UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

"CONTROLE DE QUALIDADE DE DOSES INSEMINANTES SUÍNAS: ASPECTOS QUANTITATIVOS E QUALITATIVOS"

MONIKE QUIRINO DOS SANTOS

PORTO ALEGRE
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"CONTROLE DE QUALIDADE DE DOSES INSEMINANTES SUÍNAS: ASPECTOS QUANTITATIVOS E QUALITATIVOS"

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Tese apresentada como requisito parcial para obtenção de grau de Doutora em Ciências Veterinárias na área de Fisiopatologia da Reprodução de Suínos

Orientador: Prof. Fernando Pandolfo Bortolozzo

Coorientadores: Dr. Marin Schulze
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Título: Controle de qualidade de doses inseminantes suínas: aspectos quantitativos e qualitativos

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RESUMO

Apesar dos atuais e expressivos esforços empregados pelas centrais de inseminação artificial para garantir a qualidade das doses inseminantes suínas, ainda existem lacunas a serem preenchidas bem como oportunidades a serem exploradas no que diz respeito ao processo de controle de qualidade. Desta forma, foram conduzidos cinco experimentos para melhor compreender a influência de fatores quanti e qualitativos sobre a qualidade espermática e, também, para otimizar o processo de avaliação da qualidade das doses inseminantes suínas. O primeiro estudo avaliou o efeito de doses de sêmen suíno com diferentes concentrações espermáticas em 50 mL de Androstar Plus® (APlus; 20, 30, 60 e 100 × 10^6 células/mL) sobre a qualidade espermática e observou-se aumento da motilidade total (MT) à medida que a concentração espermática aumentou de 20 para 30 × 10^6 células/mL. Porém, a MT diminuiu conforme a concentração de células aumentou para 60 e 100 × 10^6/mL (*P* quadrático < 0,01). Além disso, os valores limite para atingir pelo menos 70% de MT às 120 h de armazenamento foram 4 e 59 × 10^6 células/mL, enquanto o valor que não afetou mais a MT foi de 50 × 10^6 células/mL (72% de MT). O segundo estudo comparou doses com baixa concentração espermática (16,7 × 10^6 células/mL em 90 mL) e doses mais concentradas (33,3 × 10^6 células/mL em 45 mL) produzidas com BTS ou APlus. Os dados demonstraram que, após 300 min de incubação a 38°C (teste de termorresistência; TTR), as doses de baixa concentração perderam 2 a 3 vezes menos pontos percentuais (pp) de MT em relação às doses com 33,3 × 10^6 células/mL (*P* < 0,01) independentemente do tipo de diluente. No terceiro estudo, foi verificada a influência de diferentes proporções de espermatozoides mortos (25%, 50% e 75%) na qualidade de doses de sêmen suíno ao longo do TRT (30 e 300 min). No início do teste, os tratamentos apresentaram medianas para MT de 77,6%, 50,2% e 25,6%, respectivamente. As doses com 25%, 50% ou 75% de células mortas perderam uma proporção semelhante de MT ao longo do TTR: 25% = -11,9%, 50% = -16,0% e 75% = -17,5% (*P* = 0,31). Além disso, a variação relativa dos parâmetros analisados por citometria de fluxo (CF) sugeriu que a presença das células mortas não alterou substancialmente a estrutura espermática ao longo do TTR. Por fim, o último estudo desenvolveu um painel de CF com cinco fluorocromos para analisar simultaneamente atividade mitocondrial (A), grau de desorganização lipídica da membrana plasmática (B), integridade da membrana plasmática (C) e *status* acrossomal (D) em doses de sêmen suíno, adicionando-se um marcador de DNA (E). Fortes correlações e valores

moderados e substanciais de CCC entre os ensaios individuais para cada fluorocromo e o ensaio multicolor foram encontrados para todos os parâmetros (0,99/0,99; 0,96/0,96; 0,93/0,92; 0,98/0,98 e 0,99/0,99 para A, B, C, D e E; $P < 0,01$, respectivamente), e a diferença média entre as abordagens não ultrapassou 0,84.
Palavras-chave: Controle de qualidade, Citometria de fluxo, Doses inseminantes, Sêmen suíno.

ABSTRACT

Despite the current and significative efforts applied by artificial insemination centers to guarantee boar semen does quality, there are still gaps to be filled and opportunities to be explored regarding the quality control process. Therefore, five experiments were performed to understand better the influence of some quantitative and qualitative factors on sperm quality and optimize the evaluation of boar semen doses quality. The first study evaluated the effect of boar semen doses with different sperm concentrations, in 50 mL of Androstar Plus® (APlus; 20, 30, 60 e 100 × 10^6 células/mL) on sperm quality, and it was observed an increase in total motility (TM) as the sperm concentration increased from 20 to 30 × 10^6 cells/mL. However, it decreased as the sperm concentration increased to 60 and 100 × 10^6 cells/mL (P quadratic < 0.01). Limit values to reach at least 70% TM at 120 h of storage were defined as 4 and 59 × 10^6 cells/mL, and the value which no longer affected the TM was 50 × 10^6 cells/mL (72% TM). The second investigation further assessed the sperm concentration effect on boar sperm quality, but focusing on the sperm resilience and the extender type. Low-concentrated doses (16.7 × 10^6 cells/mL in 90 mL) and higher concentrated doses (33.3 × 10^6 cells/mL in 45 mL) produced with BTS or APlus were compared. After 300 min of incubation at 38°C (thermo-resistance test; TRT), the low-concentrated doses lost 2 to 3-fold less pp of TM than the doses with 33.3 × 10^6 cells/mL (P < 0.01), regardless of the extender type. In the third study, the influence of different proportions of dead spermatozoa (25%, 50% and 75%) on the quality boar semen doses over a TRT was verified (30 and 300 min). Doses with 25%, 50% or 75% of dead cells lost similar proportion of TM over the TRT: 25% = -11.9%, 50% = -16.0% and 75% = -17.5% (P = 0.31). Regarding the flow cytometry parameters (FC), the relative variation suggested that dead sperm did not substantially change sperm structure over the TRT. Finally, the last study developed a 5-color FC panel to concurrently analyze mitochondrial activity (A), degree of lipid disorder of plasma membrane (B), integrity of plasma membrane (C), acrosomal status (D) in boar semen doses, while adding a DNA-marker (E). Strong correlations as well as moderate and substantial CCC values were found between the single staining assays and the 5-color staining assay regarding all parameters (0.99/0.99; 0.96/0.96; 0.93/0.92; 0.98/0.98, and 0.99/0.99) for A, B, C, D, and E; P < 0.01, respectively), and the mean difference between the methods did not overtake 0.84.

Keywords: *Flow cytometry, Quality control, Semen doses, Boar semen.*

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1. INTRODUÇÃO

A inseminação artificial (IA) é considerada a biotécnica mais importante na produção de suínos, permitindo inúmeras vantagens, como ganhos genéticos com o emprego de machos geneticamente superiores, maior segurança sanitária, redução nos custos de cobertura, melhor aproveitamento das instalações, eliminação de ejaculados impróprios para uso, etc. (BORTOLOZZO *et al.*, 2005). Atualmente, estima-se que mais de 90% das matrizes suínas de países com produção tecnificada são inseminadas artificialmente, com uso da inseminação artificial (IA) em 100% do plantel dos principais países produtores de carne suína (BORTOLOZZO *et al.*, 2015).

Apesar das vantagens e dos avanços obtidos ao longo dos anos na criopreservação de sêmen, o sêmen suíno congelado apresenta eficiência limitada. Dessa forma, a IA na produção de suínos ocorre majoritariamente com o uso de sêmen refrigerado, armazenado a 15 – 18°C por até 3 – 7 dias (WABERSKI *et al.*, 2019; MELLAGI *et al.*, 2022). Considerando esse cenário, as centrais têm empregado esforços para controlar os pontos críticos desde a coleta do ejaculado, sua diluição e envase. Além disso, tem-se realizado o monitoramento da capacidade de manutenção da longevidade das doses produzidas, avaliando-se, ao longo do período de validade, a motilidade de doses mantidas como contraprova na própria central (ALKMIN, 2019). Ainda, algumas centrais realizam um controle adicional (externo), a partir do envio periódico de doses a laboratórios de referência para análises de motilidade, morfologia e concentração.

A identificação de potenciais fatores a influenciar na qualidade espermática somente é possível devido ao avanço dos estudos desenvolvidos acerca da qualidade espermática ao longo das últimas décadas. Na Europa, nos últimos anos, um programa de controle de qualidade, considerado *science-based*, tem sido aplicado em cerca 40 centrais de IA do continente, em cooperação com dois laboratórios credenciados da Alemanha e a Associação de Pesquisas Bioeconômicas da Alemanha, Áustria e Suíça (SCHULZE *et al.*, 2022). No Brasil, embora se estime uma produção anual de doses de aproximadamente 9,5 milhões (BENNEMANN *et al.*, 2018), compreendendo cerca de 42 centrais registradas (MAPA, 2020), ainda há grandes oportunidades para implementar sólidos programas de controle de qualidade.

Apesar de todos os avanços obtidos até o momento, ainda existem lacunas acerca da real influência e importância de alguns parâmetros sobre a qualidade do espermatozoide suíno, como o efeito da taxa de diluição do ejaculado, o qual está diretamente relacionado à concentração espermática das doses. Os resultados das investigações até então realizadas não foram consistentes, e a maioria dos estudos não acompanhou a evolução técnica obtida ao longo dos últimos anos, principalmente em termos de diminuição do número total de células na dose (MARCOS *et al.*, 1991; ALEXOPOULOS *et al.*, 1996; WEITZE *et al.*, 2011). Dessa forma, a determinação e o monitoramento da concentração espermática das doses produzidas atualmente ocorre de acordo com o alvo de células totais e volume final da dose determinados pela central, sem levar em conta qualquer possível efeito direto da concentração sobre a qualidade espermática.

Outro potencial parâmetro a influenciar a qualidade das doses de sêmen suíno e que ainda pode ser melhor explorado é o percentual de células mortas presentes na dose. O efeito negativo das células mortas sobre as células vivas contemporâneas parece estar relacionado à ocorrência de estresse oxidativo (SHANNON & CURSON, 1972; BRINSKO *et al.*, 2003), e já foi reportado inclusive em sêmen suíno, mas as investigações limitaram-se a amostras criopreservadas (MARTINEZ-ALBORCIA, *et al.*, 2012; ROCA *et al.*, 2013). Para sêmen suíno refrigerado, a possível influência das células mortas sobre a qualidade espermática faz-se relevante principalmente no atual contexto de armazenamento das doses por longos períodos, devido ao processo de envelhecimento espermático (JOHNSON *et al.*, 2000). Nesse sentido, também se ressalta a execução de certas estratégias pelas centrais que elevam o percentual de células mortas nas doses, como a prática de compensação da baixa motilidade de ejaculados, através do aumento do número total de células na dose inseminante (BROEKHUIJSE *et al.*, 2012).

Ainda, deve-se reforçar a relevância do desenvolvimento de tecnologias e novas técnicas para avaliação *in vitro* dos espermatozoides, principalmente a serem aplicadas nos laboratórios de referência para controle externo de qualidade. Nesse cenário, destaca-se a citometria de fluxo, tecnologia que tem sido considerada uma das análises padrão-ouro de laboratórios de andrologia, juntamente com o sistema computadorizado de análise de sêmen - CASA (NIŻAŃSKI *et al.*, 2016). Apesar de já ter sido validada para mensurar a concentração espermática de ejaculados de diversas espécies (HANSEN *et al.*, 2002;

CHRISTENSEN, *et al.*, 2004a; CHRISTENSEN, *et al.*, 2004b; CHRISTENSEN *et al.*, 2005; HANSEN *et al.*, 2006; ANZAR *et al.*, 2009), a citometria de fluxo não foi implementada na rotina dos laboratórios de andrologia animal para determinar o parâmetro em doses inseminantes suínas, existindo ainda limitações referentes à precisão e execução da técnica, e também aos custos dos protocolos. Somado a isto, a tecnologia ainda se encontra limitada no que diz respeito a uma de suas principais vantagens: a avaliação simultânea de diferentes estruturas celulares a partir de ensaios multicolor (PETRUNKINA & HARRISON, 2011). O uso de painéis multicolor pode representar grandes avanços na avaliação de doses, uma vez que permite a identificação de subpopulações espermáticas com um mesmo atributo, fomentando o entendimento sobre a fisiologia espermática e a qualidade funcional das células (BUCHER *et al.*, 2019).

Portanto, o objetivo geral desse projeto foi desenvolver estudos para melhor compreender a influência de alguns fatores quanti e qualitativos sobre a qualidade espermática, e expandir o uso de tecnologias, como a citometria de fluxo, na avaliação da qualidade de doses inseminantes suínas. Para isso, foram conduzidos quatro estudos que serão apresentados em três diferentes capítulos ao longo desta tese.

2. CAPÍTULO I – Influência da concentração espermática sobre a qualidade de doses inseminantes suínas

2.1 Revisão bibliográfica

A qualidade espermática das doses inseminantes suínas está relacionada a diversos fatores, como a taxa de diluição (LOPEZ RODRIGUEZ *et al.*, 2017). A taxa de diluição refere-se à proporção entre o volume de diluente e de ejaculado, sendo determinada diretamente pela concentração espermática das doses inseminantes. A concentração espermática das doses de sêmen é dependente do número total de espermatozoides a se obter e o volume final da dose. Estes valores são definidos de acordo com a técnica de inseminação a ser executada e com os avanços obtidos na área de reprodução suína ao longo dos anos (WABERSKI *et al.*, 2019). O parâmetro de concentração espermática é um fator determinante a otimização do uso de ejaculados e obtenção de resultados satisfatórios reprodutivos a campo.

Devido à disponibilidade de componentes dos diluentes na dose ou a alterações metabólicas, doses muito ou pouco concentradas podem influenciar negativamente a qualidade espermática (FLOWERS, 1997) e, consequentemente, no desempenho reprodutivo. Alguns estudos foram realizados no passado com o intuito de sugerir taxas de diluição apropriadas para se obter a melhor qualidade espermática das doses (MARCOS *et al.*, 1991; ALEXOPOULOS *et al.*, 1996; WEITZE *et al.*, 2011). Contudo, os resultados são inconsistentes e as metodologias não acompanharam os avanços obtidos ao longo dos últimos anos no que diz respeito à diminuição do número total de células por dose, além das alterações de volume, conforme as diferentes técnicas de deposição das doses no trato reprodutivo feminino (BORTOLOZZO *et al.*, 2015; RIESENBECK *et al.*, 2015; WABERSKI *et al.*, 2019).

Portanto, ainda é necessário entender e elucidar o real efeito de diferentes taxas de diluição sobre a qualidade espermática, de forma a propiciar o estabelecimento de limites referentes à concentração espermática para assegurar a qualidade das doses produzidas. Nesse sentido, uma breve revisão acerca do assunto será desenvolvida a seguir.

2.1.1 Evolução da concentração espermática das doses de sêmen suíno

Desde a implementação da IA na suinocultura, o número de células por dose, bem como seu volume, vêm sendo alterados com base na técnica de IA a ser realizada e também na tentativa de se obter o máximo aproveitamento de cada ejaculado, diminuindo-se os custos de produção e potencializando o avanço genético. Nos anos 80, as centrais holandesas, por exemplo, produziam doses para IA cervical (IAC) contendo ~ 40 × 10^6 espermatozoides/mL (4 bilhões em 100 mL). Cerca de vinte anos após, as mesmas centrais passaram a produzir doses com praticamente metade dessa concentração: ~ 19 × 10^6 espermatozoides/mL (1,5 bilhão em 80 mL; RIESENBECK *et al.*, 2015).

No Brasil, uma evolução de 25 – 40 × 10^6 espermatozoides/mL (2,5 a 4,0 bilhões em 100 mL) para 31 – 38 × 10^6 espermatozoides/mL (2,5 a 3,0 bilhões em 80 mL) foi observada nos últimos 15 anos (BORTOLOZZO *et al.*, 2015). Atualmente, na Europa, de forma geral se observa a produção de doses para IAC contendo 18 – 29 × 10^6 espermatozoides/mL (1,5 a 2,5 bilhões em ~ 85 mL; WABERSKI *et al.*, 2019). Já em relação às doses para IA pós-cervical (IAPC), o objetivo atual tem sido atingir 1,0 a 2,0 bilhões em um volume final de ~ 45 mL, o que leva à produção de doses com concentração de 22 – 44 × 10^6 espermatozoides/mL BORTOLOZZO *et al.*, 2015; WABERSKI *et al.*, 2019).

É preciso considerar que a concentração espermática das doses inseminantes pode ser influenciada por determinadas estratégias adotadas pelas centrais, como a prática de compensação, a qual resulta no aumento da concentração espermática na tentativa de compensar ejaculados de baixa motilidade ou com alto percentual de defeitos espermáticos (BROEKHUIJSE *et al.*, 2012). Adicionalmente, pode-se citar a diluição do ejaculado com base no seu percentual de células móveis (viáveis) e não no número de células totais (WABERSKI *et al.*, 2019). Nestes dois cenários, a concentração espermática estará acima dos valores geralmente considerados.

Existem também cenários nos quais o alvo de concentração espermática não pode ser atingido, ficando abaixo dos valores desejados. A redução do número de células por dose, neste caso, seria uma consequência da ocorrência de surtos sanitários em uma ou mais centrais de uma empresa, por exemplo. Como plano de contingência, as centrais não afetadas tendem a produzir doses inseminantes com menor número de células justamente

para conseguir atingir a demanda de coberturas de todas as granjas da rede (BENNEMANN, 2008).

Por fim, é necessário considerar ainda os possíveis erros durante o processamento do ejaculado, como erros de avaliação da concentração espermática do ejaculado (problemas na homogeneização da amostra, falhas da técnica utilizada, etc.; BRITO *et al.*, 2016), levando à superestimação ou subestimação do número total de células na dose inseminante e, consequentemente, afetando a concentração espermática. Erros também podem ocorrer durante o processo de diluição do ejaculado; por isso, é necessário assegurar que um adequado volume de diluente seja adicionado, evitando-se o aumento ou a diminuição da taxa de diluição e, consequentemente, alterando a concentração espermática esperada na dose. Alguns diluidores automáticos já estão disponíveis a fim de se assegurar o volume a ser adicionado, evitando erros comuns da diluição manual. Durante a etapa de envase das doses inseminantes, também podem ocorrer como a má homogeneização do sêmen diluído também devem ser evitadas, evitando a sedimentação dos espermatozoides.

2.1.2 Efeitos da concentração espermática das doses inseminantes suínas

2.1.2.1 Sobre a qualidade espermática

Segundo FLOWERS (1997), as células espermáticas de doses com alta concentração espermática podem ter sua viabilidade prejudicada, devido à proporção insuficiente de substrato energético bem como de substâncias tamponantes. Da mesma forma, o autor discute que doses pouco concentradas também podem ser prejudiciais aos espermatozoides devido à ocorrência de choque osmótico, o qual provavelmente reduz a eficiência de processos bioquímicos das células que são fundamentais para a sua sobrevivência.

MARCOS *et al.* (1991) observaram maior motilidade em doses contendo entre 18 e 26 × 10^6 espermatozoides/mL comparado a doses contendo de 36 a 28 × 10^6 espermatozoides/mL ou 14 a 3 × 10^6 espermatozoides/mL. No estudo de WEITZE *et al.* (2011), doses contendo 31 × 10^6 espermatozoides/mL apresentaram melhores resultados de motilidade em comparação às doses menos concentradas: 6 ou 12 × 10^6 espermatozoides/mL. Ao comparar doses de 100 mL contendo 10, 30 ou 50 × 10^6 espermatozoides/mL, ALEXOPOULOS *et al.* (1996) relataram piores resultados de

motilidade nas doses mais concentradas (50 × 10^6 espermatozoides/mL). Já no estudo de LIPENSKÝ *et al.* (2013), maiores valores de motilidade progressiva após 60 min de incubação a 38°C foram observados para doses contendo ~ 84 × 10^6 espermatozoides/mL em comparação às doses mais diluídas, contendo ~ 25 e 46 × 10^6 espermatozoides/mL.

Nesse sentido, verifica-se que o real efeito da concentração espermática sobre a qualidade das doses de sêmen ainda não está bem estabelecido e que pode ser, ainda, influenciado por outros fatores, como o tipo de diluente utilizado, conforme observador por WEITZE *et al.* (2011) e LIPENSKÝ *et al.* (2013). Portanto, estudos mais robustos devem ser conduzidos para melhor investigar essa relação.

2.1.2.2 Sobre os parâmetros reprodutivos

A maioria dos estudos da área não conseguem investigar de fato o efeito da concentração espermática sobre a fertilidade, uma vez que as metodologias aplicadas até o momento variaram o número total de células nas doses dos diferentes tratamentos. Dessa forma, torna-se mais complexo ainda avaliar se o possível efeito negativo das altas ou baixas taxas de diluição do ejaculado sobre a qualidade das células espermáticas pode influenciar de alguma forma o processo de fecundação. Nos anos 90, por exemplo, ALEXOPOULOS *et al.* (1996) relataram que fêmeas inseminadas com doses de concentração 50 × 10^6 espermatozoides/mL (total de 5 bilhões de células em 100 mL) apresentaram maior taxa de retorno e menor taxa de parto em relação aos animais inseminados com doses menos concentradas: 10 ou 30 × 10^6 espermatozoides/mL (total de 1 ou 3 bilhões de células em 100 mL). Ao realizar a IA pós-cervical de fêmeas suínas com 0,25; 0,50 ou 1,0 bilhão de células totais (12, 25 e 50 × 10^6 espermatozoides/mL, respectivamente), MEZALIRA *et al.* (2005) não observaram diferenças na taxa de prenhez.

Além disso, nem todos os estudos trazem todas as informações necessárias para auxiliar na interpretação dos resultados. Ao avaliar a influência de diversos fatores relacionados à qualidade espermática sobre o desempenho reprodutivo de fêmeas inseminadas, FLOWERS (2005) observou que doses muito ou pouco concentradas, apresentando taxa de diluição abaixo de 1:5 ou acima de 1:15 contribuíram com uma redução média de 11% na taxa de parto e de 1,0 leitão no número total de leitões nascidos.

