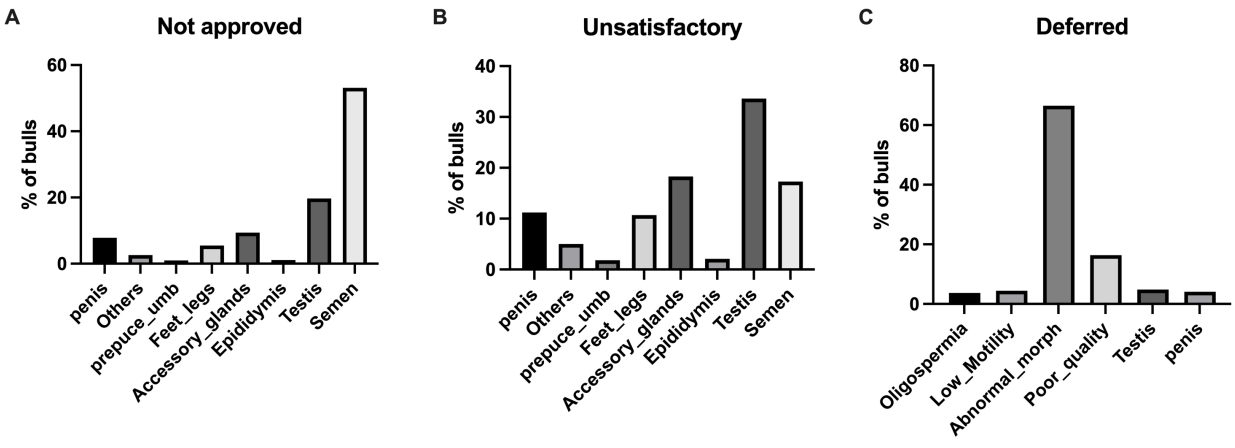


Fig. 1. Reasons for breeding soundness evaluation failure in young Nellore bulls. Data from 46,566 bull BSE classified as A) not approved (Unsatisfactory potential breeders and deferred) B) Unsatisfactory potential breeder and C) Deferred.

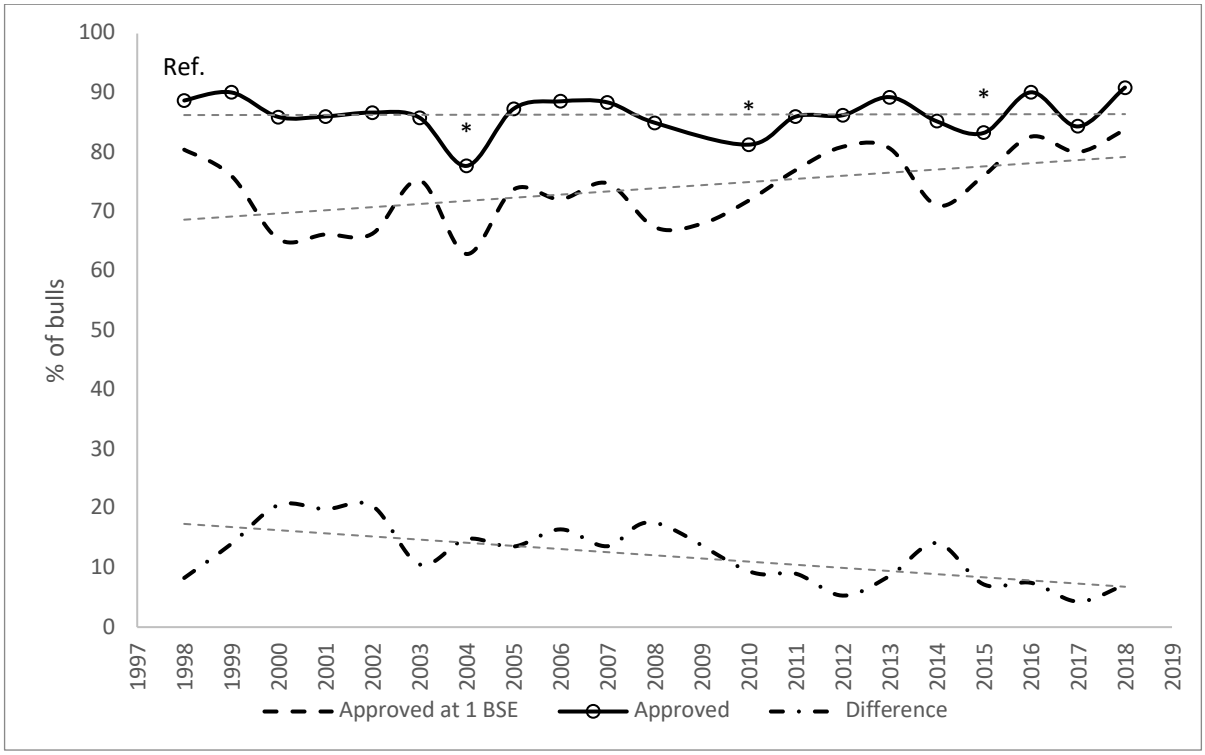


Fig. 2. Overall percentage of Nellore bulls approved, and percentage of bulls approved at the first breeding soundness evaluation and difference between them by year. Approved bulls include the satisfactory potential breeders and the bulls qualified for natural breeding service. The year 1998 and the farm SP were the references for comparison. *Asterisks indicate significant difference in the percentage of approved bulls with the reference year by Tukey-Kramer test (P-value <0.05).

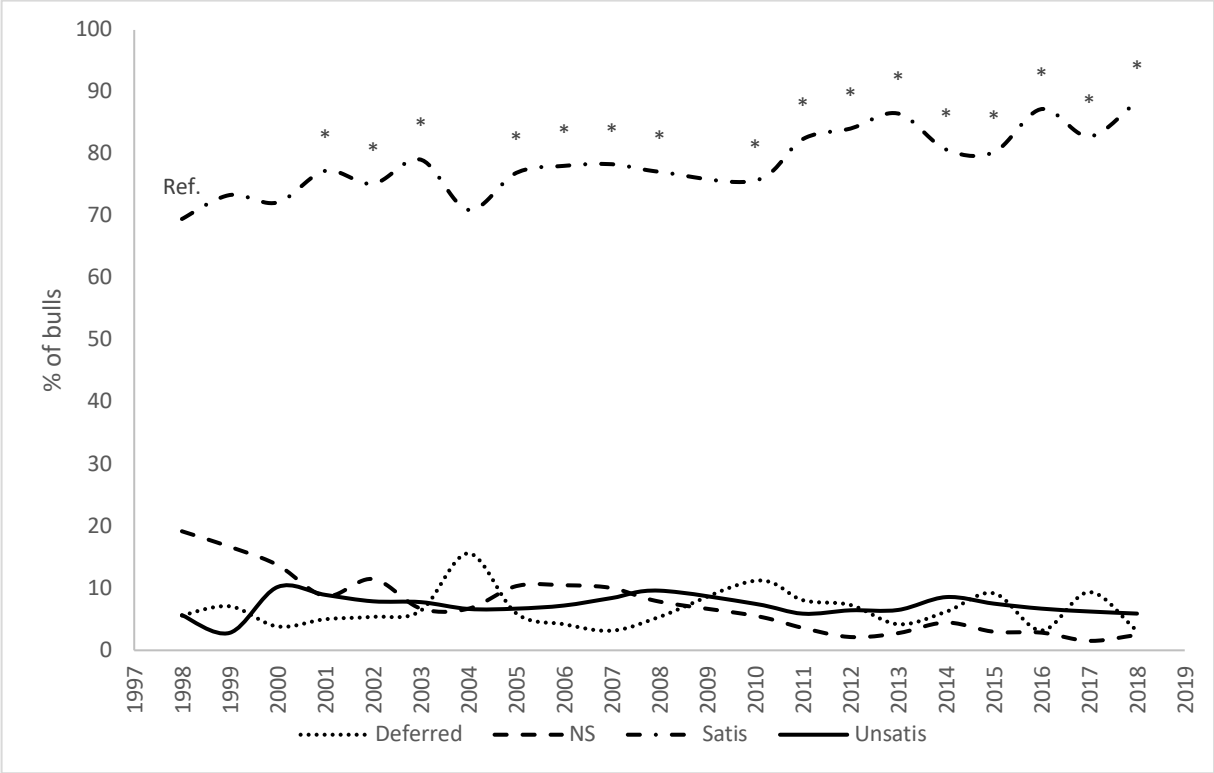


Fig. 3. Percentage of Nellore Bull Breeding Soundness Evaluation classification by year. Bulls were classified as a satisfactory (Satis), qualified for natural breeding service (NS), deferred and unsatisfactory potential breeder (Unsatis). *Asterisks indicate significant difference with the reference year by Tukey-Kramer test (P-value <0.05).

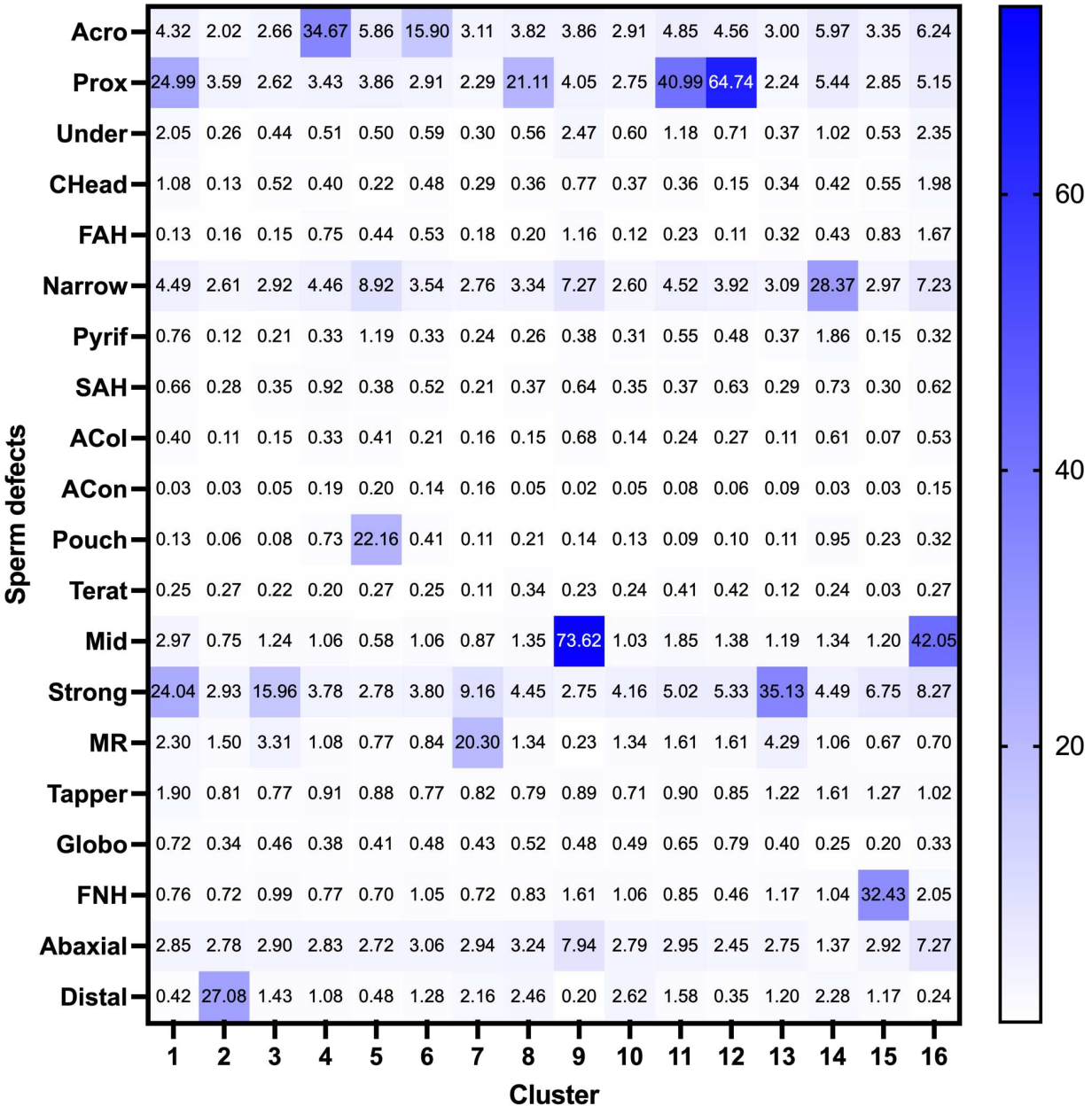

Sperm defects
Acro 4.32 2.02 2.66 34.67 5.86 15.90 3.11 3.82 3.86 2.91 4.85 4.56 3.00 5.97 3.35 6.24
Prox 24.99 3.59 2.62 3.43 3.86 2.91 2.29 21.11 4.05 2.75 40.99 64.74 2.24 5.44 2.85 5.15
Under 2.05 0.26 0.44 0.51 0.50 0.59 0.30 0.56 2.47 0.60 1.18 0.71 0.37 1.02 0.53 2.35
CHead 1.08 0.13 0.52 0.40 0.22 0.48 0.29 0.36 0.77 0.37 0.36 0.15 0.34 0.42 0.55 1.98
FAH 0.13 0.16 0.15 0.75 0.44 0.53 0.18 0.20 1.16 0.12 0.23 0.11 0.32 0.43 0.83 1.67
Narrow 4.49 2.61 2.92 4.46 8.92 3.54 2.76 3.34 7.27 2.60 4.52 3.92 3.09 28.37 2.97 7.23
Pyrif 0.76 0.12 0.21 0.33 1.19 0.33 0.24 0.26 0.38 0.31 0.55 0.48 0.37 1.86 0.15 0.32
SAH 0.66 0.28 0.35 0.92 0.38 0.52 0.21 0.37 0.64 0.35 0.37 0.63 0.29 0.73 0.30 0.62
ACol 0.40 0.11 0.15 0.33 0.41 0.21 0.16 0.15 0.68 0.14 0.24 0.27 0.11 0.61 0.07 0.53
ACon 0.03 0.03 0.05 0.19 0.20 0.14 0.16 0.05 0.02 0.05 0.08 0.06 0.09 0.03 0.03 0.15
Pouch 0.13 0.06 0.08 0.73 22.16 0.41 0.11 0.21 0.14 0.13 0.09 0.10 0.11 0.95 0.23 0.32
Terat 0.25 0.27 0.22 0.20 0.27 0.25 0.11 0.34 0.23 0.24 0.41 0.42 0.12 0.24 0.03 0.27
Mid 2.97 0.75 1.24 1.06 0.58 1.06 0.87 1.35 73.62 1.03 1.85 1.38 1.19 1.34 1.20 42.05
Strong 24.04 2.93 15.96 3.78 2.78 3.80 9.16 4.45 2.75 4.16 5.02 5.33 35.13 4.49 6.75 8.27
MR 2.30 1.50 3.31 1.08 0.77 0.84 20.30 1.34 0.23 1.34 1.61 1.61 4.29 1.06 0.67 0.70
Tapper 1.90 0.81 0.77 0.91 0.88 0.77 0.82 0.79 0.89 0.71 0.90 0.85 1.22 1.61 1.27 1.02
Globo 0.72 0.34 0.46 0.38 0.41 0.48 0.43 0.52 0.48 0.49 0.65 0.79 0.40 0.25 0.20 0.33
FNH 0.76 0.72 0.99 0.77 0.70 1.05 0.72 0.83 1.61 1.06 0.85 0.46 1.17 1.04 32.43 2.05
Abaxial 2.85 2.78 2.90 2.83 2.72 3.06 2.94 3.24 7.94 2.79 2.95 2.45 2.75 1.37 2.92 7.27
Distal 0.42 27.08 1.43 1.08 0.48 1.28 2.16 2.46 0.20 2.62 1.58 0.35 1.20 2.28 1.17 0.24
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Cluster
60
40
20

Fig. 4. Heat map of the canonical means of the sperm abnormalities in the clusters. Each cluster was denominated by the main sperm abnormality, which is the sperm abnormality with a higher mean value. There were clusters with an association of defects (i.e., Cluster 1 had an association of proximal droplets and strongly coiled tail sperm), and clusters with the same main defect (i.e., cluster 8, 11 and 12 had proximal droplets). Clusters 1 represent proximal droplets and strongly coiled tail, 2 distal droplet, 3 and 13 strongly coiled tail, 4 and 6 acrosome, 5 pouch formation, 7 midpiece reflex, 8, 11 and 12 proximal droplets, 9 and 16 midpiece defects, 14 narrow at the base, 15 free normal heads, and 10 bulls that were not grouped or displayed random defects. *Sperm defects were classified as major and minor and include: Acrosome (Acro), proximal droplets (prox), underdeveloped (under), teratologic forms (terat), Coiled in the head (CHead), pouch formation (Pouch), pyriform sperm (Pyrif), narrow at the base (Narrow), abnormal color (ACol), abnormal contour (ACon), small abnormal heads (SAH), free abnormal heads (FAH), and midpiece defects (Mid), strongly coiled tail (Strong), Distal midpiece reflex (DMR), narrow heads (Tapper), giant and short broad heads (Globo), free normal heads (FNH), abaxial implantation (Abaxial), distal droplet (Distal).*

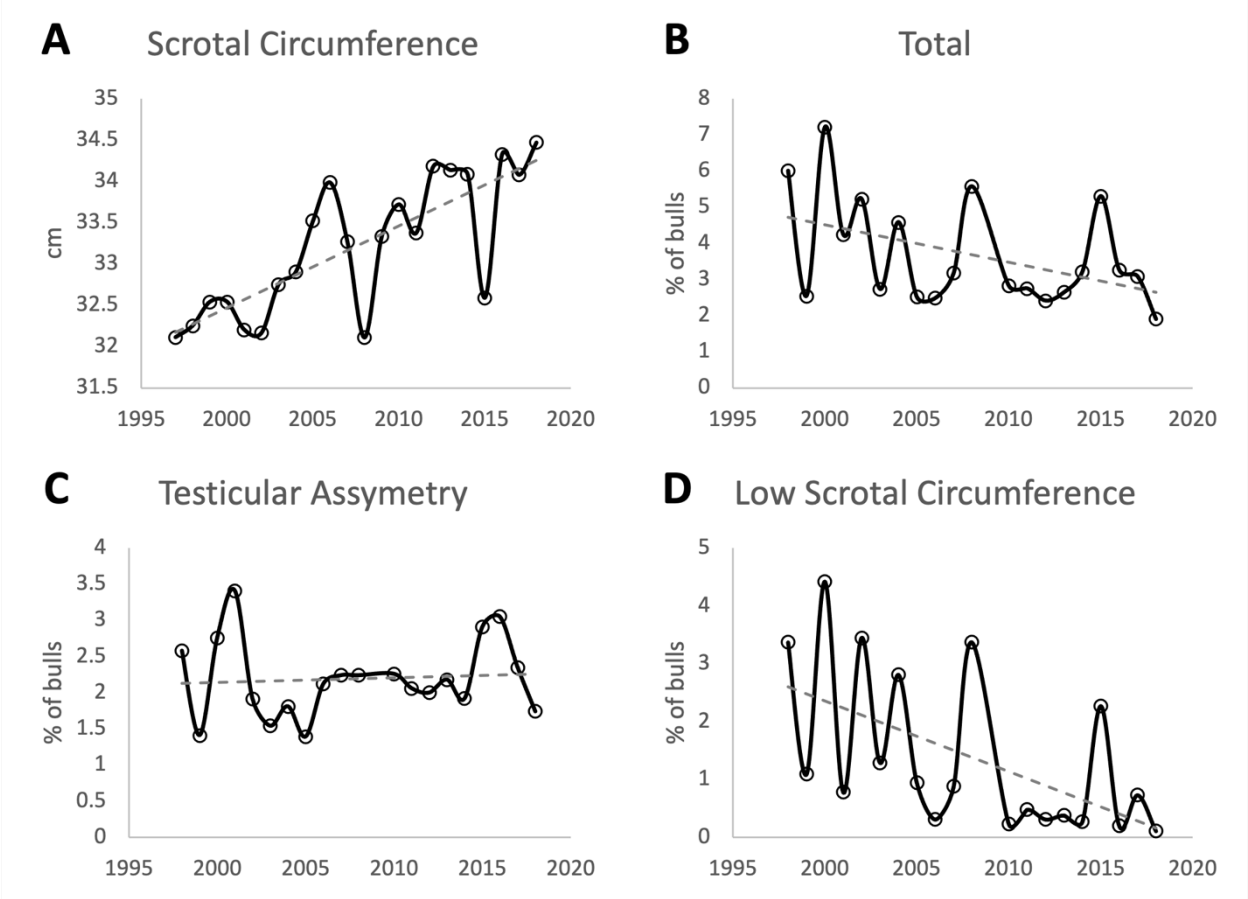
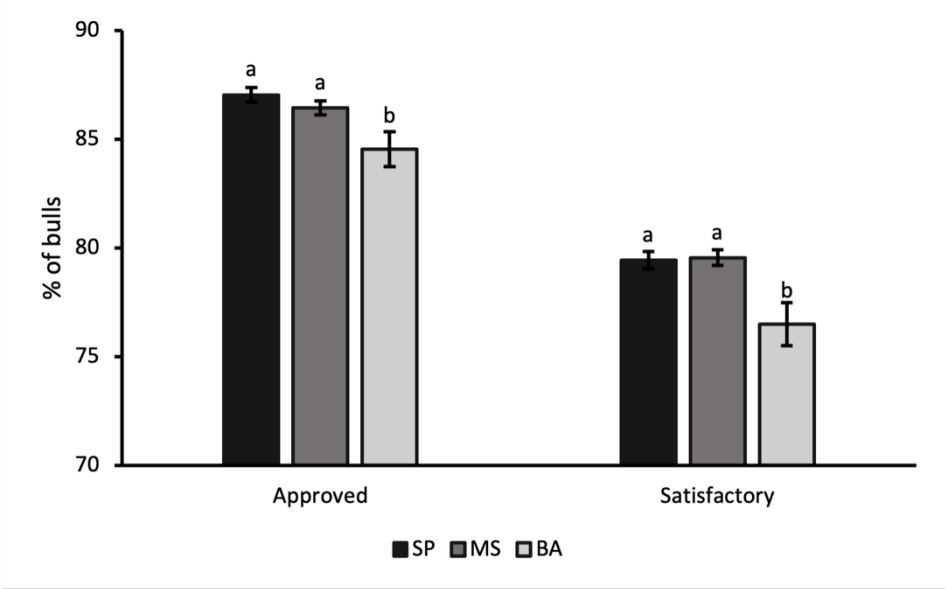
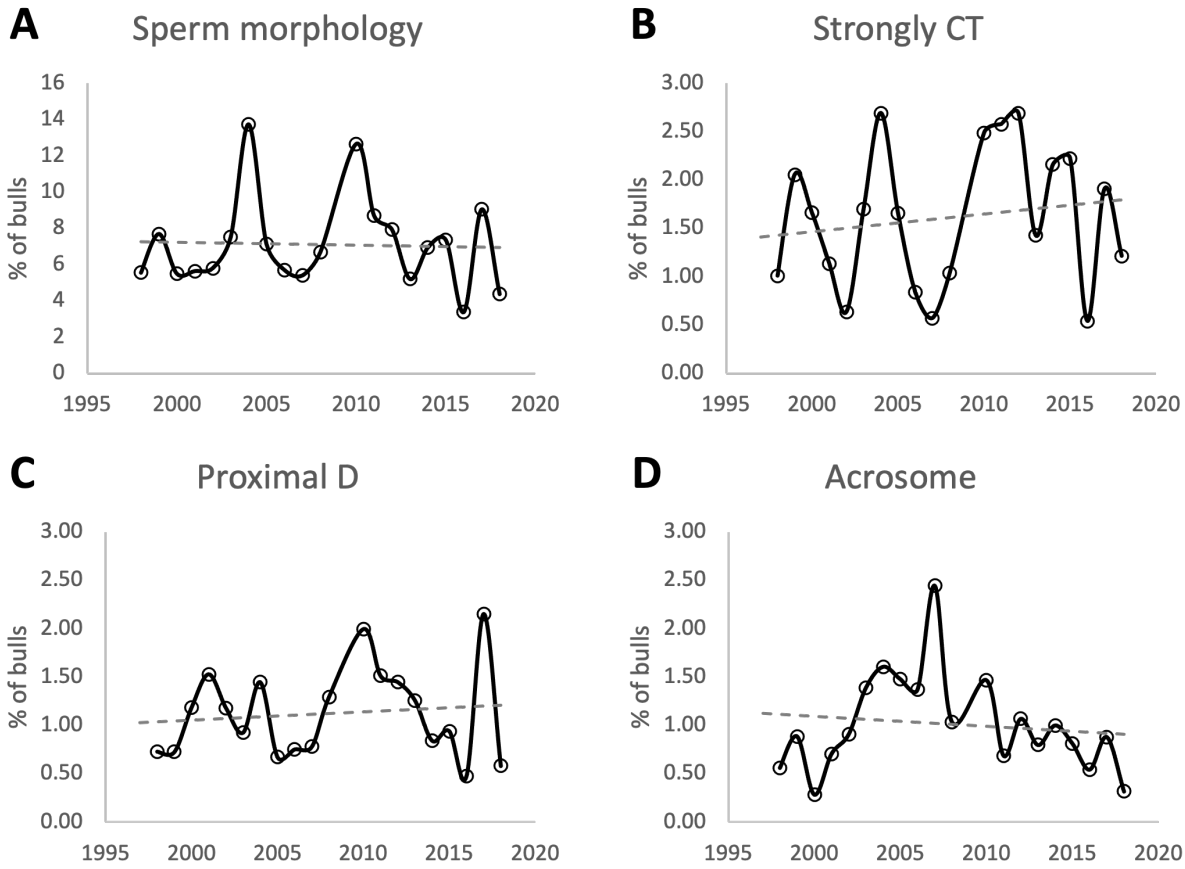


Fig. 5. Testicular measurements and frequencies of testicular abnormalities in Nellore bulls by year. A) Scrotal circumference (SC) of the bull population over the years, B) Frequency of bulls culled because of testicular abnormalities (Total) C) testicular asymmetry, and D) Low SC. 1,634 bulls born from 1998 to 2018 were diagnosed with either testicular asymmetry or low SC. Bulls were considered to have testicular asymmetry when the testis had a difference of ≥1cm in width or length, and bulls with <28cm of SC were considered to have low SC.