Contudo, o autor não informou mais detalhes sobre as doses, como concentração espermática, volume ou número total de células.

É importante destacar ainda que as metodologias aplicadas até então normalmente empregaram um alto controle de fatores influenciadores, como adequado protocolo de detecção de estro, controle do momento entre a última IA e a ovulação, tipo de IA realizada, qualidade da dose, etc. Dessa forma, a interpretação dos resultados acaba sendo limitada até mesmo quando se deseja avaliar propositalmente o efeito do número total de células nas doses. Isto enfatiza ainda mais a complexidade de se investigar a influência da concentração espermática sobre a fertilidade controlando fatores que podem mascarar os dados.

2.2 Primeiro artigo científico

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ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Estimation of sperm concentration limits to produce intrauterine insemination doses in swine

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Running head: Sperm concentration limits for swine semen doses

Estimation of sperm concentration limits to produce intrauterine insemination doses in swine

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ABSTRACT

This study evaluated the effect of sperm concentration of boar semen doses, for intrauterine artificial insemination (IUAI), on semen quality and established concentration limits for their production. Twenty ejaculates from four crossbred mature PIC® boars were collected to produce 50 mL-semen doses in a split-sample, reaching the following sperm concentrations: ~ 20; 30; 60 and 100 × 10^6 cells/mL. Doses were produced using Androstar Plus®, stored at 17 °C, and evaluated until 120 h of storage. There was a linear decrease in sperm motility as the sperm concentration increased (*P* linear < 0.01). The concentration

which no longer affected the total and progressive motility was 59 and 55 × 10^6 cells/mL, respectively (corresponding to 71 and 62%, respectively). The pH linearly decreased as the sperm concentration increased ($P < 0.01$); however, at 72 and 120 h the parameter dramatically reduced in boar semen doses with 60 and 100 × 10^6 cells/mL. The percentage of cells with intact plasma and acrosomal membranes or with high mitochondrial membrane potential was not influenced by the sperm concentration ($P \geq 0.15$) In conclusion, sperm motility was negatively affected in highly (60 and 100 × 10^6 cells/mL) concentrated doses. To achieve suitable sperm motility, boar semen doses may not surpass the sperm concentration of 55 × 10^6 cells/mL. The effect of low-concentrated boar semen doses on sperm quality still needs to be better evaluated, mainly considering the influence of extender type and thermo-resistance conditions.

Keywords: Semen dilution; Boar semen; Quality control; Sperm concentration; Sperm structure.

1. Introduction

Artificial insemination (AI) has contributed to the significant progress of the reproductive performance in the swine industry, promoting the use of males of high genetic value and optimizing the number of females inseminated per boar (Knox, 2014). It is estimated that more than 90% of swine females are artificially inseminated with cooled semen in most main pork-producing countries (Waberski et al., 2019), which reinforces AI as a fundamental biotechnique for current pork production. One of the key points influencing AI success is the semen dose quality, which is related to several factors, including the dilution rate (Lopez Rodriguez et al., 2017). It corresponds to the proportion

between the extender and ejaculate volumes (v/v), related to the sperm concentration of semen doses.

The use of low-concentrated semen doses was reported to negatively affect sperm quality (Marcos et al., 1991; García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013). This detrimental effect of decreasing the sperm concentration seemed to be related to metabolic changes in the medium (Flowers, 1997) or a negative dilution effect of important seminal plasma compounds (Weitze et al., 2011). However, most of these results may not be related to the progress that has taken place over the last few years in terms of the total number of sperm cells per dose or the evolution in boar genetics.

Furthermore, it is also important to mention the effect of high sperm concentrations, which has not been considered by most available studies. Despite current efforts to reduce the number of sperm cells per dose, some AI centers increase the total sperm number of semen doses, and consequently the sperm concentration, to compensate for problems of sperm quality, such as defective or immotile boar sperm (Broekhuijse et al., 2012). In addition, some boar studs produce doses based on the proportion of motile sperm instead of the total sperm number of ejaculates (Waberski et al., 2019). In these cases, the final volume of semen doses is not adjusted, then the sperm concentration increases and consequently, the proportion of substrate and buffer compounds is lower, which could also negatively affect sperm quality (Flowers, 1997).

Additionally, it is important to mention that even the recent investigations did not consider doses used for intrauterine AI (IUAI) (Marcos et al., 1991; García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013). This biotechnique shows a widespread commercial use for sows (García-Vázquez et al., 2019), and is

currently being performed with semen doses containing 25 to 40 × 10^6 cells/mL (1.0–2.0 × 10^9 sperm cells in 40–50 mL; Bortolozzo et al., 2015; Waberski et al., 2019), thus less diluted compared to the CAI doses already tested in the previous studies (6 to 10 × 10^6 cells/mL; Marcos et al., 1991; García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013).

In this sense, the real effect of sperm concentration of semen doses on boar sperm quality is not clear yet. Thus, in light of all the aforementioned, this study aimed to evaluate the effect of different sperm concentrations on the quality of boar semen doses for IUAI, using a long-term extender, and estimate limits for this parameter.

2. Material and methods

The study protocol was approved by the Veterinary Research Committee (COMPESQ-Vet) of the Federal University of Rio Grande do Sul (UFRGS), under process No. 39395.

2.1 Chemicals

All chemicals used in this study were of analytical grade unless otherwise stated. The fluorescein-isothiocyanate conjugated Pisum sativum agglutinin (FITC-PSA) and the carbonyl cyanide 3-chlorphenylhydrazone solution (CCCP) were purchased from Sigma-Aldrich (USA), while propidium iodide (PI) and SYBR-14 were obtained from Life Technologies – Thermofisher (LIVE/DEAD™ Sperm Viability Kit; USA). The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from BD Pharmingen™ (BD™ MitoScreen kit; USA). The semen extender (Androstar® Plus) was acquired from Minitüb (GmbH, Tiefenbach, Germany).

2.2 Animals and facilities

Over five weeks, ejaculates from four crossbred boars (Pietrain × Duroc; Agroceres PIC, Laranjeiras do Sul, Brazil) were collected weekly, totaling 20 ejaculates. The boars were healthy, mature (> 24-months old), housed in 6 m^2 individual pens and were collected for routine semen dose production at weekly intervals. The animals were fed 2.2 kg/day of a commercial corn-soybean meal diet (3.02 Mcal metabolizable energy/kg, 12.0% crude protein, and 0.7% total lysine), having *ad libitum* access to water.

2.3 Semen collection and processing

Ejaculates were collected by the gloved hand method using a pre-warmed (38 °C) collection cup. The pre-sperm phase of each ejaculate was discarded, and the gel fraction was removed by using a filter during collection. Raw semen was evaluated for volume, and 90 µL of each ejaculate was diluted in 810 µL of warmed extender (34 °C; Androstar® Plus) for sperm motility and concentration analysis using a computer-assisted semen analysis system (CASA system; AndroVision®, Minitüb GmbH). A 3-µL sample was placed by capillarity in preheated Leja chambers (Leja® 20 µm chamber depth, Nieuw Vennep, The Netherlands), and five fields were analyzed at 200 × magnification under a phase-contrast microscope with a warm stage set at 37 °C (Axio Scope A1, Zeiss® Germany). All ejaculates showed more than 70% sperm motility after collection. Regarding the settings for cell detection, sperm with amplitude lateral head displacement < 1 mm and curvilinear velocity < 24 mm/s were considered immotile. Progressive motility was defined as curvilinear velocity > 40 mm/s and straight-line velocity > 10 mm/s (Menezes et al., 2020).

Based on the sperm concentration of each ejaculate, semen doses were produced in a split-sample to achieve a total sperm number of 1.0×10^9, 1.5×10^9, 3.0×10^9, or 5.0×10^9 in a final volume of 50 mL, and consequently the following sperm concentrations: 20; 30; 60 and 100×10^6 cells/mL, respectively. Dilution was performed using Androstar® Plus at 34 °C. Extended semen was stored in 100-mL plastic bottles (Minitüb GmbH), removing the air content. After 90 min under room temperature (22–24 °C), semen doses were stored in a temperature-controlled cabinet at 17 °C for 120 h. To avoid an effect of manipulation, a different sealed bottle was used at each time point of analysis.

2.4 Semen dose evaluation

2.4.1 Sperm motility and concentration

At 24, 72, and 120 h of storage, motility was analyzed using the CASA system. One milliliter of extended semen was incubated, in a thermal block, for 10 min at 37 °C. Immediately after the incubation period, the samples from treatments with 30, 60 and 100×10^6 cells/mL were briefly diluted in Androstar® Plus (37 °C) to achieve ~ 20×10^6 cells/mL (2 parts of extended semen:1 part of the extender; 1:3 and 1:5, respectively). This step was carried out to standardize the number of cells analyzed by the CASA system among the treatments and also to allow the proper identification of all the sperm cells, considering mainly the samples containing 60 or 100×10^6 cells/mL. Then a 3-µL sample was placed by capillary flow in preheated Leja chambers and was analyzed as described for raw semen analysis. The sperm concentration at 24 h, assessed by the CASA system, was also recorded.

2.4.2 Flow cytometric assessment

2.4.2.1 Sample staining

Plasma membrane and mitochondrial status of boar spermatozoa were assessed at 24, 72, and 120 h of storage over four weeks of the experiment. Samples of semen doses were diluted in Androstar® Plus to achieve a final concentration of 5.0×10^6 sperm cells/mL. Then, 150 µL-samples were stained with FITC-PSA (final concentration: 2.5 µg/mL) and PI (final concentration: 12 µM), to assess plasma and acrosomal membrane integrity. After staining, samples were incubated for 10 min at 37 °C, and prior to analysis, 150 µL of Androstar® Plus was added, achieving a final concentration of 2.5×10^6 sperm cells/mL. For assessing the mitochondrial membrane potential, the 150 µL-samples were stained using the BD™ MitoScreen kit (JC-1), achieving a final volume of 400 µL (1.9×10^6 sperm cells/mL), according to the manufacturer's instructions. The samples were incubated for 10 min at 37 °C and then analyzed.

2.4.2.2 Sample analyses

Analysis was performed using a BD Accuri C6 Plus (BD Biosciences, San Jose, USA) equipped with lasers, blue (488 nm, 20 mW) and red (640 nm, 12.5 mW), containing four detectors: 533/30 nm (FL1, band pass filter), 585/40 nm (FL2, band pass filter), 670 nm (FL3, long pass filter), and 675/25 nm (FL4, band pass filter). A total of 10,000 events were obtained per sample, and all the fluorescence signals were plotted on logarithmic scales. The sperm population was gated according to the expected forward- and side-scatter signals, also plotted on logarithmic scales, based on a previous pool semen sample stained with SYBR-14.

For establishing the gates for the fluorochromes, a pooled sample from diluted semen doses was obtained and used for preparing the unstained, positive, and negative controls for all fluorochromes. For fluorochromes FITC-PSA and PI, the negative control consisted of the original pool stained with each fluorochrome individually, while the positive control was the pool submitted to three rounds of flash-freezing process in liquid nitrogen, followed by rapid thawing at 37 °C and subsequent staining. For JC-1, the controls were performed using an original pool sample and a pool sample stained with JC-1 after being stained and incubated (37 °C for 5 min) with 1.0 µL of CCCP solution (at 150 mM).

Fluorescence signals of FITC-PSA and PI were detected by FL1 and FL3 channels, respectively, while JC-1 signals were detected by FL1 and FL2 channels. The percentage of spermatozoa with intact plasma and acrosome membranes was recorded, as well as the percentage of spermatozoa with high mitochondrial membrane potential. All data were recorded and analyzed using the BD Accuri C6 Plus software. The equipment was calibrated prior to each routine to verify the cytometer's optical alignments and fluidic system, using specific calibration beads provided by the manufacturer.

2.4.3 Monitoring of pH

Semen doses' pH were determined at 24, 72, and 120 h of storage using a previously calibrated digital pH-meter (K39-2014B Kasvi®, São José dos Pinhais, PR, Brazil). The pH-meter calibration was performed using three buffer solutions with a pH of 4.01, 7.00, and 10.01. The pH electrode was thoroughly washed with distilled water and wiped between samples.

2.4.4 Thermo-resistance test

During two weeks of the experimental period, a thermo-resistance test (TRT) was performed after 120 h of storage. Subsamples of 10 mL of each semen dose were incubated at 38 °C for up to 300 min, according to an adapted protocol from Schulze et al. (2013). Total and progressive motility were analyzed using the CASA system after 60 min (TRT60) and 300 min (TRT300) incubation. The motility analysis procedure was performed as described previously.

2.5 Statistical analysis

Data were analyzed using the Statistical Analysis System software (SAS, version 9.4; SAS® Institute Inc., USA), presented as observed means or LSMeans ± standard error of the mean and considered significant at a $P \leq 0.05$. For comparing the sperm concentration, total sperm number and the dilution rate among treatments, the GLIMMIX procedure was used, comparing the mean values with Tukey's test.

As semen evaluations were performed at different time points (24, 72, and 120 h of storage), the analyses of response variables (total and progressive motility, plasma and acrosomal membrane integrity, mitochondrial membrane potential, and pH) were performed as repeated measures considering a 4 (sperm concentration) × 3 (storage times) factorial design. As the correlation between observations along storage time on the same subject could be not mutually independent, the adequate covariance structure was selected for each model. Polynomial orthogonal contrasts were used to determine the linear and quadratic effects, as well as the effects of their interaction with sperm concentration and time of storage. The IML procedure of SAS was used to adjust linear and quadratic coefficients after accounting for unequally spaced sperm concentration.

The fixed effects included in the models were sperm concentration (20, 30, 60 and 100 × 10^6 cells/mL), time of storage (24, 72, and 120 h), and their interactions, while boar and week were included as random effects. When the sperm concentration × storage time interaction was significant, differences in linear or quadratic responses between the storage times were investigated using the corresponding contrast coefficients for this comparison pair. Tukey-Kramer test was used for multiple comparisons regarding the storage time.

Regarding the TRT data, the linear and quadratic effects of varying the sperm concentration on sperm motility, polynomial orthogonal contrasts were also used in the GLIMMIX procedure, considering the sperm concentration as a fixed effect and including boar and week as random effects. For all models, a binomial distribution was fit to the motility (total and progressive), plasma and acrosomal membrane integrity, and mitochondrial membrane potential data, whilst a normal distribution was fitted to pH data.

Furthermore, to better evaluate the sperm concentration effect on sperm motility, we fitted three competing dose-response mixed models to total and progressive motility: quadratic polynomial model (QP), Broken-line linear descending model (BLL) and Broken-line quadratic descending model (BLQ). The GLIMMIX procedure was used to fit the base and QP models and the NLMIXED procedure was used to fit the BLL and BLQ models (Gonçalves et al., 2016). All the models took into account three treatments (30 to 100 × 10^6 sperm cells/mL), including two segments: 30 to 60 × 10^6 sperm cells/mL and 60 to 100 × 10^6 sperm cells/mL. The mean total or progressive motility considering all the storage times was included as fixed effect and the boar was included as random effect. Fit between competing these models was compared using a maximum likelihood-based Bayesian information criterion (BIC; smaller is better), according to Schwarz (1978). Then, a BLL model was applied to the total and progressive motility data (Formula 1).

Formula 1: BLL predictive equation for total and progressive motility

$$For\ X_i \geq 30 \times 10^6 and < \omega_{BLL}: y_{ij} = \varphi_{BLL} + \beta_{BLL} \times (\omega_{BLL} - X_i) + b_j + e_{ij}$$

$$For\ X_i > \omega_{BLL}: y_{ij} = \varphi_{BLL} + b_j + e_{ij}$$

X_i: $sperm\ concentration\ (\times 10^6/mL)$
ω_{BLL}: $Break - point$
y_{ij}: $Motility, \%$
φ_{BLL}: $Plateau\ in\ y$
β_{BLL}: $Regression\ coefficient$
b_j: $Boar\ effect$
e_{ij}: $Residual$

The competing dose-response mixed models were also fitted to pH data, according to the storage time. The models included the four treatments, considering two segments: 20 to 30 × 10^6 sperm cells/mL and 60 to 100 × 10^6 sperm cells/mL, and the boar was included as random effect. A QP model was applied to the pH at 24 and 120 h (Formula 2), while a BLL model better fitted to pH at 72 h (Formula 3).

Formula 2: QP predictive equation for pH at 24 and 120 h

$$y_{ij} = \beta_{0,QP} + \beta_{1,QP} X_i + \beta_{2,QP} {X_i}^2 + b_j + e_{ij}$$

X_i: $sperm\ concentration\ (\times 10^6/mL)$;
y_{ij}: pH
$\beta_{0,QP}$: $Intercept$
$\beta_{1,QP}$: $Linear\ regression\ coefficient$
$\beta_{2,QP}$: $Quadratic\ regression\ coefficient$
b_j: $Boar\ effect$
e_{ij}: $Residual$

Formula 3: BLL predictive equation for pH at 72 h

$$For\ X_i > \omega_{BLL}: y_{ij} = \varphi_{BLL} + \beta_{BLL} \times (\omega_{BLL} - X_i) + b_j + e_{ij}$$

$$For\ X_i < \omega_{BLL}: y_{ij} = \varphi_{BLL} + b_j + e_{ij}$$

X_i: $sperm\ concentration\ (\times 10^6/mL)$
ω_{BLL}: $Break - point$
y_{ij}: pH
φ_{BLL}: $Plateau\ in\ y$
β_{BLL}: $Regression\ coefficient$

$b_j : Boar\ effect$
$e_{ij} : Residual$

3. Results

The mean values for total and progressive motility in raw semen were 95.6 ± 0.6% and 93.2 ± 0.7%, respectively. Overall, the number of spermatozoa per mL (× 10^6) and raw semen volume (mL) were 458.4 ± 33.0 and 203.2 ± 14.2, respectively (**Table 1S**).

The difference in the sperm concentration and total number of cells per dose among the treatments was statistically significant ($P < 0.01$), ensuring the treatments were successfully achieved. The treatments 20; 30; 60 and 100 × 10^6 cells/mL had an average of 19.9; 29.0; 60.7; and 105.2 × 10^6 sperm cells/mL, respectively, corresponding to the following total sperm numbers: 0.95, 1.45, 3.03, and 5.26 × 10^9 cells per dose. Regarding the dilution rate, the highest value was observed for semen doses containing 20 × 10^6 cells/mL (22 parts of extender:1 part of semen), while the lowest rate was registered for doses with 60 and 100 × 10^6 cells/mL (7:1 and 4:1, respectively, $P < 0.01$; **Table 2S**).

The pH value was affected by the linear interaction between the sperm concentration and the storage time ($P < 0.01$). The parameter linearly decreased as the sperm concentration increased; however, the slope of each storage time differed, as shown in **Figure 1S**. Contrarily to 24 h, pH at 72 and 120 h dramatically reduced in boar semen doses with 60 and 100 × 10^6 cells/mL. Based on the dose-response models applied to pH data (**Figure 1S**), a plateau on pH of semen doses stored for 72 h occurred from 20 to 30 × 10^6 cells/mL (7.21; break-point: 29.9 × 10^6 cells/mL). From this point on, the pH was estimated to decrease ~ 0.02 for each increase of 5 × 10^6 cells/mL ($y = 7.21 + 0.0034 \times (29.9 - X)$).

For total and progressive motility, no significant interaction between the storage time and sperm concentration was observed ($P \geq 0.29$); however, the values were higher at 72 h of storage compared to 120 h ($P < 0.01$). Irrespective of storage time, as the sperm concentration increased, motility parameters linearly decreased ($P < 0.01$; **Table 1**). According to the BLL model applied to total motility data, a break-point was observed when the sperm concentration of 59.4×10^6 cells/mL was achieved, corresponding to 71.4% total motility (**Figure 1**). Based on the BLL predictive equation, total motility = 71.1 + 0.40 × (59.4 – sperm concentration) if sperm concentration ≥ 30 and < 59.4×10^6 cells/mL; or = 71.1 if sperm concentration > 59.4×10^6 cells/mL. For progressive motility, the plateau occurred when the sperm concentration of 54.8×10^6 cells/mL was achieved (62.1%; **Figure 1**). The following BLL predictive equation was estimated: progressive motility = 62.1 + 0.57 × (54.8 – sperm concentration) if sperm concentration ≥ 30 and < 54.8×10^6 cells/mL, or = 62.1 if sperm concentration > 54.8×10^6 cells/mL.

When the TRT was performed (**Figure 2**), it was observed at 60 min that as the sperm concentration increased, total and progressive motility quadratically increased ($P < 0.01$). A quadratic effect ($P < 0.01$) was also observed in sperm motility (total and progressive) at 300 min of incubation, but at this moment the motility decreased according to the increase on sperm concentration. No significant effect of the sperm concentration or sperm concentration × storage time was found on the percentage of cells with intact plasma and acrosomal membranes or on the percentage of spermatozoa with a high mitochondrial potential ($P \geq 0.15$; **Table 1**). However, greater proportion of sperm with intact membranes was observed at 72 h of storage ($P < 0.01$), and the mitochondrial membrane potential was higher at 72 h compared to 24 h of storage ($P = 0.01$).

4. Discussion

To better understand the real effect of sperm concentration on quality of semen doses, this study compared the sperm parameters of boar semen doses produced with different total sperm numbers in 50 mL: 1.0; 1.5; 3.0; or 5.0×10^9 sperm cells (20; 30; 60 and 100×10^6 cells/mL, respectively). Our results showed no effect of sperm concentration was observed on membrane integrity (plasma and acrosomal) or mitochondrial membrane potential, sperm concentration seems to have no impact on the sperm structure in boar semen doses. However, we could observe that increasing the sperm concentration negatively affected the motility of IUAI doses (total and progressive), since the parameters linearly decreased according to the increase on the number of cells/mL.

In this sense, the effect of high sperm concentrations ($> 60 \times 10^6$ cells/mL) on the quality of semen doses should be a concern for boar studs, mainly when performing the compensation strategy. This method allows the use of ejaculates presenting motility lower than the minimum expected (70%) by increasing the total sperm number per dose and, consequently, the sperm concentration (Broekhuijse et al., 2012). Sperm cells from doses with high sperm concentration might present lower motility due to an insufficient amount of energetic substrate (Flowers, 1997) or to a lower proportion of buffer substances since the amount of lactic acid produced is proportional to the sperm concentration (Marcos et al., 1991). This seems to be the case in CAI doses containing 50×10^6 cells/mL (5 billion cells in 100 mL; Alexopoulos et al., 1996) as well as the IUAI doses we produced in this study, with 60 and 100×10^6 cells/mL, which presented lower motility and pH values, mainly at 72 and 120 h of storage. Thus, to avoid the negative effects of high sperm concentrations, boar studs should also consider adjusting the final volume of semen doses when performing the compensation strategy. It must be noted that even making this

adjustment, the studs must still take into account that this strategy is also related to the proportional increase of dead sperm cells in semen doses, and its effect on sperm quality is not entirely clear yet (Quirino et al., 2022).

According to the BLL fitted to total motility data, semen doses with > 59 × 10^6 cells/mL would present total motility lower than 71%., while progressive motility values lower than 62% would be expected for doses with > 55 × 10^6 cells/mL. It suggests an upper limit of ~ 55 × 10^6 cells/mL to achieve suitable motility in boar semen doses. Doses produced with this high concentration are not commercially used in the swine industry (Waberski et al., 2019). Currently, it occurs only when the compensation strategy is performed or in errors scenario during the dilution of ejaculate or filling of semen doses. For this reason, we reinforce the importance of quality control programs focused on the sperm concentration of doses to guarantee sperm quality. Moreover, in a near future, some boar studs may apply more efforts to further reduce the semen dose volume (Waberski et al., 2019). Therefore, precise control of sperm concentration in semen doses would be necessary to avoid decreased sperm motility.

Interestingly, the sperm motility values did not seem to linearly decrease when the sperm concentration increased from 60 to 100 × 10^6 cells/mL, suggesting the occurrence of a breaking point, which corresponds to the value of sperm concentration that would no longer influence sperm motility. This point was estimated to be 59 × 10^6 cells/mL for total motility and 55 × 10^6 cells/mL for progressive motility. We believe that from this point onward, the competition for substrate and the metabolic alterations may have reached a plateau, thus, controlling the possible negative effects on total sperm motility.

It is logical to hypothesize that low-concentrated semen doses would have better sperm quality since they are submitted to a higher availability of nutrients and buffers (high

dilution rate). However, high dilutions could cause osmotic shock (Flowers, 1997) or lead to a proportional reduction of protective seminal plasma components (Weitze et al., 2011). Thus, doses with a low sperm concentration could also have sperm motility negatively affected. Indeed, as reported in other studies with CAI doses (Weitze et al., 2011; Lipenský et al., 2013), we observed that IUAI doses containing 20×10^6 cells/mL had lower total and progressive motility than doses with 30×10^6 cells/mL. Unfortunately, there is a lack of studies assessing the effect of low-concentrated boar semen doses. It has already been investigated the effect of low total sperm number ($\leq 0.3 \times 10^9$ cells) on reproductive performance; however, the semen doses used contained ~ 30×10^6 cells/mL (Belstra et al., 2020). In this way, further studies focused on the sperm quality of low-concentrated semen doses ($< 20 \times 10^6$ cells/mL) are required for better estimating lower limits of sperm concentration to be related to the sperm quality.

Still considering the low sperm concentration effect, we should highlight the results from the TRT, which is a fundamental tool for predicting semen fertility that simulates the time of spermatozoa in the female genital tract (Schulze et al., 2019, 2021). At the on-test, greater results were observed for doses with 30×10^6 cells/mL. However, after 300 min of incubation, there was a linear decrease of motility according to the increase of sperm concentration, suggesting a better resistance for the low concentrated doses (20×10^6 cells/mL). A higher extender proportion must probably better control sperm metabolism due to a greater concentration of membrane stabilizers in the final semen dose. In this sense, it is possible that low-concentrated semen doses would demand more incubation time to reactivate the sperm metabolism better. However, additional investigations must be performed to further evaluate the sperm concentration effect over a TRT. Consequently, we recommend that boar studs consider the limit values we have calculated for sperm

concentration when decreasing the total sperm number per dose, a common strategy applied in recent years for producing cost-efficient semen (Waberski et al., 2019).

A long-term extender was used in our methodology, which might play an important role in the relationship between sperm concentration and sperm quality. Weitze et al. (2011) and Lipenský et al. (2013) reported that complex extenders minimized the negative effects of altering the sperm concentration in CAI doses. Long-term extenders may probably be able to counteract a possible effect of low sperm concentrations due to their ability to provide better membrane protection and stabilization. As observed, the pH dramatically decreased in boar semen doses with 60 and 100 × 10^6 cells/mL after 72 h of storage. At 120 h, the parameter already started decreasing when sperm concentration increased from 20 to 30 × 10^6 cells/mL. In this sense, the use of complex extenders could also be fundamental for doses highly concentrated, mainly considering the lactic acid production (Marcos et al., 1991), since they may offer a greater buffer proprieties for better controlling the medium's pH. Indeed, no effect of sperm concentration on the boar sperm membranes and mitochondrial potential was observed in this study. Moreover, if simpler extenders are used, the negative impact of high sperm concentrations on sperm motility may be even more significant in semen doses than we observed. For this reason, it is important to investigate the influence of the extender type on the sperm concentration effect for boar semen doses.

This study provides important preliminary results regarding the relationship between sperm concentration and sperm quality in boar semen doses. However, the effect of sperm concentration on reproductive performance still must be investigated. We may also mention that we did not identify a relationship between the ejaculate parameters (such as motility or sperm concentration) and its resilience according to the different sperm

concentrations. In this sense, further studies, with greater variability of boars and genetics, are needed to assess whether there are ejaculate properties capable of suggesting it can be highly or poorly diluted. Furthermore, with a larger number of boars, we could identify whether there is any effect of boars influencing the sperm concentration response and verify if it would be attributed to possible differences in seminal plasma composition.

5. Conclusion

In conclusion, boar semen doses highly concentrated ($60 - 100 \times 10^6$ cells/mL) negatively impaired the sperm motility. Moreover, boar semen doses may not surpass the sperm concentration of 55×10^6 cells/mL to reach suitable sperm motility. Additional studies investigating the sperm concentration effect are still necessary to understand the relationship between low sperm concentration and sperm quality in boar semen doses, mainly considering the sperm resilience over a TRT, extender type influence, boar effect, and reproductive performance.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

MQ: Methodology, Investigation, Formal analysis, Writing – original draft. **GTR:** Investigation **TCS:** Investigation. **WRV:** Investigation. **RRU:** Methodology, Supervision, Review & Editing. **MLB:** Formal analysis. **MS:** Supervision, Review & Editing. **APGM:** Methodology, Formal analysis, Supervision, Review & Editing. **FPB:** Conceptualization, Methodology, Supervision, Review & Editing, Funding acquisition, Project administration. All authors read, revised, and approved the final manuscript. **Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Effect of sperm concentration and storage time on motility of boar semen doses (50 mL) stored for 120 h at 17°C.

	Sperm concentration ($\times 10^6$ cells/mL)					*P*-value			
	20	**30**	**60**	**100**	**Mean**	**ST**	**SC, Linear**	**SC, Quadratic**	**ST × SC**
Total motility, %									
24 h	80.3	83.9	72.2	75.5	78.4[ab]	< 0.01	< 0.01	0.10	0.29
72 h	79.1	86.3	73.7	74.4	78.8[b]				
120 h	78.7	82.7	69.6	68.5	75.4[a]				
Mean	79.2	84.1	72.0	73.6	.				
Progressive motility, %									
24 h	74.1	77.3	63.6	65.5	70.5[ab]	< 0.01	< 0.01	0.14	0.61
72 h	73.4	79.5	66.0	65.0	71.1[b]				
120 h	72.3	75.8	60.2	58.1	67.0[a]				
Mean	72.9	77.2	63.4	63.6	.				
Plasma and acrosomal membrane integrity, %									
24 h	73.9	73.4	75.6	72.4	73.8[a]	< 0.01	0.17	0.15	0.21
72 h	77.7	78.8	80.4	80.3	79.3[b]				
120 h	70.2	72.0	72.7	73.9	72.3[a]				
Mean	74.1	74.9	76.4	75.7	.				
Mitochondrial membrane potential, %									
24 h	67.2	70.5	70.1	69.4	69.3[a]	0.01	0.07	0.22	0.60
72 h	70.6	70.6	72.0	73.7	71.3[b]				
120 h	67.8	70.4	71.9	71.8	70.5[ab]				
Mean	68.5	70.5	71.4	71.7	.				

ST: Storage time; SC: Sperm concentration

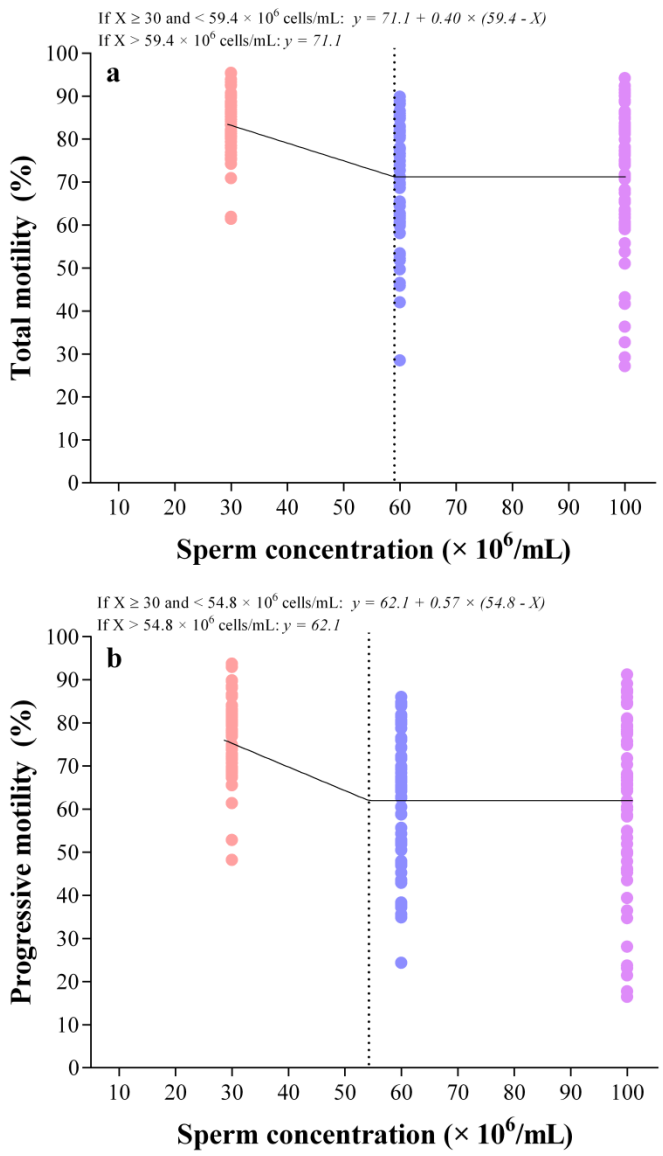

Fig. 1. Nonlinear broken-line linear mixed model for sperm motility (y), including mean predictions (descending and horizontal lines) and estimated sperm concentration (X) break-point (vertical line). **a)** Total motility; **b)** Progressive motility.

The models considered three treatments (30 to 100 × 10^6 sperm cells/mL), including two segments: 30 to 60 × 10^6 sperm cells/mL and 60 to 100 × 10^6 sperm cells/mL. Therefore, the equation for the descending segment applies only to sperm concentrations ≥ 30 × 10^6 and < plateau value (59.4 and 54.8 × 10^6 sperm cells/mL for total and progressive motility, respectively).

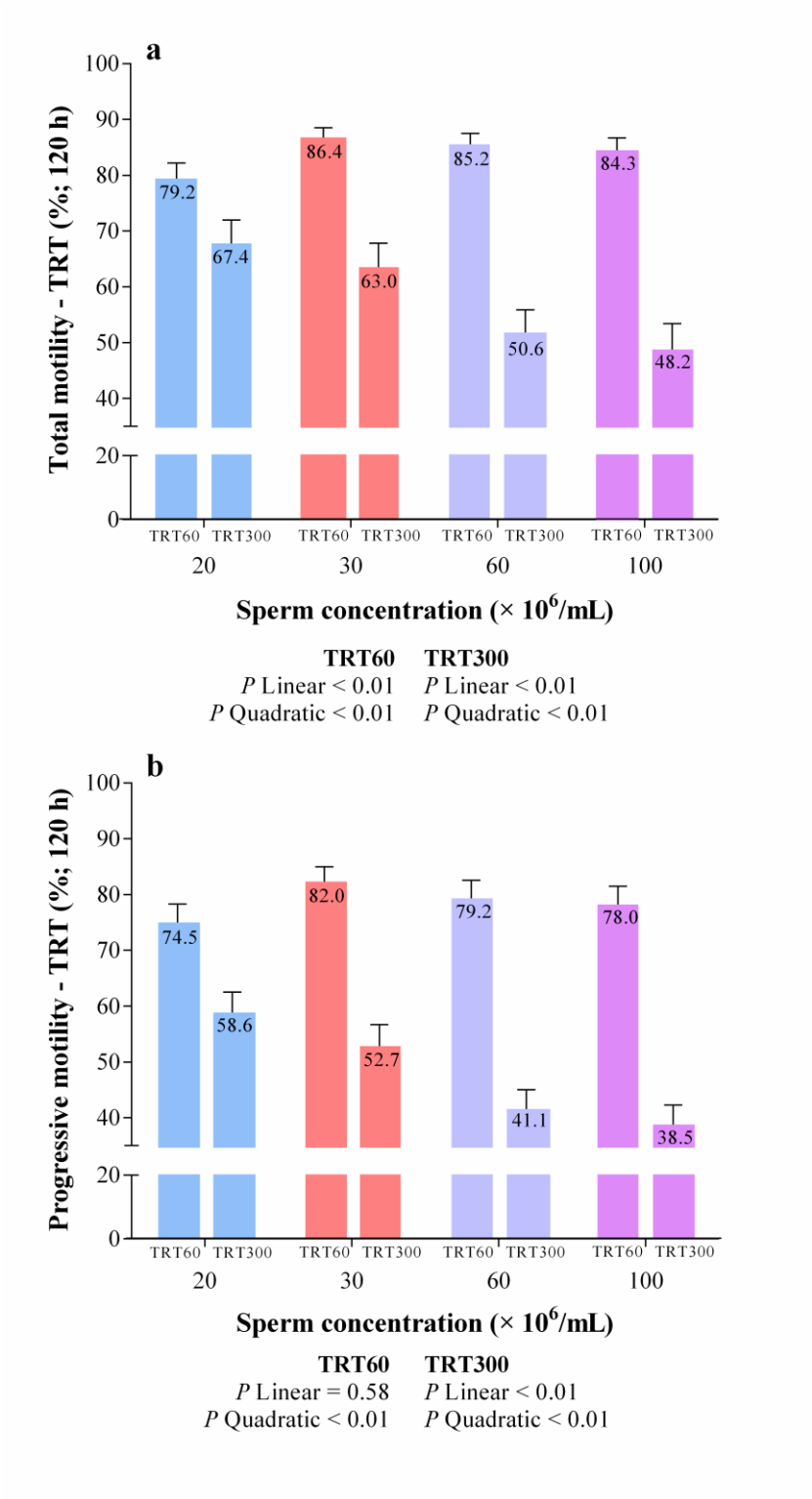

Fig. 2. Effect of sperm concentration on total (**a**) and progressive (**b**) motility of boar semen doses (50 mL) submitted to a thermo-resistance test (TRT) after 120 h of storage at 17 °C. TRT60: 60 min; TRT300: 300 min.

SUPPLEMENTARY MATERIAL

Table 1S. Sperm parameters of boar ejaculates used for producing the intrauterine insemination semen doses containing different sperm concentrations.

Characteristics	*n*	Mean	SD	SEM	Min	Max	1st Quartile	Median	3rd Quartile
Total motility (%)	20	95.6	2.7	0.6	88.1	98.5	94.5	96.4	97.5
Progressive motility (%)		93.2	3.2	0.7	83.7	96.8	92.0	93.7	95.4
Concentration (10^6/mL)		458.4	147.6	33.0	203.7	723.0	351.9	446.5	524.1
Volume (mL)		203.2	63.6	14.2	80	375.0	173.8	197.5	230.0

SD: standard-deviation; SEM: standard error of the mean; Min: minimum; Max: maximum

Table 2S. Sperm concentration, total sperm number, and dilution rate (mean ± standard error of mean) of boar semen doses, for intrauterine artificial insemination (50 mL), according to the stablished treatments for sperm concentration.

	Sperm concentration of 50 mL-semen doses (cells/mL)			
	20×10^6	30×10^6	60×10^6	100×10^6
Real sperm concentration, $\times 10^6$ cells/mL	19.9 ± 0.76^a	29.0 ± 1.03^b	60.7 ± 1.17^c	105.2 ± 2.82^d
Target total sperm number, $\times 10^9$ cells	1.0	1.5	3.0	5.0
Real total sperm number, $\times 10^9$ cells	0.95 ± 0.04^a	1.45 ± 0.05^b	3.03 ± 0.08^c	5.26 ± 0.19^d
Dilution rate, extender/semen	21.9 ± 1.65^a	14.3 ± 1.09^b	6.7 ± 0.54^c	3.6 ± 0.32^c

Different letters indicate differences among treatments ($p < .01$).

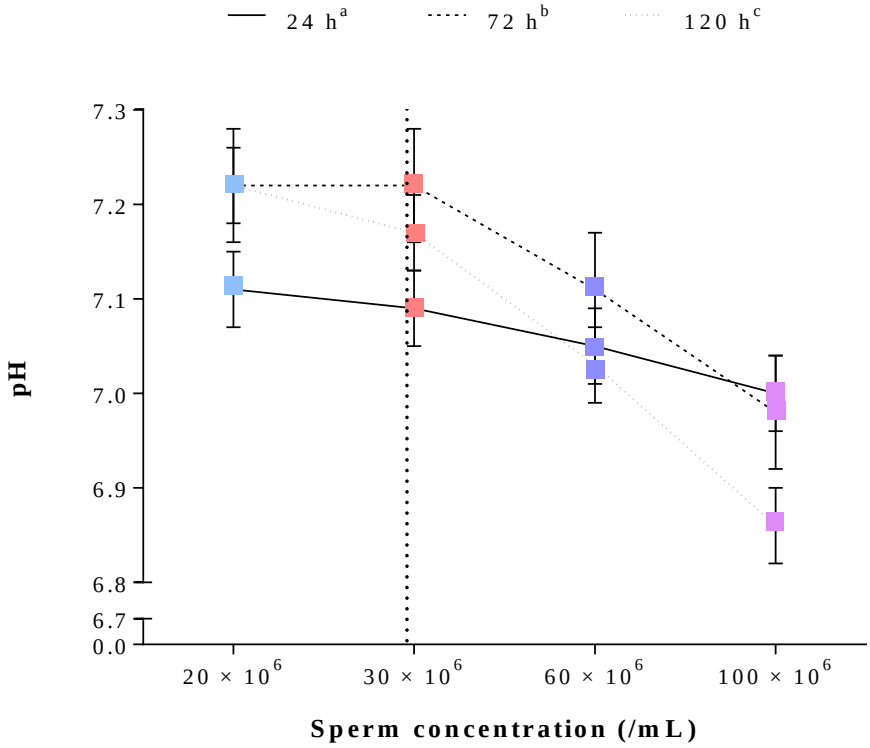

Fig. 1S. Comparison of slope responses regarding the pH values according to the interaction between sperm concentration and storage time of boar semen doses stored at 17°C (p = .01).

[abc]Different letters indicate that responses to sperm concentration were different among the storage time ($p \leq .04$).

24 h (Quadratic polynomial predictive equation):

$y = 7.14 – 0.0018 \times (\text{sperm concentration}) + 0.0000040 \times (\text{sperm concentration})^2$

72 h (Broken-line linear predictive equation):

if sperm concentration < 29.9 × 10^6/mL: $y = 7.21$;

if sperm concentration > 29.9 × 10^6/mL: $y = 7.21 + 0.0034 \times (29.9 – \text{sperm concentration})$.

Vertical line = break-point.

120 h (Quadratic polynomial predictive equation):

$y = 7.33 – 0.0056 \times (\text{sperm concentration}) + 0.0000091 \times (\text{sperm concentration})^2$

2.3 Segundo artigo científico

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Sperm concentration of boar semen doses and sperm quality: Novel perspectives based on the extender type and sperm resilience

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Running head: Concentration of boar semen doses and sperm quality

Sperm concentration of boar semen doses and sperm quality: Novel perspectives based on the extender type and sperm resilience

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ABSTRACT

This study evaluated the effect of sperm concentration of boar semen doses on their capacity to maintain its motility over the thermo-resistance test (TRT; sperm resilience) and verified if the extender type (short or long-term) could influence this effect. Thirty ejaculates from five crossbred mature PIC® boars were used, and a factorial design was followed to produce semen doses with 1.5 billion cells in 45 or 90 mL, using Beltsville Thawing Solution (BTS) or Androstar® Plus (APlus). Then, low-concentration doses (16.7 × 10^6 cells/mL in 90 mL) and higher-concentration doses (33.3 × 10^6 cells/mL in 45 mL) with BTS or APlus were produced and stored at 17 °C for 168 h. At 72 h, during the TRT, the low-concentration doses (16.7 × 10^6 cells/mL) lost three-fold less motility than doses with 33.3 × 10^6 cells/mL ($P < 0.01$), regardless of the extender type (11. 5% vs. 30.5% of

initial motility, respectively). Similar results were found when the TRT was carried out at 168 h, with low-concentration doses losing two-fold less motility (11.4%) than highly concentrated doses (25.9%; $P < 0.01$). No sperm concentration effect was observed on membrane integrity or mitochondrial membrane potential ($P \geq 0.23$). The osmolarity was not affected by the sperm concentration ($P = 0.56$), only by the extender and the storage time ($P < 0.01$). In conclusion, the sperm concentration effect on sperm quality was not influenced by extender type, and the data suggest that a low concentration of semen doses positively affects sperm resilience.

Keywords: Dose volume; Extender; Semen dilution; Sperm resistance; Swine spermatozoa.