SUPPLEMENTARY MATERIAL



Suppl. Fig. 1. Differences of farm Least Squares Means effect in the percentage of bulls approved (satisfactory potential breeders and qualified for NS) and the percentage of bulls classified as satisfactory potential breeders in the breeding soundness evaluation (BSE). Different letters indicate significant differences across farm by Tukey-Kramer test (P-value <0.05).



Suppl. Fig. 2. Frequency of Nellore bulls culled due to the sperm morphology analysis and frequency of the top 3 defects by year. A) Frequency of Nellore bulls culled due to the sperm morphology, B) a high percentage of strongly coiled tails (Strongly CT) (Cluster 3 and 13), C) proximal droplets (Proximal D) (Cluster 8, 11 and12) and D) Acrosome defects (Acrosome) (Cluster 4 and 6). Data from the cluster analysis of 3641 bulls born between 1998 to 2018 were used.

Suppl. Table 1. Solution for fixed effects and Odds Ratio Estimates for multivariate logistic regression analysis of the overall percentage of Nellore bulls approved (satisfactory potential breeder and qualified for NS) at the breeding soundness evaluation (BSE) according to the year and farm.

Effect	Estimate	t Value	Pr > |t|	Odds ratio	OR 95% CL	
					Lower	Upper
Intercept	2.067 ± 0.07	27.57	<.0001	.	.	.
1998 (ref.)	0	.	.	.	.	.
1999	0.148 ± 0.10	1.47	0.1405	1.16	0.952	1.413
2000	-0.255 ± 0.10	-2.61	0.009	0.775	0.64	0.938
2001	-0.239 ± 0.09	-2.53	0.0113	0.787	0.655	0.947
2002	-0.182 ± 0.10	-1.86	0.0635	0.834	0.688	1.01
2003	-0.255 ± 0.10	-2.56	0.0104	0.775	0.638	0.942
2004	-0.813 ± 0.09	-9.13	<.0001	0.444	0.373	0.528
2005	-0.133 ± 0.10	-1.35	0.1756	0.875	0.722	1.061
2006	-0.009 ± 0.10	-0.09	0.9251	0.991	0.814	1.205
2007	-0.013 ± 0.10	-0.13	0.8986	0.987	0.804	1.212
2008	-0.290 ± 0.10	-2.99	0.0028	0.748	0.618	0.905
2010	-0.543 ± 0.10	-5.64	<.0001	0.581	0.481	0.702
2011	-0.186 ± 0.10	-1.83	0.0679	0.831	0.681	1.014
2012	-0.163 ± 0.10	-1.59	0.111	0.85	0.695	1.038
2013	0.116 ± 0.11	1.06	0.2871	1.123	0.907	1.392
2014	-0.246 ± 0.11	-2.27	0.023	0.782	0.632	0.967
2015	-0.380 ± 0.10	-3.67	0.0002	0.684	0.559	0.838
2016	0.211 ± 0.12	1.71	0.0871	1.234	0.97	1.571
2017	-0.316 ± 0.11	-2.98	0.0029	0.729	0.592	0.898
2018	0.300 ± 0.12	2.54	0.0112	1.349	1.07	1.701
SP (ref.)	0	.	.	.	.	.
BA	-0.205 ± 0.07	-2.76	0.0058	0.815	0.705	0.942
MS	-0.052 ± 0.05	-1.1	0.2729	0.949	0.865	1.042

Suppl. Table 2. Solution for fixed effects and Odds Ratio Estimates for multivariate logistic regression analysis of the percentage of Nellore bulls classified as satisfactory potential breeders at the breeding soundness evaluation (BSE) according to the year and farm.

Effect	Estimate	t Value	Pr > |t|	Odds ratio	OR 95% CL	
					Lower	Upper
Intercept	0.825 ± 0.052	16	<.0001	.	.	.
1998 (ref.)	0	.	.	.	.	.
1999	0.189 ± 0.069	2.74	0.0061	1.207	1.055	1.382
2000	0.129 ± 0.071	1.82	0.0687	1.138	0.99	1.307
2001	0.399 ± 0.070	5.71	<.0001	1.491	1.3	1.71
2002	0.285 ± 0.071	3.99	<.0001	1.33	1.156	1.529
2003	0.505 ± 0.076	6.65	<.0001	1.657	1.428	1.924
2004	0.070 ± 0.068	1.03	0.3052	1.072	0.939	1.224
2005	0.381 ± 0.072	5.29	<.0001	1.463	1.271	1.685
2006	0.445 ± 0.072	6.14	<.0001	1.56	1.354	1.798
2007	0.459 ± 0.077	5.97	<.0001	1.582	1.361	1.84
2008	0.408 ± 0.073	5.55	<.0001	1.503	1.302	1.736
2010	0.323 ± 0.074	4.35	<.0001	1.381	1.194	1.597
2011	0.729 ± 0.079	9.19	<.0001	2.072	1.774	2.421
2012	0.853 ± 0.081	10.54	<.0001	2.347	2.003	2.751
2013	1.039 ± 0.087	11.95	<.0001	2.827	2.384	3.353
2014	0.621 ± 0.086	7.24	<.0001	1.862	1.573	2.203
2015	0.607 ± 0.082	7.4	<.0001	1.835	1.562	2.155
2016	1.112 ± 0.100	11.07	<.0001	3.039	2.497	3.7
2017	0.759 ± 0.086	8.82	<.0001	2.136	1.805	2.529
2018	1.215 ± 0.096	12.72	<.0001	3.371	2.795	4.065
SP (ref.)	0	.	.	.	.	.
BA	-0.171 ± 0.065	-2.62	0.0087	0.843	0.742	0.958
MS	0.006 ± 0.039	0.16	0.8696	1.006	0.932	1.087

CHAPTER 2

Title: "Calving date as a potential predictor for the probability of approval in the first breeding soundness evaluation of Nellore bulls"

HIGHLIGHTS

-The probability of approval of bulls at the breeding soundness evaluation decrease when the calving date increase.

-Bulls born on day 0 of the calving season were 1.26 times more likely to pass the breeding soundness evaluation than bulls born on the date 21.

-To achieve an 80% probability of approval in 20-22 mo. Bulls at the breeding soundness evaluation, the calving date should not exceed 47 days.

ABSTRACT

Globally, the beef production system primarily uses natural service (NS) for breeding. However, a significant number of bulls used for NS are subfertile, limiting the profitability of the cow-calf operation. Therefore, producers should select bulls based on breeding soundness evolutions (BSE) to ensure higher pregnancy rates. Several factors can affect the bull ability to pass a BSE. We hypothesize that factors such as the calving date affects the bull probability of approval at the first BSE. For this purpose, a multivariate logistic regression in a dataset of 14,737 BSEs from young bulls was used. Correlations between calving date, biometrics, and semen traits were evaluated using Pearson`s correlation coefficient and a multivariate linear regression was performed to estimate the scrotal circumference (SC) according to the calving date, farm, and age group. Our results demonstrate that the calving date affected the probability of approval at the first BSE (P-value <0.05). Hence, bulls born on day 0 of the calving season have 1.26 more chances to be approved at the first BSE than bulls born 21 days later. In addition, the strongest correlation with the calving date was with SC, which decreased as the calving date increased. Therefore, the calving date could be used as a predictor of the outcome of the first BSE in young bulls. In that way, producers can make crucial management decisions such as choosing the bulls with a higher chance of approval at the BSE to be tested and those with less chance to be culled, saving time and cost of testing.

Keywords: Natural service; bull fertility; breeding season; Scrotal circumference.

1. Introduction

The demand for animal protein will likely continue to increase during the next 50 years given that the world population will increase by 35%. Therefore, beef protein will remain as an essential source of nutrients for people around the world, and producers have to continue increasing their efficiency [1]. Although artificial insemination was introduced to the cattle industry in the 1930s, the NS remains one of the most important management strategies because it is the predominant breeding method in beef herds [2-4]. For example, around 84% of the beef female cattle population available for reproduction in Brazil are subjected to NS breeding [5]. This number is not so different from the ones reported in other countries such as the US (~90%) and Australia (~98%) [6-9]. Therefore, improving bull fertility for NS is very important for cow-calf operations worldwide. Lamb et al. [10] estimated that infertility costs the producers USD 75 per exposed cow in total or USD 5 per exposed cow for every 1% decrease in pregnancy rate, meaning that the cost of infertility to the entire U.S. Industry with a cow population of 42.5 million likely losses revenue of more than $1.06 billion annually.

Efforts to increase cattle profitability and sustainability include using controlled breeding seasons. This allows for optimum timing for heifers and cows to be bred in accordance with the best time for feed and environmental conditions for the dams to calve [11,12]. This aids the body condition of the dams and their reproductive efficiency. Other advantages of the breeding season include a more uniform calf crop, increased calf survival, and better pre-wean growth of calves. It also improves management planning and labor resources, and simplifies the management workload (nutritional, health, and reproductive) [13]. Additionally, it is used as a selection strategy for fertility by culling open cows at the end of the breeding season. In that sense, the calving date is one of the key reproductive traits in beef genetic programs. The calving date also displays high heritability, which positively influences the longevity and

lifetime productivity of the female [14,15]. Several studies have shown that early calving dams produce calves with high weaning weight and pre-breeding heifer weight, which increases the chances of the heifers reaching precocious puberty and cyclicity, thus becoming pregnant and calving earlier [16-19]. While the effect of early calving on the reproductive performance of future dams has been investigated, such an effect on future sires remains to be elucidated.

The BSE is one of the most reliable methods to evaluate the reproductive potential of a sire. It should be a routinely applied test because approximately 40% of bulls may have reduced fertility, with some of them being completely sterile [20]. Various/multiple factors can impact the approval rate of young bulls in a BSE, such as low SC, unacceptable sperm morphology, low sperm motility, poor libido, and physical defects, among others. These factors can also be influenced by the environment, nutrition, age, breed, and genetics [21]. The SC is the most commonly used and perhaps the most important reproductive trait in young bulls; it has strong and favorable correlations with several reproductive traits measured in both the bull (semen quality) and their female progeny (age at puberty, age at first calving, probability of pregnancy at 14 months, pregnancy rate, and ovulation rate) [22-24]. Although these factors have been reported and investigated, the effect of the calving date on the sperm quality and BSE result of future sires remains to be elucidated. This is an important trait of study since the sales of young bulls represent an important outcome in the cow-calf enterprise.

Thus, this study aimed to evaluate the effect of the time of calving in the calving season on several reproductive traits of young bulls to be selected as future sires for reproduction in breeding programs. Our main hypothesis was that the males calved earlier in a 5-month calving season are more likely to be approved in their first breeding soundness evaluation when compared to males calved later in the same calving season. Biologically, this will be reflected in better reproductive parameters such as scrotal circumference, and sperm motility and morphology.

2. Materials and methods

2.1. Animals and location

Data from 14,737 Nellore bulls (*Bos taurus indicus*) calved in yearly seasons (August to December) over seven years (2012 through 2018) were used in this study. Management of these animals has been described previously [24]. Briefly, animals were calved and raised in grazing conditions *(Urochloa spp)*, with *ad libitum* access to water and mineral salt. Calves were kept with their dams and weaning was performed between 7-8 months of age. After weaning, the calves were kept on pastures until 16 months of age, when they were confined in feedlots before submission to the first BSE.

All bulls were calved and raised in two private farms in Brazil. One farm (MS) is located 20.2947° South, 55.4454° West with annual average temperature of 23.3 °C and annual rainfall of 1,400 mm. Another farm (BA) is located in 13.3411° South, 44.6346° West with annual average temperature of 24.4 °C and annual rainfall of 924 mm.

2.2 Breeding soundness evaluation (BSE) and scrotal circumference (SC)

The first BSE of most of the bulls in this study was at approximately 21 months of age, that is, between June and July of the second year after birth. However, a few bulls had their first BSE between August and September, which is the date of the second BSE of the bulls that did not initially meet the BSE parameters. For research purposes, we only used and reported data from the first BSE.

The BSE followed the standards determined by the Brazilian College of Animal Reproduction [25]. First, a rigorous clinical evaluation was conducted to determine the normality of testicular and epididymal function, as well as of the genital organs [26]. The semen was collected by electroejaculation and stored in prewarmed, graded, conical plastic tubes protected (by a polystyrene cover) from light, cold shock, and rapid temperature changes.

Immediately after semen collection, mass motility, sperm motility, and sperm vigor were subjectively assessed using light microscopy. Sperm morphology was evaluated by phase-contrast microscopy of wet-mount semen fixed in isotonic formol saline [27] using major and minor defects classification [28]. Moreover, all morphological abnormalities in the sperm cell were recorded. This means that more than one abnormality within a single sperm was considered in the evaluation and the analysis.

The SC was measured in the region of the largest diameter of the testes and included both testes positioned symmetrically side by side, leaving the scrotal skin distended. Testicular width and length were obtained by using a pachymeter and testicular volume was calculated as the equation proposed by Bailey et al. [29]:

$$TV = \frac{4}{3}\pi\left(\frac{TL}{2}\right)\left(\frac{TW}{2}\right)\left(\frac{TW}{2}\right)$$

TV = testicular volume (cm^3); TL = testicular length (cm); and TW = testicular width (cm).

Bulls were considered as a satisfactory potential breeder when they met all of the following BSE criteria: (1) presented a healthy and sound status; (2) SC ≥ 28 cm; (3) sperm motility ≥ 50%; (4) major sperm defects ≤ 10%; (5) minor sperm defects ≤ 20%; (6) major individual defects ≤ 5%; (7) minor individual defects ≤ 10%; and (8) total sperm defects ≤ 30%. If failing to meet one or more of the previous criteria, bulls were considered unsatisfactory potential breeders.

2.3 Data curation and statistical analysis

The original dataset included 48,698 Nellore bulls calved between 1996 and 2018 and submitted to an initial and a second (if not approved in the initial) BSE; that is, a total of 58,516 BSE. The second BSE (n = 9,818) was removed from the dataset. Moreover, 33,769 animals calved before 2012 were removed because the breeding season and subsequently calving season

were not well established in both farms at that time. We also excluded bulls born out of the breeding season (n = 192) since they were mostly obtained through *in vitro* embryo production, represented outliers, or had missing information. After data curation, a total of 14,737 bulls submitted to a single BSE were included in the dataset: 13,220 (89.7%) bulls belonged to the MS farm and 1,517 (10.3%) to the BA farm.

For data analysis, the Statistical Analysis System [30] was used. Initially, we carried out a collinearity diagnosis. Categorical explanatory variables (nominal and ordinal) were dichotomized and, together with quantitative variables, evaluated in Reg Procedure. The VIF (variation inflation proportion) values were recorded. A VIF value higher than 5 was considered as potentially problematic variable.

We evaluated the probability of a bull to be approved in the first BSE based on explanatory variables: calving date (in days), age group (bi-monthly based), farm, and year of birth. For calving date, we considered the date of the first calving of the calving-season as day 0 (zero).

The explanatory variables were evaluated for their potential prognostic value, based on univariate logistic regression analyses using the Logistic Procedure (data not shown). For multivariate logistic regression analysis, the order of the explanatory variables in the forward selection was designed according to prognostic value. The Akaike's information criterion (AIC) was estimated in each step of the forward selection and chosen to balance model fit and parsimony. If the AIC decreased, the respective independent variable was kept in the model in the next step.

The effects of the explanatory variables on the bull approval in the BSE were evaluated using GLIMMIX Procedure, with each bull as an experimental unit, explanatory variables (calving date, age group, and farm) as fixed effects, and the intercept for each year of birth as a random effect. Probabilities were calculated using the general formula [31].

$$P = \frac{e^{\beta_0+\beta_1 x_1+\cdots+\beta_p x_p}}{1+e^{\beta_0+\beta_1 x_1+\cdots+\beta_p x_p}}$$

The calving date for a threshold of 80% of approval was calculated with the following formula:

$$X_1 = \frac{\ln\left(\frac{P}{e^{\beta_0}.e^{\beta_2 x_2}.(1-P)}\right)}{\beta_1}$$

Where: X_1: Calving date; P: Probability of approval at the suggested threshold (80%); β_0, β_1 e β_2: coefficients estimated by the logistic regression.

Correlations between calving date, scrotal measurements and semen traits were evaluated by Pearson`s correlation coefficient (CORR Procedure).

Additionally, a multivariate analysis using GLIMMIX Procedure was performed to evaluate the effects of the calving date, age group, farm (fixed effects), and intercept for each year of birth (random effect) on SC.

3. Results

The overall percentage of bulls classified as satisfactory potential breeders in the first BSE was 79.4%, which corresponded to 11,701 bulls. Table 1 shows the average, minimum, and maximum values for bulls' calving date, SC, testicular volume, and sperm parameters such as motility and sperm defects of the bulls in the first BSE. Additionally, Suppl. Tables 1, 2 and 3 provide the same information divided by farm, age group, and year of birth.

Tests for collinearity showed values of VIF lower than 5 for all explanatory variables (Suppl. Tables 4 and 5).

The effects of calving date, year of birth, and age group at the BSE showed better prognostic value by reducing the values of AIC, but the farm effect showed the opposite effect (Table 2). The effects kept for multivariate mixed logistic regression were bull's age group at

the first BSE and calving date (Table 3). Table 4 shows the results of the multivariate mixed logistic regression to predict probability of approval in Nellore young bulls at the first BSE. Results for random effects are in Suppl. Table 6.

Fig. 1 depicts the probability of approval at the first BSE according to the calving date and age group. The probability of approval decreased when the calving date increased. The chances of approval in the first BSE of a bull that was born on the day 0 of the calving season were 1.26, 1.61, 2.04, and 2.59 times higher than for bulls born 21, 42, 63, and 84 days later in the calving season, respectively (Table 5; 21-d calving periods). It follows that to achieve an 80% probability of approval of Nellore Bulls at the first BSE, the calving date should not exceed 42, 47, 66, and 38 days in the age groups 18-20, 20-22, 22-24, and 24-26 months, respectively.

Fig. 2 shows the correlation coefficient between calving date and sperm motility, sperm defects (major, minor, and total), proximal cytoplasmatic droplet (PCD), SC, and testicular volume. Values for major, minor, and total sperm defects, and PCD were weakly but positively correlated with the calving date ($P < 0.05$). Values for sperm motility were also weakly, but negatively correlated with the calving date. The SC and testicular volume had the strongest correlations with the calving date.

Table 6 shows that the calving date, farm, and age group affect the SC in young Nellore bulls. The estimates for all effects are represented in Table 7. There were differences in the SC between the farms and the age group ($P < 0.05$; Table 8). Hence, Fig. 3 illustrates the estimated SC according to the calving date, farm, and age groups. Thus, the scrotal circumference decreased as the calving date increased. Results for random effects are shown in Suppl. Table 7.

4. Discussion

Calving date has been shown to be a key reproductive trait in beef female genetic improvement programs [15]. Heifers calved at the beginning of the season have better pre-

breeding body weight, cyclicity, and pregnancy rates when compared to the herd mates calved in the late days of the calving season [32]. Calving date also influences the reproductive performance in the subsequent calving seasons; a survival analysis of heifers that calved within the first 21 days of their first calving season showed that they remained in the herd longer than heifers that calved later. They also had an increase in weaning weight that amounted to the production of an extra calf during their lifetime [33]. Other studies showed that heifers that calved earlier produced one more calf in their lifetime than those that calved in the later periods [34]. Thus, each delay of 20 days in a previous calving date means 6.1% more cows not calving in the next season [35].

The hypothesis of our study was supported: males calved earlier in a 5-month calving season are more likely to be approved in their first breeding soundness evaluation when compared to males calved later in the calving season. Data to support the hypothesis was provided by the linear regression showing how the calving date affected the SC, decreasing as the calving date in the bulls increased. Indeed, the SC had the best correlation with the calving date, although the Pearson correlation between the calving date and sperm quality was low. Furthermore, the SC was influenced by the farms and the age group, even though, there was less than a centimeter difference in the SC between the age groups and farms. Low SC in bulls can be reflected in a delay of puberty and poor semen quality and may affect the BSE outcome [36,20], explaining part of the decrease in the probability of approval at a late calving date.

Other factors could potentially explain the greater probability of Nellore bulls being approved at the first BSE related to the calving date. For example, we can suggest that precocious dams produce precocious bulls [37,38]. Also, the calving date influences animal health, nutrition, and range; animals born at the beginning of the calving season are healthier and have better average daily gain than animals born at the end of the calving season [39]. Another reason for the decreased probability of approval of young bulls in their first BSE could

be the epigenetic effect of the maternal environment [40]. That is, the breeding season is usually when females experience a plenitude of available nutrients, and cows that calved at the beginning of the calving season, conceived at the beginning of the breeding season. In this sense, early pregnancy is when testis development occurs, and any maternal dietary protein perturbation during conception and early gestation may delay puberty in the offspring [40].

Most studies suggest that the age at the BSE is the main factor influencing the outcome of the BSE in young bulls, since semen quality is related to puberty and sexual maturity in bulls [41]. In Nellore bulls, puberty is reached by 12-13 mo. [42,43] and the age at the first BSE in this study varied from 18 to 26 mo. This means there is enough time for the bulls to reach sexual maturity and consequently be approved in the BSE. However, multiple bulls do not achieve this. Therefore, the idea of including the calving date as a predictor of the probability of approval may result in more accurate estimates, perhaps because in a well-established breeding season, the calving date will influence all the factors mentioned above. This includes the SC, the age at the first BSE, the health and body weight, and may also benefit from indirect selection for fertility since the cows that calved early in the breeding season tend to be more fertile than the cows that calved later in the breeding season. Furthermore, the curve of the probability of approval of Nellore bulls at the first BSE according to the calving date in the 24-26 month age group was lower than in the younger age groups. This finding supports the idea that the age at evaluation is not the only factor influencing the BSE outcome and, in this study, could be explained by bulls with delayed puberty being evaluated later in the BSE season.

Interestingly, in our 21-d calving period analysis, bulls born on day 0 of the calving season were 1.26 times more likely to pass the first BSE than bulls born on the 21st day. The significant decrease in the odds ratio of success in the first BSE can be translated into significant losses in the operation. Similar to open cows after the breeding season, bulls not approved for reproduction before the breeding season are usually culled from the herd, representing larger

losses in sustainability and profitability. To achieve an 80% approval in the first BSE of 20-22 mo. Nellore bulls, the calving date threshold should not exceed 47 days. Under the conditions of present study, such a measure could help the cow calf producer choose the future bulls for BSE, thereby saving the time and money spent on the testing and sorting of the low probable bulls for early culling.

The novelty of this study is in showing that the calving date affects the probability of candidate breeding bulls to be approved in the first BSE. This is important information to producers, with two potential outcomes. First, producers can decide to select bulls with a higher probability of being approved in the BSE based on their calving date within the BSE season. Second, producers can select cows that calve earlier in the calving season. Finally, producers can choose to use biotechnologies that help to concentrate calving at the beginning of the season, producing not only more profitable heifers, but also more productive young bulls. Some of these strategies include timed artificial insemination and resynchronization programs to increase the percentage of calving during the first 21 to 30 days of the calving season or to reduce the length of the calving season and alter the distribution of calves born early in the calving season [44,9].

5. Conclusion

In conclusion, this study shows that the calving date potentially affects the reproductive performance of male calves. Although we did not measure reproductive efficiency directly, indirect measurements of reproduction performance were used in this retrospective study to evaluate the probability of approval of Nellore bulls at their first BSE according to the calving date within the calving season of two farms located in Brazil. Thus, the calving date is a potential predictor of the outcome of the first BSE in young bulls. That is, Brazilian Nellore bulls calved at the beginning of the calving season had more chances to be approved at their

first BSE than bulls born later at the calving season. Therefore, producers can make important management decisions based on this information. Moreover, there is a need to estimate the magnitude of heritability and genetic correlations for this trait in young bulls for the purpose of genetic selection aimed at the genetic improvement of herd reproduction.

CRediT authorship contribution statement

Edgar Diaz-Miranda: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft preparation **Jurandy Penitente-Filho**: Conceptualization, Methodology, Validation, Formal analysis, Writing – review and editing **Victor Gomez-Leon:** Visualization, Investigation, Writing - Review & Editing **Camilo Lopez:** Investigation **Faider Villadiego:** Investigation **Denise Okano:** Investigation **Tamires Neto:** Resources **Simone Guimarães:** Supervision, Writing - Review & Editing **Jeanne Siqueira:** Investigation **José Guimarães:** Conceptualization, Supervision, Writing – review and editing, Project administration, Funding acquisition.

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The authors declare no conflict of interest.

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TABLES

Table 1. Descriptive statistics of the calving date, and biometric and semen traits from young Nellore bulls at the first breeding soundness evaluation (BSE).

Variable	n	Mean (SD)	Minimum	Maximum
Calving date (days)	14737	49.57 (27.33)	0	140
Scrotal circumference (cm)	14688	33.54 (2.60)	23.7	44.6
Testicular volume (cm^3)	14439	354.75 (79.01)	108.48	899.86
Motility (%)	13648	69.01 (12.68)	0	95
Total defects (%)	13634	19.02 (13.22)	3	168
Major defects (%)	13634	14.12 (11.71)	1	146
Minor defects (%)	13634	4.90 (4.75)	0	71

Table 2. Comparison of Akaike information criterion (AIC) values for the forward selection of variables for the logistic model and the contribution of each variable to model fit.