1. Introduction

Artificial insemination (AI) is a fundamental biotechnique for current pork production (Waberski et al., 2019), and its success has mainly been associated with semen dose quality (Bortolozzo et al., 2015; Knox, 2016). In this sense, it is known that semen dose quality can be influenced by several factors, such as the dilution rate (Lopez Rodriguez et al., 2017), which is related to the sperm concentration of the doses. Furthermore, according to Flowers (1997) and Weitze et al. (2011), low-concentration semen doses could have their quality impaired due to metabolic changes in the medium or even the dilution of important seminal plasma compounds. Most studies assessing the sperm concentration effect on boar semen quality, however, did not provide consistent results nor practical recommendations regarding the theme (Marcos et al., 1991; García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013). Furthermore, these investigations did not address the important changes in the sperm concentration of

semen doses with which swine production has been dealing in recent years, mainly due to the reduction in the total number of sperm cells per dose to optimize the use of ejaculates (Waberski et al., 2019).

Recently, we evaluated the sperm concentration effect on semen doses for intrauterine artificial insemination (IUAI) containing 20, 30, 60, or 100 × 10^6 cells/mL in a final volume of 50 mL. The results showed that sperm concentration of semen doses does impair sperm quality since semen doses with 30 × 10^6 cells/mL, incubated for 10 or 30 min, presented higher motility compared to lower (20 × 10^6 cells/mL) and higher concentration doses (60 or 100 × 10^6 cells/mL; Quirino et al., 2023). These semen doses, however, were produced with a long-term extender, containing a complex composition that may have minimized the negative effect observed, as reported in the past in semen doses for cervical AI (CAI; Weitze et al., 2011; Lipenský et al., 2016). In this sense, there is still room to further verify the relation between the sperm concentration effect and the extender type in boar semen doses, mainly considering the use of long-term extenders as a potential strategy to minimize the impairment of sperm quality.

Considering the current variation of total sperm number and volume for boar semen doses according to the type of semen dose (IUAI or CAI) and the AI center location, it is also reasonable to question if doses produced around the world exhibit comparable results of sperm quality. Some AI centers in Europe have currently produced CAI semen doses with 20 or even 16 × 10^6 cells/mL (1.8 or 1.5 billion cells in ~ 90 mL; Waberski et al., 2019), while IUAI semen doses usually produced in South America present ~ 30 × 10^6 cells/mL (1.5 billion cells in ~ 50 mL; Bortolozzo et al., 2015). As observed in our preliminary study, boar semen doses containing ~ 20 × 10^6 cells/mL presented lower total and progressive motility compared to ~ 30 × 10^6 cells/mL (Quirino et al., 2023). So far,

however, the sperm quality of IUAI and CAI semen doses were only compared considering different total sperm numbers and similar sperm concentrations (Luongo et al., 2020).

Another fundamental and still unclear point about the sperm concentration effect in boar semen doses is its influence on the sperm capacity to maintain its motility (*in vitro* sperm resilience). This parameter is usually assessed by performing a thermo-resistance test (TRT), which simulate the temperature of the female genital tract before fertilization and, therefore, plays an important role in predicting semen fertility (Schulze et al., 2013; 2019). Unfortunately, none of the published investigations concerning this topic performed a TRT (Marcos et al., 1991; García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013). Despite our previous results showing that a low sperm concentration (20×10^6 cells/mL) negatively affected sperm motility after 30 min of incubation, the sperm motility was less reduced than in higher concentration doses (60 or 100×10^6 cells/mL) at the end of the test (300 min; Quirino et al., 2023). In this way, it is still necessary to verify if low-concentration boar semen doses could, in fact, positively influence sperm resilience.

Based on the aforementioned reasons, the aim of this study was to assess the influence of extender type on the effect of sperm concentration on boar semen dose quality, compare the sperm quality of IUAI and CAI semen doses produced with different sperm concentrations, and further evaluate the sperm resilience of low-concentration semen doses. For this, the sperm quality of AI semen doses containing 1.5 billion cells in 45 or 90 mL (33.3 and 16.7×10^6 cells/mL) and produced with two different extenders (short or long-term) was compared.

2. Material and Methods

The study protocol was approved by the Veterinary Research Committee (COMPESQ-Vet) of the Federal University of Rio Grande do Sul (UFRGS) – Brazil, under process No. 39395.

2.1 Chemicals

All chemicals used in this study were of analytical grade unless otherwise stated. The fluorescein-isothiocyanate conjugated Pisum sativum agglutinin (FITC-PSA) and the carbonyl cyanid 3-chlorphenylhydrazone solution (CCCP) were purchased from Sigma-Aldrich (USA), while propidium iodide (PI) and SYBR-14 were obtained from BD Pharmingen™ and Life Technologies – Thermo Fisher scientific (LIVE/DEAD™ Sperm Viability Kit; USA), respectively. The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was also purchased from Life Technologies – Thermo Fisher scientific (USA). Semen extenders (Beltsville Thawing Solution; BTS, and Androstar® Plus; APlus) were acquired from Minitüb GmbH (Germany).

2.2 Animals and facilities

Thirty ejaculates from five healthy and mature (> 24-months old) crossbred boars (Pietrain × Duroc; Agroceres PIC, Laranjeiras do Sul, Brazil) were collected weekly over the period of six weeks. The animals were housed in 6 m^2 individual pens and were collected for routine semen dose production, at weekly intervals. The boars were fed 2.2 kg/day of a commercial corn-soybean meal diet (3.02 Mcal metabolizable energy/kg, 12.0% crude protein, and 0.7% total lysine), and *ad libitum* access to water was provided.

2.3 Semen collection and processing

Using a pre-warmed (38 °C) collection cup, ejaculates were collected by the gloved hand method. The pre-sperm phase was discarded and the gel fraction was removed by using a filter during collection, therefore, the semen collection included the sperm rich and poor fractions. Raw semen was evaluated for volume, and 90 µL of each ejaculate was diluted in 810 µL of warmed extender (34 °C; BTS) for sperm motility analysis using a computer-assisted semen analysis system (CASA system; AndroVision®, Minitüb GmbH). A 3-µL sample was placed by capillarity in preheated Leja chambers (Leja® 20 µm chamber depth, Nieuw Vennep, The Netherlands), and five fields were analyzed at 200 × magnification using a phase-contrast microscope with a warm stage set at 37 °C (Axio Scope A1, Zeiss® Germany). A single sample was analyzed for each ejaculate (~ 1,200 cells/sample) and sperm with amplitude lateral head displacement < 1 mm and curvilinear velocity < 24 mm/s were considered immotile. Progressive motility was defined as curvilinear velocity > 40 mm/s and straight-line velocity > 10 mm/s (Menezes et al., 2020).

Only ejaculates having at least an average of 70% total sperm motility were used. In addition, an aliquot of 100 µL was taken from each ejaculate and diluted in 9.9 mL of formalin-citrate solution (formalin at 4% and sodium citrate at 2.9%) for sperm concentration analysis, using the hemocytometer chamber (improved Neubauer). For each ejaculate, a single sample was analyzed, then, based on the sperm concentration of each ejaculate, semen doses containing a total sperm number of 1.5×10^9 were produced in a split-sample. Two types of extenders were used (BTS and APlus), considering two different final volumes (45 or 90 mL). In this way, the following 2 × 2 factorial design was performed: doses with 1.5×10^9 cells in 45-mL of BTS (BTS 45); doses with 1.5×10^9 cells in 90-mL of BTS (BTS 90); doses with 1.5×10^9 cells in 45-mL of APlus (APlus 45),

and doses with 1.5×10^9 cells in 90-mL of APlus (APlus 90). Consequently, the sperm concentration of semen doses containing 1.5×10^9 cells in 45 and 90 mL was 33.3 and 16.7 $\times 10^6$ cells/mL, respectively.

Isothermic dilution was performed (34 °C), and semen doses were stored in 100 mL-plastic bottles (Minitüb, Germany), removing the air content. After 90 min under room temperature (22–24 °C), the doses were stored in a temperature-controlled cabinet at 17 °C for 168 h. In each time point of analysis (storage time), a different sealed bottle of semen was used, avoiding manipulation effects.

2.4 Semen doses evaluation

2.4.1 Sperm motility

At 24, 72, and 120 h of storage, total and progressive motility were analyzed using the CASA system. One mL of semen dose was incubated, in a thermal block, for 20 min at 37 °C. After the incubation period, a 3 µL-sample was placed by capillary flow in preheated Leja chambers (37 °C), and the motility analysis was carried on as described for ejaculate analysis. The analysis was carried out using single samples from semen doses.

2.4.2 Thermo-resistance test (TRT)

The TRT was performed at 72, 120, and 168 h of storage. Subsamples of 15 mL were incubated at 38 °C for up to 300 min. Total and progressive motility were analyzed after 30 and 300 min of incubation (Schulze et al., 2013), using the CASA system and following the same procedure and settings described for ejaculate analysis.

2.4.3 Flow cytometric assessment

2.4.3.1 Sample dilution and staining

Plasma membrane and acrosome integrity as well as mitochondrial status of sperm cells were assessed at 72 and 120 h of storage using single samples of semen doses. An aliquot from semen doses was diluted in BTS or APlus, according to the treatment, to achieve a final concentration of 5.0×10^6 spermatozoa/mL. For assessment of plasma membrane and acrosome integrity, 150 µL of diluted samples were stained with FITC-PSA (final concentration: 2.5 µg/mL) and PI (final concentration: 3.3 µg/mL). For mitochondrial membrane potential analysis, the 150 µL of diluted samples were stained with JC-1 (final concentration: 1 µM) and PI (final concentration: 3.3 µg/mL). For both assays, the stained samples were incubated for 10 min at 37 °C.

2.4.3.2 Sample analyses

Immediately prior to analysis, each stained sample was diluted in 150 µL of BTS or APlus, according to its treatment, obtaining a final concentration of 2.5×10^6 spermatozoa/mL. Analysis was performed using a BD Accuri C6 Plus (BD Biosciences, San Jose, USA) equipped with lasers, blue (488 nm, 20 mW) and red (640 nm, 12.5 mW), containing four detectors: 533/30 nm (FL1, band pass filter), 585/40 nm (FL2, band pass filter), 670 nm (FL3, long pass filter), and 675/25 nm (FL4, bandpass filter). A total of 25,000 events were obtained per sample, and all the fluorescence signals were plotted on logarithmic scales. The sperm population was gated according to the expected forward- and side-scatter signals, also plotted on logarithmic scales, being based on a previous pool semen sample stained with SYBR-14 (final concentration: 100 nM).

To establish the gates for the fluorochromes, a pool-sample from diluted semen doses was obtained and used for preparing the unstained, positive, and negative controls for all fluorochromes. With respect to fluorochromes FITC-PSA and PI, the negative control

consisted of the original pool stained with each fluorochrome individually, while the positive control was the pool submitted to three rounds of a flash-freezing process in liquid nitrogen, followed by rapid thawing at 37 °C and subsequent staining. For JC-1, the controls were performed using an original pool-sample and a pool-sample stained with JC-1 after being stained and incubated (37 °C for 5 min) with CCCP (final concentration: 1 mM).

Fluorescence signals of FITC-PSA and PI were detected by FL1 and FL3 channels, respectively, while JC-1 signals were detected by FL1 and FL2 channels. The percentage of spermatozoa with intact plasma and acrosome membranes was recorded, as well the percentage of spermatozoa with high mitochondrial membrane potential. All data were recorded and analyzed using the BD Accuri C6 Plus software. The equipment was calibrated prior to each routine to verify the cytometer's optical alignments and fluidic system, using specific calibration beads provided by the manufacturer.

2.4.4 Monitoring of pH and osmolarity

Using a calibrated digital pH-meter (K39-2014B Kasvi®), the pH of semen doses was determined, at 24, 72, and 120 h of storage. The pH-meter calibration was performed using three buffer solutions with a pH of 4.01, 7.00, and 10.01, then single samples of semen doses were analyzed. The pH electrode was thoroughly washed with distilled water and wiped between samples. For osmolarity evaluation, aliquots of 50 µL from doses were analyzed in duplicate, immediately after dilution, and at 24 and 120 h of storage, using a calibrated Micro Osmometer (Precision Systems Osmette™ Micro-Osmette Osmometer). The osmolarity of the extenders used to produce the semen doses was also registered.

2.5 Statistical analysis

The Statistical Analysis System software (SAS, version 9.4; SAS® Institute Inc., Cary, NC, USA) was used for analyzing the data. Regarding the total motility over the TRT, for each storage time, a repeated measures analysis was performed, based on the incubation time: 30 or 300 minutes, were carried out using the GLIMMIX procedure. The model took into account the effects of extender type, the final volume of semen doses, incubation time, and their interaction. For the other parameters, the repeated measures analyses were based on the storage time, including the type of extender, the final volume of semen doses, the storage time, and their interaction as fixed effects.

The relative loss (%) of total motility over the TRT was also analyzed using the GLIMMIX procedure, according to the storage time. For this model, the extender type, final volume of semen doses, and their interaction were considered. The parameters were defined according to the following formula:

$$\frac{|Value\ at\ TRT300\ -\ Value\ at\ TRT30|}{Value\ at\ TRT30} \times 100$$

For all models, the boar and week were included as random effects, and a binomial distribution was fitted to the motility and flow cytometry data. The results are presented as LSMeans ± standard error of the mean (SEM) and were considered significant at a $P \leq 0.05$.

3. Results

3.1 Ejaculates

Overall, in raw semen, the number of spermatozoa and volume were 364.7 ± 21.7 × 10^6/mL and 220.0 ± 14.8 mL, and the mean values for total and progressive motility were 96.0 ± 3.5% and 93.6 ± 4.6%, respectively.

3.2 Semen doses

3.2.1 Dilution rate and sperm concentration

Regarding the semen doses with 1.5×10^9 cells in 45 mL, the mean dilution rate was 9.9:1 (9.9 parts of extender for 1 part of semen; SEM: ± 0.64), achieving a sperm concentration of 35.1 and 33.6×10^6/mL for the BTS and APlus-doses, respectively. The mean dilution rate for doses with 1.5×10^9 cells in 90 mL was 21:1 (SEM: ± 1.3), obtaining 18.3 and 18.1×10^6 sperm cells/mL for BTS-doses and APlus-doses, respectively.

3.2.2 Sperm motility

An interaction between the volume of semen doses and storage time was observed for total motility. The 90 mL-semen doses had slightly higher total motility at 72 h compared to 24 h of storage time (84.2 vs. 83.6%; $P = 0.02$). For doses with 45 mL, no differences throughout storage occurred. Still, there was an effect of extender type in the total motility, and semen doses produced with APlus had a higher value (89.3%) than the BTS-doses (85.7; $P < 0.01$). The progressive motility was also influenced by the extender type since semen doses produced with APlus showed a higher value (85.7%) compared to BTS-doses (81.7%; $P < 0.01$; **Table S1**).

3.2.3 TRT

Considering the total motility registered over the TRT performed at 72 h of storage (**Figure 1a**), there was an effect of the interaction between volume and incubation time (30 and 300 min; $P < 0.01$). At TRT30, the 45-mL doses had higher motility than the 90-mL doses (88.5 vs. 85.1%), but at TRT300 the motility for 45-mL doses was lower compared to 90-mL doses (61.7 vs. 75.6%). An effect of the extender was also observed, indicating that

total motility was higher for APlus compared to BTS (81.4 vs. 77.2%). For the TRT at 120 h, there was an effect of the three-way interaction: extender, volume, and incubation time (P = 0.01; **Figure 1b**). At TRT30, APlus 45 showed the highest value (88.2%) while the lowest motility was observed for BTS 90 (79.8%); however, at TRT300, APlus 90 and BTS 90 presented higher values (68.5 and 70.8%, respectively) than APlus 45 and BTS 45 (64.5 and 58.6%, respectively). At 168 h, an effect of the extender (APlus: 78.5 vs. BTS: 76.1%) and of the interaction between volume and incubation time was also observed in TRT results (P < 0.01). At TRT30, the 45-mL doses showed higher motility than the 90-mL doses (87.1 vs. 80.8%), and at TRT300 the motility higher for 90-mL doses than for 45-mL doses (64.9 vs. 71.8%; **Figure 1c**).

Regarding the TRT results as a relative loss of total motility, an effect of the volume for the TRT performed at 72 h (P < 0.01) was observed. The 45-mL doses lost 30.5% of their initial motility over the period of incubation, while the 90-mL doses lost only 11.5% (**Figure 2a**) At 120 h, there was an effect of volume and extender interaction (P < 0.01). The highest loss was found for APlus 45 (27.5%) and BTS 45 (32.3%), while the lowest loss was observed for BTS 90 (11.2%; **Figure 2b**). At 168 h, the relative loss was affected by the volume (P < 0.01) since the 45-mL doses lost 25.9% of initial motility, and the 90-mL doses lost only 11.4% (**Figure 2c**).

3.2.4 Flow cytometric assessment

With respect to the sperm structure, there was a significant interaction between extender and storage time on the percentage of sperm cells with plasma and acrosomal membrane integrity (P < 0.01; **Figure 3a**). For the APlus-doses, a higher proportion of intact cells was observed at 72 h than at 120 h (92.1 vs. 91.4%; P = 0.03); for BTS-doses,

however, no difference between storage times was found (88.9 vs. 89.7%, for 72 and 120 h, respectively; $P = 0.07$). There was an extender effect regarding the mitochondrial membrane potential, since APlus-doses presented a higher percentage of cells with high mitochondrial membrane potential than BTS-doses (86.4 vs. 81.6%; $P < 0.01$; **Figure 3b**).

3.2.5 pH and osmolarity

The pH value was affected by the interaction between the volume of semen doses and the storage time. The 45-mL doses had similar pH values to the 90-mL doses at 24 h, but at 72 h, the 45-mL doses had a higher pH value (7.31 vs. 7.25), as well as at 120 h (7.25 vs. 7.22; $P < 0.01$; **Table S1**). The osmolarity was affected by the type of extender since semen doses produced with APlus showed a lower osmolarity compared to those with BTS (303 vs. 308 mOsm; $P < 0.01$). An effect of storage time was also observed and at 24 h of storage, the osmolarity was lower (308 mOsm) than at 0 and 120 h (311 and 312 mOsm, respectively; $P < 0.01$; **Figure S1**). No effect of extender type and storage time interaction was observed in the osmolarity.

4. Discussion

Efforts have been applied to reduce the total number of sperm cells per dose and, consequently, optimize the use of ejaculates. In some scenarios, however, the concurrent decrease in the final volume is not performed, mainly considering the semen doses for CAI (Riesenbeck, 2011; Waberski et al., 2019), which leads to doses with a low sperm concentration. To further understand the effect of low-concentration boar semen doses on sperm quality, we investigated the extender type influence (short or long-term) as well as the possible effects on sperm resilience after the TRT. Doses with a low sperm concentration are expected to have negatively affected sperm motility (Marcos et al., 1991;

García, 1994; Alexopoulos et al., 1996; Weitze et al., 2011; Lipenský et al., 2013; Quirino et al., 2023). This has been addressed by possible metabolic changes leading to alterations in osmolarity (Flowers, 1997); no data, however, regarding the osmolarity of low-concentration boar semen doses have previously been reported. Only effects of the storage time and the extender type on osmolarity were observed, contradicting the referred hypothesis.

According to Weitze et al. (2011), the negative effect of a low concentration in boar semen doses would be associated with the dilution effect of important seminal plasma compounds, which seem to be essential for sperm membrane stabilization (Harrison et al., 1982; Watson, 1995). The authors also reported supplementing boar semen doses with seminal plasma as an efficient strategy to mitigate sperm concentration impairment. Nevertheless, our results showed that the sperm structure, including the membrane integrity, was not even altered by the different sperm concentrations. Some studies also observed that using long-term extenders was a potential strategy to counteract this negative effect, probably due to their better ability to protect and stabilize the sperm membrane (Weitze et al., 2011; Lipenský et al., 2016). Seminal plasma supplementation may not be a functional recommendation for AI centers and, despite being more costly, the use of complex extenders could be considered a more practical strategy. The extender type, however, did not influence the sperm concentration effect in this study for any parameter.

It is noteworthy that this is the first investigation comparing the sperm quality of IUAI and CAI boar semen doses with different sperm concentrations but the same total sperm number. Usually, both types of boar semen doses are produced with the same sperm concentration, and no differences in sperm quality are expected. Recently, Luongo et al. (2020) compared boar semen doses with 2.7×10^9 cells in 80 mL and 1.5×10^9 cells in

45 mL, (corresponding to 33–34 × 10^6 cells/mL), and no differences were found regarding sperm parameters. The comparison, however, may come off when considering the current standards for producing IUAI semen doses in South America (1.5 billion cells in ~ 50 mL; ~ 30 × 10^6 cells/mL; Bortolozzo et al., 2015) and those applied for CAI semen doses in Europe (1.5–1.8 billion cells in ~ 90 mL; ~ 17–20 × 10^6 cells/mL; Waberski et al., 2019).

Regarding sperm motility, our initial results showed that semen doses with a low concentration (16.7 × 10^6 cells/mL) had decreased total motility (less 11−12% of initial motility) compared to the more concentrated doses (33.3 × 10^6 cells/mL). Nevertheless, to further assess the effect of sperm concentration on semen dose quality, we evaluated spermatozoa resilience through a TRT, focusing mainly on the loss of motility over the process, as already done by our research group (Quirino et al., 2022). Despite Schulze et al. (2019) reporting no effect of higher dilution rates on TRT when trying to identify factors for explaining the variation in boar sperm thermo-resistance, none of the previous studies on sperm concentration effect in boar semen doses evaluated sperm resilience. Based on our data, after 300 min of TRT, the lowest motility values were found for the more concentrated doses (33.3 × 10^6 cells/mL in 45 mL) at all storage times. In our preliminary investigation, a similar behavior was reported when comparing IUAI doses with 20 and 30 × 10^6 cells/mL: a loss of 11.8 vs. 23.4 percentage points (Quirino et al., 2023), also suggesting that sperm resilience is greater in low-concentration semen doses.