Variable added to the model	Value of AIC	Change in AIC value after adding a variable [1]
Intercept only	14979.706	NA [2]
Calving date	14703.239	-276.467
Year of birth	14558.702	-144.537
Age group	14554.578	-4.124
Farm	14555.306	+0.728

[1] In the forward selection, variables were added to the logistic model in descending order (starting with the "Intercept only" model and ending with adding "Farm"). In each step, the AIC after adding a variable was compared with the AIC of the previous step.

[2] Not applicable.

Table 3. Type III tests of fixed effects for multivariate mixed logistic regression analysis of the first breeding soundness evaluation (BSE) outcome according to the calving date and age group.

Effect	F Value	Pr > F
Calving date	90.28	<.0001
Age group	3.25	0.0209

Table 4. Solution for fixed effects for multivariate mixed logistic regression analysis of the first breeding soundness evaluation (BSE) outcome according to the calving date and age group.

Effect	Estimate	t Value	Pr > |t|	95% CL	
				Lower	Upper
Intercept	1.8631 ± 0.1692	11.01	<.0001	1.4491	2.2772
Calving date	-0.01136 ± 0.001196	-9.50	<.0001	-0.01370	-0.00902
Age group (18_20) ref.	0	.	.	.	.
Age group (20_22)	0.06424 ± 0.08131	0.79	0.4295	-0.09514	0.2236
Age group (22_24)	0.2719 ± 0.1189	2.29	0.0222	0.03882	0.5050
Age group (24_26)	-0.04142 ± 0.2693	-0.15	0.8777	-0.5692	0.4864

Table 5. Odds ratio from multivariate mixed logistic regression analysis of the first breeding soundness evaluation (BSE) outcome in Nellore bulls.

Effect	Odds ratio	Lower 95% CL	Upper 95% CL
Age group			
18_20 (ref.)	-	-	-
20_22	1.066	0.909	1.251
22_24	1.312	1.040	1.657
24_26	0.959	0.566	1.626
Calving date			
0 vs 21	1.269	1.333	1.209
0 vs 42	1.610	1.779	1.460
0 vs 63	2.045	2.370	1.764
0 vs 84	2.597	3.165	2.132

Table 6. Type III tests of fixed effects for multivariate analysis of the scrotal circumference (SC) according to the farm, calving date, and age group.

Effect	F Value	Pr > F
Farm	71.47	<.0001
Calving date	257.92	<.0001
Age group	50.52	<.0001

Table 7. Solution for fixed effects for multivariate analysis of the scrotal circumference (SC) according to the farm, calving date, and age group.

Effect	Estimate	t Value	Pr > |t|	95% CL	
				Lower	Upper
Intercept	33.8817 ± 0.3089	109.70	<.0001	33.1259	34.6375
Farm (MS) ref.	0	.	.	.	.
Farm (BA)	0.5789 ± 0.06848	8.45	<.0001	0.4447	0.7131
Calving date	-0.01913 ± 0.001191	-16.06	<.0001	-0.02147	-0.01680
Age group (18_20) ref.	0	.	.	.	.
Age group (20_22)	0.4851 ± 0.08430	5.75	<.0001	0.3199	0.6503
Age group (22_24)	1.1763 ± 0.1176	10.00	<.0001	0.9459	1.4068
Age group (24_26)	1.9786 ± 0.2423	8.17	<.0001	1.5037	2.4536

Table 8. LS-means of the scrotal circumference (SC) according to the farm and age group.

Variable	Estimate	Standard Error
Farm		
BA	34.4225 [a]	0.2934
MS	33.8436 [b]	0.2872
Age group		
18_20	33.2230 [d]	0.2937
20_22	33.7081 [c]	0.2837
22_24	34.3994 [b]	0.2884
24_26	35.2017 [a]	0.3561

Different letters within a column indicate significant differences $P < 0.05$ by Tukey–Kramer test.

FIGURES

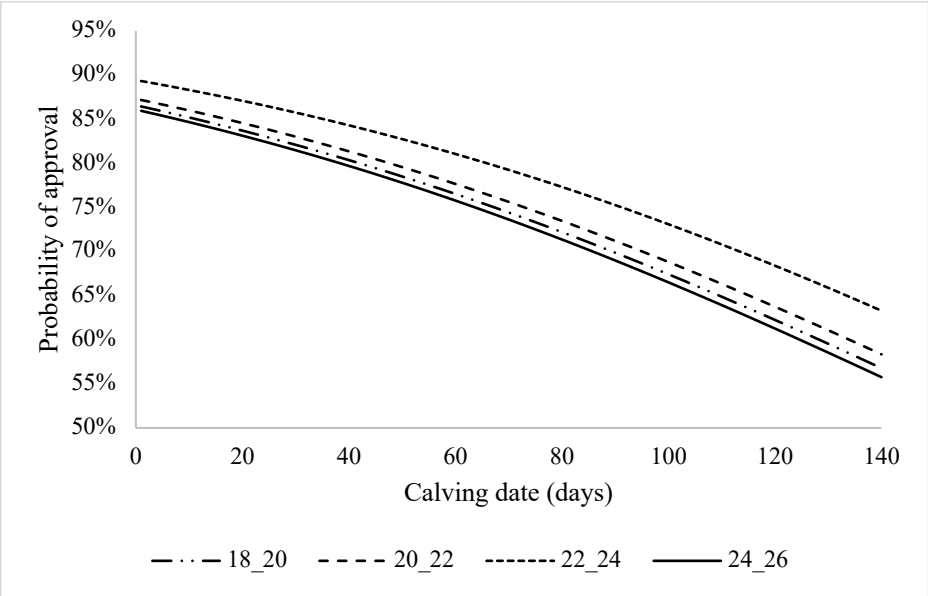

Fig. 1. Probability of approval of Nellore bulls at the first breeding soundness evaluation (BSE) according to the calving date and age group.

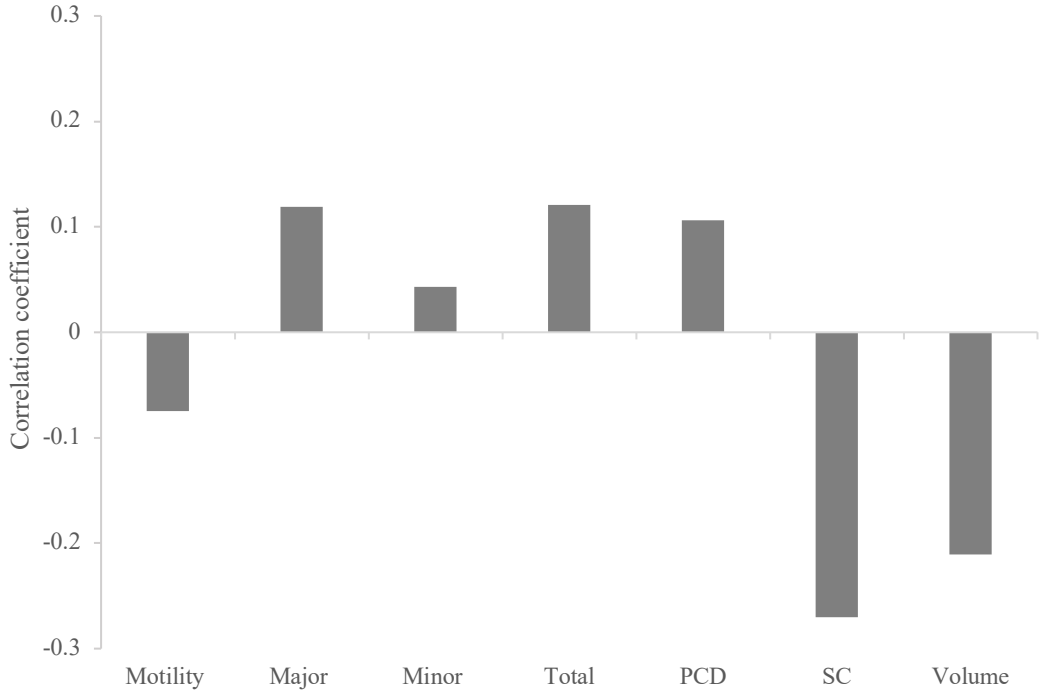

Fig. 2. Correlation coefficient of the Biometric and semen traits with the calving date in young Nellore bulls at the first breeding soundness evaluation (BSE). Major and minor classification (Blom, 1973); Total: Total defects; PCD: Proximal cytoplasmatic droplet; SC: Scrotal circumference; Volume: Testicular volume (Bailey et al., 1998).

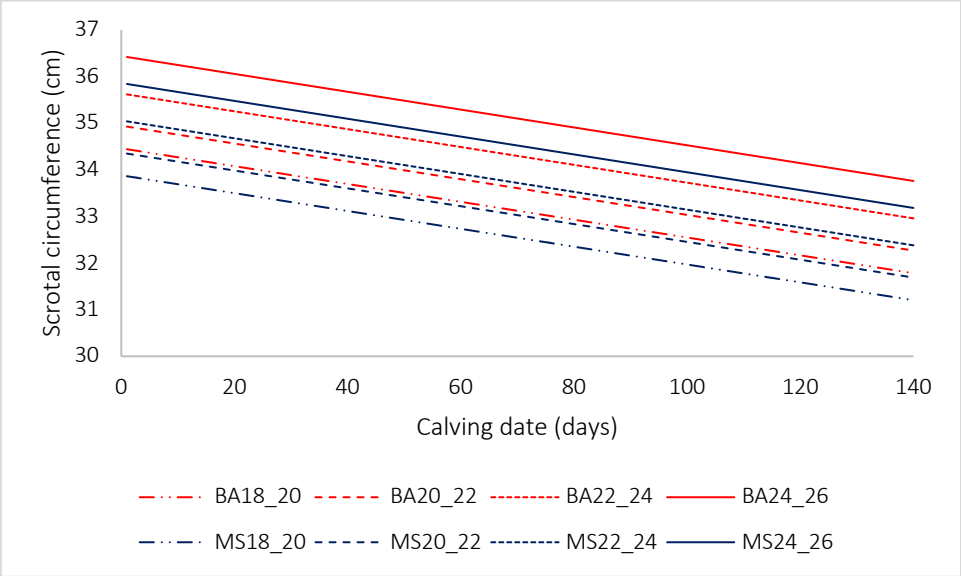

Fig. 3. Scrotal circumference (SC) of Nellore Bulls at the first breeding soundness evaluation (BSE) according to the calving date, farm, and age group.

SUPPLEMENTARY MATERIAL

Suppl. Table 1. Descriptive statistics of the calving date, biometric and semen traits from young Nellore bulls at the first breeding soundness evaluation (BSE) by farm.

Farm	n	Variable	Mean (SD)	Minimum	Maximum
BA	1517	Calving date (days)	70.9 (27.3)	4	138
	1517	Age (months)	20.6 (1.1)	17.9	23.9
	1512	Scrotal circumference (cm)	33.3 (2.6)	24.3	43.3
	1484	Testicular volume (cm^3)	342.6 (76.8)	108.5	812.2
	1344	Motility (%)	70.1 (12.7)	0	95
	1340	Total defects (%)	19.4 (12.5)	4	101
	1340	Major defects (%)	14.4 (11.0)	2	93
	1340	Minor defects (%)	5.1 (4.7)	0	50
MS	13220	Calving date (days)	47.1 (26.2)	0	140
	13220	Age (months)	21.3 (1.0)	18.0	25.0
	13176	Scrotal circumference (cm)	33.6 (2.6)	23.7	44.6
	12955	Testicular volume (cm^3)	356.2 (79.1)	108.6	899.9
	12304	Motility (%)	68.9 (12.7)	0	95
	12294	Total defects (%)	19.0 (13.3)	3	168
	12294	Major defects (%)	14.1 (11.8)	1	146
	12294	Minor defects (%)	4.9 (4.8)	0	71

Suppl. Table 2. Descriptive statistics of the calving date, biometric and semen traits from young Nellore bulls at the first breeding soundness evaluation (BSE) by year.

Year	n	Variable	Mean (SD)	Minimum	Maximum
2012	2892	Calving date (days)	54.3 (29.7)	0	140
	2892	Age (months)	21.6 (1.3)	18.2	25.0
	2856	Scrotal circumference (cm)	33.8 (2.7)	25.0	44.0
	2812	Testicular volume (cm^3)	363.8 (86.1)	142.8	791.1
	2725	Motility (%)	69.2 (10.6)	0	95
	2723	Total defects (%)	17.7 (12.0)	4	112
	2723	Major defects (%)	13.1 (10.9)	2	103
	2723	Minor defects (%)	4.6 (3.7)	0	53
2013	2382	Calving date (days)	46.3 (27.0)	0	138
	2382	Age (months)	21.3 (0.9)	18.3	22.8
	2379	Scrotal circumference (cm)	33.7 (2.6)	25.4	43.9
	2335	Testicular volume (cm^3)	358.6 (84.2)	111.9	827.6
	2221	Motility (%)	67.7 (11.3)	0	95
	2226	Total defects (%)	19.7 (12.8)	3	112
	2226	Major defects (%)	14.7 (11.5)	3	105
	2226	Minor defects (%)	5.0 (4.1)	0	57
2014	1714	Calving date (days)	41.7 (26.2)	0	132
	1714	Age (months)	21.4 (0.9)	18.5	22.8
	1713	Scrotal circumference (cm)	33.1 (2.5)	25.0	42.5
	1701	Testicular volume (cm^3)	309.2 (75.4)	108.5	679.0
	1582	Motility (%)	68.1 (13.7)	0	95

	1574	Total defects (%)	22.5 (14.1)	5	100
	1574	Major defects (%)	17.8 (12.9)	4	93
	1574	Minor defects (%)	4.8 (3.9)	0	59
2015	2338	Calving date (days)	51.9 (28.5)	0	136
	2338	Age (months)	21.0 (1.0)	18.2	24.4
	2332	Scrotal circumference (cm)	32.2 (2.5)	23.7	43.3
	2230	Testicular volume (cm^3)	345.4 (72.7)	185.5	812.2
	2042	Motility (%)	70.7 (12.1)	0	95
	2047	Total defects (%)	19.0 (12.4)	4	106
	2047	Major defects (%)	14.0 (10.8)	2	88
	2047	Minor defects (%)	5.0 (4.9)	0	64
2016	1474	Calving date (days)	46.3 (23.2)	0	130
	1474	Age (months)	21.2 (0.8)	18.4	23.6
	1474	Scrotal circumference (cm)	34.0 (2.6)	24.9	44.6
	1455	Testicular volume (cm^3)	368.1 (76.1)	164.9	899.9
	1382	Motility (%)	67.0 (13.2)	0	95
	1379	Total defects (%)	17.6 (12.1)	3	135
	1379	Major defects (%)	12.7 (10.2)	2	122
	1379	Minor defects (%)	4.8 (5.4)	0	71
2017	2044	Calving date (days)	51.3 (26.2)	0	128
	2044	Age (months)	20.9 (0.9)	18.4	22.6
	2043	Scrotal circumference (cm)	33.9 (2.4)	24.3	42.5
	2023	Testicular volume (cm^3)	360.8 (67.5)	146.6	651.1
	1878	Motility (%)	71.9 (15.8)	0	95

	1874	Total defects (%)	18.8 (14.1)	4	152
	1874	Major defects (%)	13.9 (12.6)	2	146
	1874	Minor defects (%)	4.9 (5.0)	0	66
2018	1893	Calving date (days)	51.3 (25.5)	0	131
	1893	Age (months)	20.6 (0.9)	17.9	22.4
	1891	Scrotal circumference (cm)	34.2 (2.4)	26.7	42.8
	1883	Testicular volume (cm^3)	371.9 (68.3)	189.9	686.8
	1818	Motility (%)	67.7 (12.1)	0	95
	1811	Total defects (%)	18.5 (14.7)	4	168
	1811	Major defects (%)	13.2 (12.6)	1	142
	1811	Minor defects (%)	5.4 (6.3)	0	64

Suppl. Table 3. Descriptive statistics of the calving date, biometric and semen traits from young Nellore bulls at the first breeding soundness evaluation (BSE) by age group.

Age group	n	Variable	Mean (SD)	Minimum	Maximum
18_20	1853	Calving date (days)	97.3 (15.1)	65	140
	1853	Age (months)	19.4 (0.4)	17.9	20.0
		Scrotal circumference			
	1849	(cm)	32.2 (2.6)	24.3	43.3
		Testicular volume	321.8		
	1770	(cm^3)	(70.1)	108.6	812.2
	1609	Motility (%)	66.9 (13.7)	0	95
	1609	Total defects (%)	22.2 (15.7)	4	120
	1609	Major defects (%)	17.0 (14.3)	2	117
	1609	Minor defects (%)	5.2 (5.2)	0	51
20_22	9917	Calving date (days)	48.4 (17.5)	5	135
	9917	Age (months)	21.1 (0.5)	20.0	22.0
		Scrotal circumference			
	9884	(cm)	33.5 (2.5)	23.7	43.0
		Testicular volume	352.9		
	9734	(cm^3)	(76.5)	108.5	814.6
	9208	Motility (%)	68.9 (12.9)	0	95
	9189	Total defects (%)	18.9 (13.3)	3	168
	9189	Major defects (%)	14.0 (11.7)	1	146
	9189	Minor defects (%)	4.9 (4.9)	0	71
22_24	2831	Calving date (days)	23.7 (21.3)	0	93
	2831	Age (months)	22.4 (0.4)	22.0	24.0

		Scrotal circumference			
	2820	(cm)	34.5 (2.5)	25.2	44.6
		Testicular volume	378.4		
	2799	(cm^3)	(82.7)	122.5	899.9
	2699	Motility (%)	70.4 (11.1)	0	95
	2704	Total defects (%)	17.5 (11.0)	4	125
	2704	Major defects (%)	12.9 (9.7)	2	108
	2704	Minor defects (%)	4.6 (3.9)	0	55
24_26	136	Calving date (days)	20.5 (7.3)	3	33
	136	Age (months)	24.4 (0.2)	24.0	25.0
		Scrotal circumference			
	135	(cm)	35.6 (2.6)	28.4	43.4
		Testicular volume	429.8		
	136	(cm^3)	(89.0)	229.1	700.8
	132	Motility (%)	73.7 (12.0)	5	95
	132	Total defects (%)	17.1 (10.4)	7	76
	132	Major defects (%)	12.0 (9.4)	4	70
	132	Minor defects (%)	5.1 (3.5)	1	23

Suppl. Table 4. Collinearity diagnosis. Parameter estimates, standard errors, and variance inflation values for explanatory variables. Dependent variable: Breeding soundness evaluation (BSE) outcome (0, not approved; 1, approved).

Variables	Parameter Estimates	Standard Error	Variance Inflation
Intercept	0.86198	0.02012	0
Calving date	-0.00179	0.00018661	2.39336
Farm (MS)	0	.	.
Farm (BA)	0.00064603	0.01127	1.07932
Age group (18_20)	0	.	.
Age group (20_22)	0.02034	0.01352	3.70151
Age group (22_24)	0.03696	0.01803	4.64177
Age group (24_26)	0.02030	0.03824	1.23013

Suppl. Table 5. Collinearity diagnosis. Parameter estimates, standard errors, and variance inflation values for explanatory variables. Dependent variable: Scrotal circumference (SC).

Variables	Parameter Estimates	Standard Error	Variance Inflation
Intercept	33.97585	0.12592	0
Calving date	-0.01910	0.00117	2.40093
Farm (MS)	0	.	.
Farm (BA)	0.30628	0.07048	1.07980
Age group (18_20)	0	.	.
Age group (20_22)	0.41053	0.08460	3.70792
Age group (22_24)	0.96680	0.11286	4.65132
Age group (24_26)	2.05014	0.23955	1.23003

Suppl. Table 6. Solution for random effects of the first breeding soundness evaluation (BSE) outcome analysis.

Effect	Subject	Estimate	Std Err Pred
Intercept	year 2012	0.1227	0.1206
Intercept	year 2013	0.02084	0.1204
Intercept	year 2014	-0.5530	0.1211
Intercept	year 2015	-0.1584	0.1194
Intercept	year 2016	0.1621	0.1258
Intercept	year 2017	0.07011	0.1214
Intercept	year 2018	0.3357	0.1241

Suppl. Table 7. Solution for random effects of the scrotal circumference (SC) analysis.

Effect	Subject	Estimate	Std Err Pred
Intercept	year 2012	0.2105	0.2852
Intercept	year 2013	0.03868	0.2852
Intercept	year 2014	-0.7102	0.2863
Intercept	year 2015	-1.2835	0.2852
Intercept	year 2016	0.4340	0.2870
Intercept	year 2017	0.4790	0.2857
Intercept	year 2018	0.8315	0.2861

CHAPTER 3

Title: "Proteomic and morphofunctional study of bull infertility with emphasis on the aplastic midpiece defect"

ABSTRACT

Abnormal sperm morphology is a major factor contributing to male infertility. Although there is a growing list of genetic sperm defects, in most cases, its etiology remains unknown. Therefore, we aimed to understand the origin and factors contributing to the abnormal sperm production of the aplastic midpiece defect (AMD). Six dairy Gyr bulls obtained from the same farm and under identical management were castrated to conduct a sperm morphofunctional and proteomic study, and to evaluate the redox state of reproductive tissues. The bulls were divided into two groups consisting of three bulls with AMD and three control bulls characterized by the breeding soundness evaluation as satisfactory potential breeders. The morphological study showed that the AMD spermatozoa originated in the testis, and there was a reduction in the percentage of abnormal cells that reached ejaculation in both groups. Surprisingly, the control bulls also produced AMD spermatozoa, but it was removed prior to epididymal passage. The defective sperm removal process probably generated mild oxidative stress in the caput epididymis milieu, reflected in an increase of malondialdehyde and catalase levels in the control group. Nevertheless, the oxidative stress was controlled before reaching the cauda epididymis. There were no changes in the gross testicular morphometric parameters. However, there was an increase of acridine orange positive cells in all the epididymis epithelium regions of the AMD bulls. The proteomic analysis suggested deficient sperm maturation in the AMD group due to an alteration in the protein expression of the epididymal sperm. Sperm collected from the caput epididymis of the AMD group showed a downregulation (log2-fold change cutoff ≥ 3) of proteins involved in the oxidative phosphorylation and sperm-egg interactions, and an

upregulation of proteins involved in proteolysis in spermatozoa collected from the cauda epididymis. These alterations were also evident in the KEGG pathway analysis of the unique proteins between groups, where the control group showed an enrichment of the oxidative phosphorylation during epididymal transit. Furthermore, the AMD group had an enrichment in the proteasome and phagosome pathways, probably related to epididymal sperm quality control. On the other hand, testicular upregulated proteins in AMD spermatozoa that participate in the aforementioned pathways, including PSME2 and NEDD8, could be targeted for further studies of this mechanism. This study allowed us to have a better understanding of the processes involved for abnormal sperm production.

Keywords: sperm morphology, oxidative stress, bull fertility.

1. Introduction

Sperm morphology is a key determinant of semen quality and male fertility. Several morphological and molecular changes occur during spermiogenesis for spermatids to become spermatozoon, including nuclear condensation and elongation, acrosome and tail formation, organelle reorganization, and elimination of excess residual cytoplasm upon spermiation. An error in one of these steps, caused by spermatogenesis-specific gene mis-expression/mutation could result in the production of aberrant spermatozoa, and ultimately in infertility (YAN, 2009; HERMO et al., 2010). The sperm tail is divided into four segments defined by the nature of the sheaths that envelope the core complex of the axoneme and outer dense fibers. (FAWCETT, 1975). One of these segments is the midpiece, characterized by the mitochondrial sheath. Several factors may influence the correct formation of the mitochondrial sheath, including sufficiently structured and mature mitochondria, the protein transport of all components, and their structural assembly (SUZUKI-TOYOTA et al., 2007; LEHTI and SIRONEN, 2017).

Indeed, any failure of these steps can contribute to asthenoteratozoospermia or male infertility due to mitochondrial sheath aplasia because a well-organized mitochondrial sheath plays a role as a support structure, as well as an energy generator for sperm motility and hyperactivation, which is essential for sperm capacitation.

Within this framework, it is crucial to understand the mechanisms of the formation of abnormal mitochondrial sheath, mainly when these defects occur naturally and are not induced as knockout or mutant models (TAYLOR et al., 2018). Thus, various types of sperm abnormalities are a consequence of defective spermiogenesis. However, some tail and midpiece defects may also result from the incomplete sperm maturation during epididymal passage or from the conjunction of the two factors (KOEFOED-JOHNSEN and PEDERSEN, 1971; SUZUKI-TOYOTA et al., 2007). Factors that maintain a normal (by morphological criteria) tail and mitochondrial sheath structure prior to ejaculation can be studied by proteomic approaches. Identifying the origin of sperm abnormalities and how specific proteins modulate the pathways that influence these phenotypes could be a target for improved diagnostics and treatment of male infertility.

Spermatozoa are specialized and polarized cells produced in the testis in a complex process called spermatogenesis (TURNER, 2003). After the spermatozoa are released from the testis, they enter and travel down the epididymis, where they undergo the maturation process in a complex series of events that lead to the establishment of capacity for progressive motility and fertility (HINTON et al., 1996). The places where spermatogenesis and sperm maturation occur are vulnerable to oxidative stress and, as a result, spermatozoa are susceptible as well. However, in order to maintain the redox equilibrium, the testis and the epididymis are equipped with a complex array of defense systems, including enzymatic and non-enzymatic antioxidants (VERNET et al., 2004; AITKEN and ROMAN, 2008).

There are important sources of reactive oxygen species (ROS) in the testicular and epididymal environment, including phagocytic cells such as Sertoli cells and polymorphonuclear leukocytes in the testis that physiologically produce ROS at 1000-fold greater levels than spermatozoa (BAUCHÉ et al., 1994; PLANTE et al., 1994). This ROS production can result from normal physiological processes or be a consequence of cell activation, like immunological responses or apoptosis (VERNET et al., 2004; HENKEL, 2011). The testis, for example, is very rich in highly unsaturated fatty acids (AITKEN and ROMAN, 2008). In the epididymal caput, there is a higher vascularization and blood flow than in the more distal regions (HINTON et al., 1995). Therefore, particular features make some organs of the male genital tract vulnerable to oxidative stress.

Previous studies reported the removal of abnormal spermatozoa prior to ejaculation (ROUSSEL et al., 1967; CRABO et al., 1971; SINOWATZ et al., 1979; RAO et al., 1980; GOYAL, 1982; SUTOVSKY et al., 2015). So far, the most widely accepted explanation is that the defective spermatozoa become ubiquitinated on their surface during epididymal sperm maturation, making the prone to intraluminal andor endocitotic recycling. Then, these abnormal spermatozoa could be broken down intraluminally during epididymal passage by epididymal fluid proteases, and some of the resultant debris phagocytosed by epididymal dendritic cells (SUTOVSKY et al., 2015). However, some spermatozoa escape this mechanism, making the ubiquitination of sperm surface a negative male fertility biomarker, reflecting the semen content of defective spermatozoa (SUTOVSKY et al., 2015a).

A previous study hypothesized that ejaculates containing a high percentage of spermatozoa with aplastic midpiece defect were under oxidative stress (DIAZ-MIRANDA et al., 2019). Therefore, the present study aimed to identify the origin of the aplastic midpiece defect, the sperm protein profile, and morphological changes during the testicular sperm release

and epididymal transit, in addition to analyzing the microscopic and redox state changes in testis and epididymis of bulls with AMD.

2. Materials and methods

2.1 Ethics and animals

The experiment was conducted as described in our previous study (DIAZ-MIRANDA et al., 2019). Six dairy Gyr bulls (*Bos taurus indicus*; 24 to 36 months of age) were obtained from the same herd and classified into two groups by Breeding Soundness Evaluation (BSE). The control group (n = 3) was composed of animals classified as satisfactory potential breeders, while bulls exhibiting a high percentage of aplastic midpiece defect (AMD) and classified as unsatisfactory potential breeders (n = 3) were grouped in the AMD group. The experiment was conducted at the dairy cattle research facility at the Universidade Federal de Viçosa (UFV), Viçosa, Brazil. Animal care procedures and research protocols were approved by the Ethics Committee of Animal Use (CEUA, protocol no. 44/2018).

2.2 Semen and reproductive organs collection

Bulls were on a routine semen collection using electroejaculation before this investigation. Eight ejaculates were collected from each bull, and an aliquot of each sample was fixed in isotonic formol saline (HANCOCK, 1957) for the sperm morphology analysis. The bulls were weighed and castrated as described by SILVA et al. (2003), and the gonads were weighed and collected. Segments of the testis and epididymis were used to collect spermatozoa. Pieces of tissue were also fixed for histological analysis, as well as flash frozen in liquid nitrogen and then stored at -80 ^{o}C for the oxidative stress analysis. After collecting the above samples, testis from each animal was frozen and dissected to determine the percentage occupied by the tunica albuginea and the testicular mediastinum (AMANN, 1970).

2.3 Sperm collection from testis and epididymis tissue

For the proteomic analysis, spermatozoa were harvested from testis, and caput and cauda epididymis. Spermatozoa from testis reflects the gamete proteome after spermatogenesis, while sperm from caput and cauda epididymis represents the beginning of the maturation phase (immature gamete) and the end of this physiological event (mature gamete), respectively. Pieces of testis, caput and cauda epididymis were rinsed in sterile normal saline solution to remove the blood. Subsequently, the pieces were transferred to a sterile Petri dish containing a prewarmed (37 ºC) Bigger, Whitten and Whittingham medium (BWW). Then, they were gently crushed and poked using a sterile needle to facilitate the release of spermatozoa. After 30 min of incubation, the sperm-containing medium was filtered with a 100 µm Cell Strainer (Corning®). The spermatozoa were separated from testicular/epididimal fluid and debris by centrifugation through single-layer 30% Percoll, which was prepared by diluting a 90% Percoll (90 mL of Percoll with 10 mL 10× Ham's F10) with BWW (1:3). The 30% Percoll was loaded in a 15 mL plastic Falcon tube and gently overlaid with the semen sample, and then centrifuged at 500× g for 20 min. The sperm pellet was resuspended in red blood cells (RBC) Lysis buffer (10 mg EDTA, NH4Cl 2.075 g/NaHCO3 0.5 g in 250 mL pH 7.4) and washed three times at 700×g for 15 min until the red blood cells were removed. Finally, sperm samples were washed twice in BWW at 500×g for 5 min before proceeding with the protein extraction. Aliquots from the isolated spermatozoa were fixed in isotonic formol saline (HANCOCK, 1957) for the sperm morphology analysis.

2.4 Sperm morphology analysis

Two morphological analyses were performed. The first analysis aimed to determine whetheher the AMD originated in the testes; therefore, ejaculated and testicular spermatozoa from the control and AMD groups were compared. Based on the results of the first analysis, a second analysis in one bull was performed to investigate the sperm morphology changes from

the testis to the epididymis. After sperm fixation, the sperm morphology was assessed by phase-contrast microscopy of wet mount and classified according to the subcellular localization of the sperm defect. In the testicular spermatozoa, the cytoplasmatic droplets were excluded since they were considered physiological and not removed until epididymal passage. All morphological abnormalities were recorded in order to consider more than one abnormality within a single spermatozoa in the evaluation and analysis

2.5 Sperm protein extraction and digestion

Sperm samples stored at -80 ºC were resuspended in acetic acid and acetonitrile solution. Subsequently, the samples from each bull were pooled according to the experimental group (AMD vs control), dried in a speed vac, and stored at − 20 ◦C for further analysis. Protein digestion was performed as described previously (VIANA et al., 2018). After reduction and alkylation, all samples were digested with trypsin (Promega, Fitchburg, WI, USA) at 37 ◦C for 18 h, with a 1/50 (w/w) enzyme/substrate ratio. Then, a solution of 1% trifluoroacetic acid was added to stop the tryptic activity.

2.6 Mass spectrometry

An Easy nLC II nanoflow HPLC system (Thermo Scientific) was coupled to an LTQ Orbitrap XL mass spectrometer (Thermo, Bremen, Germany). Tryptic sperm peptides from each animal were loaded onto a trap column (2 cm, 100 µm internal diameter) packed in house with C18 resin (5 µm, 100Å pore, Magic C18 AQ, Bruker, Auburn, CA) and fractionated on an RP-HPLC column (30 cm, 75 µm internal diameter), in two technical replicates. The chromatography was conducted by applying the gradient condition of 2-40% of 0.1% trifluoroacetic acid (v/v) in acetonitrile solution for 162 min and 80% of the same solution for the last 4 min. To equilibrate the column, the 80% solution was maintained for 2 min.

The eluted peptides were analyzed by an LTQ Orbitrap XL MS, with a resolving power of 60,000, generating MS1 spectra between 300 m/z to 1700 m/z. After exclusion of singly charged ions, the ten most intense precursor ions were selected for fragmentation (MS2) by collision-induced dissociation (CID), with a normalized collision energy of 35%, and a threshold for detection of 10,000 counts (COUTO-SANTOS et al., 2021).

2.7 Database search and Analysis of functional clusters

The data searches were performed using Peaks Studio 8.5. against Uniprot-bovine repositories in September 2019, using the following parameters: two missed enzymatic cleavage, Carbamidomethyl-Cys residues, and oxidized-Met as fixed modification, whereas phosphorylation (P) was used as a variable modification. In addition, a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 20 ppm were also set. The PEAKS decoy fusion approach was used to estimate the False Discovery Rates (FDR). The criteria used to establish FDR values at peptide and protein levels included a peptide-spectrum match FDR of $p<0.01$ and a minimum of one unique peptide per protein.

In order to determine which key pathways were involved in the development or maturational changes of spermatozoa with AMD, functional clusters were analyzed through the DAVID platform (DAVID Functional Annotation Bioinformatics Analysis - https: //david.ncifcrf.gov; HUANG et al., 2009). The analysis was conducted separately for the groups of proteins detected in the control and AMD groups. They were compared with the metabolic pathways annotated in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, clustered and defined according to enrichment scores and p-values.

2.8 Testis and epididymis histology

Pieces of testis and epididymis regions (caput, corpus, and cauda; n = 3/group) were immersed in paraformaldehyde solution (4% PF in 0.1 M phosphate buffer, pH 7.2) for 12

hours, dehydrated in ascending ethanol series, and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Sections with a thickness of 3 μm were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany), and stained with toluidine blue-sodium borate (1%). Images were obtained using light microscopy (Olympus CX40, Tokyo, Japan) and analyzed with ImageJ software (National Institute of Health, USA).

Other histological sections of 1 μm from testis and epididymis were stained with acridine orange (AO) and propidium iodide (PI) (DE ALMEIDA LIMA et al., 2018). PI stains only dead cells, while AO is a vital dye that stains living and apoptotic cells. It can also stain acidic vesicular organelles, such as lysosomes, where it is trapped due to its property of being permeable in basic media but impermeable after protonated in acidic media. Therefore, it has been used as an autophagy/phagocytosis marker (THOMÉ et al., 2016). Digital images were captured by photomicroscope EVOS fl (Life Technologies, Carlsbad, Canada) and analyzed with ImageJ software according to Oberholzer et al., 1996. A total area of 30×10^4 μm^2 was quantified for each group.

2.9 Testicular histomorphometry

The gonadosomatic index (GSI, %) was calculated from the division of the testicular weight by the body weight, multiplied by 100 (AMANN, 1970). The weight of the testicular parenchyma was calculated by subtracting the weights of the tunica albuginea and the mediastinum from the testicular weight. The mean tubular diameter was obtained by randomly measuring 30 tubular cross-sections that were most circular per animal. The same sections were also used to measure the epithelium height, based on the distance between the basal membrane and the tubular lumen. The epithelium height for each tubule was estimated by the average of two diametrically opposed measurements (SOUZA et al., 2016). The volumetric proportions of the tubular and intertubular compartments were estimated using a square grid and counting

2660 points on 10 images from the histological slides. Points in the tubular compartment (tunica propria, epithelium, and lumen) and the intertubular compartment (Leydig cell and stroma) were recorded. The percentage of points in each component was calculated using the formula: volumetric proportion (%) = (number of points in tubule or intertubule/2660 total points) × 100. The diameters of 30 Leydig cells nuclei were measured in each animal, choosing the ones with the most spherical nuclei and evident nucleoli. The following formulas were used to calculate nuclear volume (NV), cytoplasmic volume (CV) and each Leydig cell volume (LCV): NV= 4/3 πR^3 (R: average nuclear diameter/2); CV= % of cytoplasm x NV/% of nucleus; LCV= NV + CV. These values were expressed in μm^3.

2.10 Oxidative stress markers in the testis and epididymis

The redox markers were evaluated using 100 mg of frozen testis and epididymis (caput, corpus, and cauda) homogenized in 0.1 mol/L PBS pH 7.4 (n = 3/group). Each homogenate was centrifuged at 10,000× g for 10 min at 4 °C. The supernatant was used for the analysis of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) in addition to the lipid peroxidation marker malondialdehyde (MDA). The pellets were used for analyses of protein oxidation. The SOD activity was determined by the pyrogallol method based on the ability of this enzyme to catalyze the superoxide and hydrogen peroxide reactions (DIETERICH et al., 2000). The SOD activity was then calculated as units (U) per milligram of protein, where one U of SOD is defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. The CAT activity was evaluated by measuring the rate of H_2O_2 decomposition, according to AEBI (1984). The extinction coefficient of ε_{240} = 0.036 mmol × L^{-1} × cm^{-1} was used for calculations. One unit of CAT activity was defined as the amount of enzyme that decomposes one mmol H_2O_2 for 1 min. The CAT activity was calculated as U per milligram of protein. The GST activity was estimated spectrophotometrically, as described by HABIG et al. (1974) and assayed according to the formation of glutathione-

conjugated 2,4-dinitrochlorobenzene (CDNB). The molar extinction coefficient used for CDNB was ε340 = 9.6 mmol × L-1 × cm-1. One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of one μmol of product × min-1 × mL-1. The GST activity was expressed as U per milligram of protein. Finally, lipid peroxidation was assessed in these organs analyzing the levels of malondialdehyde (MDA) by a standard curve from known concentrations of 1,1,3,3-tetramethoxypropane (TMPO; BUEGE and AUST, 1978). The results were expressed as nmol per mg of protein. Protein oxidation was analyzed by the quantification of protein carbonyls from the homogenates, using the 2,4-dinitrophenylhydrazine (DNPH) method (LEVINE et al., 1990). The results were expressed as nmol per mg of protein, based on the molar extinction coefficient of ε_{370} = 22 mmol x L^{-1} × cm^{-1}. The total protein level in the reproductive tissue was measured using the Bradford method (BRADFORD, 1976).

2.11 Mineral Microanalysis

The mineral content in the testis, caput, corpus, and cauda epididymis (n = 3/group) was assessed by energy dispersive X-ray spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with an x-ray detector system (Tracor TN5502, Middleton, WI, USA), according to NOVAES et al. (2013). Briefly, tissues previously fixed in paraformaldehyde solution were dehydrated in ethanol series, submitted to critical point drying (CPD 030, Bal-tec, Witten, North RhineWestphalia, Germany), and coated with evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The analysis was performed at a magnification of ×150, using an accelerating voltage of 20 kV and a working distance of 10 mm. The proportion of the co-factors of antioxidant enzymes including selenium (Se), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) was evaluated by EDS and expressed as a mean value.

2.12 Statistical analysis

Statistical Analysis System version 9.0 (SAS Institute Inc., Cary, NC, USA) was used to conduct data analysis. Data were submitted to Kolmogorov-Smirnov and Bartlett tests to verify normality of errors and homogeneity of variances, respectively. Percentage data were submitted to arcsine transformation ($y' = arcsin\sqrt{y}$). Data from SOD activity in the cauda epididymis were submitted to inverse transformation ($y' = 1/y$). Data were submitted to ANOVA at 5% probability and presented as mean and standard error. Proteins were considered up- or down-regulated using a log2-fold change (FC) cutoff ≥ 3, trying to avoid the risk of reporting false-positive hits particularly when comparing pooled samples.

3. Results

3.1 Sperm morphology analysis

We tested the hypothesis that the aplastic midpiece defect was produced as a result of defective spermiogenesis and not from the epididymal transit by using sperm morphology analysis in the AMD group. However, these results also provided an interesting revelation: the abnormal production of the same defect by the testis in the control group and the absence of the AMD defect in the control bulls ejaculates. Nonetheless, the control group produced significantly fewer spermatozoa with AMD than the AMD group ($p > 0.05$). Furthermore, a significant decrease in all the categories of sperm abnormalities in the testicular spermatozoa compared with ejaculated spermatozoa was observed in the control group ($p < 0.05$; Figure 1). When the morphology of the testicular spermatozoa was compared with the ejaculated spermatozoa in the AMD group, a significant decrease was observed only in the acrosome, tail, and total abnormalities. Therefore, both groups showed the same pattern of decreased percentages of sperm abnormalities in most of the evaluated categories between the testicular and ejaculated spermatozoa.

Due to the fact that control bulls did not have the AMD in ejaculated spermatozoa, but there was a high percentage of this abnormality in their testicular spermatozoa, we decided to perform a sperm morphology analysis on gametes harvested from the testis, epididymis (caput and corpus), and ejaculates from a control bull. Figure 2 shows a decrease in the aplastic midpiece sperm defect and most other defects in the caput of the epididymis; in other words, the elimination of defective spermatozoa appeared to occur in the rete testis and/or efferent duct.

3.2 Oxidative stress markers and mineral microanalysis in the testis and epididymis

Regarding the oxidative balance, in the testis of the control group had a higher the GST activity compared to the AMD group. In contrast, the CARB protein levels were significantly increased in the AMD group ($p < 0.05$; Figure 3a). No differences between the groups were observed in the SOD and CAT activities, or in the MDA production in the testis ($p > 0.05$; Figure 3a). In the caput epididymis, control bulls exhibited a significant increase in the MDA level and CAT activity compared to the AMD group ($p < 0.05$; Figure 3b). However, there were no significant differences in the SOD and GST activities, as well as in the CARB protein levels between groups ($p > 0.05$; Figure 3b). In the corpus epididymis, the CAT activity increased in the AMD group ($p < 0.05$; Figure 3c). However, no differences in the other enzymes and products were found between groups ($p > 0.05$; Figure 3c). On the other hand, in the cauda epididymis, no significant differences were observed in any enzyme or product among groups ($p > 0.05$; Figure 3d).

The content of Se in the testis of the AMD group was significantly higher than in the control group ($p < 0.05$; Figure 4a), with no differences in the proportion of the other minerals ($p > 0.05$; Figure 4a). Contrarily, the proportion of Cu was significantly higher in the caput epididymis of the control group when compared with the AMD group ($p < 0.05$; Figure 4b).

No differences were observed in the mineral proportion of the corpus and cauda epididymis from both groups ($p > 0.05$; Figure 4c and 4d).

3.3 Testicular histomorphometry epididymis histology

Regarding histology, no alterations in the germ cells presence and distribution patterns in the seminiferous epithelium were observed between the AMD and control group. The seminiferous epithelium presented Sertoli cells supporting the different types of germ cells, such as spermatogonia, spermatocytes, and spermatids, as well as spermatozoa in the lumen (Supp. Fig. 1). Although we notice an increase presence of vacuoles in the seminiferous epithelium in the AMD group (Supp. Fig. 1C and D) when compared to the control group (Supp. Fig. 1A and B), we did not observe significant changes in all the testicular histomorphometry parameters evaluated ($p > 0.05$; Table 1). However, increased AO staining was observed in all epididymal regions of the AMD bulls compared with the control bulls ($p < 0.05$; Figure 5).

3.4 Sperm proteomic analysis

We identified 215 proteins from both testicular and epididymal spermatozoa in the control group (Figure 6A) and 320 in the AMD group (Figure 6B), totaling 354 proteins, of which 181 proteins were found in both sperm phenotypes (Figure 6C). Among the 354 proteins, 34 were found to be unique to control spermatozoa, and 139 were identified only in spermatozoa from the AMD group (Figure 6C) (Supplementary Table 1).

Figure 7 shows the functional clustering analysis, which revealed an overall decrease in the oxidative phosphorylation pathways, while pathways related to protein degradation and tissue remodeling were enriched in the AMD spermatozoa. Synaptic vesicle cycling and collecting duct acid secretion pathways were also enriched in the AMD spermatozoa. By analyzing the functional clustering pathways from sperm harvested in every gonadal region, is possible to identify how the oxidative phosphorylation pathways, Huntington and Parkinson

disease pathways, and metabolic pathways increase during the sperm journey in the control bulls (Supplementary Table 2).

Several subunits of the NADH ubiquinone oxidoreductase, isoforms of the ATP synthase (ATP5F1A, ATP5F1D, ATP5PB), proteins of the succinate dehydrogenase complex (SDHA), and cytochrome C and ubiquinol-cytochrome C reductase (UQCRQ, UQCRFS1, UQCR10) were detected in the oxidative phosphorylation pathway of the spermatozoa from control group but absent in the AMD group. Some of these proteins also participate in the Huntington and Parkinson disease pathways. Nevertheless, there were also cytoskeleton proteins not detected from this pathway in the AMD group such as dynein axonemal intermediate chain (DNAI1). Proteins of the autophagosome pathway such as the complement C3, subunits of the ATPase H+ transporting V1 (ATP6V1F, ATP6V1G1) and some tubulins were unique to the AMD group in addition to some proteins in the ubiquitin-proteasome system such as ADRM1, PSMA2, PSMB2, PSMB6, and POMP. Relative to the metabolic pathway, major proteins such as Glycerol kinase and Serine racemase were not identified in the AMD group.

Among the 181 proteins common to both phenotypes, 35 were upregulated, while 28 were downregulated in spermatozoa with AMD compared to control (Log2 Fold Change ≥ 3; Table 1). Most of the upregulated proteins were found in the testicular spermatozoa (25 proteins) and in those from the cauda epididymis (5 proteins). Interestingly, most of the proteins were downregulated in the caput epididymis (16 proteins). In the testicular spermatozoa, the upregulated proteins were involved in transcription regulation (Enhancer of rudimentary homolog, Myristoylated alanine-rich C-kinase substrate, Ropporin-1, Peptidyl-prolyl cis-trans isomerase A), the maintenance of the redox state (Superoxide dismutase [Cu-Zn], N(G) N(G)-dimethylarginine dimethylaminohydrolase 1, and Glutathione S-transferase P), oxidative phosphorylation (mitochondrial ATP synthase-coupling factor 6, Cytochrome b5,

mitochondrial NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, Heme-binding protein 1), and in proteasomal activity (Proteasome activator complex subunit 2 and NEDD8). Other upregulated proteins had chaperone activities, like CALR and CLU, or they were cytoskeleton components (CENTRIN1, Tubulin beta-4B chain, cytoplasmic Dynein light chain 2, Tropomyosin alpha-1 chain, cytoplasmic Actin 2). On the other hand, downregulated proteins had three main functions, i.e., regulation of proteins transcription (Peptidyl-prolyl cis-trans isomerase FKBP1A, Calmodulin, and C-type natriuretic peptide), transcription chaperones (Prefoldin subunit 1 and mitochondrial 10 kDa heat shock protein) and cytoskeleton components (Actin alpha skeletal muscle, Protein chibby homolog 2, Myosin light polypeptide 6).

There were substantial biological differences in the functions and abundance of the sperm protein during epididymal transit. The proteins upregulated in caput epididymis spermatozoa played roles in maintaining the redox state (mitochondrial Peroxiredoxin-5), chromatin structure (Uncharacterized protein C7orf61 homolog), cytoskeleton components (cytoplasmic Actin 2), and metabolism (testis-specific Glyceraldehyde-3-phosphate dehydrogenase). In contrast, downregulated proteins participated in functions such as oxidative phosphorylation (Cytochrome c oxidase subunit 5B, mitochondrial, mitochondrial Acyl carrier protein, Cytochrome c oxidase subunit 5A, mitochondrial, Cytochrome b-c1 complex subunit 6, mitochondrial, NADH-cytochrome b5 reductase 1, mitochondrial ATP synthase subunit beta), sperm-egg interaction (Sperm acrosome membrane-associated protein 3, Arylsulfatase A, Disintegrin and metalloproteinase domain-containing protein 2/ADAM2, IQ domain-containing protein F1, Insulin-like peptide INSL6, Lysozyme-like protein 4), and mitophagy (FUN14 domain-containing protein 2). However, the upregulated proteins from spermatozoa stored in the cauda epididymis were mainly involved in biological functions such as proteolysis (Isoaspartyl peptidase/L-asparaginase and Lysosomal Pro-X carboxypeptidase), and proteins

involved in the sperm-egg interactions (Sperm acrosome membrane-associated protein 3 and Spermadhesin Z13). The only downregulated proteins were a cytoskeleton component (Dynein light chain 2, cytoplasmic) and metabolic enzyme (L-lactate dehydrogenase A chain).

4. Discussion

This study indicates that the aplastic midpiece defect originated in the testis as opposed to being induced in the epididymis. It also showed strong evidence of the intrinsic presence of the aplastic midpiece defect in the testis of control bulls, which could explain why there was no difference in the histomorphometry parameters evaluated in the testis between the control and the AMD groups, although the AMD group produced more AMD spermatozoa. However, the AMD defect in the control group appeared to be mitigated before epididymal passage and consequently, the bulls were classified as satisfactory potential breeders in the BSE. In addition, the same control bulls showed normal sperm parameters and traits in a previous trial (DIAZ-MIRANDA et al., 2019). Furthermore, our investigation supports the critical role of the epididymis in regulating the redox state after the elimination of the abnormal spermatozoa.

Our morphological analyses revealed that the elimination of the abnormal spermatozoa in the control group occurs either before or very early during the epididymal transit. Therefore, the removal of the abnormal spermatozoa must begin in the rete testis and/or efferent duct, places where phagocytosis of spermatozoa has been described (SINOWATZ et al., 1979; GOYAL, 1982). Even though sperm removal has been previously reported, the mechanism and the site where this process occurs are under debate because literature also suggests that the epididymis is equipped with machinery to target abnormal spermatozoa for intraluminal degradation (JRAD-LAMINE et al., 2011; AITKEN and BAKER, 2013; SMITH et al., 2014; SUTOVSKY, 2015). In support of our results, CRABO et al. (1971) described a high incidence of malformed and detached sperm heads in the rete testis, most of which had disappeared before

the proximal part of caput epididymis was reached, presumably due to phagocytosis by the efferent ducts. Furthermore, the semen characteristics of ejaculates from the bull in CRABO et al (1971) experiment, as well as the ejaculates from our control group, were within physiological limits, which indicates that the elimination of the defective spermatozoa can mask suboptimal or abnormal testicular functions.

The increased red-to-green ratio of the AO staining in the epididymal epithelium from the AMD group may be related to phagocytotic activity rather than apoptosis of the cells in the epithelium. The AMD group also showed a decreased in the percentage of ejaculated abnormal spermatozoa compared with the testicular spermatozoa. The epididymis participates in spermatozoa's transport, maturation, and storage (ROBAIRE and HINTON, 2015). However, it may have a role as the last checkpoint of the sperm quality control to ensure that only the fittest, most functional and structurally sound cells are contributed to the ejaculate (AITKEN and BAKER, 2013). This idea is supported by the fact that the established negative male fertility biomarkers, such as ubiquitin, bind abnormal spermatozoa in the epididymis (SUTOVSKY et al., 2015a). However, the removal of abnormal spermatozoa by the epididymis appears to be compensatory; in other words, it may occur only when the number of abnormal spermatozoa exceeds the absorptive capacity of the rete testis and efferent ducts (RAO et al., 1980; CHACON et al., 1999).