Over the TRT, the metabolism increases, leading to more rapid utilization of extender compounds. Therefore, it is logical to expect that semen doses with fewer cells/mL would have better sperm resistance. Furthermore, a scenario of fewer cells/mL in the extended semen provides higher availability of nutrients and buffers for spermatozoa,

for better regulating the energy depletion and chemical alterations in the medium caused by the active metabolism of boar sperm (e.g., production of CO_2 and other metabolites; Jones, 1997). Despite this, it is not clear why the semen doses with 16.7×10^6 cells/mL showed lower motility than those with 30×10^6 cells/mL when incubated for a short period at 37–38 °C (≤ 30 min). We believe there is greater control of sperm metabolism in low-concentration doses due to the higher availability of membrane stabilizers. Thus, low-concentration semen doses may demand more incubation time to reactivate the cell metabolism properly.

Overall, this investigation grants important insights regarding the real effect of low sperm concentration and the current quality of boar semen doses. Despite this, we also highlight that only *in vitro* evaluations were performed in this study. The TRT has already been proven to be predictive for semen fertility in boars (Schulze et al., 2013; 2021), but the female productive tract contains many substances which may influence sperm maintenance up to fertilization time (Vianna et al., 2009). Thus, the sperm concentration effect must still be further evaluated for boar semen doses, mainly considering its influence on reproductive performance.

5. Conclusion

The use of long-term extenders did not influence the sperm concentration effect in this study, suggesting that their use could not be a potential strategy to counteract the effect of sperm concentration on boar semen dose quality. In addition, semen doses with 16.7×10^6 cells/mL positively affected sperm resilience compared to more concentrated doses (33.3×10^6 cells/mL), showing a lower loss of motility over the TRT. In this way, the intentional production of low-concentration semen doses apparently may not be a concern

for AI centers with respect to sperm quality. Still, supplementary studies concerning the sperm concentration effect are required to investigate the low-concentration effect on the *in vivo* performance.

Author contributions

Monike Quirino: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Victória Nunes Pereira:** Investigation. **Mayara Souza Costa Tamanini:** Investigation. **Rafael da Rosa Ulguim:** Supervision, Review & Editing. **Martin Schulze:** Supervision, Review & Editing. **Ana Paula Gonçalves Mellagi:** Methodology, Supervision, Review & Editing. **Fernando Pandolfo Bortolozzo:** Methodology, Supervision, Review & Editing. All authors read, revised, and approved the final manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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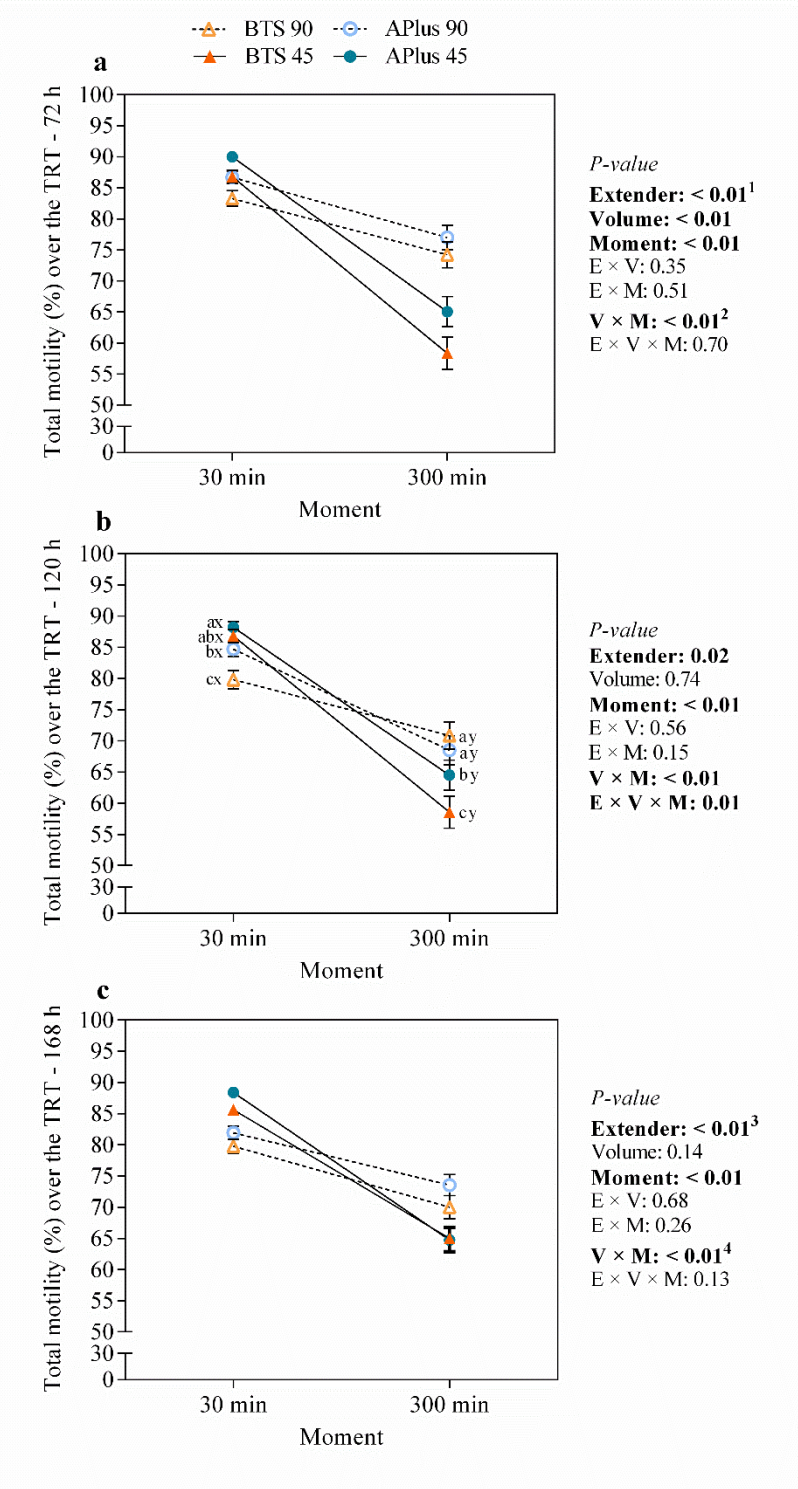

Fig. 1. Total motility of boar semen doses, with different sperm concentrations in two types of extender, submitted to thermo-resistance tests at 72, 120, and 168 h of storage, respectively.

Data are presented as least squares mean ± standard error of the mean. 1.5 billion cells in 45 or 90 mL of BTS (BTS 45; BTS 90) or Androstar® Plus (APlus 45; APlus 90). [abc]Indicate significant differences between the groups within the moment; [xy]Indicate significant differences between the moments within the groups; [ABC]Indicate significant difference among the groups ($P < 0.05$). [1]APlus > BTS (81.4 vs. 77.2%). [2]30 min: 45 mL > 90 mL (88.5 vs. 85.1%); 300 min: 45 mL < 90 mL (61.7 vs. 75.6%). [3]APlus > BTS (78.5 vs. 76.1%). [4]30 min: 45 mL > 90 mL (87.1 vs. 80.8%); 300 min: 45 mL < 90 mL (64.9 vs. 71.8%).

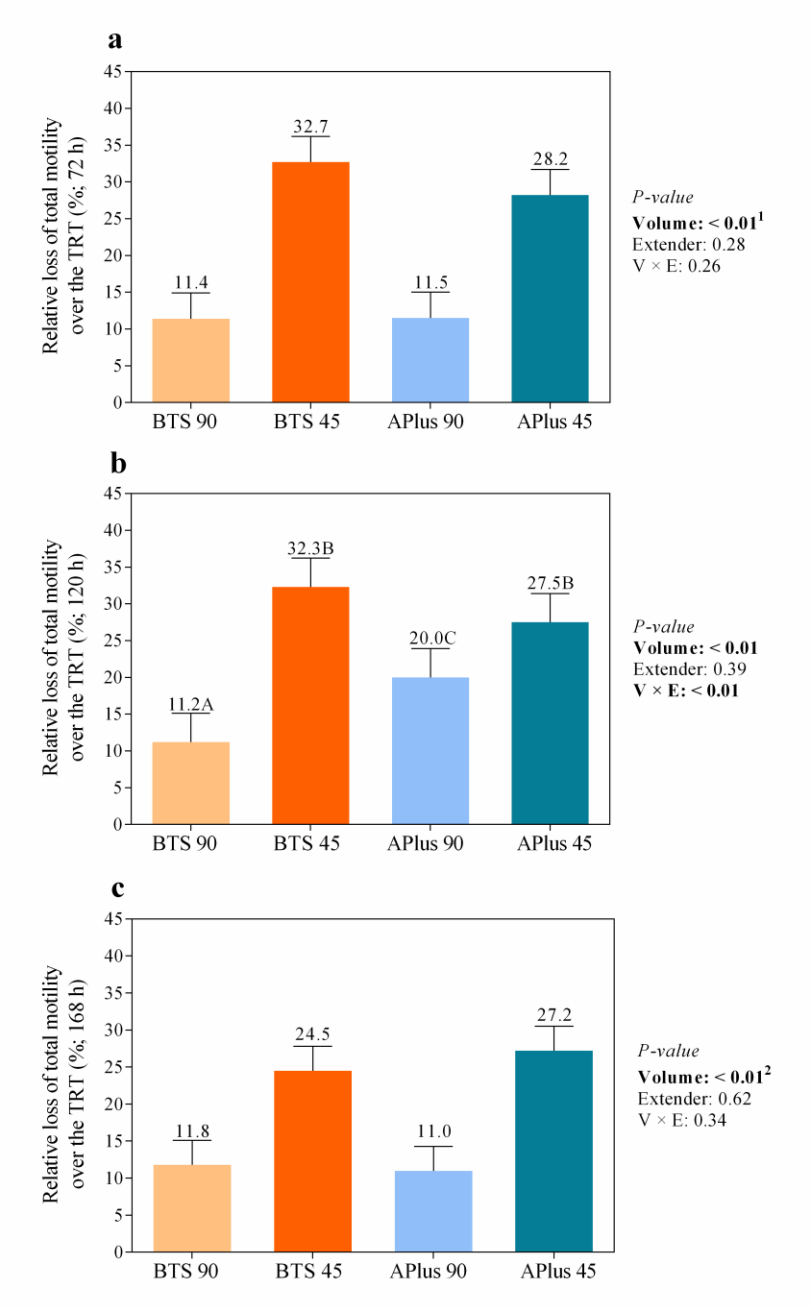

Fig. 2. Relative loss of total motility* of boar semen doses, with different sperm concentrations in two types of extender, submitted to thermo-resistance tests at 72, 120, and 168 h of storage, respectively.

Data are presented as least squares mean ± standard error of the mean. 1.5 billion cells in 45 or 90 mL of BTS (BTS 45; BTS 90) or Androstar® Plus (APlus 45; APlus 90). *Relative loss of total motility: |(TRT300min – TRT30 min)|/TRT30 min ×100. [1]45 mL > 90 mL: 30.5 vs. 11.5%. [2]45 mL > 90 mL: 25.9 vs. 11.4%.

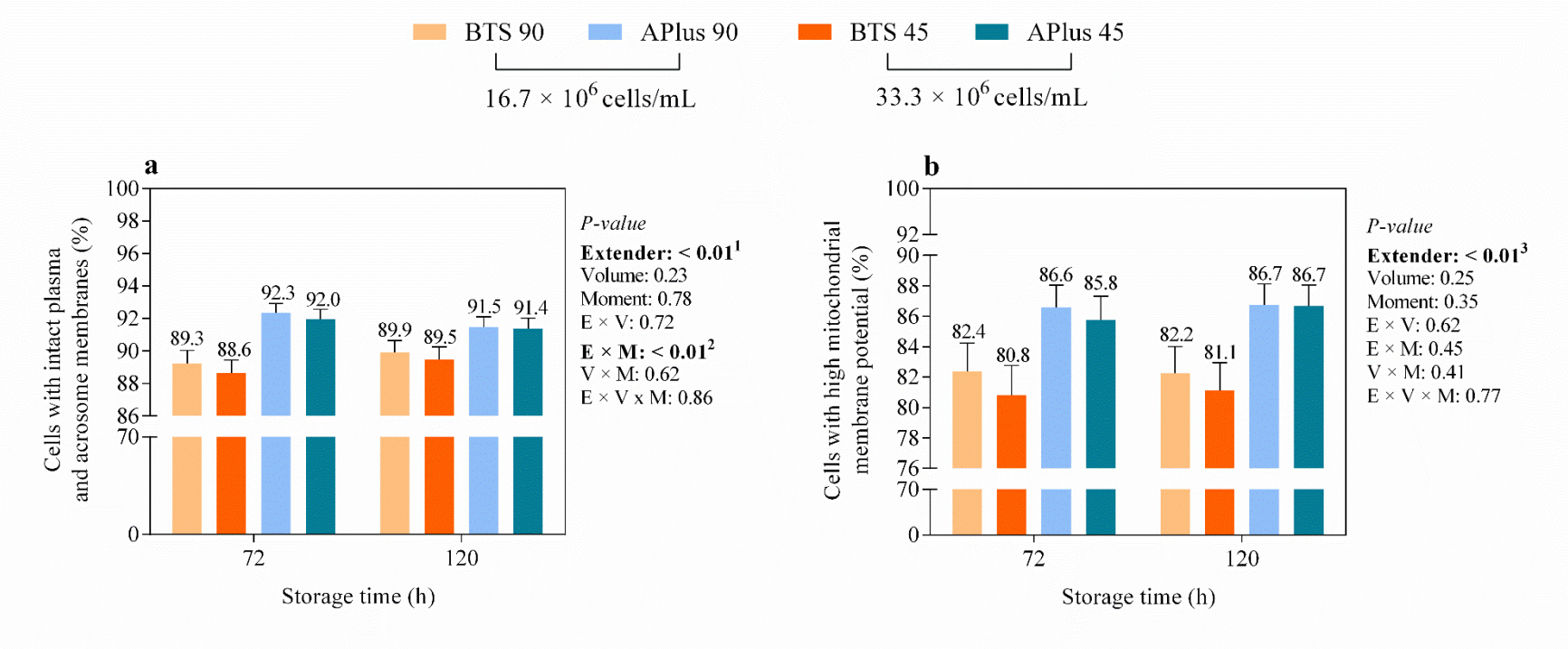

Fig. 3. Sperm structure of boar semen doses with different sperm concentrations in two types of extender and stored for 120 h at 17 °C. **a**: Plasma and acrosomal membrane integrity; **b**: Mitochondrial membrane potential.

Data are presented as least squares mean ± standard error of the mean. 1.5 billion cells in 45 or 90 mL of BTS (BTS 45; BTS 90) or Androstar® Plus (APlus 45; APlus 90). [1]APlus > BTS (91.8 vs. 89.3%). [2]BTS: 72 h = 120 h (88.9 vs. 89.7%) and APlus: 72 h = 120 h (92.1 vs. 91.4%). [3]APlus > BTS (86.4 vs. 81.6%).

SUPPLEMENTARY MATERIAL

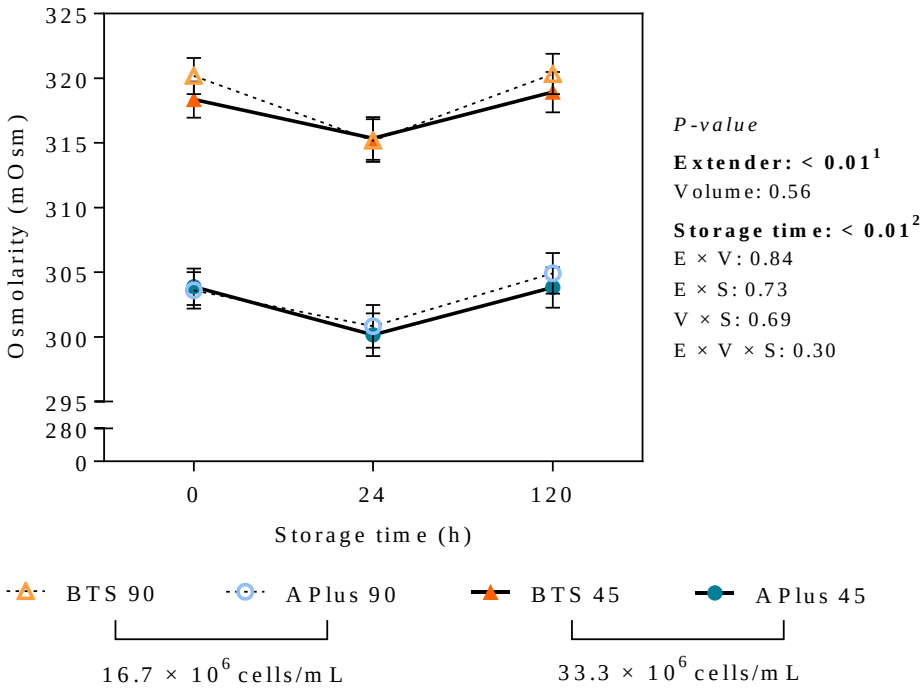

Fig. S1. Osmolarity of boar semen doses with different sperm concentrations in two types of extender and stored for 120 h at 17 °C. 1.5 billion cells in 45 or 90 mL of BTS (BTS 45; BTS 90) or Androstar® Plus (APlus 45; APlus 90). Data are presented as least squares mean ± standard error of the mean.

[1]APlus < BTS (303 vs. 308 mOsm);

[2]24 h < 0 and 120 h (308 vs. 311, and 312 mOsm, respectively).

Table S1. Sperm motility and pH (LSmean ± SEM) of boar semen doses with different sperm concentrations* in two types of extender and stored for 120 h at 17 °C.

Storage time	1.5×10^9 cells				Significative *P*-values
	BTS		APlus		
	90 mL	45 mL	90 mL	45 mL	
Total motility, %					
24 h	83.6 ± 1.2	88.9 ± 0.8	85.9 ± 1.0	91.2 ± 0.7	Extender Volume Volume × Storage time[1]
72 h	84.2 ± 1.1	88.0 ± 0.9	88.3 ± 0.9	91.4 ± 0.7	
120 h	83.9 ± 1.2	88.4 ± 0.9	86.4 ± 1.0	91.4 ± 0.7	
Total	86.3 ± 0.8		89.3 ± 0.7		
Progressive motility, %					
24 h	79.9 ± 1.4	83.4 ± 1.2	82.9 ± 1.3	87.3 ± 1.0	Extender Volume Volume × Storage time[2]
72 h	80.4 ± 1.4	83.1 ± 1.2	85.4 ± 1.1	87.6 ± 0.9	
120 h	79.9 ± 1.4	83.3 ± 1.2	82.9 ± 1.2	87.7 ± 0.9	
Total	81.7 ± 1.1		85.7 ± 0.9		
pH					
24 h	7.18 ± 0.02	7.21 ± 0.02	7.11 ± 0.02	7.15 ± 0.02	Extender Volume Storage time Extender × Storage time[3] Volume × Storage time[4]
72 h	7.28 ± 0.02	7.37 ± 0.02	7.22 ± 0.02	7.25 ± 0.02	
120 h	7.26 ± 0.02	7.34 ± 0.02	7.17 ± 0.02	7.24 ± 0.02	
Total	7.23 ± 0.01		7.19 ± 0.01		

LSmean: least squares mean; SEM: standard error of the mean; APlus: Androstar® Plus

90 mL doses: 16.7×10^6 cells/mL; 45 mL doses: 33.3×10^6 cells/mL.

[1]45 mL doses: 24 h = 72 h = 120 h; 90 mL doses: 72 h > 24 h (86.4 vs. 84.8%);

[2]45 mL doses: 24 h = 72 and 120 h; 90 mL doses : 72 h > 24 and 120 h (83.0 vs. 81.4 and 81.4%);

[3]APlus doses: 72 h = 120 h (7.2 vs. 7.2), BTS doses: 72 h = 120 h (7.3 vs. 7.3);

[4]24 h: 45 mL doses = 90 mL (7.18 vs. 7.14); 72 h: 45 mL doses > 90 mL doses (7.31 vs. 7.25); 120 h: 45 mL doses > 90 mL doses (7.29 vs. 7.22).

3. CAPÍTULO II – Células espermáticas mortas e seus possíveis efeitos na qualidade de doses inseminantes suínas

3.1 Revisão bibliográfica

A definição de morte celular não se limita a um conceito devido à complexidade dos processos que levam e caracterizam esse evento (SEN, 1992; BLANC-LAYRAC *et al.*, 2000; LOOS & ENGELBRECHT, 2009). Contudo, células espermáticas podem ser consideradas como mortas quando perdem a integridade de sua membrana plasmática, e por conseguinte perdem sua homeostase e motilidade, tornando-se células não viáveis (ROCA *et al.*, 2016). Como esse tipo de célula está presente em todos os ejaculados, independentemente da espécie (ROCA *et al.*, 2016), é esperado que todas as doses inseminantes suínas apresentem alguma proporção de células mortas.

A principal preocupação envolvendo a presença de células mortas em doses inseminantes suínas refere-se aos efeitos negativos que esse tipo de célula pode ocasionar às células vivas contemporâneas. Tal efeito, principalmente associado ao estresse oxidativo, já foi observado em outras espécies (SHANNON & CURSON, 1972; BRINSKO *et al.*, 2003); contudo, em suínos os estudos somente abordaram o efeito em sêmen criopreservado (MARTINEZ-ALBORCIA, M. J. *et al.*, 2012; ROCA *et al.*, 2013). Dessa forma, há ainda a necessidade de se investigar o efeito de células mortas em sêmen suíno refrigerado.

Uma melhor compreensão da relação entre células espermáticas mortas e vivas nas doses de sêmen atualmente comercializadas na produção de suínos pode permitir o estabelecimento de limites aceitáveis para a presença de células mortas a serem considerados nos programas de controle de qualidade do processamento de sêmen e das doses produzidas. Para isso, é necessário revisar brevemente os principais fatores que levam à presença de espermatozoides mortos em ejaculados e doses inseminantes, relembrando também os efeitos diretos e indiretos ocasionados por esse tipo de célula, e discutindo possíveis estratégias para prevenir a morte espermática ou pelo menos controlar seus efeitos deletérios.