Higher levels of protein oxidation in the testis of the AMD group may be explained by a higher phagocytosis rate of abnormal, apoptotic germ cells and could be avoided in the control group by higher activity of the GST, which is a key ROS scavenger in the testis (KAUR et al., 2006; AITKEN and ROMAN, 2008). The testis is highly sensitive to oxidative damage, and the germ cells are more susceptible since they are associated with the free radical generating, phagocytic Sertoli cells (KAUR et al., 2006). On the other hand, Sertoli cells nurture and protect germ cells via blood-testis barrier. It is also well known that more than a half of the

differentiating germ cells undergo apoptosis during spermatogenesis (NAKANISHI and SHIRATSUCHI, 2004), which could further increase in the bulls with impaired spermatogenesis (CHACON et al., 1999) such as the bulls examined in the present study. This redox state shifts in the epididymal caput, where the control bulls showed a higher MDA production with a higher activity of CAT. The lipid peroxidation should result from the elimination of abnormal spermatozoa by the rete testis and efferent ducts, probably by phagocytosis. This may activate the antioxidant defense mechanisms, resulting in higher activity of CAT within the epididymal epithelium. The phagocytosis of spermatozoa may be silenced in the epididymis of fertile males so that no reactive oxygen species (ROS) or pro-inflammatory cytokines are produced at higher levels (AITKEN and BAKER, 2013). Thus, the epididymis plays another vital function, protecting the maturing spermatozoa from oxidative damage (HINTON et al., 1995; VERNET et al., 2004). The role of the CAT in the epididymis seems to be important only in cases of oxidative stress and not in the sequestration of ROS when produced physiologically, because it acts in conjunction with high concentrations of H_2O_2 or with SOD expression (VERNET et al., 2004). Although the SOD activity showed no significant differences between control and AMD cohorts in our study, CAT followed the same behavior as SOD activity in most regions.

Contrary to caput epididymis, the antioxidant activity in the corpus of the epididymis shifted, exhibiting higher CAT activity in the AMD group compared to the control group, without significant differences in the other analytes. Furthermore, the fact that the cauda epididymis showed no significant analyte differences between groups suggests that the oxidative stress was under tight control, and the redox state was balanced. This is an important adjustment, as it is in this region that spermatozoa are stored until ejaculation in a metabolically suspended state. The epididymis has the ability to ensure that spermatozoa are protected as they mature along the epididymal duct, where each region or segment of the epididymis has

developed its own sperm protective mechanisms (ROBAIRE and HINTON, 2015). The mild lipid peroxidation in the epididymal caput was rapidly and effectively reduced by the antioxidant mechanisms. The CAT appeared to be the antioxidant with the highest biological activity. Surprisingly, the SOD activity did not differ between the groups in any region, and the GST enzyme appeared to be irrelevant to the protection of bovine epididymis.

The high availability of Se in the testis of the AMD groups may indicate that the enzyme controlling the oxidative stress in this region is the Se-dependent glutathione peroxidase (Se-GPx). Further research should be conducted to prove this hypothesis; however, it has been established that Se-GPx is an essential constituent of the testis defense system (AITKEN and ROMAN, 2008). In addition, it is recognized that when there is a low content of Se, there is an improvement in GST activity, as detected in the control group (MEHLERT and DIPLOCK, 1985). The epididymis possesses two forms of the SOD, the cytosolic (Cu/Zn) as well as the extracellular (SOD-Ex) form, both of which catalyze the dismutation of O^{2-} (VERNET et al., 2004). The higher Cu proportion in the epididymal caput means more availability of this mineral, which suggests a possible role of the Cu/Zn SOD in maintaining of the redox balance. Our analysis showed this tendency but no significant differences between groups and epididymal segments. Thus, it make sense that the Cu/Zn SOD is highly expressed along the epididymis (JERVIS and ROBAIRE, 2001) and plays an important role in immune response (MARIKOVSKY et al., 2003). Together the micromineral proportion and the analysis of oxidative stress display shared trends, with significant differences in the testis and caput epididymis and lesser or no differences in the corpus and cauda epididymis. This showed that the redox balance in the epididymal epithelium is reestablished after the transit in the caput epididymis.

The overall enrichment in the proteasome and phagosome pathways proteins in the AMD spermatozoa supports the idea that the abnormal spermatozoa are marked in the

epididymis to be targeted for elimination prior to ejaculation. In addition, the overall enrichment in oxidative phosphorylation pathways in the control group was not a surprise, since spermatozoa with AMD lack mitochondria resulting in a decrease in the number and expression of proteins that participate in such a vital process. Furthermore, the increase in the -log p-value in the oxidative phosphorylation pathway, and the Huntington and Parkinson's disease pathways during the sperm journey through the epididymis suggest that these pathways may be critical in the sperm maturation. A recent study (ZIGO et al., 2022) showed enrichment in the same pathways in a boar sperm zincoproteome study focused particularly on sperm capacitation process. This may be due to the epididymal activation of mitochondrial function playing a crucial role in capacitation process after sperm deposition in the female reproductive system (AITKEN et al., 2007). Thus, the AMD spermatozoa may be deficient in achieving capacitation due to the lack of mitochondria. Another important change uncovered by the KEGG pathways analysis was the enrichment in lysosome pathways from the epididymal caput sperm of the AMD group. This enrichment also supports the idea of proteolysis and sperm degradation by epididymal fluid proteases prior to phagocytosis of sperm derived debris by the epididymal clear and/or dendritic cells.

In this context, the quantitative analysis corroborated the functional analyses of the unique peptides from both groups, including the downregulation of proteins involved in the oxidative phosphorylation and sperm-egg function in the caput epididymis spermatozoa from the AMD group. During sperm maturation, the spermatozoa must undergo a series of changes, requiring the incorporation of new molecules from the epididymal fluid and exosomes (epididymosomes) as well as post-translational modifications of proteins synthesized during spermiogenesis (GERVASI and VISCONTI, 2017; ZHANG et al., 2021). The diminished sperm content of proteins involved in the aforementioned pathways confirmed the deficiency in the maturation of the AMD spermatozoa. Furthermore, there was also an upregulation of

proteins involved in the proteasomal assembly and activity in the AMD spermatozoa from the testis as well as in the general proteolysis pathways in cauda epididymis AMD spermatozoa, again supporting the idea that the abnormal spermatozoa could be targeted early during epididymal passage and undergo proteolysis during their descent down from caput to cauda epididymis.

The proteins and pathways involved in the removal of the abnormal sperm prior to epididymal passage in the control group remain to be elucidated. For instance, chaperones and proteasomal proteins were upregulated in the AMD spermatozoa from the testis, and those pathways have also been linked to abnormal sperm. The sperm proteasome plays an important role in sperm functions, including capacitation and acrosome exocytosis, as well as sperm-zona pellucida penetration; they are responsible for the degradation of proteins via non-lysosomal proteolytic pathways. The subunit PSME2 is part of the 11S proteasome regulatory complex or REG. This complex can associate with the 20S proteasome core complex and may be able to degrade proteins that are not tagged with ubiquitin (SUTOVSKY, 2011). NEDD8 is a small ubiquitin-like protein modifier; the process of NEDD8 conjugation to a substrate, called neddylation, plays fundamental roles in signal transduction, cell division, morphogenesis, and embryogenesis (WEI et al., 2020). Furthermore, neddylation is involved in regulating the morphology, tracking, and function of mitochondria (ZHOU et al., 2021). Both proteins are attractive subjects for future studies on the regulation of epididymal sperm quality, especially PSME2, which has been implicated in the regulation of autophagy in some cancers (WANG et al., 2021).

More research should be needed to decipher the defective sperm removal mechanisms and how the abnormal spermatozoa is targeted and recognized prior to epididymal transit, especially in the bovine species, since there are many variations between species. For example, a considerable part of the stallion, goat, and bull's epididymal caput is composed of vascular

cones of the efferent ducts. In contrast, the rat, rabbit, and mouse caput is composed mainly of the epididymal duct (MARTAN, 1969). In this context, if phagocytosis is the sperm removal mechanism, there is still no explanation for why it occurs in some specimens and not in others (GOYAL, 1982).

In conclusion, we found evidence that the AMD spermatozoa is a consequence of aberrant spermiogenesis. Our morphological analysis also showed that the control bulls have a mechanism of removal of the defective spermatozoa prior to or very early during the epididymal transit. This mechanism generates mild oxidative stress in the testis and caput epididymis; however, the redox state of the epididymal milieu is reestablished in the corpus and cauda epididymis. Conversely, our proteomics suggest that the quality control of defective sperm in the bulls with the AMD may be rely on sequential targeting, proteolysis, and probably phagocytosis of the abnormal spermatozoa during the epididymal transit, which comes short to remove all defective sperm. Finally, our qualitative and quantitative proteomic analysis also showed that the sperm maturation of the bulls with the AMD was likely deficient as they had differences in their proteomes and a inadequate enrichment of pathways critical for sperm fertility.

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FIGURES

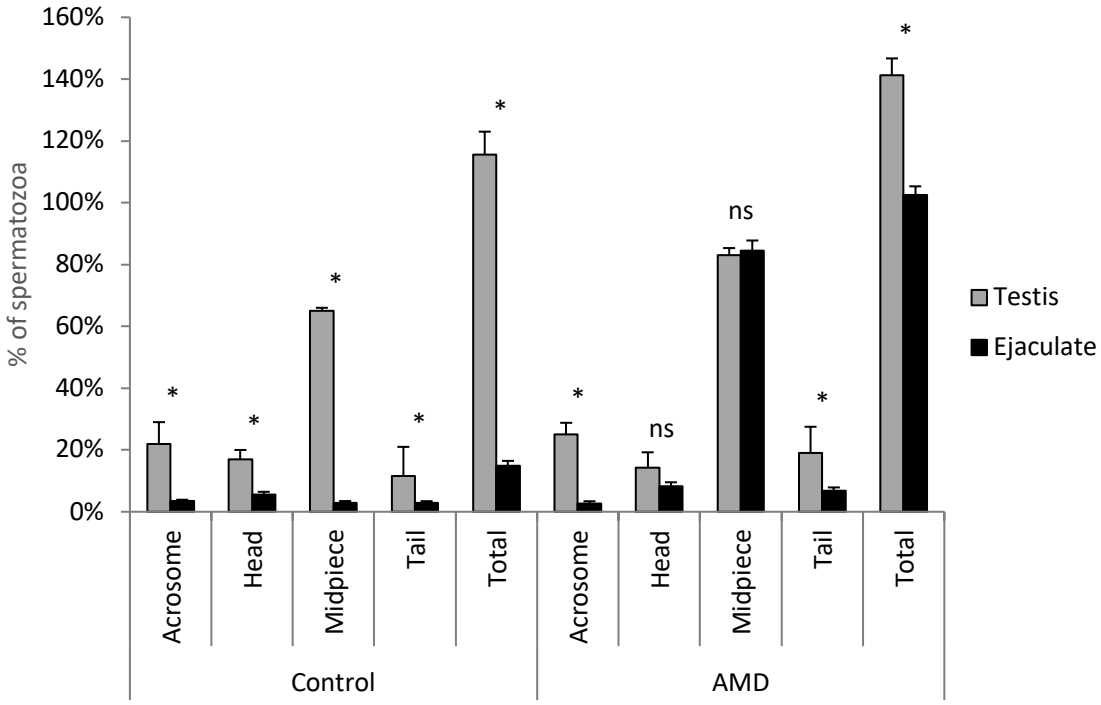


Figure 1. Sperm morphology analysis of testicular and ejaculated spermatozoa from the control and AMD group. Sperm defects were classified according to the subcellular localization (acrosome, head, midpiece and tail) and added up as total defects. Cytoplasmatic droplets were excluded from the analysis of testicular spermatozoa wherein they were considered physiological. All morphological abnormalities in each spermatozoon were recorded, meaning that more than one abnormality within an individual spermatozoon was considered in the evaluation and in the analysis. Mean ± SE; *asterisks indicate significant difference between testicular and ejaculated sperm (P-value < 0.05).

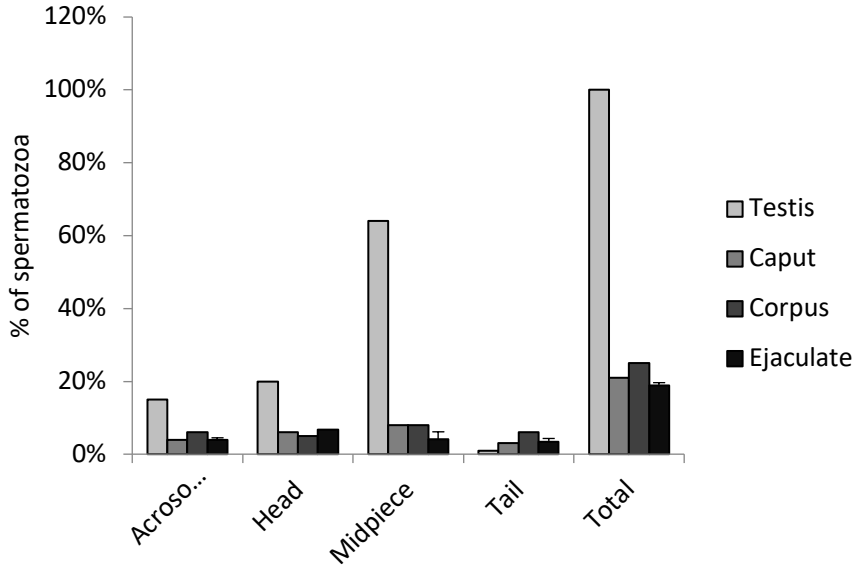


Figure 2. Sperm morphology analysis of the testicular and epididymal spermatozoa from a control bull. Sperm defects were classified according to the subcellular localization (acrosome, head, midpiece and tail) and total defects. All morphological abnormalities in each spermatozoon were recorded, meaning that more than one abnormality within an individual spermatozoon was considered in the evaluation and in the analysis.

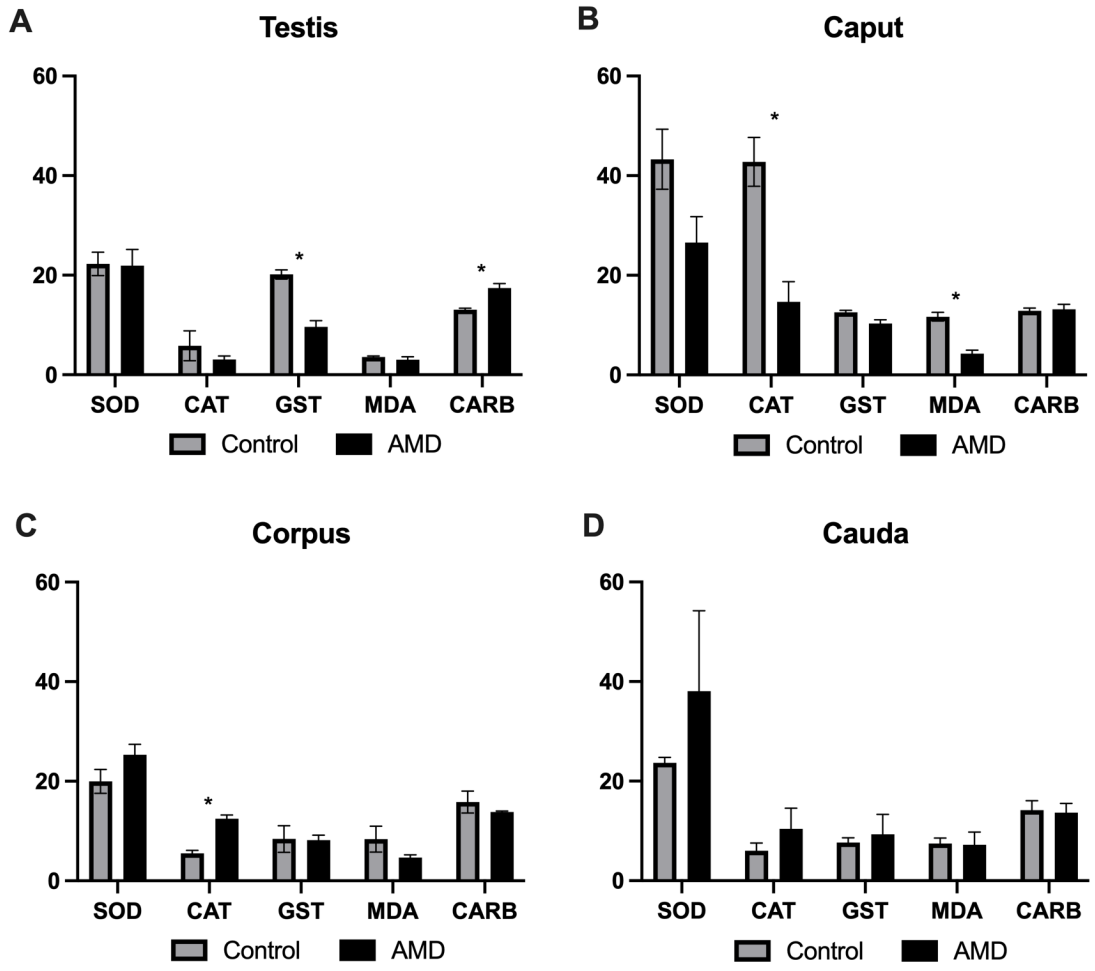


Figure 3. Superoxide dismutase (SOD), glutathione-s-transferase (GST) and catalase (CAT) activities, malondialdehyde (MDA) and carbonyl protein (CARB) levels in the testis (A) and epididymal caput (B), corpus (C), and cauda (D) from the control and AMD group. SOD and CAT levels were expressed in U/mg of protein. GST levels was expressed in μmol $min^{-1}g^{-1}$. MDA levels was estimated as nmol/mg of protein and CARB levels as nmol/mL. Mean ± SE; *asterisks indicate significant difference between the groups (*P*-value < 0.05).

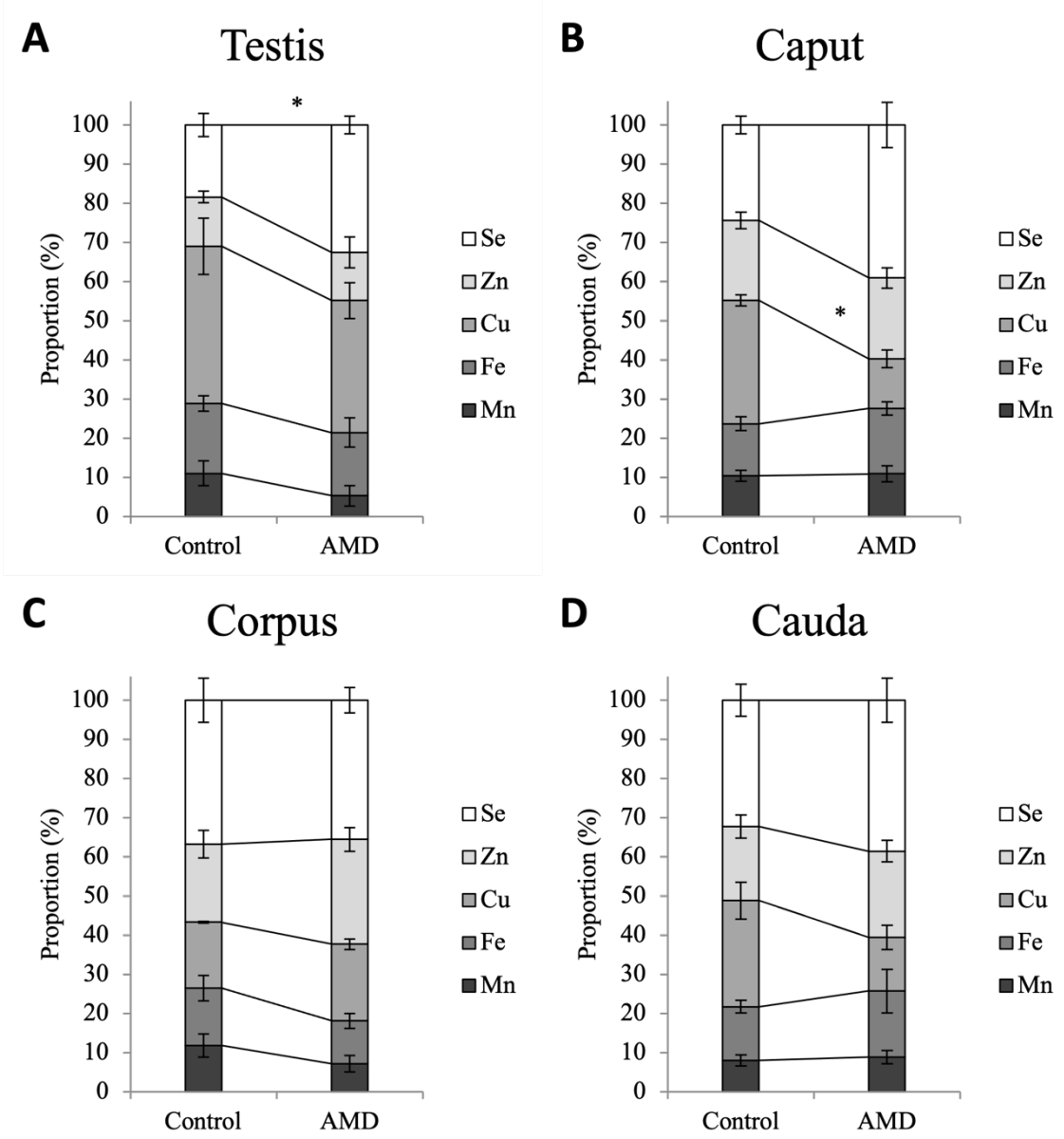
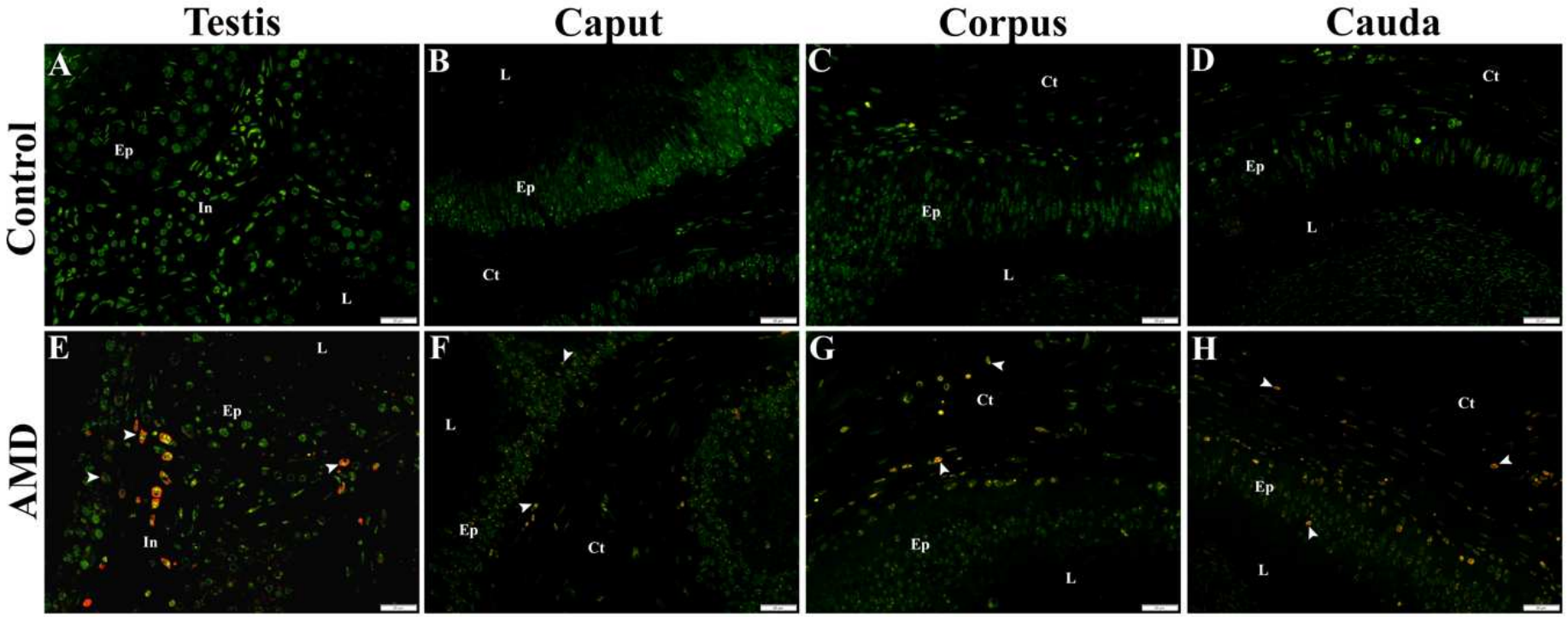


Figure 4. Mineral distribution in the testis (A) and epididymal caput (B), corpus (C), and cauda (D) from the control and AMD group. Se: Selenium; Zn: zinc; Cu: copper; Fe: iron; and Mn: manganese. Mean ± SE; *asterisks indicate significant difference between the groups (*P*-value < 0.05).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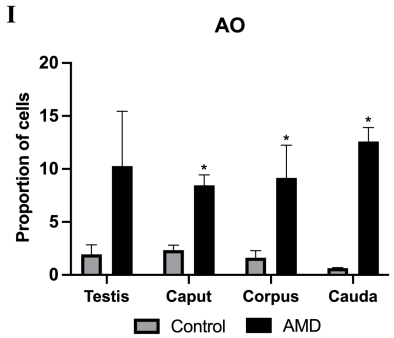
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Figure 5. Representative images of the Acridine Orange (green-orange) and Propidium Iodide (red) double staining in the testis and epididymis from the control (A, B, C, D) and AMD (E, F, G, H) group (bar scale = 20 μm). Ep-Epithelium, L-Lumen, In-Interstitium, Ct-Intertubular compartment. Arrows point to cells showing yellow-red stain reflective of acidic organelles, indicative of phagocytosis. (I) Proportion of AO-stained cells in the testis and epididymis from the control and AMD group. Mean ± SE; *Asterisks indicate significant difference between the groups (*P*-value < 0.05).