3.1.1 Morte celular pré-ejaculação

Células espermáticas mortas fazem parte do ejaculado de indivíduos saudáveis de todas as espécies, como bovinos, ovinos, equinos, galos e suínos (ROCA *et al.*, 2016). O exato mecanismo que leva à morte de células anteriormente à ejaculação ainda não está totalmente elucidado; contudo, já se provou que a apoptose ocorre ao longo de todo o período de espermatogênese (HUCKINS, 1978). O principal objetivo desse processo de morte programada é equilibrar a proliferação espermática, promovendo a fagocitose das células espermáticas apoptóticas pelas células de Sertoli (SHAHA, 2007). A apoptose parece ocorrer, ainda, no epidídimo, no qual um mecanismo de controle de qualidade celular identifica as células apoptóticas ou já mortas (JONES, 2004; D'AMOURS *et al.*, 2012). Contudo, um certo número de espermatozoides mortos ainda não removidos podem permanecer na cauda do epidídimo, de forma a participar da população de células liberadas em cada ejaculação (CORREA-PÉREZ *et al.*, 2004; FERNÁNDEZ-SANTOS *et al.*, 2011).

Há, ainda, a possibilidade de algumas células morrerem durante o trajeto pelo ducto deferente e pela uretra. Acredita-se que a força hidrostática ocorrida ao longo do trajeto ou até mesmo a mistura das células com as secreções das glândulas acessórias poderiam levar a alterações de pH e osmolaridade, possibilitando a morte celular (AHMADI & NG, 1999). Nesse sentido, tais eventos poderiam justificar o fato de ejaculados de indivíduos saudáveis apresentarem de 10 a 40% de células mortas (JANETT *et al.*, 2003; SANCHO *et al.*, 2004; BARKAWI *et al.*, 2006; AZAWI & ISMAEEL, 2012; MURPHY *et al.*, 2013), destacando-se a espécie humana, na qual ejaculados com mais de 40% de células mortas ainda se encontram dentro dos limites fisiológicos considerados para indivíduos férteis (COOPER *et al.*, 2010). Em suínos, recomenda-se que apenas ejaculados com pelo menos 70% de células móveis sejam utilizados na rotina de produção de doses inseminantes, uma vez que na prática células móveis são consideradas espermatozoides vivos/viáveis (WABERSKI *et al.*, 2019).

3.1.2 Morte celular pós-ejaculação

3.1.2.1 Processamento do sêmen

Considerando o número de células definido por dose, o ejaculado deve ser processado. De forma geral, esse processamento deve ser realizado de forma a minimizar a

velocidade na qual ocorrem as perdas celulares, evitando-se, por exemplo, a morte de espermatozoides que já passaram por algum processo de envelhecimento celular antes da ejaculação. A diluição é realizada em meio específico contendo principalmente substrato energético e substâncias tamponantes e protetivas para atender a demanda metabólica das células, manter sua homeostase e impedir danos à membrana plasmática (JOHNSON *et al.*, 2000; BUSSALLEU & TORNER, 2013). Porém, o número de células mortas pode, ainda, aumentar durante a fase de diluição propriamente dita. Na produção de doses de sêmen suíno, pode-se destacar potenciais falhas como o atraso para iniciar o processo de diluição, preparo inadequado do diluente (SCHULZE *et al.*, 2015), temperatura inadequada do diluente (RIESENBECK *et al.*, 2015) e baixas ou altas taxas de diluição (FLOWERS, 1997; WEITZE *et al.*, 2011).

3.1.2.2 Transporte e recebimento das doses de sêmen

Para preservar a funcionalidade e capacidade fecundante do sêmen suíno ao longo do tempo, as doses inseminantes são armazenadas a 15 – 18°C por 3 a 7 dias, de forma a reduzir o metabolismo espermático. O transporte deve ocorrer a uma faixa de temperatura próxima à temperatura de armazenamento, para evitar possíveis choque térmicos e consequentemente influenciar na viabilidade das células espermáticas (MELLAGI *et al.*, 2022). Esse é um ponto que se torna ainda mais crítico em países cuja centralização das centrais de inseminação já é realidade, levando a longas distâncias de transporte, reforçando a necessidade de aplicação de condições ótimas de transporte e monitoramento (SCHULZE *et al.*, 2019).

Os sistemas de rastreio também são indicados para informar sobre o momento esperado de entrega e, dessa forma, controlar outro potencial ponto crítico para a sobrevivência espermática: a variação de temperatura do momento de chegada das doses de sêmen até o seu armazenamento propriamente dito (MELLAGI *et al.*, 2022). Uma flutuação de 2–3°C na temperatura de doses inseminantes armazenadas foi capaz de reduzir sua vida útil em um dia (ROZEBOOM, 2003). Esse efeito deve ser ainda mais pronunciado se as variações ocorrerem de forma inconsistente, levando as células espermáticas a reajustarem sua atividade metabólica (FLOWERS, 1997), provocando assim dano e, possivelmente, morte celular.

O transporte de doses inseminantes suínas também pode afetar o percentual de células mortas devido ao efeito das vibrações emitidas ao longo do trajeto (MELLAGI *et al.*, 2022). Já foi observado que doses inseminantes suínas produzidas com diluentes de curta ou longa duração submetidas a determinadas frequências de vibração (200 a 300 rpm), por longos períodos (4 a 12 h) apresentaram redução significativa da motilidade espermática (SCHULZE *et al.*, 2018; TAMANINI *et al.*, 2022) e integridade de membrana (SCHULZE *et al.*, 2018; PASCHOAL *et al.*, 2021; SEBASTIÁN-ABAD *et al.*, 2021). Apesar de necessárias, estratégias de controle dos efeitos da vibração durante o transporte ainda não foram estabelecidas, uma vez que o efeito do transporte é dependente de diversos fatores (SCHULZE *et al.*, 2018).

3.1.2.3 Armazenamento das doses de sêmen

É importante ressaltar que na faixa de 15 – 18°C o metabolismo celular é apenas reduzido, não pausado. Portanto, algumas células podem não sobreviver ao armazenamento devido ao seu envelhecimento celular (JOHNSON *et al.*, 2000), contribuindo para o aumento do número de células mortas na dose. Conforme já mencionado, as variações na temperatura também podem contribuir para esse evento, validando a importância dos sistemas de manutenção e monitoramento da temperatura também ao longo do processo de armazenamento.

Estudos recentes têm aplicado o armazenamento hipotérmico de doses inseminantes suínas (~ 5°C) com o principal foco de melhor controlar o crescimento bacteriano e, assim, promover um uso mais prudente de antibióticos na produção animal (DE ALCANTARA MENEZES *et al.*, 2020; JÄKEL *et al.*, 2021). Apesar dos resultados promissores quanto ao controle da carga bacteriana e ao desempenho reprodutivo, não se pode assegurar que essa abordagem não aumentaria o percentual de células mortas dada às condições de baixa temperatura, uma vez que o sucesso do armazenamento hipotérmico tem sido dependente do uso diluentes complexos, contendo potentes protetores de membrana, além de um rígido controle de temperatura durante as etapas de resfriamento, armazenamento e transporte (PASCHOAL *et al.*, 2020; MELLAGI *et al.*, 2022).

No caso do sêmen congelado, o qual representa apenas 1% do sêmen suíno produzido mundialmente (WABERSKI *et al.*, 2019), o metabolismo espermático é

pausado, propiciando melhor controle dos efeitos do envelhecimento celular. Além disso, há um melhor controle de manutenção da temperatura. Contudo, o próprio método de criopreservação torna-se responsável pela morte de 30 a 50% da população espermática da dose, independentemente da espécie (THURSTON & WATSON, 2002).

3.1.3 Efeitos dos espermatozoides mortos na qualidade das células vivas

Estudos envolvendo sêmen bovino e equino refrigerado já demonstraram o efeito negativo dos espermatozoides mortos sobre os espermatozoides vivos (SHANNON & CURSON, 1972; BRINSKO *et al.*, 2003). A interação negativa também já foi verificada em sêmen suíno congelado, confirmando a toxicidade das células mortas sobre as células vivas (MARTINEZ-ALBORCIA, M. J. *et al.*, 2012; ROCA *et al.*, 2013), porém o efeito dessa interação ainda não havia sido investigado em doses de sêmen suíno armazenado a ~ 17°C.

A citotoxicidade das células mortas parece ser mediada pela liberação de metabólitos citotóxicos, como as espécies reativas de oxigênio (ERO), mais especificamente o H_2O_2 (ROCA *et al.*, 2013). Em bovinos, ovinos e equinos já foi demonstrado que as células espermáticas mortas apresentaram maiores níveis da enzima LAAO (L-aminoácido oxidase; (SHANNON & CURSON, 1972; UPRETI *et al.*, 1998; AITKEN *et al.*, 2015), responsável por catalisar a produção de H_2O_2, o qual é sintetizado na mitocôndria ou no citoplasma da cauda espermática das células mortas (AITKEN & KOPPERS, 2011). Posteriormente, o H_2O_2 é transportado por difusão ao longo da membrana destas células, havendo sua liberação para o meio extracelular, o que permite seu contato com as células vivas contemporâneas, ocasionando estresse oxidativo. Além disso, existe a possibilidade de o H_2O_2 gerar outros radicais livres, os quais podem ser ainda mais agressivos ao espermatozoide, como a hidroxila (ROCA *et al.*, 2016).

A ocorrência de peroxidação lipídica das células vivas também pode ser umas das consequências espontâneas da liberação de ERO pelas células já mortas ou em processo de morte, uma vez que os radicais livres tendem a atingir os ácidos graxos poli-insaturados da membrana plasmática (MANN & LUTWAK-MANN, 2012; ROCA *et al.*, 2016). A fragmentação de DNA também já foi observada em amostras de sêmen suíno

criopreservado com altas proporções de células mortas (75%; ROCA *et al.*, 2013). Contudo, ainda não está elucidado se a integridade do DNA pode ser afetada devido da ação direta da ERO liberada pelos espermatozoides mortos, ou por sua ação indireta, ou seja: a ERO inicialmente liberada gerando danos às células vivas, de forma a desencadear uma alta produção de ERO por estas células, levando à fragmentação de DNA por apoptose ou necrose (ROCA *et al.*, 2016).

Pode-se considerar que a extensão dos efeitos negativos das células mortas sobre a qualidade do sêmen é dependente da quantidade de ERO liberadas pelos espermatozoides mortos (ROCA *et al.*, 2016). Logo, deve-se ressaltar que uma maior atividade da LAAO foi registrada na presença de gema de ovo nas amostras de sêmen bovino (SHANNON & CURSON, 1972), o que propicia uma maior extensão dos efeitos das células mortas em sêmen congelado. Estudos reportaram que níveis significativos de ERO em amostras de sêmen congelado de diferentes espécies podem ser obtidos com somente 25% de células mortas na população, enquanto para sêmen refrigerado, seria necessário ter o dobro dessa proporção para atingir os mesmos níveis (BRINSKO *et al.*, 2003; BALL, 2008; MARTINEZ-ALBORCIA, M. *et al.*, 2012; ROCA *et al.*, 2013).

A relação entre células mortas e vivas também é dependente do período de exposição dos espermatozoides vivos e sua sensibilidade às ERO (ROCA *et al.*, 2013). Nesse sentido, destaca-se que o H_2O_2 é um dos metabólitos considerados mais citotóxicos para o espermatozoide suíno (AWDA *et al.*, 2009), agravando pelo fato do sêmen suíno apresentar uma capacidade limitada de eliminar tal ERO (TOSIC & WALTON, 1950). Outro fator chave é a presença de substâncias capazes de sequestrar as ERO, o que também reforça a relação entre sêmen criopreservado e os efeitos das células mortas já que o plasma seminal é removido em amostras a serem congeladas (ROCA *et al.*, 2016).

3.1.4 Efeitos diretos e indiretos dos espermatozoides mortos sobre o processo de fecundação

Poucos estudos têm investigado os efeitos dos espermatozoides mortos sobre o processo de fecundação *in vitro* ou *in vivo*, mas os dados disponíveis demonstram que amostras de sêmen suíno congelado contendo 50% ou mais de células mortas diminuíram as taxas de fecundação *in vitro* ou quantidade de células espermáticas ligadas aos oócitos

em avaliações *in vivo* (BAKER & DEGEN, 1972; ROCA *et al.*, 2013). Esse efeito direto pode ser em decorrência principalmente dos efeitos negativos que as células mortas causam na integridade do DNA das células vivas (ROCA *et al.*, 2013).

Além do efeito direto, é necessário atentar-se para os efeitos indiretos, como a ocorrência de inflamação no trato uterino, a qual influencia negativamente o processo de fecundação. Em éguas, inseminações com doses contendo altas proporções de espermatozoides mortos levou a um quadro de excessiva inflamação uterina (ALGHAMDI *et al.*, 2004), porém em fêmeas suínas essa reposta ainda não tem sido bem elucidada (LESSARD *et al.*, 2003). Ressalta-se, ainda, que o tipo de morte celular exerce papel fundamental sobre esse efeito colateral (ROCA *et al.*, 2016). Células espermáticas que sofreram morte programada (apoptose) tendem a externalizar a fosfatidilserina, um fosfolipídeo da membrana interna, as quais são posteriormente fagocitadas durante um processo silencioso, não inflamatório (AITKEN & BAKER, 2013). Consequentemente, não se esperam efeitos negativos sobre o processo de fecundação.

Já os espermatozoides que morreram através do processo de necrose, são capazes de promover a síntese de citocinas pró-inflamatórias pelo endométrio, levando a uma resposta imune agressiva (HOLMSTRÖM & FINKEL, 2014; ROCA *et al.*, 2016) e, possivelmente, reduzindo as chances de fecundação. Este seria o cenário para as células vivas que após contato com as ERO sintetizadas pelas células mortas acabam por produzir alta quantidade de ROS, bem como das células mortas adicionadas nos tratamentos da maioria dos estudos acerca do tema (após consecutivos ciclos de congelamento; SHANNON & CURSON, 1972; BRINSKO *et al.*, 2003; ROCA *et al.*, 2013; LÜTTGENAU *et al.*, 2021). Portanto, ao investigar o impacto de células mortas sobre a qualidade do sêmen e seus efeitos na fecundação é importante considerar quais eventos foram responsáveis pela morte celular: apoptose, necrose ou ambos.

3.1.5 Prevenção e controle dos efeitos negativos ocasionados pelos espermatozoides mortos

A prevenção dos efeitos deletérios ocasionados pela presença de células mortas é dependente da forma de armazenamento do sêmen: refrigerado ou criopreservado. No caso do sêmen criopreservado, estratégias para remoção das células mortas logo após a coleta do

ejaculado (por filtragem, centrifugação com coloides, etc.) podem ser alternativas aplicáveis, embora seja necessário avaliar a segurança da técnica, já que alguns materiais utilizados para a separação podem ser deletérios aos espermatozoides (AITKEN *et al.*, 2014). Já para o sêmen refrigerado, tais abordagens não se mostram práticas tampouco econômicas. Para doses inseminantes suínas refrigeradas, a prevenção consiste basicamente no adequado controle das etapas de processamento do sêmen, transporte e armazenamento, de modo a evitar o aumento do percentual de células mortas na dose. Nesse contexto, destaca-se a importância do estabelecimento de limites aceitáveis quanto à proporção de células mortas em uma dose de sêmen, principalmente ao considerar o emprego de estratégias de compensação ou de diluição de sêmen com base no percentual de células viáveis no ejaculado (BROEKHUIJSE *et al.*, 2012; WABERSKI *et al.*, 2019). Dessa forma, será possível fomentar e aplicar um adequado controle de todo o processo de produção de doses.

Quando o objetivo é minimizar os efeitos deletérios ocasionados pelas ERO liberadas pelas células mortas já presentes no sêmen e nas doses inseminantes, é possível utilizar estratégias de suplementação com antioxidantes enzimáticos ou não, seja perfazendo a suplementação dos animais (vitamina C, vitamina E; BATHGATE, 2011), do diluente (catalase, glutationa peroxidase, vitamina E; AGARWAL *et al.*, 2014) ou possivelmente de ambos (ROCA *et al.*, 2016). O exato mecanismo dos antioxidantes envolve um complexo processo ainda não totalmente conhecido, porém, eles parecem atuar de forma a modificar a composição dos ácidos graxos da membrana plasmática, tornando-a mais resiliente à peroxidação lipídica (LIU *et al.*, 2014). O uso dessas substâncias representa uma estratégia potencial para sêmen criopreservado, já que para esse tipo de preservação os componentes do plasma seminal são removidos previamente ao processo de congelamento, podendo, também ser alternativas viáveis para o sêmen suíno refrigerado.

É valido ressaltar, ainda, que o percentual de células mortas na dose também pode ser influenciado pela estratégia aplicada para determinar o número de células por dose. Centrais de inseminação que aplicam a estratégia de compensação (aumento do número de células na dose) para contornar problemas de motilidade ou morfologia do ejaculado (BROEKHUIJSE *et al.*, 2012) acabam por produzir doses com uma maior proporção de células mortas. Além disso, há centrais que produzem as doses com base no percentual de

células móveis (WABERSKI *et al.*, 2019), o que também pode levar à produção de doses com maior quantidade de células mortas, uma vez que células não móveis podem já estar mortas ou em processo de morte. Nesse sentido, deve-se também ponderar o uso dessas estratégias quando se deseja controlar os efeitos negativos das células mortas sobre a qualidade das doses de sêmen suíno

3.2 Terceiro artigo científico

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Live cells are not affected by dead sperm in liquid boar semen: New insights based on a thermo-resistance test

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Running head: Dead sperm effect in the quality of boar semen doses

Live cells are not affected by dead sperm in liquid boar semen: new insights based on a thermo-resistance test

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ABSTRACT

This study evaluated the effect of different proportions of dead spermatozoa on the quality of liquid boar semen during a thermo-resistance test (TRT). After three days of storage (17°C), 54 conventional AI semen doses (~ 23×10^6 sperm/mL in ~ 88 mL of BTS) were split into three 15 mL-treatments (25%, 50% and 75% dead sperm cells) by mixing two subsamples containing 75% (I) and 0% (II) of live cells. Spermatozoa were evaluated after TRT at 30 (on-test) and 300 min (off-test) incubation at 38°C. At the on-test, treatments 25%, 50% and 75% dead sperm cells showed medians for total sperm motility of 77.6%, 50.2% and 25.6%, respectively. Considering the absolute variation of sperm motility during TRT, doses with 25% dead sperm lost more percentage points (pp) (-9.4 pp) compared to doses containing 50% (-8.2 pp) and 75% dead sperm (-4.5 pp). The lowest loss was observed for doses with 75% dead sperm ($P < 0.01$). However, data showed that treatments lost similar proportion of motile cells over the TRT: 25% dead sperm = -11.9%, 50% dead sperm = -16.0% and 75% dead sperm = -17.5% ($P = 0.31$). Regarding the flow cytometry parameters (plasma and acrosomal membrane integrity, mitochondrial activity of cells with intact plasma membrane, high degree of lipid disorder and apoptotic cells), the absolute variations did not surpass values of -1.8 pp, 3.4 pp, -5.4 pp and 4.7 pp, respectively. Moreover, the relative variation suggested that dead sperm did not substantially change their values over the TRT. In conclusion, dead sperm cells did not

influence the quality of contemporary live cells during the period and in conditions of a TRT.

Keywords: Boar semen; Dead spermatozoa; Semen quality; Thermo-resistance; Viable sperm.

INTRODUCTION

Artificial insemination (AI) is the technique nearly exclusively used for sow fertilization by pork-producing countries (Waberski et al., 2019). To achieve the greatest possible output, the temperature-sensitive boar ejaculates are preserved in liquid AI semen doses stored at 17 °C. However, even in high-quality ejaculates, it is assumed that a certain number of dead sperm cells will be present in freshly-produced AI semen doses, since dead sperm cells can be found in the cauda epididymis, which are then released during ejaculation (Sancho et al., 2004). Moreover, the population of weaker sperm in the ejaculate is more sensitive to the semen process and storage, i.e., dilution, aging, and cooling (Roca et al., 2016).

The percentage of dead sperm cells in AI doses can vary greatly, depending on some strategies and criteria used by boar studs. A higher percentage is found when preparing the AI semen doses using the "compensation strategy". This method allows for the use of ejaculates presenting motility lower than the minimum expected (70%; Waberski et al., 2019) by increasing the total sperm number per AI dose. However, as a consequence, the proportion of dead sperm cells in the AI doses is also increased (Broekhuijse et al., 2012). In addition, some boar studs produce AI doses based on the proportion of motile sperm instead of the total sperm number of ejaculates (Waberski et al., 2019), which also lead to an increase on the percentage of dead sperm cells per AI dose.

For these reasons, it is important to investigate the effects of dead sperm cells on contemporary viable sperm since it is possible for dead cells to release cytotoxic metabolites (Roca et al., 2016), negatively affecting sperm quality. This harmful cytotoxic effect of dead sperm cells has already been proven in bull and stallion semen (Shannon and Curson, 1972; Brinsko et al., 2003). Similar findings have already been reported in boars; however, the investigations have been limited to cryopreserved semen (Martinez-Alborcia et al., 2012; Roca et al., 2013). Moreover, previous studies based on cryopreserved semen

also did not evaluate the effect of dead sperm considering the thermo-resistance of cells. The thermo-resistance is known for playing an important role in predicting semen fertility (Schulze et al., 2013b; Schulze et al., 2021) and represents a challenging situation for the sperm as it simulates the conditions found in the female genital tract before fecundation (Schulze et al., 2019). In this way, the use of a thermo-resistance test (TRT) for assessing the dead sperm effect in cooled boar semen could be a first step to start investigating the relationship between these cells and their contemporary live cells. Based on the aforementioned reasons, this study aimed to evaluate if dead sperm cells could affect the viable sperm cells in liquid boar semen during a TRT.

MATERIAL AND METHODS

Chemicals

Chemicals for spermatological analyses were purchased from Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Propidium iodide (PI) was obtained from Thermofischer (Eugene, USA), whereas Rhodamine 123 (R123), fluorescein-isothiocyanate conjugated peanut agglutinin (FITC-PNA), fluorescein-isothiocyanate conjugated Pisum sativum agglutinin (FITC-PSA), YoPro-1 and Merocyanine 540 (M540) were purchased from Sigma-Aldrich (Steinheim, Germany). Semen extenders were acquired from Minitüb GmbH (Tiefenbach, Germany).