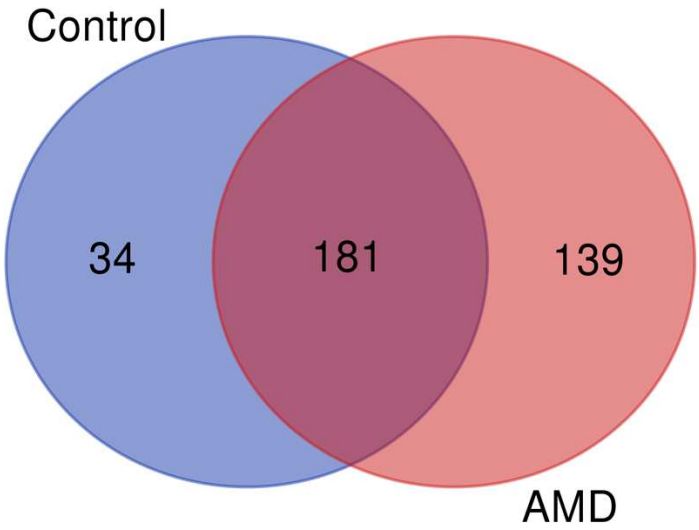


Figure 6. Venn diagram of the proteins identified in the control and AMD groups. From a total of 354 proteins, 34 were identified in exclusively spermatozoa from the control group and 139 were identified only in the sperm from the AMD group. A total of 181 overlapping proteins were identified.

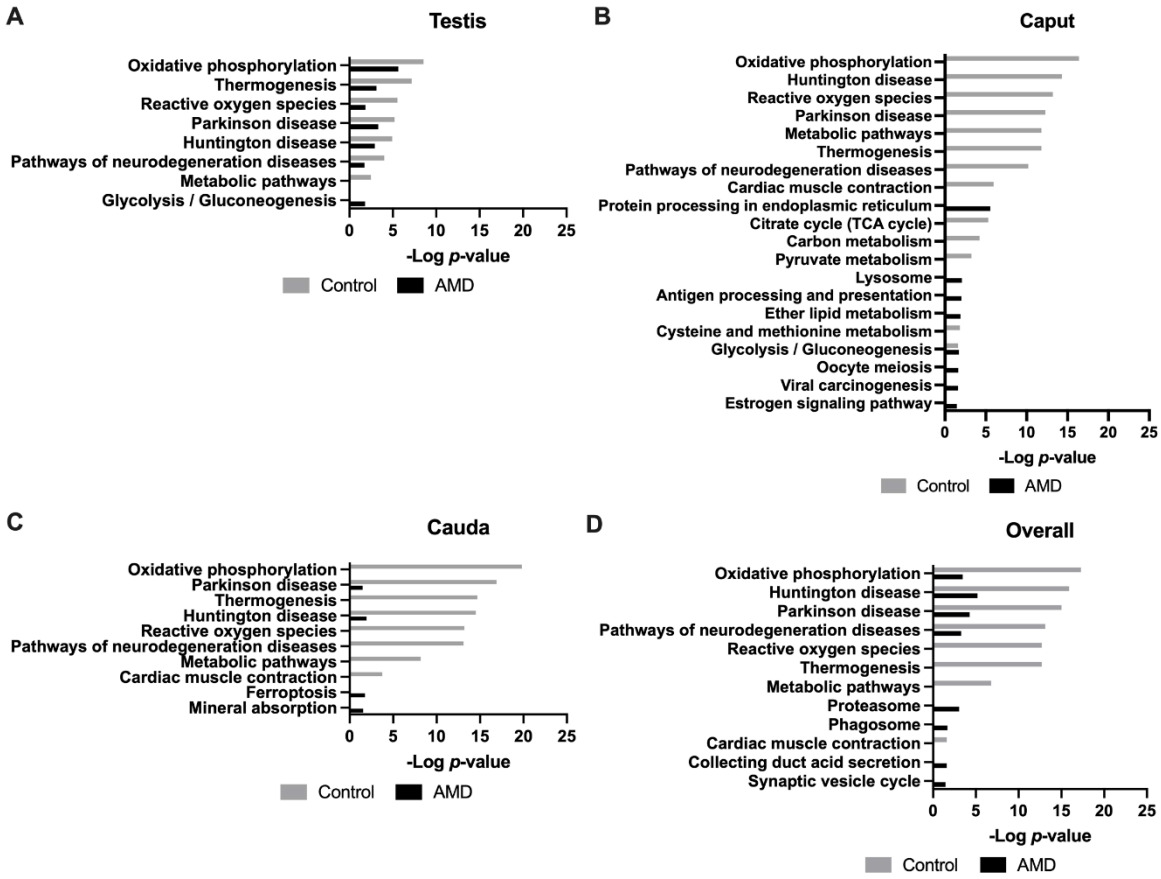


Figure 7. Functional annotations [-log (p-value)] of protein clusters identified in the spermatozoa from the testis (A), and caput epididymis (B), cauda epididymis (C), and pooled proteomes (D) of the control and AMD groups according to the KEGG pathway analysis. Protein data were clustered and defined according to enrichment scores and p-values using the DAVID platform.

TABLES

Table 1. Testicular biometric parameters in dairy Gyr bulls comparing the aplastic midpiece defect (AMD) and control groups.

Parameters	Control (n=3)	AMD (n=3)
Body weight (Kg)	525.0 ± 125.0	565.67 ± 48.32
Testis (g)	454.02 ± 81.31	503.25 ± 28.58
Gonadosomatic index (%)	0.08 ± 0.005	0.09 ± 0.006
Tubular diameter (μm)	279.22 ± 16.76	294.40 ± 7.92
Epithelium height (μm)	95.76 ± 1.61	91.28 ± 2.89
Tubular Compartment (%)	81.22 ± 0.72	77.90 ± 2.26
Epithelium (%)	65.73 ± 1.69	61.08 ± 3.05
Tunica propria (%)	4.15 ± 0.22	3.91 ± 0.37
Lumen (%)	11.34 ± 1.80	12.91 ± 2.57
Intertubular Compartment (%)	18.78 ± 0.72	22.10 ± 2.26
Leydig cell (%)	6.17 ± 1.53	7.91 ± 0.34
Nuclear percentage (%)	1.39 ± 0.30	1.67 ± 0.07
Cytoplasm percentage (%)	4.78 ± 1.37	6.24 ± 0.31
Stroma (%)	12.61 ± 1.89	14.19 ± 2.20
Leydig cell volume (μm^3)	942.49 ± 237.69	1093.57 ± 88.03
Nuclear volume (μm^3)	208.71 ± 24.76	235.81 ± 11.47
Cytoplasm volume (μm^3)	733.78 ± 217.86	857.75 ± 76.74

Mean ± SE; Different letters within rows indicate significant differences $p < 0.05$ by Kruskal-Wallis test.

Table 2. List of up and down-regulated proteins of testicular and epididymal (caput and cauda regions) spermatozoa from bulls with aplastic midpiece defect (AMD).

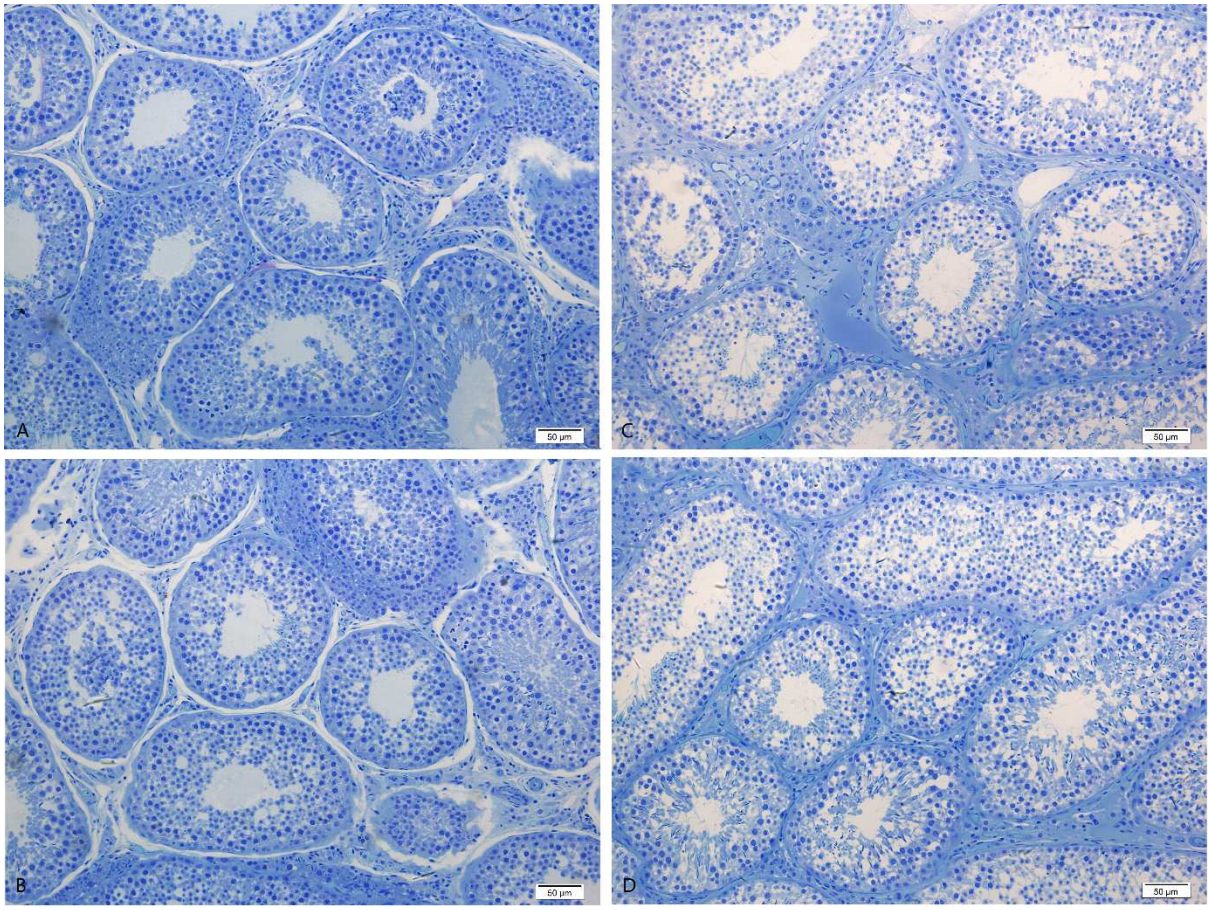
Accession	Protein description	Regulation	Log2FC
Testicular sperm			
Q3SZC0	Enhancer of rudimentary homolog	Up	7.17
P52193	Calreticulin	Up	6.94
P00442	Superoxide dismutase [Cu-Zn]	Up	6.66
Q32LE3	Centrin-1	Up	6.60
P56965	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	Up	5.90
P02721	ATP synthase-coupling factor 6, mitochondrial	Up	5.02
P12624	Myristoylated alanine-rich C-kinase substrate	Up	4.87
Q5E9G3	Proteasome activator complex subunit 2	Up	4.83
Q3MHM5	Tubulin beta-4B chain	Up	4.62
P61282	NEDD8	Up	4.52
P68401	Platelet-activating factor acetylhydrolase IB subunit alpha2	Up	4.38
Q148C9	Heme-binding protein 1	Up	4.33
P61283	Barrier-to-autointegration factor	Up	4.29
P28801	Glutathione S-transferase P	Up	4.26
P00171	Cytochrome b5	Up	3.94
P17697	Clusterin	Up	3.87
P23709	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	Up	3.73
P69678	Protein CutA	Up	3.49

Q3MHR3	Dynein light chain 2, cytoplasmic	Up	3.38
Q3T064	Ropporin-1	Up	3.36
Q5KR49	Tropomyosin alpha-1 chain	Up	3.24
Q1LZF3	UPF0449 protein C19orf25 homolog	Up	3.22
P63258	Actin, cytoplasmic 2	Up	3.16
P07107	Acyl-CoA-binding protein	Up	3.09
P62935	Peptidyl-prolyl cis-trans isomerase A	Up	3.06
P18203	Peptidyl-prolyl cis-trans isomerase FKBP1A	Down	-3.10
P68138	Actin, alpha skeletal muscle	Down	-3.39
P62157	Calmodulin	Down	-3.47
Q3SZE2	Prefoldin subunit 1	Down	-5.21
P00423	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Down	-6.86
P61603	10 kDa heat shock protein, mitochondrial	Down	-6.97
O97680	Thioredoxin	Down	-6.99
A6QQS3	Protein chibby homolog 2	Down	-7.32
P55206	C-type natriuretic peptide	Down	-7.41
P60661	Myosin light polypeptide 6	Down	-8.49
Epididymal sperm (caput region)			
Q9BGI1	Peroxiredoxin-5, mitochondrial	Up	9.14
Q2T9X5	Uncharacterized protein C7orf61 homolog	Up	5.20
P63258	Actin, cytoplasmic 2	Up	4.28
O46415	Ferritin light chain	Up	4.01
Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	Up	3.20

P63103	14-3-3 protein zeta/delta	Down	-3.11
P00428	Cytochrome c oxidase subunit 5B, mitochondrial	Down	-3.17
P52505	Acyl carrier protein, mitochondrial	Down	-3.25
P00426	Cytochrome c oxidase subunit 5A, mitochondrial	Down	-3.36
P00126	Cytochrome b-c1 complex subunit 6, mitochondrial	Down	-3.78
Q3MHW9	NADH-cytochrome b5 reductase 1	Down	-4.08
A6QQ77	Sperm acrosome membrane-associated protein 3	Down	-4.40
P00829	ATP synthase subunit beta, mitochondrial	Down	-4.56
Q32LE5	Isoaspartyl peptidase/L-asparaginase	Down	-4.66
Q08DD1	Arylsulfatase A	Down	-4.98
O77780	Disintegrin and metalloproteinase domain-containing protein 2	Down	-5.01
Q3SYS7	IQ domain-containing protein F1	Down	-5.07
Q32L79	Insulin-like peptide INSL6	Down	-5.62
Q2YDI9	Ferritin, mitochondrial	Down	-5.95
Q2T9N7	Lysozyme-like protein 4	Down	-6.45
Q8MJN0	FUN14 domain-containing protein 2	Down	-11.96
Epididymal sperm (cauda region)			
Q32LE5	Isoaspartyl peptidase/L-asparaginase	Up	8.14
A6QQ77	Sperm acrosome membrane-associated protein 3	Up	6.16

P82292	Spermadhesin Z13	Up	5.40
P62157	Calmodulin	Up	5.11
Q2TA14	Lysosomal Pro-X carboxypeptidase	Up	4.99
Q3MHR3	Dynein light chain 2, cytoplasmic	Down	-3.34
P19858	L-lactate dehydrogenase A chain	Down	-6.49

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Images of the testes from the control (A and B) and AMD (C and D) groups.

CHAPTER 4

Title: "Binder of sperm (BSP) proteins abundance is negatively associated with sperm quality".

ABSTRACT

Binder of sperm (BSP) proteins, secreted mainly by the accessory sex glands, are the major protein family found in bovine seminal plasma and on the ejaculated sperm surface. BSPs are multifunctional proteins. However, previous studies have also shown that BSP proteins are enriched in defective spermatozoa. Therefore, we aimed to characterize the localization and abundance of BSP1, BSP3, and BSP5 in the supernatant and pellet fractions of cryopreserved bull spermatozoa after density gradient separation, to determine whether BSP protein enrichment correlates with changes in acrosomal membrane integrity and membrane damages induced by incubation with digitonin (0.025%). These studies were performed by image-based flow cytometry (IBFC) and indirect immunofluorescence. The supernatant fraction was enriched with spermatozoa with high intensity of BSP when compared to the gradient pellet fraction for all BSP proteins investigated (BSP1, BSP3, BSP5; $P<0.05$). In the pellet fraction, BSP1 and BSP3 bound predominately to the acrosomal region, whereas BSP5 had a high affinity for the midpiece of the sperm tail. However, in the supernatant fractions, all BSP proteins bound to the entire spermatozoon with no clear regional specificity. Differences in the localization of specific BSPs may be due to differential binding affinities, since BSP1 and BSP3 have the same affinity for choline phospholipids, and BSP5 also binds phosphatidylserine. Furthermore, high BSP proteins intensity was found to correlate with acrosome and membrane damage induced by permeabilization. Overall, these findings suggest that spermatozoa highly enriched with BSPs, have compromised membrane integrity, likely reducing their fertilizing potential.

Keywords: Seminal plasma, bull fertility, acrosome damage, lectin.

1. INTRODUCTION

Upon ejaculation, spermatozoa rapidly pass through the ductus deferens and mix with the seminal plasma. Seminal plasma plays a crucial role in male fertility as it modulates sperm viability, fertilizing potential, and function [1]. The major proteins constituents of bovine seminal plasma are the Binder of Sperm proteins (BSPs), characterized by a unique N-terminal domain and two fibronectin (Fn2) type II domains, which confer their binding and functional properties [2, 3]. In bull semen, BSP proteins have been shown to regulate multiple spermatozoan functions, such as the ability to form the sperm reservoir and maintain motility before capacitation [4], undergo plasma membrane cholesterol and lipid efflux upon capacitation [5], and act as decapacitating factors [6].

The BSP1, BSP3, and BSP5 make up the family of BSP proteins in bovine seminal plasma [7]. Both BSP1 and BSP3 bind specifically to the phosphorylcholine group of plasma membrane phospholipids, whereas BSP5 can bind to both phospholipids containing phosphorylcholine and phosphatidylserine [8]. Concerning glycosylation, BSP1 has two distinct isoforms based on differential glycosylation, BSP3 has no glycosylation, and BSP5 is the most glycosylated BSP species [9, 10]. There are also differences in BSP protein size; BSP1 and BSP3 have molecular weights ranging from 13 to 15 kDa, whereas BSP5 has a molecular weight of 30 kDa [11]. These variations may result in differential binding properties and colocalization on spermatozoa. However, some studies have reported that bovine BSP proteins have no preferential localization and bind the entire sperm head and tail [2, 10, 12, 13].

Quantifying BSP proteins on the surface of sperm is complicated by their tendency to form aggregates, and to date, the best-proposed solution is to use immunofluorescence microscopy [14]. Nowadays, technology combining the power of light microscopy with high throughput

fluorometry and rapid image acquisition such as image-based flow cytometry may help in BSP studies [15]. Furthermore, BSP protein abundance has also been shown to be influenced by semen processing techniques such has cryopreservation, with some studies showing an increase in BSP proteins on the sperm surface [16], while others have found a significant decrease (up to 80%) in sperm bound BSP proteins. These divergent observations were attributed to BSP sequestration by semen extenders components [12, 17], which sequester BSPs from the seminal plasma, preventing membrane changes and protecting spermatozoa from cryodamage [18]. Since the binding of seminal BSPs to the sperm plasma membrane is rapid, specific, and stable after freeze-thawing [19], any membrane changes that occur during semen processing can influence the binding properties and abundance of BSP proteins, ultimately affecting spermatozoon function [16, 20, 21].

Previous studies have shown enrichment of BSP proteins in the spermatozoa from low fertility bulls, as well as in defective, immotile, and dead spermatozoa [12, 22-26]. The proposed mechanism of this differential BSP affinity to morphologically normal vs. defective spermatozoa may be due to their ability to extract phospholipids and cholesterol from the sperm plasma membrane in a time- and concentration-dependent manner [14], thus destabilizing the plasma membrane and causing premature sperm capacitation [27]. In this study, we aimed to characterize the binding properties of the BSP proteins in frozen-thawed bull semen and hypothesized that after discontinuous gradient separation, the dead/defective sperm supernatant fraction would show enrichment of all BSP proteins compared to the pellet fraction, which contains mostly viable, motile spermatozoa. Furthermore, we postulated that the increased concentrations of BSP proteins would correlate with cryocapacitation-induced sperm membrane changes, predominately observed in defective/dead spermatozoa of the gradient supernatant fraction.

2. MATERIALS AND METHODS

2.1 Sample acquisition

During this study, all semen straws used were donated by either Select Sires Inc. (Great Plains, OH) or SEMEX (Madison, WI).

2.2 Influence of gradient separation on BSP content

Semen from seven fertile AI sires was used to evaluate the localization and abundance of BSP proteins after sperm separation on a discontinuous gradient. Three technical replicates were performed for each animal. Spermatozoa were purified using a discontinuous gradient [50% (v/v) and 90% (v/v)] of Isolate (Irvine Scientific, Santa Ana, CA) [28]. After centrifugation, the 50% fraction and the supernatant were pooled, and the pellet fraction was processed separately. The respective fractions were washed in TL-HEPES by centrifugation at 700 × g for 5 minutes [29]. Subsequently, spermatozoa were incubated with PNA-AF647 (1:2500, Cat# L32460, Invitrogen) in TL-HEPES for 15 minutes at 37 ºC, shielded from light, before being fixed in 2% formaldehyde in PBS for 20 minutes. Fixed spermatozoa from each fraction were blocked using 5% normal goat serum in phosphate buffered saline (NGS-PBS) for 25 minutes at RT before being incubated overnight with custom-made primary antibodies for BSP1, BSP3, or BSP5 [16], at 4ºC and shielded from light. The next day, each sample was washed using 1% NGS-PBS before incubation with a secondary antibody (goat-anti-rabbit IgG-FITC, 1:200, Cat# 62-6111, Invitrogen) and the nuclear stain DAPI (4’,6-diamidino-2-phenylindole, 1:1,000) for 40 minutes at RT. After secondary antibody incubation, spermatozoa were washed twice in 1% NGS-PBS, and once with PBS only before being analyzed by image-based flow cytometry (IBFC).

2.3 Influence of sperm permeabilization on BSP Content

Straws of cryopreserved spermatozoa from fertile AI bulls were thawed for 45 seconds at 37ºC, diluted 1:1 with TL-HEPES, and centrifuged at 700 x g for 5 minutes. The supernatant (extender and seminal plasma) was removed and kept at 37ºC for later use. The sperm pellet was subsequently washed in 1mL of TL-HEPES (700 x g for 5 min) and resuspended in TL-HEPES. An aliquot of the sperm suspension was incubated for only 10 minutes with PNA-AF647 at 37ºC, shielded from light, then diluted in 1 mL of TL-HEPES, centrifuged, and fixed in 2% formaldehyde for 20 minutes as a reference or time 0 (T0). The effect of permeabilization was induced through sperm incubation with 0.025% digitonin [30] in TL-HEPES for either 20 or 40 minutes at 37ºC. Aliquots of non-permeabilized spermatozoa at the same incubation period were used as timed controls. At the end of each incubation period, samples were diluted in 1ml of TL-HEPES, and spun down for 5 minutes at 700 x g. Each sample was subsequently incubated in the extender/seminal plasma mixture reserved after the first centrifugation, supplemented with PNA-AF647, for 20 minutes at 37ºC while shielded from light. After extender/seminal plasma incubation, sperm samples were diluted in 1 ml TL-HEPES, spun down for 5 minutes at 700 x g, and fixed in 2% formaldehyde for 20 minutes. Samples were then blocked using 5% NGS-PBS for 25 minutes at RT before being incubated overnight with primary antibodies for BSP1, BSP3, or BSP5 [16] at 4 ºC shielded from light. The next day, each sample was washed using 1% NGS-PBS before incubation with secondary antibody (goat-anti-rabbit IgG-FITC, 1:200) and DAPI (1:1,000), for 40 minutes at RT. After secondary antibody incubation, spermatozoa were washed twice with 1% NGS-PBS, and once with PBS only then analyzed by IBFC.

2.4 Image-Based Flow Cytometry (IBFC)

Samples were analyzed by an Amnis FlowSight imaging flow cytometer (EMD Millipore Corp., Seattle, WA, United States) fitted with a 20X microscope objective (numerical aperture

of 0.5) and an imaging rate of up to 2,000 events/sec as described previously [29]. Raw image data were acquired using INSPIRE® software (Amnis-Millipore). Samples were analyzed using four lasers simultaneously: a 405-nm line with intensity set to 30mW; a 488-nm line with intensity set to 15 mW; a 642-nm line with intensity of 30 mW and a 785-nm line (side scatter) with intensity set to 50 mW. A total of 10,000 events were collected per sample.

Data analysis of the raw images was performed using the IDEAS® software (Version 6.3; Amnis-Millipore). A single cell-focused population gate was used for the histogram display of mean pixel intensities by frequency for collected channels. Intensity histograms and scatterplots of individual channels were then used for gating of subpopulations with varying intensity levels and visual confirmation. Furthermore, two masks were created to characterize the binding patterns of the BSP proteins, a sperm head mask, which was defined by taking the morphology mask of channel 7 (DAPI); and a sperm tail mask created by dilating the object mask of brightfield (channel 9) by one pixel and excluding the dilated head mask by 2 pixels.

2.5 Indirect immunofluorescence

Fixed sperm samples were suspended in 400 uL of KMT buffer (100 mM KCl, 2mM MgCl2, 10 mM Tris-HCl, 5mM EGTA, pH 7) and allowed to settle on the poly-L-lysine coated microscopy coverslips for 20 min at RT. Samples were blocked with 5% NGS- PBS for 25 minutes at RT and then incubated overnight at 4 ºC with antibodies custom-made against peptides in the N-terminal domains of BSP1, BSP3, and BSP5 (1.5 μg/mL; 3.5 μg/mL; 5 μg/mL, respectively; [16]). Coverslips were then washed twice with 1% NGS-PBS and subsequently incubated with the secondary antibody, goat anti-rabbit IgG-FITC (GAR-FITC;1:200), the nuclear stain DAPI (1:200), and lectin PNA-AF647 (1:200). Subsequently, coverslips were washed twice in PBS and mounted on glass slides with VectaShield mounting medium (Vector Laboratories, Burlingame, CA).

For sequential double-labeling of BSP1 and BSP5, fixed sperm samples suspended in 400 uL of KMT buffer were sedimented onto poly-L-lysine coated coverslips for 20 minutes, blocked with 5% NGS- PBS for 25 minutes, and then incubated with the anti-BSP1 antibody for 40 minutes all at RT. Coverslips were then washed twice with 1% NGS-PBS and immediately incubated with the first secondary antibody (GAR-FITC; 1:200). Coverslips were again washed twice in 1%NGS-PBS, re-blocked with 5% NGS for 25 minutes at RT, before being incubated with the second primary antibody, rabbit anti-BSP5, for 40 minutes at RT. Coverslips were washed twice with 1% NGS-PBS, then immediately incubated with a mixture of the second secondary antibody (GAR-TRITC; 1:200), and DAPI (1:200). Finally, coverslips were washed twice in PBS and mounted on glass slides with VectaShield mounting medium (Vector Laboratories, Burlingame, CA, United States).

All slides were examined on a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) with interference contrast (DIC) optics, Retiga QI-R6 (Teledyne QImaging, Surrey, BC, Canada), operated by MetaMorph 7.10.2.240. software (Molecular Devices Sunnyvale, CA, USA).

2.6 Western blotting

Semen straws from three fertile AI bulls were thawed in a water bath (37°C) for 45 seconds. Four straws per bull were pooled to obtain a sufficient number of spermatozoa per treatment. A subset of semen was washed by centrifugation in TL-HEPES at 700 x g for 5 minutes (Whole Sample, WS), while the rest of the semen was placed on a discontinuous Percoll® gradient (45%/90%) and spun at 700 x g for 10 minutes to obtain supernatant and pellet sperm gradient fractions. Sperm samples were resuspended in lithium dodecyl sulfate (LDS) loading buffer [106 mM Tris HCl, 141 mM Tris Base, 2% (w/v) LDS, 10% (v/v) glycerol, 0.51 mM (0.75%) EDTA, 0.22 mM (0.075% w/v) Coomassie Blue G250, 0.175 mM (0.025% w/v) Phenol Red, pH = 8.5] supplemented with 2.5% (v/v) β-mercaptoethanol and protease and phosphatase

inhibitor cocktail (Thermo Fisher Scientific). Seminal plasma (diluted 1:1000, positive control) and 100 mg of wildtype bovine brain tissue (negative control) were also incubated in LDS loading buffer and all samples were heated to 70°C for 10 minutes. After incubation, suspensions were spun at 13,000 × g for 5 minutes at 4°C. Extracts were transferred into fresh Eppendorf tubes and used immediately.

For polyacrylamide gel electrophoresis (PAGE), the NuPAGE® electrophoresis system was used (Invitrogen, Carlsbad, CA, United States). Total protein equivalent of approximately 2 million spermatozoa or 25 µg of cow brain extract (protein concentration estimated by BCA assay, cat #23227, ThermoFisher Scientific) were loaded per lane. PAGE was performed with NuPAGE™ 4-12% Bis-Tris gel (cat# NP0329BOX, Invitrogen, Carlsbad, CA, United States) using TRIS-MOPS SDS Running Buffer [50 mM Tris Base, 50 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 0.1% SDS, 1 mM EDTA, pH = 7.7], and the anode buffer was supplemented with 5 mM sodium bisulfite to prevent the reoxidation of disulfide bonds. The molecular masses of separated proteins were estimated using Novex® Sharp Pre-stained Protein Standard (cat # LC5800, Invitrogen, Carlsbad, CA, United States) run in parallel. PAGE was carried out at 80 V constant for 3 hours to allow the samples to delve into the gel. Power was limited to 20 W. After PAGE, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) Immobilon Transfer Membrane (Millipore Sigma, Burlington, MA, United States) using an Owl wet transfer system (Thermo Fisher Scientific) at 65 V constant for 1.5 h, using Bis-Tris-Bicine transfer buffer (25 mM Bis-Tris base, 25 mM Bicine, 1 mM EDTA, pH = 7.2) supplemented with 10% (v/v) Methanol, and 2.5 mM Sodium Bisulfite.

The PVDF membrane was subsequently blocked with 10% (w/v) non-fat milk in tris buffered saline (TBS) with 0.05% (v/v) Tween 20 (TBST; Sigma-Aldrich) before incubation with custom-made BSP antibodies (BSP1 3ng/ml, BSP3 35ng/ml, BSP5 50ng/ml) and an anti-β-tubulin antibody (1:4000, Developmental Studies Hybridoma Bank) overnight at 4°C. The next

day, the membrane was washed 5 times with TBST, for 5 minutes each, before incubation with HRP-conjugated species-specific secondary antibodies (GAR-IgG-HRP, 1:8,000, cat # 31460, Invitrogen; GAM-IgG-HRP, 1:10,000, cat # 31430, Invitrogen). After incubation, the membrane was washed 4 times with TBST and once with TBS only, before being reacted with chemiluminescent substrate (Luminata Crescendo Western HRP Substrate; Millipore Sigma, Burlington, MA, United States). The blot was imaged with a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, United States), to record the protein bands. Protein bands were quantified using ImageJ, with β-tubulin serving as the loading control.

2.7 Statistical analysis

Statistical Analysis System [31] was used to conduct data analysis. Percentage data were submitted to arcsine transformation y^'=arcsin√y. Furthermore, data were analyzed using the GLM procedure considering the treatment and the bulls as fixed effects. The LS means were compared by Tukey–Kramer test. Correlations were evaluated by Pearson`s correlation coefficient (CORR Procedure). Data are presented as mean ± standard error (SE). Overall, differences were considered significant at $P < 0.05$.

3. RESULTS

3.1 BSP content and binding properties

This study investigated the abundance and localization of BSP proteins on bovine spermatozoa in correlation with sperm morphology and acrosomal integrity. Initially, we compared the content of BSP proteins by IBFC between the pellet fraction, containing predominately motile, morphologically normal spermatozoa, and the supernatant fraction, enriched with immotile spermatozoa of cryopreserved bull semen separated through discontinuous Isolate gradients. Three sperm populations were identified in all samples according to the abundance of BSP proteins: (1) basal levels of BSP (BSP-), (2) moderate intensity of BSP (BSP+), and (3) high

intensity of BSP (BSP++). Overall, in all BSP proteins investigated, we observed an enrichment of PNA+ and BSP++ sperm populations in the supernatant fractions and an absence of the BSP- population when compared to the pellet fractions (Figure 1).

To identify the localization of the BSP proteins in both supernatant and pellet sperm fractions, masks for the sperm head and tail were used to analyze the raw IBFC data and establish BSP compartmentalization patterns in the sperm head and tail. Specifically, sperm populations with moderate BSP intensity (BSPH+, BSPT+) and high intensity in the head and tail (BSPH++, BSPT++) were analyzed (Supplementary Figures S1 and S2). However, by comparing the intensity of the head mask with the tail mask, we were able to distinguish if BSP binding had regional specificity or bound to the whole spermatozoa (Supplementary Figure S3). Our findings indicate that in the supernatant fractions, there was no pattern in the intensity of the BSP proteins in the sperm head or tail but an increase in sperm bound to BSP proteins in all cell compartments. Furthermore, in the supernatant fractions, the BSP proteins were found to bind preferentially to the whole spermatozoa (Figure 2, $P<0.05$).

When comparing the BSP proteins within the pellet sperm fraction, there were fewer spermatozoa with moderate intensity of BSP3 compared to BSP1 and BSP5, and fewer spermatozoa with basal levels of BSP1 ($P<0.05$; Figure 3A). Investigations into the subcellular compartmentalization of BSP proteins abundance in the pellet fraction also showed that BSP1 and BSP3 had more intensity in the sperm head whereas BSP5 had more intensity in the sperm tail ($P<0.05$; Figure 3B, 3C). These IBFC patterns were also confirmed by immunofluorescence/epifluorescence microscopy (Figure 4A, B, C). It also showed that the BSP1 and BSP3 where localized preferentially in the acrosome region of the sperm head, whereas the BSP5 in the midpiece of the sperm tail. Sequential double labeling with anti-BSP1 and anti-BSP5 revealed that most spermatozoa were either co-labeled with both antibodies or completely free of either antibody labeling (Figure 4D).

The western blots confirm the affinity of the antibodies used in this study. Furthermore, when quantified BSP1 showed no statistical difference between the pellet and supernatant fractions, whereas sperm in the supernatant fraction has more BSP3 and BSP5 when compared to the pellet fraction (Figure 5, $P<0.05$).

3.2 Correlation of the BSP proteins with acrosome membrane integrity

The scatterplot comparison of the intensities of individual BSP proteins against the PNA intensity revealed four distinct sperm populations; BSP-PNA- (spermatozoa with basal levels of BSPs and an intact acrosome), BSP+PNA- (spermatozoa with a moderate-to-high intensity of BSPs and an intact acrosome), BSP-PNA+ (spermatozoa with basal levels of BSPs and acrosome damage), and BSP+PNA+ (spermatozoa with moderate and high intensity of BSPs and acrosome damage) (Supplementary Figure S4). The supernatant fraction had more BSP+PNA+ spermatozoa and less BSP-PNA- spermatozoa regardless of which BSP protein was sampled, when compared to the pellet fraction. Conversely, few spermatozoa in the supernatant fraction were BSP-PNA+, and only BSP3 showed an increase in BSP+PNA- spermatozoa in the supernatant fraction compared to the pellet ($P<0.05$; Figure 6).

There was a significant correlation of high intensity of BSP proteins (BSP++) with acrosome damage ($P<0.0001$; $r=0.92$, 0.91, 0.77 for BSP1, BSP3 and BSP5, respectively). This high correlation held up regardless of whether it was in the head or tail (BSPH++ and BSPT++). Furthermore, acrosomal damage was highly correlated with the percentage of spermatozoa carrying BSPs on both the head and tail (Head+Tail; $r=0.83$, 0.90, 0.86 for BSP1, BSP3 and BSP5, respectively). Inversely, basal levels of BSP proteins had a negative correlation with acrosomal damage ($r=-0.65$, -0.81, -0.64 for BSP1, BSP3 and BSP5, respectively), and there was no correlation between acrosomal damage and moderate BSP proteins intensity, except for moderate levels of BSP5 bound to the head (BSPH+; $r=0.81$) (Supplementary Table S5).

3.3 Effect of the sperm permeabilization on the BSP intensity

There was an increase in the intensities (BSP+ or BSP++) of all three BSP proteins in spermatozoa subjected to permeabilization and subsequently incubation with extender and seminal plasma ($P < 0.05$; Figure 7). However, there were no differences in the intensities of the BSP proteins according to the time of permeabilization ($P > 0.05$). This suggests that the binding of BSP proteins increases with membrane changes such as acrosome membrane integrity and membrane damage induced by permeabilization.

4. DISCUSSION

This study describes the differences in BSP proteins content of gradient-separated bull sperm fractions and depicts the unique BSP binding patterns seen in prevailing sperm phenotypes associated with these respective fractions. Nowadays, sperm manipulation is routine, and it is vital to understand the role of these proteins when using different biotechnologies. As IVP is the main source of the bovine embryo, and needs sperm separation before fertilization, it is utmost to know what is happening with the BSP after sperm preparation and before in vitro fertilization. The IBFC allowed us to accurately quantify and identify the binding properties of the BSP proteins and we found three unique sperm populations in both the supernatant and pellet fractions, which were distinguished by differential BSP content. Furthermore, when sperm populations were compared between the pellet and supernatant gradient fractions, a significantly higher proportion of the supernatant fraction spermatozoa had moderate-high BSP proteins content. These results agree with the literature suggesting that defective or low-quality sperm have an enrichment of BSP proteins [12, 22-26]. Although a subset of spermatozoa with basal levels of BSP proteins was present in the supernatant fraction, this may be due to contamination by a small sperm population at the 45-90% interface, which can contain functionally normal spermatozoa [32].

The western blot results suggested increased BSP protein abundance in the supernatant fraction compared to the pellet fraction. However, because of sample size and bull variability, there was no statistical significance for BSP1. The overall increase in abundance and coating of the entire sperm surface by BSP proteins measured by IBFC was also commensurate with a loss of subcellular localization preferences by each specific BSP protein, a phenomenon that was observed on spermatozoa in the pellet gradient fractions. These findings agree with previous reports suggesting that sperm populations with a high intensity of BSP1 were mostly composed of dead spermatozoa. It has also been suggested that BSP1 could be a negative biomarker for sperm cryo-survival because spermatozoa with moderate levels of BSP1 correlated with increased fragility of the membrane [12]. Through the use of PNA as a marker of acrosomal membrane changes, we also observed a strong positive correlation between BSP intensity and acrosomal damaged. Furthermore, there were almost no cells with acrosomal damage and basal levels of BSP (BSP-), suggesting that the BSP proteins may have increased affinity to modified/damaged plasma membranes.

The binding localizations of individual BSP proteins have been investigated by several groups and found to show no preference for specific regions of the sperm surface [2, 10, 13]. However, two separate reports state that BSP1 binds preferentially to the acrosome [12], whereas BSP5 has a greater affinity for the tail midpiece [26]. In the present study, bovine spermatozoa from the pellet fractions also showed subcellular localization preferences of BSP proteins, with BSP1 and BSP3 binding mainly to the sperm head, and BSP5 preferentially binding to the midpiece of the sperm tail. Therefore, our study agrees with D'Amours et al. [12] and Odhiambo et al. [26] and supports the notion that individual BSP proteins bind preferably to specific subregions of the sperm surface. Although the FN2 domains are highly conserved between the BSP proteins [4], the BSP1 and BSP3 preferentially bind phosphorylcholine head groups, whereas BSP5 also binds phosphatidylserine; this difference in phospholipid binding affinities may be

responsible for the differences in subcellular localization preferences and may result from differing lipid compositions of the sperm plasma membrane in the sperm head and tail [33, 34]. On the other hand, when spermatozoa are dead, the BSP proteins coat the whole surface without a clear preference for any specific subcellular region. The loss of compartmentalization may be due to irreversible membrane lipid modification during sperm death or apoptosis, increasing the binding sites for all BSP proteins [35]. Furthermore, membrane bound BSP proteins can also bind the free BSP proteins through hydrophobic interactions [18, 36], causing aggregates and influencing overall BSP protein abundance.

The binding patterns of each BSP protein were also validated by indirect immunofluorescence. Interestingly, by sequentially co-labeling spermatozoa with antibodies against both BSP1 and BSP5, when both proteins were found with moderate intensity, they were often located on the surface of the same spermatozoa, which may suggest that they could act together or that changes in different sperm membrane subdomains can increase the binding properties of different BSP proteins. BSP proteins are multifunctional proteins that control and share several sperm functions. For instance, BSP5 binds the sperm tail midpiece, which may suggest that it is implicated in sperm motility control, whereas BSP1 binds the sperm acrosome and could be involved in acrosomal function during sperm binding to zona pellucida, and sperm binding to and detachment from the oviductal sperm reservoir; both events are influenced by sperm capacitation [14]. On the other hand, it could also mean that those cells are the ones that have membrane instability and therefore attract more BSP proteins to perform its function as a decapacitating factor.

The BSP proteins content on the spermatozoa surface, could be used as a biomarker for sperm quality since fewer spermatozoa with high content of BSP proteins translate into fewer dead or defective spermatozoa per AI dose. Indeed, high BSP intensity in the sperm had a strong and positive correlation with acrosomal damage, and this was particularly true when BSP proteins

coated the whole sperm head and tail. Interestingly, a strong correlation with acrosomal damage was found when a moderate intensity of BSP5 was localized in the sperm head, suggesting that abnormal or altered localization of the individual BSP proteins can be indicative of altered sperm function. On the other hand, another study found a positive correlation between acrosomal damage and moderate intensities of BSP1 in the sperm head [12]. The positive correlations of acrosome damages with the BSP proteins bound to the sperm head also could be due to the high sensitivity of acrosomal membranes to oxidative stress [35]. When comparing the abundance of the BSP proteins in the pellet fractions, there was a larger sperm population with moderate intensities of BSP1 and BSP5 when compared to BSP3, and a limited percentage of spermatozoa with basal levels of BSP1. This could be explained by the differences in the abundance of individual BSP protein species in the seminal plasma, with BSP1 being the most abundant, followed by BSP5 and lastly BSP3 [17].

By permeabilizing the sperm membranes before co-incubation with seminal plasma, we demonstrated that the BSP proteins increase their binding after membrane integrity changes. Indeed, BSP proteins have been shown to act as decapacitating factors stabilizing the sperm plasma membrane [6, 37]. Whilst it was suggested that after mixing semen with extenders such as egg yolk or milk, the low-density lipids (LDL) and proteins of the milk sequester most of the BSP proteins from the seminal plasma, we saw an increase in the sperm-BSP binding after plasma membrane permeabilization and co-incubation with the extender/seminal plasma. The increase in the amount of sperm-bound BSP proteins may have occurred because not all BSP proteins were sequestered by the extender and the membrane damage caused by permeabilization allowed greater BSP binding availability on the sperm surface. However, increased sperm agglutination, and potential vesicularization of membrane components subsequently binding to individual spermatozoa did present a limitation in our capacity to

accurately measure BSP labeling intensities, making sperm head and tail masks a necessity in the analysis of IBFC data.

Further research should be pursued to understand the physiological events that trigger BSP binding [38]. The BSP proteins plays crucial roles in sperm function [39] such as facilitating the formation of the oviductal sperm reservoir, and the maintenance of sperm motility during storage. However, it has been suggested that BSP proteins are also responsible for inducing premature capacitation and reducing the sperm viability after cryopreservation [19, 40]. It is certain that dead and defective spermatozoa have an increased binding of BSP proteins; however, whether their increased presence provides added support to already defective spermatozoa or expedites cell death and removal by immune cells within the female reproductive tract, remains unknown.

In conclusion, the percentage of spermatozoa with higher BSP protein intensity is greater in the dead/defective sperm-rich supernatant fractions after discontinuous gradient separation, which may arise from the isolation of dead and defective sperm with compromised plasma and acrosomal membranes. We also demonstrate that BSP proteins have different binding localization; BSP1 and BSP3 preferentially bind the sperm head and BSP5 the sperm midpiece. Furthermore, high BSP protein content was correlated with acrosomal, and plasma membrane damage induced by permeabilization, which corroborates the observation that membrane changes increase the binding affinity or binding site availabilities of the BSP proteins. Therefore, the abundance and localization of the BSPs could be used as an indicator of sperm quality, due to their affinity for defective sperm.

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FIGURES.

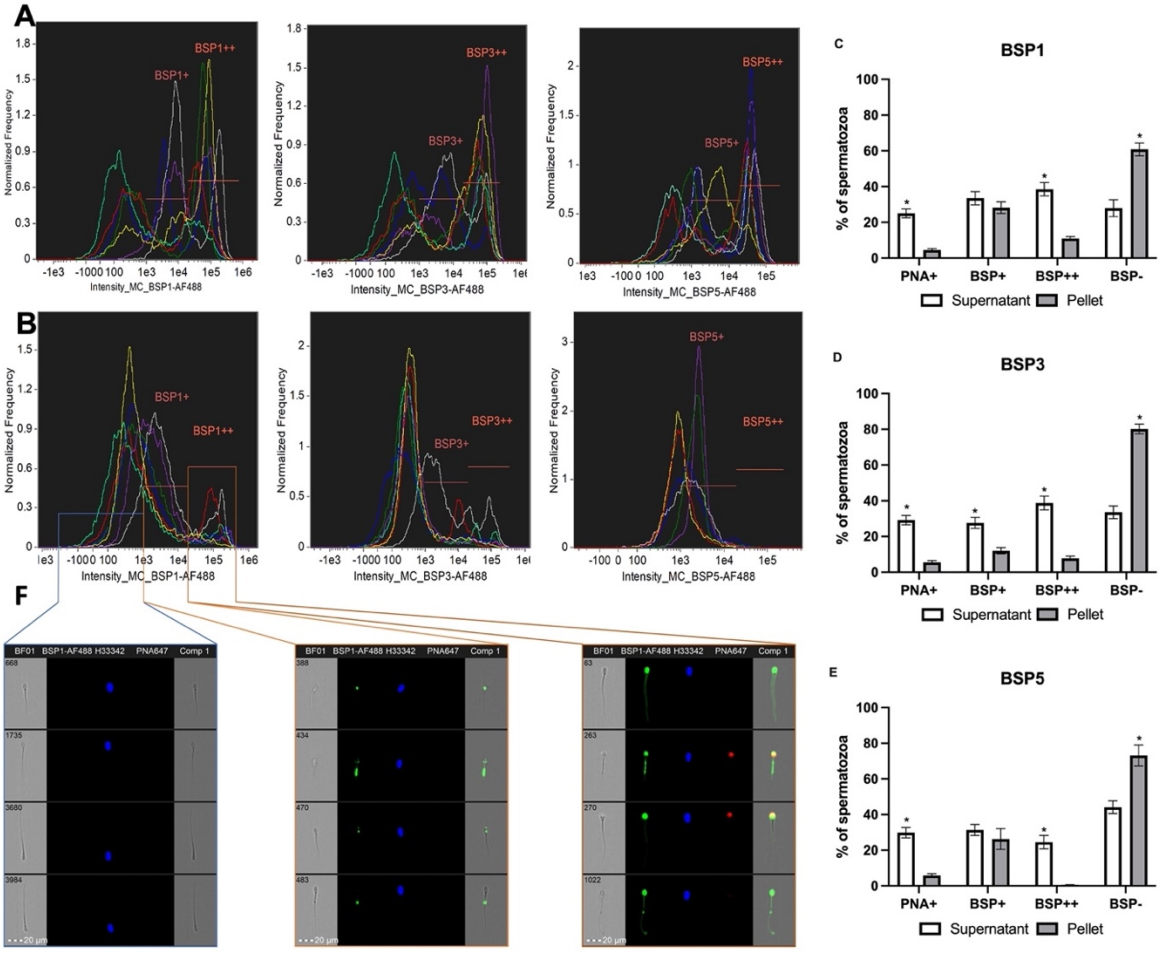


Figure 1. Representative histograms of the BSP protein-induced fluorescence intensities in the supernatant (A) and pellet (B) sperm fractions from the seven fertile AI bulls. Three populations were gated (F); BSP- (Basal levels of BSP), BSP+ (moderate intensity of BSP) and BSP++ (high intensity of BSP). Diagrams juxtaposing the percentages of acrosome damaged spermatozoa BSP1 (C), BSP3 (D), and BSP5 (E) intensities of the supernatant and pellet sperm fractions from seven fertile AI bulls. Data are presented as the mean ± SE and three technical replicates were performed per bull. *Asterisks indicate significant difference across fractions. PNA+ (acrosomal damage) (*P*-value <0.05).

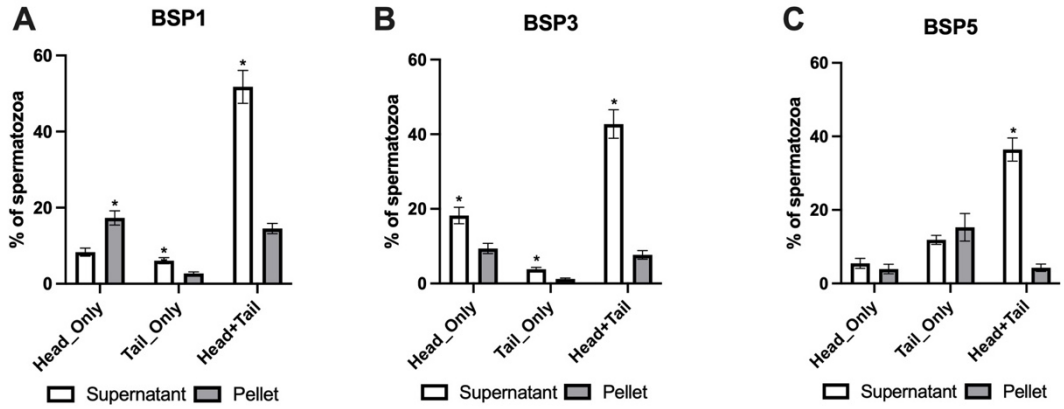


Figure 2. Differences in the BSP1 (A), BSP3 (B), and BSP5 (C) binding patterns between the supernatant and pellet sperm fractions from the seven fertile AI bulls obtained by sperm head/tail masking in the IBFC. Three populations were calculated: Head_Only (BSP bound only to the sperm head), Tail_Only (BSP bound only to the sperm tail), and Head+Tail (BSP bound to the whole spermatozoa). Data are presented as mean ± SE and three technical replicates were performed per bull. *Asterisks indicate significant difference across fractions. (*P*-value <0.05).

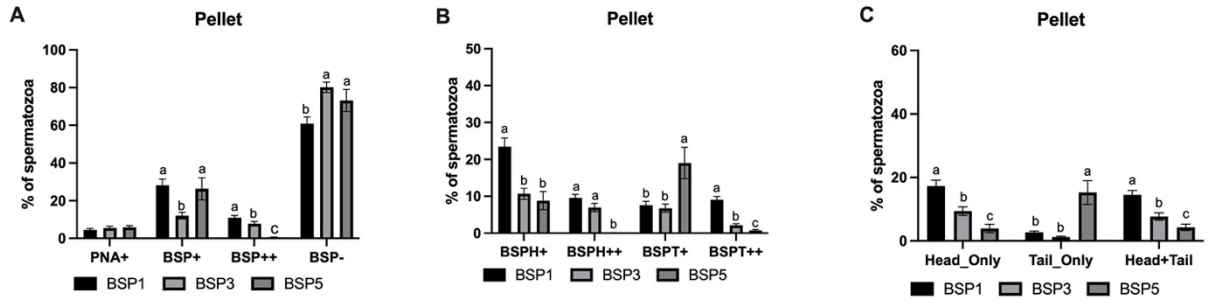


Figure 3. Comparison of the individual BSP protein fluorescence intensities in the pellet fraction from seven fertile AI bulls. (A) PNA and BSP content. (B) BSP intensity in the sperm head (BSPH+, BSPH++) and the sperm tail (BSPT+, BSPT++). (C) Differences in the BSP proteins binding patterns. Data are presented as mean ± SE and three technical replicates were performed per bull. Different letters indicate significant differences across BSP. (*P*-value <0.05).