Animals and sample collection

A total of 54 AI semen doses produced from different ejaculates (collected by the gloved-hand method) were randomly selected. The doses were obtained from 54 healthy and mature boars (Piétrain; aged 15.6 ± 3.4 months) in an AI center in Germany. They were produced with a short-term extender (BTS, Minitüb, Tiefenbach, Germany) in a final volume of ~ 85 mL. The selection was based on the following criteria at 72 hours of storage: total motility between 70–80% and sperm concentration between 20 and 30 × 10^6 cells/mL.

The sperm motility was checked with the Computer-Assisted Semen Analysis (CASA, AndroVision®, Minitüb) using preheated Leja chambers (Leja Products B.V., Nieuw Vennep, The Netherlands). The chambers were filled with 3-µL of the sample, previously incubated for 10 min at 38°C, and four fields were recorded at a rate of 30

frames per 0.5 s and under 100 × magnification. Cells were considered as motile when the curved line velocity (VCL) was > 24 µm/s and the amplitude of lateral head displacement (ALH) was > 1.0 µm, according to the manufactures' defaults setting for boar semen. A NucleoCounter SP-100^{TM} was used for the evaluation of sperm concentration in each AI semen dose as specified by Revision 1.5 of the User Guide Manual No. 991-0100 (ChemoMetec A/S, Lillerød, Denmark).

To avoid some possible effect of sperm agglutination or bacterial contamination of AI semen doses on the experimental design, the samples selected were also evaluated regarding the presence of cells agglutinated or particles suggesting bacterial contamination during the sperm motility assessment.

Sample preparation

Immediately after selection, each AI semen dose was split into three treatments: 25%, 50% and 75%, corresponding to samples, containing ~25%, ~50% or ~75% dead sperm cells in a final volume of 15 mL, respectively. For preparing these treatments, two subsamples were taken from each AI semen dose: subsample I (30 mL) and subsample II (15 mL). The subsample I was kept at 17°C while the subsample II was submitted to a flash-freezing process adapted from Roca et al. (2013) which consisted of freezing the sample in liquid nitrogen followed by immediately thawing it at 38°C in a water bath. The death of all cells was confirmed by flow cytometry (CytoFLEX; Beckman Coulter, Indianapolis, USA), using PI (final concentration: 10 µg/mL, incubation at 38°C for 5 min). After the thawed subsample achieved 16°C, the treatments were performed by mixing subsample I with subsample II in different proportions, considering that subsample I had ~75% of live cells while subsample II had no live cells (**Table 1**).

Thermo-resistance-test (TRT)

Total motility assessment was done using the CASA system over a TRT performed immediately after preparing the treatments (**Table 1**). The sperm samples (15 mL) were incubated using a water bath at 38°C (GFL 1002, Gesellschaft für Labortechnik, Burgwedel, Germany) under aerobic conditions and the motility was analyzed after 30 min (TRT30, on-test) and 300 min (TRT300, off-test) of incubation (Schulze et al., 2019). For the analysis, preheated Leja chambers were filled with 3-µL of the sample, and four fields

were recorded (30 frames per 0.5 s) under 100 × magnification. As described in the item 2.2, cells were considered as motile when presenting VCL > 2.4 µm/s and ALH > 1.0 µm.

Flow cytometric assessment

The sperm samples incubated for 30 and 300 min at 38°C (TRT30 and TRT300, **Table 1**) were analyzed using a CytoFLEX S equipped with four lasers (violet: 405nm, blue: 488 nm, yellow-green: 561 nm, and red: 638 nm). For assessment of mitochondrial activity, a 250 µL-sample of sperm was double-stained with R123 (final concentration: 0.2 µg/mL) and PI (10 µg/mL) and then incubated for 20 min at 38°C, according to the method described by Schulze et al. (2013b). The apoptosis status and the membrane lipid ordering were evaluated after staining 500 µL of sperm with YoPro-1 (75 nM) and M540 (2.0 µM) and incubating the sample for 10 min at 38°C (Schulze et al., 2020). To evaluate the integrity of acrosomal and plasma membranes, 375 µL of sperm were diluted in 125 µL of 0.5% PBS-buffered formalin and then triple-stained with FITC-PNA, FITC-PSA (incubation: 10 min at 38°C) and PI (incubation: 5 min at 38°C) as previously described by Schulze et al. (2013a). The final concentrations of FITC-PNA, FITC-PSA, and PI were: 2.5 µg/mL, 2.5 µg/mL, and 10 µg/mL, respectively. All the stained sperm samples had a concentration of ~ 23×10^6 cells/mL.

After the incubation period, 20 µL of each stained sample were diluted into 1 mL of isotherm phosphate-buffered NaCl solution, achieving a concentration of ~ 0.45×10^6 cells/mL. The sperm population was gated by referring to the expected forward- and side-scatter signals, and agglutinations were excluded by forward-scatter gating. Per sample, 10,000 events into the sperm gate were obtained. The fluorochromes were excited by blue (FITC-PNA, FITC-PSA, R123, and YoPro-1) and yellow (M540 and PI) lasers. Fluorescence signals of FITC-PNA, FITC-PSA and R123 (detected by 525/40 nm band-pass filter) were plotted on logarithmic scales, as well as the signals of M540 and PI (detected by 585/42 nm and 610/20 nm band-pass filters, respectively).

Finally, the percentage of spermatozoa with intact acrosome and plasma membranes (IAPM) were recorded (negative for PI, FITC-PNA and FITC-PSA), as well as the percentage of cells with active mitochondria and intact plasma membrane (AMIPM; positive for R123 and negative for PI). Furthermore, spermatozoa with a high degree of

membrane lipid disorder (HDLD; positive for high M540 fluorescence) as well as the percentage of apoptotic cells (APOP; positive for YoPro-1) were registered.

Statistical analysis

Using the SPSS software version 28.0 (IBM, Armonk, NY, USA), the parameters were tested for normal distribution by the Shapiro-Wilk test. Then, the values at TRT30 and TRT300, as well as the absolute and relative variations were compared among the treatments through the Friedman test followed by Bonferroni correction. The differences between the values at TRT30 and TRT300 within the treatment were calculated using the Wilcoxon signed-rank test. Results are presented as medians unless stated otherwise, and statistical significance was set at $P < 0.05$.

The absolute variation between the parameters at 30 and 300 min of TRT was calculated for all treatments using the respective formula (**formula 1**):

Formula 1 (in percentage points (pp)):

$$Value\ at\ TRT300\ -\ Value\ at\ TRT30$$

The relative variation between the parameters at 30 and 300 min of TRT was calculated for all treatments using the respective formula (**formula 2**):

Formula 2 (in %):

$$\frac{Value\ \ at\ TRT300 - Value\ \ at\ TRT30}{Value\ \ at\ TRT30} \times 100$$

RESULTS

Quality parameters of boar semen samples

The mean values and standard error of mean regarding the selected AI semen doses at 72 h of storage were 77.6 ± 0.4% total motility, 23.1 ± 0.4 cells/mL, and total volume of 88.3 ± 0.3 mL (**Table 2**). At TRT30 and TRT300, the treatments (25%, 50% and 75% dead sperm cells) were different regarding the median values of total motility, IAPM, AMIPM, HDLD, and APOP ($P < 0.01$). Moreover, the values observed at TRT30 and TRT300 were different within the treatments for all the parameters ($P < 0.01$), except for APOP, where

the percentage of apoptotic cells did not differ significantly within the treatments containing 50% and 75% of dead sperm cells ($P \geq 0.38$, **Figure 1**).

Absolute variations between the treatments

The AI semen doses with 25% dead sperm cells lost more pp of motility (-9.4 pp) compared to those containing 50% (-8.2 pp) and 75% dead sperm cells (-4.5 pp). The lowest loss was observed for doses with 75% dead sperm cells ($P < 0.01$; **Figure 2A**). For IAPM, the absolute variations were different among all treatments ($P < 0.01$). Doses containing 25% dead sperm cells had a decrease of -1.8 pp, while doses with 50% and 75% dead sperm cells showed an increase of 0.8 pp and 1.5 pp, respectively (**Figure 2B**). Considering the AMIPM, the absolute variations were similar only between doses with 50% and 75% dead sperm cells (2.7 pp and 3.4 pp, respectively, $P < 0.01$). The doses with 25% dead sperm cells showed decrease of -1.3 pp (**Figure 2C**). Regarding the percentage of cells with HDLD, the absolute variations were similar for treatments 50% and 75% dead sperm cells, with decreases of -5.4 pp and -5.3 pp, respectively. This differed from the treatment with 25% dead sperm cells, which presented a decrease of -0.8 pp ($P < 0.01$; **Figure 2D**). The treatments showed similar absolute variations for apoptosis status: 4.7 pp, 1.2 pp and 2.4 pp, respectively (**Figure 2E**).

Relative variations between the treatments

The AI semen doses with 25%, 50% and 75% dead sperm cells lost the same proportion of the on-test values for total motility: -11.9%, -16.0% and -17.5%, respectively (**Figure 3A**). Regarding the percentage of cells presenting IAPM and AMIPM, the relative variations were different between all treatments ($P < 0.01$). For IAPM, doses with 25%, 50% and 75% dead sperm cells had relative variations of -2.1%, 1.4% and 5.0%, respectively (**Figure 3B**). For AMIPM, the values were -1.5%, 4.9% and 11.8, respectively (**Figure 3C**). Considering the percentage of cells with HDLD, decreases of -7.7%, -12.6% and -7.8% during on-test were observed for the treatments 25%, 50% and 75% dead sperm cells, respectively, with significant differences between treatments 50% and 75% dead sperm cells ($P < 0.01$; **Figure 3D**). In respect to apoptosis status, all treatments showed an increase of the initial percentage of apoptotic cells, with a higher relative variation for

doses containing 25% dead sperm cells (26.2%), compared to those containing 50% (2.7%) and 75% (3.2%) dead sperm cells ($P < 0.01$; **Figure 3E**).

DISCUSSION

A negative effect caused by dead sperm cells on the contemporary live cell's motility was observed for frozen boar semen and seemed to be related to the release of cytotoxic metabolites, such as reactive oxygen species (ROS) (Martinez-Alborcia et al., 2012; Roca et al., 2013). To the best of our knowledge, no information regarding cooled semen has been previously reported. For this reason, this study aimed to investigate the effect of dead sperm cells in liquid preserved boar semen over a thermo-resistance test (TRT). Our results showed that, regardless of their proportion, the dead sperm cells did not significantly affect the motility of contemporary viable cells in liquid boar semen, based on the lack of difference in the relative loss of motility over the TRT among the treatments.

Significant differences were found in relative (between treatments) and absolute values (within treatments) during the TRT for plasma membrane and acrosome integrity (IAPM), mitochondrial activity (AMIPM), and high degree of lipid disorder (HLDL). The treatments containing 50 and 75% of dead sperm showed higher absolute differences for these parameters compared to samples with only 25%. Regarding the membrane's integrity and ordering, the samples with lower percentage of alive cells may lead to an environment with higher availability of seminal plasma for the remaining live cells. It could mediate some restoring process of membrane, since it was already reported that the membrane damage in ram sperm was restored after adsorption of seminal plasma proteins (Barrios et al., 2000). In the same sense, we could also hypothesize that mitochondrial activity would be restored over the TRT as a consequence of a higher proportional availability of substrate, since the competition for it should be lower when compared to samples with higher number of live cells. However, these are only hypotheses and the possible restoring mechanism of sperm structures is not totally clear yet, mainly considering the proportion of dead sperm cells. Moreover, the absolute variations that did not surpass values of -1.8 pp, 3.4 pp, and -5.4 pp, respectively. Thus, due to the slight differences between the on- and off-test values within each treatment for these parameters, it can be assumed for now that the dead sperm cells have a minimum impact on the sperm structure in liquid boar semen.

We evaluated the treatment effect based on the variation of results during the TRT. This approach has previously been applied to frozen boar sperm motility (Peláez et al., 2006), and it is an important strategy to interpret sperm motility resilience better. The TRT is performed after prolonged storage periods to simulate the time spermatozoa normally spend in the female genital tract. To determine the persistence of sperm to thermal stress, the motility, mostly measured by CASA systems, is evaluated. The outcomes of the TRT show a strong correlation to the fertilizing competence of ejaculates and could help to explain variations in boar fertility (Schulze et al., 2021). Unfortunately, no information regarding the boar sperm structure changes over the TRT is currently available for liquid semen. Another interesting finding in this study was, regardless the treatment, the traits evaluated by flow cytometry did not change substantially throughout the TRT. In the future, it can be recommended that sperm structure over the TRT be better evaluated.

It is important to highlight that the effect of dead sperm cells on boar semen quality can be more pronounced in cryopreserved than in liquid preserved samples, as reported in other species (Roca et al., 2016). This discrepancy could be related to several factors, such as the presence of ROS scavengers. Some antioxidant compounds of seminal fluid may provide a degree of protection against these factors (Martinez-Alborcia et al., 2012). In contrast to bull and stallion semen, the seminal fluid of boars has a higher radical reduction capacity per sperm cell (Jakop et al., 2022), which is removed from samples to be frozen but not from samples to be stored at ~17°C. Another influencing factor could be the amount of EDTA present in the extender. For bull semen, EDTA could inhibit the toxicity caused by peroxide, protecting the sperm cells against the dead sperm cell effect (Shannon and Curson, 1972). In the current study an extender with a higher concentration of EDTA (BTS: 9 mM; Menegat, 2016) was used, compared to the extenders used for freezing (BTS: 3.35 mM; Martinez-Alborcia et al., 2012; Roca et al., 2013). According to Shannon and Curson (1972), the toxic effect of dead sperm cells was enhanced by increasing the amount of egg yolk in the freezing extender for bull semen, which is probably due to a higher amount of substrate available for peroxide formation. For this reason, it can be hypothesized that egg yolk could also be related to the discrepancy in dead sperm effect in liquid to frozen boar semen.

The period of exposition of live cells to the dead sperm cells and the conditions of this contact are also essential factors. In this study, both populations were in contact during challenging conditions (incubated at 38°C). On one hand, the TRT represents a stress test for the cells. On the other hand, the fully activated metabolism at body temperature possibly enables repair mechanisms to overcome factors such as oxidative stress caused by activated antioxidative enzymes. For example, in boar semen, the antioxidative enzyme superoxide dismutase has presented high activity at temperatures of 20°C and 45°C (Orzołek et al., 2013). This effect, however, may not have been properly represented in our study, as the cells interacted under TRT conditions for only five hours. For this reason, further studies are recommended to evaluate the effect of dead sperm cells on live cells when their contact takes place over a time representative of the storage period of AI doses.

If a negative effect of dead sperm cells is observed over the storage time, it would be necessary to establish thresholds for the amount of dead and live cells present to sustain the quality of AI doses. It would also be interesting to verify if some boars are more resistant to the dead sperm effect than others. Despite not being possible to properly evaluate the boar effect in our study, our boxplots shows that not all the boars had the same behavior. Some boars did not have their sperm affected by the proportion of dead cells, while others were considerable affected. A boar effect has already been observed for frozen boar semen (Martinez-Alborcia et al., 2012).

Moreover, other possible effects could be investigated, as the dead sperm source (homologous or heterologous sperm), the boar age, mainly considering differences on seminal plasma composition between old and young boars (Koziorowska-Gilun et al., 2011; Fraser et al., 2016). As a result, new sperm quality assessments could be developed based on boar resilience against dead sperm cells. Thus, establishing a practical and suitable method to kill the sperm cells will be a necessary task. In our methodology, sperm were killed by the freezing procedure, used by most studies investigating the dead sperm effect (Shannon and Curson, 1972; Brinsko et al., 2003; Roca et al., 2013; Lüttgenau et al., 2021). Other sperm killing methods are reported, such as the heating of sperm cells at high temperatures, the exposition to media containing detergents (Triton X-100 or sodium dodecyl sulfate – SDS, (Ahmadi and Ng, 1997; Mitchell et al., 2011) or even to radiation (from ultraviolet laser or near-infrared laser; König et al., 1996). However, it would be

important to further investigate the difference in cell death mechanisms between these procedures and consider if this factor influences the dead sperm effect.

It is still necessary to regard that while there is no apparent effect on sperm quality of liquid boar semen, there may still be an impact on fertility. Dead sperm cells have already been shown to promote the gene expression of pro-inflammatory cytokines by the endometrium in mares (Lüttgenau et al., 2021). Consequently, an aggressive immune response against the sperm can be triggered, reducing the chances of fertilization (Roca et al., 2016). For sows, this response has not yet been clearly observed (Lessard et al., 2003), thus, the influence of dead sperm on the uterine response after insemination in sows requires further investigation.

CONCLUSIONS

Our results showed that dead sperm cells did not affect the motility of contemporary live cells in liquid boar semen over a TRT. A substantial influence on viable sperm structures did not occur either. How dead sperm affects stored boar semen disregarding the thermo-resistance condition remains unanswered. Therefore, further studies must investigate the dead sperm cell effect in liquid boar semen doses under storage conditions. Moreover, it is still necessary verify a possible individual boar resistance that could be influencing the relationship between dead and live sperm cells.

AUTHOR CONTRIBUTIONS

MS conceptualized the study. **MS, MQ, APGM and FPB** designed the study. **MQ** performed the acquisition, analysis and interpretation of the data, and wrote the original draft of the manuscript. **UJ, APGM, FPB, MJ** and **MS** supervised the study and critically reviewed the manuscript. **MS and FPB** acquired the financing and managed the project. All authors read, revised and approved the final manuscript.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY

The data that supported the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Overview of sample preparation and experimental design for evaluating the dead sperm effect on liquid boar semen quality during a thermo-resistance test.

Sample preparation			
Per dose	**Storage time/ temperature**	**Volume**	**% Dead sperm cells**
Subsample I	72 h/17°C	30 mL	~ 25%
Subsample II		15 mL	100%[1]
Treatments (samples)	**Volume subsample I**	**Volume subsample II**	**Final volume**
25% dead sperm cells	15 mL	0 mL	15 mL
50% dead sperm cells	10 mL	5 mL	
75% dead sperm cells	5 mL	10 mL	

Sample analysis					
Parameters	**Original sample**		**Stained sample**		**Volume analyzed**
	Incubation time/ temperature	**Incubation volume**	**Incubation time/ temperature**	**Incubation volume**	
Total motility	30 and 300 min/38°C	15 mL	n. a.	n. a.	3 µL
APMI			10 min/38°C	375 µL[2]	20 µL[3]
AMIPM			20 min/38C	250 µL	
HDLD			10 min/38°C	300 µL	
APOP			10 min/38°C	300 µL	

n. a.: not applicable

[1] Sample submitted to a flash-frozen process in liquid nitrogen, followed by thawing at 38°C

[2] Diluted in 125 µL PBS-buffered formalin

[3] 20 µL diluted in 1 mL phosphate-buffered NaCl solution

APMI: acrosomal and plasma membrane integrity; AMIPM: active mitochondria and intact plasma membrane; HDLD: high degree of lipid disorder; APOP: apoptotic cells.

Table 2. Sperm parameters of AI boar semen doses used for producing the treatments containing different dead sperm proportions.

Characteristics	***n***	**Mean**	**SD**	**SEM**	**Median**	**Min**	**Max**
Total motility (%; 72 h)		77.3	0.18	3.3	77.9	70.3	80.2
Concentration (10^6/mL; 24 h)	54	23.1	2.8	0.1	23.3	20.1	30.8
Volume (mL; 24 h)		88.3	2.6	0.3	89.0	82.9	92.9

SD: standard-deviation; SEM: standard error of the mean; Min: minimum; Max: maximum

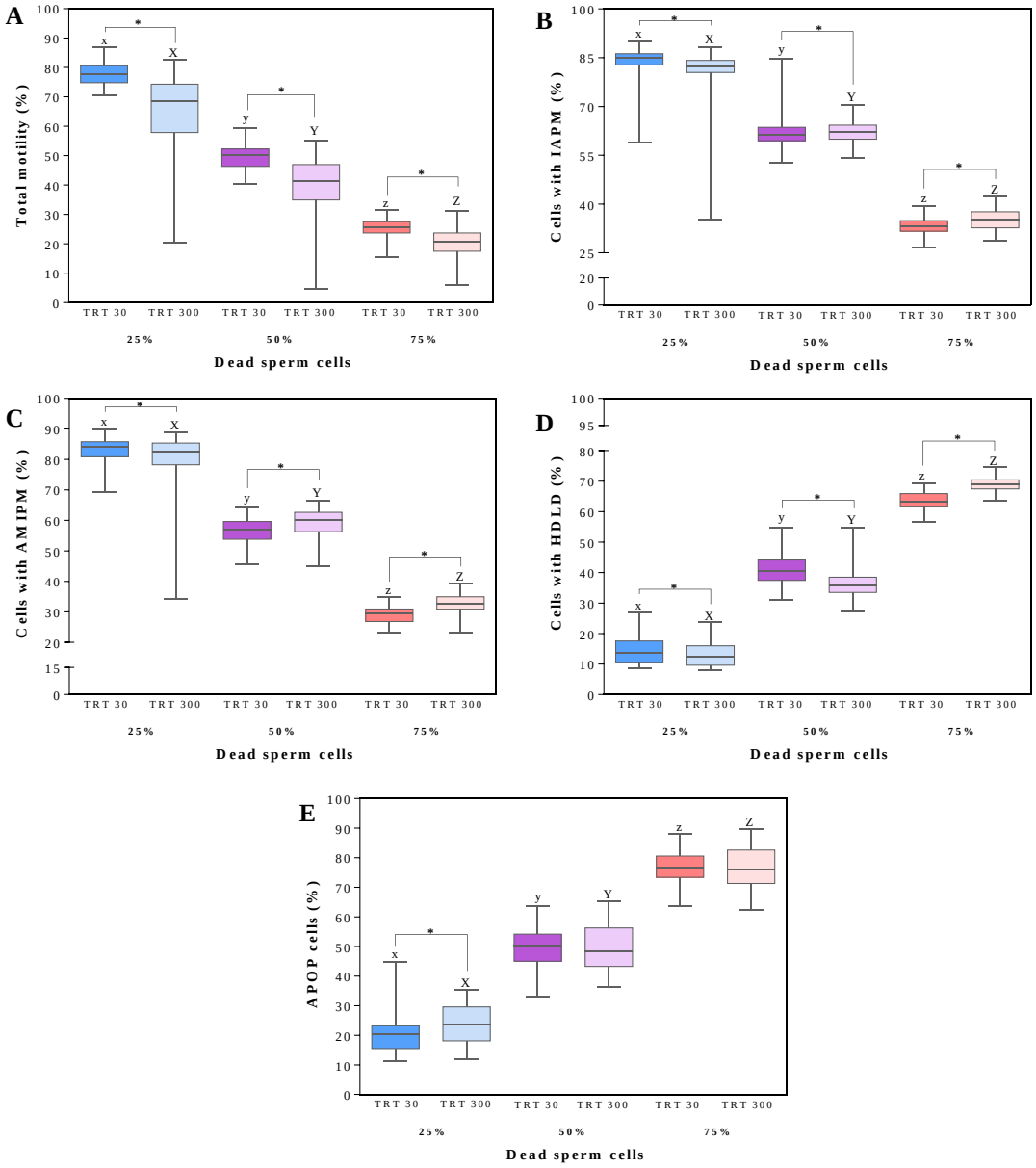

Fig. 1. Quality parameters of AI doses containing 25%, 50% or 75% of dead boar sperm cells during a thermo-resistance test (TRT). **A:** Total motility, **B:** Cells with intact acrosome and plasma membrane (IAPM), **C:** Cells with active mitochondria and intact plasma membrane (AMIPM), **D**: cells with high degree of membrane lipid disorder (HDLD) and **E**: apoptotic cells (APOP).