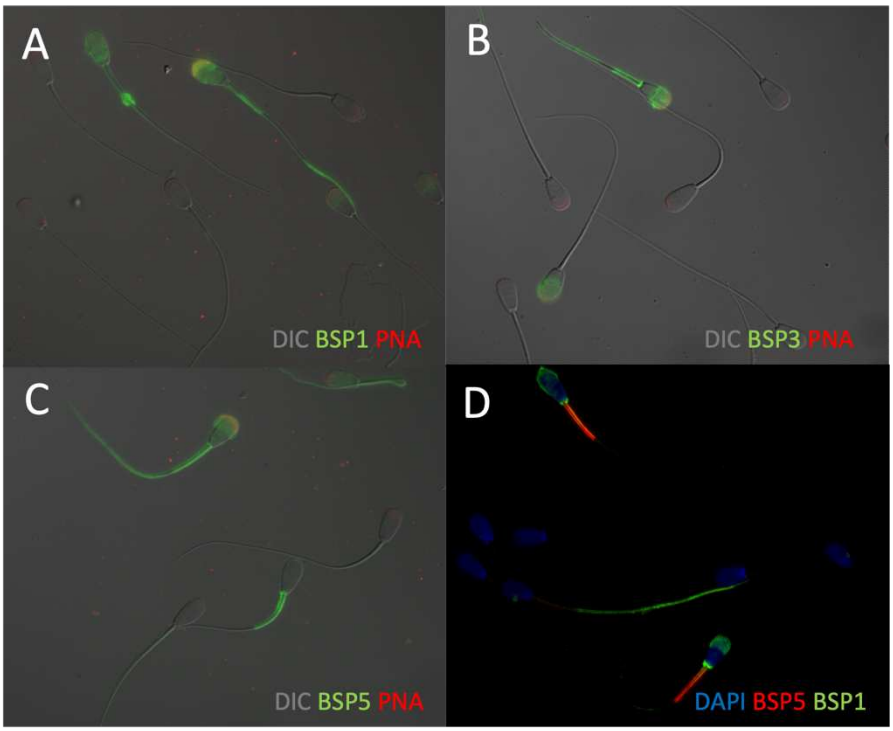


Figure 4. Representative epifluorescence microscopy images of the BSP proteins binding patterns. The BSP1 and BSP3 bind preferentially to the sperm head, whereas BSP5 binds preferentially to the sperm midpiece, and when co-incubated most cells are dually labeled or not labeled at all by anti-BSP antibodies. (A) BSP1 co-labeled with PNA-AF647. (B) BSP3 co-labeled with PNA-AF647. (C) BSP5 co-labeled with PNA-AF647. (D) Sequential double-labelling of BSP1 and BSP5. Sperm nuclei stained with DAPI.

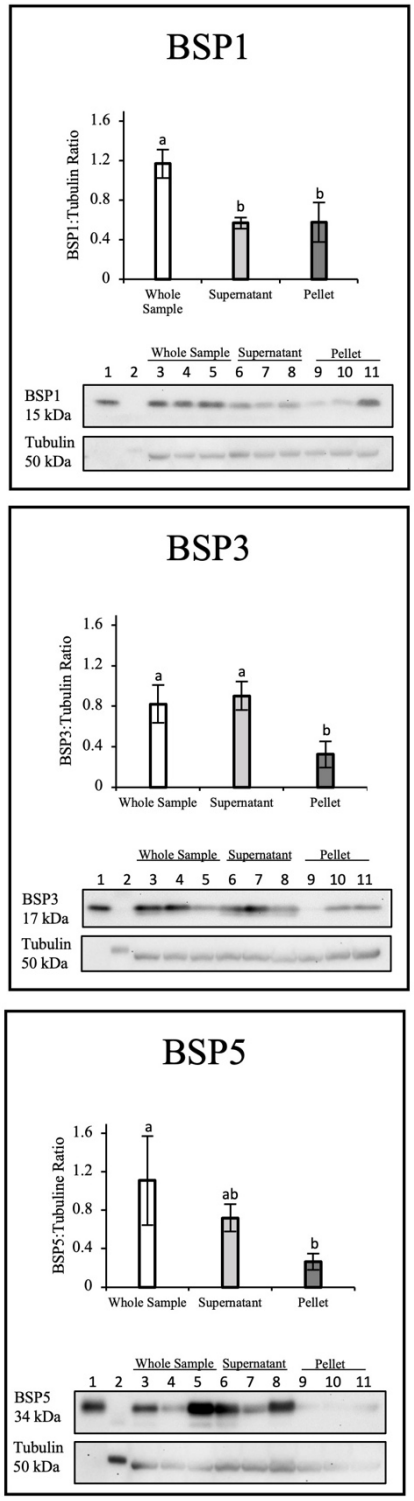


Figure 5. Western blot quantification of BSP abundance of the whole cryopreserved bovine sperm straw (Whole Sample), as well as the supernatant gradient fraction (Supernatant) and pellet gradient fraction (Pellet). For each treatment the same three bulls were used and the graphs represent the mean BSP abundance across the three bulls. Error bars indicate SEM. The

same Western blot samples were used to assess all BSP species and loaded in the same order. (1) Seminal plasma as a positive control, (2) cow brain as a negative control, (3) Bull 1 Whole Sample, (4) Bull 2 Whole Sample, (5) Bull 3 Whole Sample, (6) Bull 1 Supernatant Fraction, (7) Bull 2 Supernatant Fraction, (8) Bull 3 Supernatant Fraction, (9) Bull 1 Pellet Fraction, (10) Bull 2 Pellet Fraction, (11) Bull 3 Pellet Fraction. Tubulin was used as a loading control for quantification.

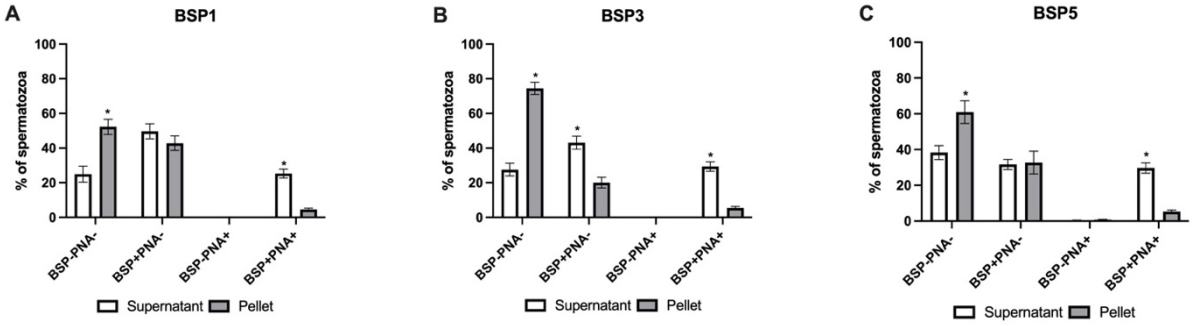


Figure 6. Percentage of sperm subpopulations in the supernatant and pellet sperm fractions from the seven fertile AI bulls, distributed according to acrosomal integrity and fluorescence intensities of BSP1 (A), BSP3 (B), and BSP5(C). BSP-PNA- (spermatozoa with basal levels of BSP and an intact acrosome), BSP+PNA- (spermatozoa with moderate BSP and an intact acrosome), BSP-PNA+ (spermatozoa with basal levels of BSP and acrosomal damage), and BSP+PNA+ (spermatozoa with moderate-to-high levels of BSP and acrosomal damage). Data are presented as mean ± SE and three technical replicates were performed per bull. *Asterisks indicate statistically significant differences across fractions. (*P*-value <0.05).

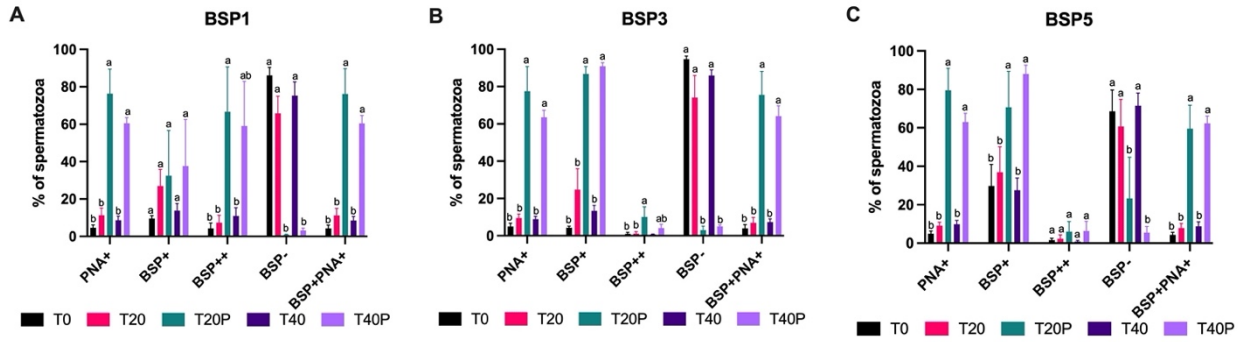
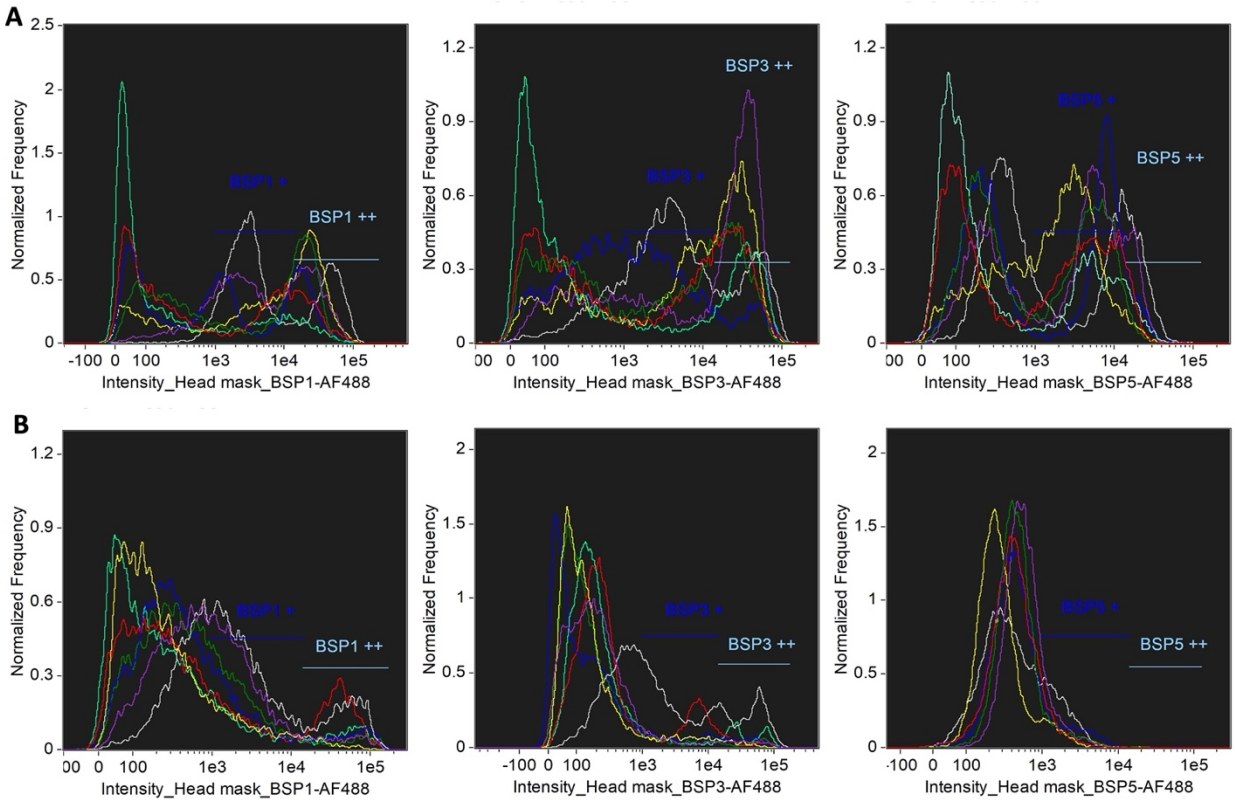
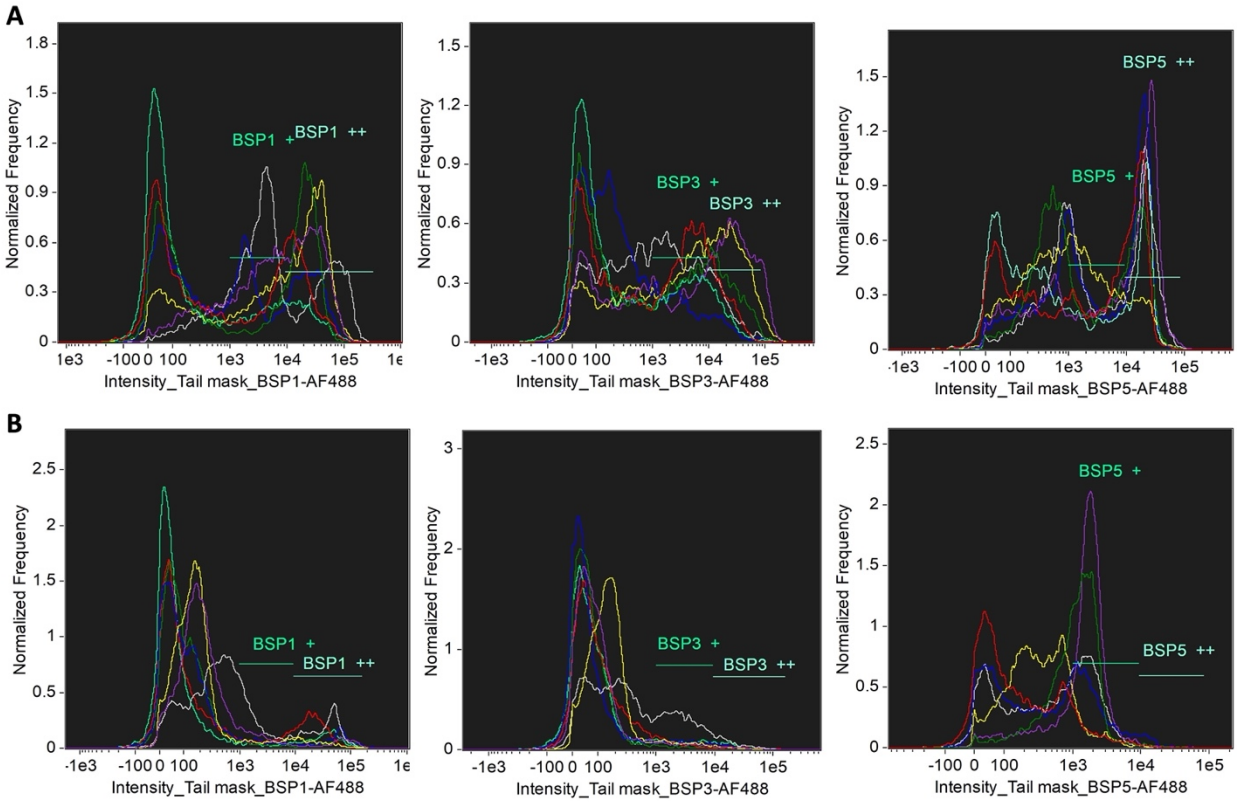


Figure 7. Effect of membrane permeabilization (0.025% digitonin) on the incidence of acrosomal damage and fluorescence intensities of BSP1 (A), BSP3 (B) and BSP5 (C) in cryopreserved bull spermatozoa. PNA+ (acrosomal damage), BSP- (basal levels of BSP), BSP+ (moderate intensity of BSP), BSP++ (high intensity of BSP), and BSP+PNA+ (spermatozoa with high levels of BSP and acrosomal damage). Sperm was evaluated immediately after thawing and permeabilization for either 20 or 40 minutes. Aliquots of non-permeabilized spermatozoa at the same incubation period were used as timed controls. Data are presented as mean ± SE and three biological replicates were performed. Different letters indicate significant difference across treatments. (P value <0.05).

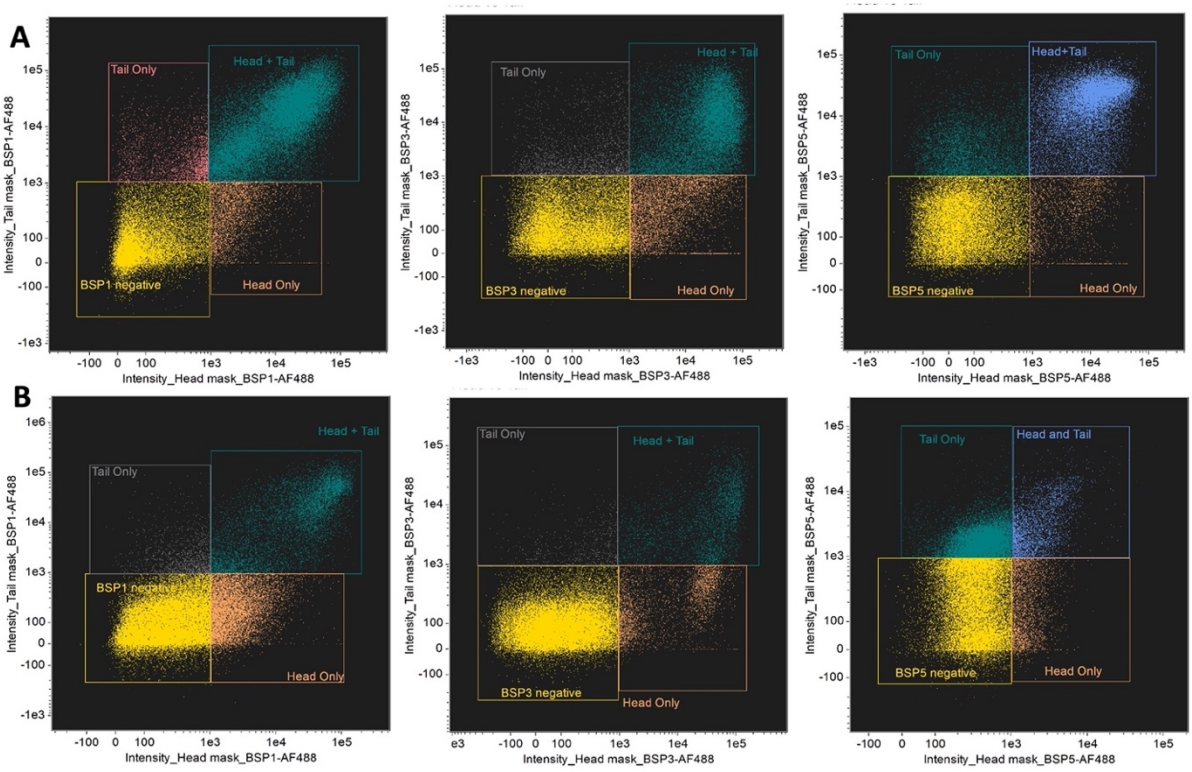
SUPPLEMENTARY MATERIAL



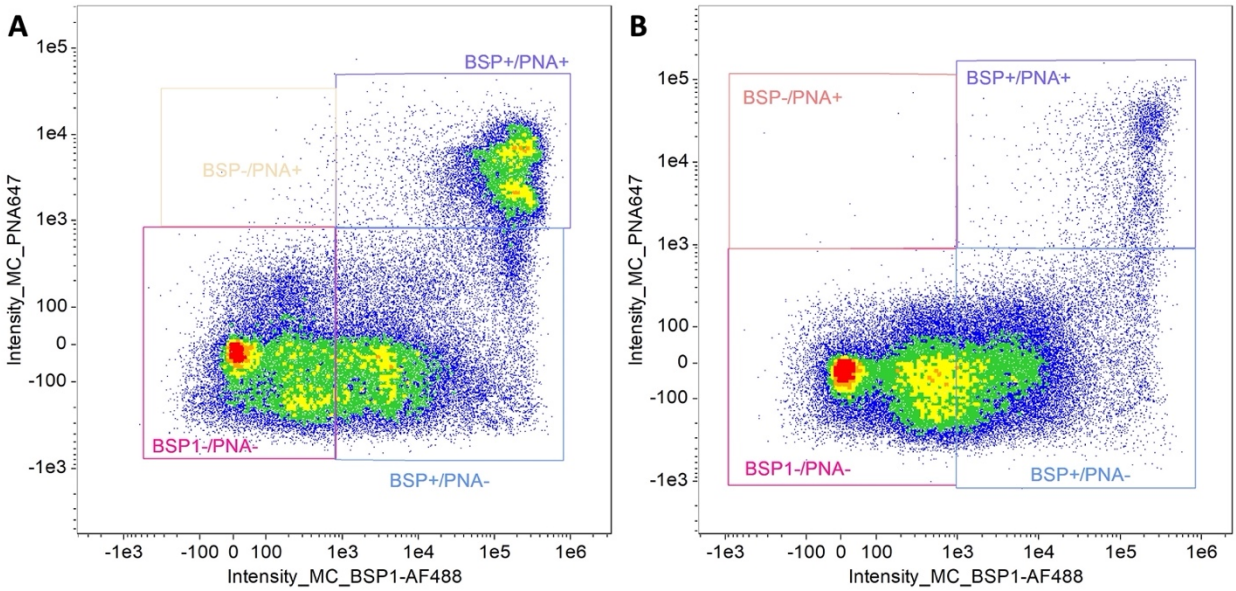
Supplementary Figure S1. Representative histograms of the BSP protein intensity within the head mask in the supernatant (A) and pellet (B) sperm fractions from seven fertile AI bulls. Three populations were gated; BSP- (Basal levels of BSP), BSP+ (moderate intensity of BSP), and BSP++ (high intensity of BSP).



Supplementary Figure S2. Representative histograms of the BSP protein intensity in the tail mask in the supernatant (A) and pellet (B) sperm fractions from seven fertile AI bulls. Three populations were gated; BSP- (Basal levels of BSP), BSP+ (moderate intensity of BSP) and BSP++ (high intensity of BSP).



Supplementary Figure S3. Representative scatterplots of the BSP protein fluorescence intensities from the sperm tail and head masks in the supernatant (A) and pellet (B) sperm fractions from seven fertile AI bulls. Data were gated as BSP proteins bound to the sperm head (Head Only), sperm tail (Tail only), whole spermatozoa (Head and Tail) or BSP negative. BSP1 and BSP3 localize preferentially to the sperm head in the pellet fraction, while BSP5 binds to the sperm tail. (C) In the supernatant fractions, both BSP proteins preferentially bind to the whole spermatozoa (both heads and tails).



Supplementary Figure S4. Representative scatterplots of fluorescence intensities of BSP1 and acrosomal integrity in the supernatant (A) and pellet (B) sperm fractions from seven fertile AI bulls. The scatterplots separate four sperm subpopulations: BSP-PNA- (spermatozoa with basal levels of BSP and an intact acrosome), BSP+PNA- (spermatozoa with moderate BSP levels and with and intact acrosomes), BSP-PNA+ (spermatozoa with basal levels of BSP and acrosomal damage), and BSP+PNA+ (spermatozoa with high levels of BSP and acrosomal damage).

Supplementary Table S5. Correlation coefficient of acrosome damage (PNA+) with the parameters evaluated from sperm fractions relative to BSP1 (A), BSP3 (B), and BSP5 (C). ***P < 0.0001; ** P < 0.001; *P < 0.05.

	PNA+		
	BSP1	**BSP3**	**BSP5**
BSP+	-0.05	0.27	0.17
	ns	ns	ns
BSP++	0.92	0.91	0.77
	***	***	***
BSP-	-0.65	-0.81	-0.64
	***	***	***
BSPH+	0.31	0.45	0.81
	*	**	***
BSPH++	0.85	0.90	0.52
	***	***	**
BSPT+	0.43	0.71	0.25
	**	***	ns
BSPT++	0.90	0.76	0.76
	***	***	***
Head Only	-0.49	0.18	0.23
	**	ns	ns
Tail Only	0.23	0.41	-0.09
	ns	**	ns
Head+Tail	0.83	0.90	0.86
	***	***	***

CRITICAL CONSIDERATIONS

- The breeding soundness evaluation (BSE) is still the best resource to assess the reproductive potential for bulls prior their use for natural service or cryopreservation; it's use is critical because around 14% of the bulls in selected populations have low fertility.
- The strict use of the BSE can improve the reproductive fitness of replacement bulls overtime and can control the dissemination of undesirable defects.
- Bulls born earlier in the calving season have better chances to be approved during the BSE and is reflected in better scrotal circumference.
- The aplastic midpiece defect (AMD) is a result of defective spermiogenesis. Bulls with a high percentage of AMD had an abnormal protein expression pattern during sperm maturation when compared to control bulls.
- The epididymis had an important function controlling any oxidative imbalance, protecting the sperm during transit and storage.
- Binder of sperm (BSP) proteins are multifunctional proteins. BSP1 and BSP3 bind preferentially the acrosome sperm head domain, while BSP5 bind preferentially the tail sperm midpiece.
- Sperm from the supernatant fractions have an increase abundance of BSP proteins in their surface.
- Sperm permeabilization induces BSP proteins binding, and acrosome damage correlates with a high abundance of BSP proteins.
- BSP proteins abundance could be a potential biomarker for defective sperm.