TRT30: 30 min of TRT, TRT300: 300 min of TRT.

* Indicate difference between the moments within the treatment ($P < 0.01$)

x, y, z Indicate difference among the treatments within the moment TRT30 ($P < 0.01$)

[X, Y, Z] Indicate difference among the treatments within the moment TRT300 ($P < 0.01$)

The boxplots show the following parameters: minimum value, first quartile, median, third quartile, and maximum value.

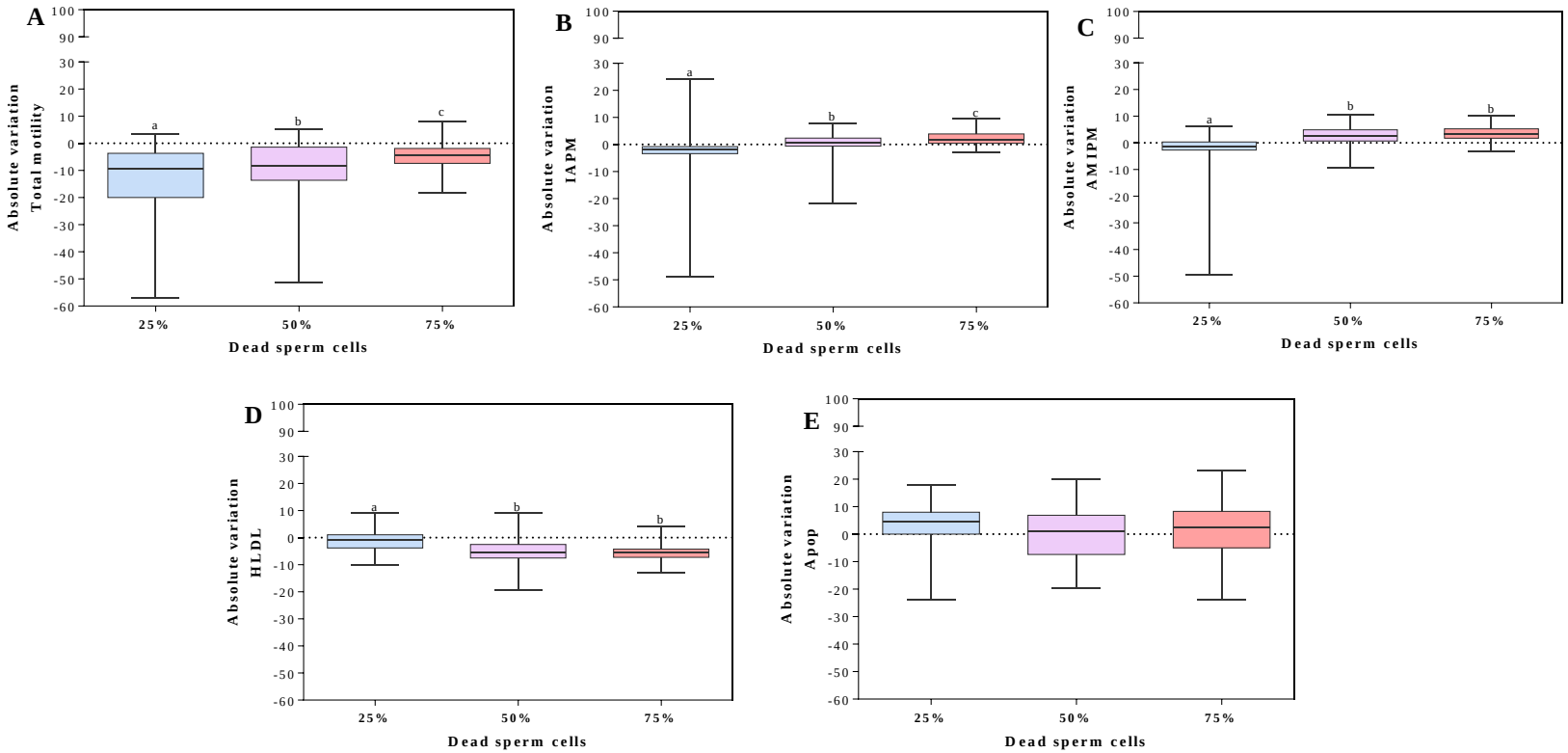

Fig. 2. Absolute variation (in percentual points; pp)* of quality parameters in AI doses containing 25%, 50% or 75% of dead boar sperm cells during a thermo-resistance test. **A:** Total motility, **B:** Cells with intact acrosome and plasma membrane (IAPM), **C:** Cells with active mitochondria and intact plasma membrane (AMIPM), **D**: cells with high degree of membrane lipid disorder (HDLD) and **E**: apoptotic cells. [a, b, c] Indicate difference among the treatments ($P < 0.01$). *(Value at 300 min of TRT – Value at 30 min of TRT) The boxplots show the following parameters: minimum value, first quartile, median, third quartile, and maximum value.

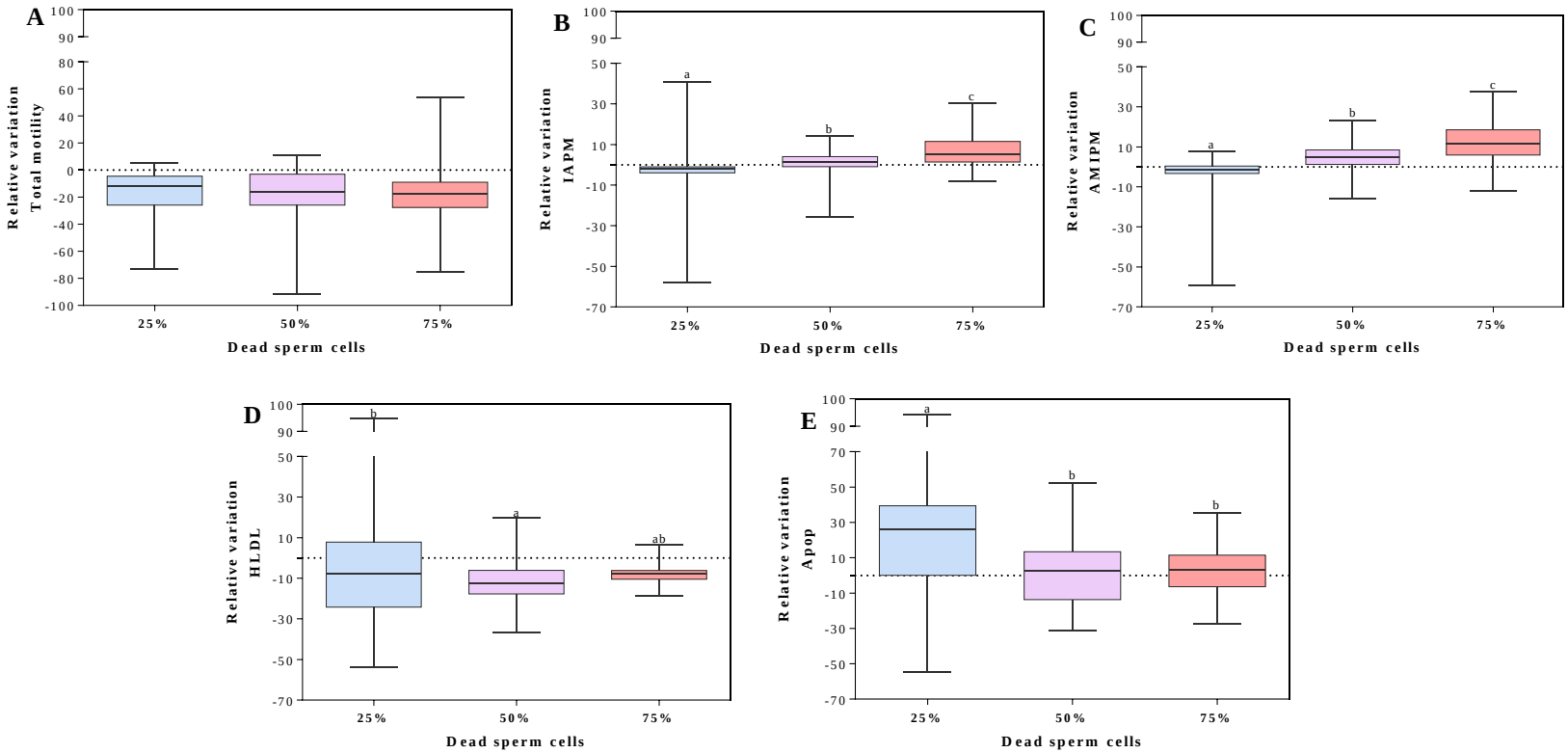

Fig. 3. Relative variation (%)* of quality parameters in AI doses containing 25%, 50% or 75% of dead boar sperm cells during a thermo-resistance test. **A:** Total motility, **B:** Cells with intact acrosome and plasma membrane (IAPM), **C:** Cells with active mitochondria and intact plasma membrane (AMIPM), **D**: cells with high degree of membrane lipid disorder (HDLD) and **E**: apoptotic cells.

[a, b, c] Indicate difference among the treatments ($P < 0.01$). *(Value at 300 min of TRT – Value at 30 min of TRT)/Value at 30 min of TRT × 100. The boxplots show the following parameters: minimum value, first quartile, median, third quartile, and maximum value.

4. CAPÍTULO III – Aplicabilidade da citometria de fluxo para o controle de qualidade de doses inseminantes suínas

4.1 Revisão bibliográfica

A citometria de fluxo (CF) começou a ser utilizada na andrologia animal e humana a partir da década de 70, com foco na marcação do DNA espermático. Ao longo dos anos subsequentes, a tecnologia foi se estabelecendo gradativamente, de forma a substituir técnicas de fluorescência consideradas menos práticas e mais suscetíveis a erros, como a microscopia de fluorescência (MARTÍNEZ-PASTOR *et al.*, 2010).

Atualmente, a CF já é considerada como uma das análises padrão-ouro de laboratórios de andrologia, juntamente com o sistema CASA (NIŻAŃSKI *et al.*, 2016). A capacidade da CF em promover análise de milhares de eventos, considerando múltiplas características espermáticas de forma objetiva, acurada e rápida (PEÑA, 2015), contribuiu significativamente para a implementação da tecnologia na análise de sêmen, assim como o fato de os espermatozoides da maioria das espécies animais encontrarem-se naturalmente em uma suspensão fluida e monocelular (BOE-HANSEN & SATAKE, 2019). Para facilitar o entendimento sobre a atual aplicabilidade da CF bem como seus avanços, perspectivas e oportunidades na análise de sêmen, principalmente suíno, uma breve revisão será abordada neste capítulo.

4.1.1 Citometria de fluxo para determinar a concentração espermática

A determinação do número de células foi uma das primeiras aplicações da CF (BOE-HANSEN & SATAKE, 2019); contudo, com foco em amostras contendo células sanguíneas vermelhas. Somente a partir de 1990, a abordagem começou a ser testada e validada para células espermáticas (EVENSON *et al.*, 1993). Independentemente do tipo celular, a determinação da concentração de células através da CF sempre se baseou em duas diferentes abordagens: a quantificação do número de eventos na amostra a partir do uso de *beads* de referência ou da contagem de eventos em um volume fixo a ser adquirido, também conhecida por *true volume counting* (BRITO *et al.*, 2016).

4.1.1.1 Concentração espermática do ejaculado e de doses inseminantes

Até o presente momento, todos os estudos que tentaram validar a CF para quantificar o número de células espermáticas utilizaram amostras de ejaculado ou de fluido epididimal. O estudo pioneiro na área de andrologia utilizou a espécie bovina como modelo e, posteriormente, foram desenvolvidas metodologias envolvendo amostras de sêmen de roedores e humanos. Em 2004, o primeiro estudo validando a técnica para ejaculado de suínos foi desenvolvido por CHRISTENSEN *et al.* (2004a)

A validação geralmente é realizada através da comparação da técnica de CF com métodos convencionais de determinação da concentração espermática, como a contagem utilizando a câmara hemocitométrica, o sistema CASA, o NucleoCounter e espectrofotômetros (**Tabela 1**). Apesar de não ser amplamente aplicado na rotina de avaliação da concentração espermática de centrais para produção de doses de sêmen suíno ou de laboratórios relacionados, a técnica tem sido aplicada e aprimorada em nível de pesquisa, principalmente levando em consideração a capacidade que a CF oferece de excluir componentes não celulares e de analisar rapidamente um alto número de eventos (BRITO *et al.*, 2016).

Conforme pode-se observar na **Tabela 1**, ainda não foram desenvolvidos estudos que abordem o uso da técnica em programas de controle de qualidade para mensurar a quantidade de células espermáticas em doses inseminantes de suínos. Porém, é irrefutável a relevância e o impacto que a CF pode ter enquanto técnica de avaliação da concentração espermática para esses programas. Além da acurácia relacionada ao grande número de células contabilizadas por amostras e da praticidade, a aplicação poderia propiciar como rotina a avaliação simultânea da concentração e estrutura espermática das doses, otimizando a rotina e agregando resultados.

4.1.1.2 Limitações da citometria de fluxo para avaliar a concentração espermática

Apesar da atual disponibilidade de citômetros de fluxo aptos a realizarem análises com base em volume fixo, a maioria dos protocolos já desenvolvidos, independentemente da espécie, analisaram as amostras adquirindo um certo número de eventos, demandando o uso das *beads* de referência. Nesse sentido, é possível que o custo da análise tenha se

tornado um dos fatores limitantes para a aplicação da tecnologia na rotina dos laboratórios, mesmo aqueles que já possuem o equipamento. Logo, a necessidade de desenvolvimento e validação de novos protocolos é iminente. Além disso, a tecnologia de CF também demanda qualificação por parte de seus operadores, os quais devem ser capacitados para preparar as amostras, conduzir as análises e interpretar os resultados, de forma minimizar erros e outras variações (BRITO *et al.*, 2016).

Tabela 1. Principais protocolos de determinação da concentração espermática utilizando a técnica de citometria de fluxo.

Referência	**Amostra**	**Espécie**	**Protocolo**
EVENSON *et al.* (1993)	Ejaculado	Bovinos	A) Acridine orange + *Beads* B) PI + *Beads*
YAMAMOTO *et al.* (1998)	Fluido epididimal	Ratos	PI
EUSTACHE *et al.* (2001)	Ejaculado	Humanos	A) *Gate* pré-determinado + *Beads* B) PI + *Beads* C) *Gate* pré-determinado + PI + *Beads*
CHRISTENSEN,*et al.* (2004a)	Ejaculado	Suínos	SYBR-14 + PI + *Beads*
CHRISTENSEN *et al.* (2004b)	Ejaculado	Suínos, ovinos, roedores, aves e humanos	SYBR-14 + PI + *Beads*
CHRISTENSEN *et al.* (2005)	Ejaculado	Bovinos	SYBR-14 + PI + *Beads*
HANSEN *et al.* (2006)	Ejaculado	Suínos	SYBR-14 + PI + *Beads*
PRATHALINGAM *et al.* (2006)	Ejaculado	Bovinos	*Gating* (sem fluorocromos)
ANZAR *et al.* (2009)	Ejaculado	Bovinos	Thiazole orange + PI + *Beads*

4.1.2 Citometria de fluxo para avaliar as estruturas espermáticas

A partir dos anos 90, as aplicações da CF foram além da marcação de DNA e se estenderam à avaliação de estruturas como a membrana plasmática (PEÑA, 2015). Atualmente, uma variedade de aspectos funcionais da célula espermática pode ser analisada pela CF, como: integridade acrossomal, atividade mitocondrial, peroxidação e grau de desorganização lipídica da membrana plasmática, estresse oxidativo, status de capacitação espermática e apoptose, absorção de cálcio e integridade de cromatina (MARTÍNEZ-PASTOR *et al.*, 2010; BOE-HANSEN & SATAKE, 2019). Destaca-se que alguns destes parâmetros são considerados supostos preditores de fertilidade em suínos (SCHULZE *et al.*, 2013; SCHULZE *et al.*, 2021).

4.1.2.1 Painéis simples e multicolor

No passado, a indisponibilidade de citômetros de fluxo mais modernos assim como a limitação nas opções de fluorocromos eram um dos principais fatores limitantes para o desenvolvimento e uso de painéis mais complexos na área. Dessa forma, a maioria das análises de andrologia realizadas por CF baseavam-se no uso de painéis simples, utilizando apenas duas ou três cores no mesmo ensaio e obtendo informações de, no máximo, dois parâmetros espermáticos simultaneamente (PETRUNKINA & HARRISON, 2011). No entanto, apesar dos avanços na modernização dos equipamentos e disponibilidade de novos fluorocromos, os painéis simples ainda compreendem a maioria dos ensaios realizados atualmente por CF em laboratórios de andrologia, principalmente veterinária (PETRUNKINA & HARRISON, 2013).

A possibilidade de analisar diversos parâmetros de um mesmo evento é uma das principais vantagens da técnica de CF (MARTÍNEZ-PASTOR *et al.*, 2010) e painéis multicolor com mais de 10 cores são comumente aplicados na área da imunologia (MACIOROWSKI *et al.*, 2017). A associação de fluorocromos em uma mesma amostra permite a rápida e simultânea avaliação de diversos atributos da célula, diminuindo o tempo de análise, bem como custos dispendidos com materiais de consumo. No cenário atual da andrologia, o uso desses painéis representa, portanto, solução promissora para contornar uma das históricas limitações enfrentadas nas análises de sêmen, principalmente no que diz

respeito à predição de fertilidade: a incapacidade de testar simultaneamente múltiplas características de uma única célula (PEÑA, 2015).

Até o momento, poucos painéis multicolor foram desenvolvidos para análise de sêmen animal (**Tabela 2**). Nesse contexto, acredita-se que ainda existam fatores limitantes, como os custos de aquisição de equipamentos mais avançados, além de restrições dos laboratórios quanto à realização da compensação da sobreposição de espectro entre os fluorocromos (PETRUNKINA & HARRISON, 2011). Contudo, a adaptação dos laboratórios ao uso de ensaios multicolor é uma necessidade para o futuro desenvolvimento das análises de sêmen (PETRUNKINA & HARRISON, 2013; PEÑA *et al.*, 2018), auxiliando no melhor entendimento da fisiologia espermática e sua relação com a fertilidade.

4.1.3 Perspectivas da citometria de fluxo na análise de sêmen

Avanços na variedade de características estruturais e metabólicas a serem analisadas pela CF são esperados, principalmente devido à variedade de novos fluorocromos disponibilizados nos últimos anos. Consequentemente, evoluções de painéis multicolor também são uma expectativa para a andrologia (PETRUNKINA & HARRISON, 2011; PETRUNKINA & HARRISON, 2013). Ressalta-se, ainda, que já estão disponíveis métodos computacionais (citometria de fluxo computacional; ORTEGA-FERRUSOLA *et al.*, 2017a) para possibilitar a análise dos resultados obtidos a partir de ensaios com muitas cores associadas, representando um grande potencial para novas descobertas na área de sêmen (BOE-HANSEN & SATAKE, 2019).

Além disso, nos próximos anos, a citometria de fluxo convencional tende a ser substituída pela citometria de fluxo de imagem (BOE-HANSEN & SATAKE, 2019). Esse avanço poderá otimizar o desenvolvimento de novos protocolos de citometria para avaliação da concentração espermática bem como a validação de novos painéis multicolor.

Tabela 2. Exemplos de painéis multicolor de citometria de fluxo validados para análise de sêmen animal.

Referência (espécie)	Cores	Número de parâmetros	Parâmetros	Fluorocromos
PEÑA (2015) (equinos)	4	3	Permeabilidade de membrana Integridade de membrana Potencial mitocondrial	YoPro-1 PI Mitotracker deep red +Hoechst 33342
			Permeabilidade de membrana Integridade de membrana Estresse oxidativo	YoPro-1 PI CellRox deep red +Hoechst 33342
TORRES *et al.* (2016) (suínos)	4	3	Integridade acrossomal Integridade de membrana Potencial mitocondrial Marcação celular	FITC-PSA JC-1 PI +Hoechst 33342
JÄKEL *et al.* (2021) (suínos)	4	3	Permeabilidade celular Fluidez de membrana Integridade acrossomal	YoPro-1 Merocianina 540 PNA-Alexa Fluor 647 +Hoechst 33342
			Potencial mitocondrial Influxo de cálcio Viabilidade celular	JC-1 Calbryte 630 Sir700-DNA+Hoechst 33258
KANNO *et al.* (2016) (bovinos)	4	3	Integridade de membrana Integridade acrossomal Potencial mitocondrial	SYBR-14 PE-PNA PI Mitotracker Deep Red

Tabela 2 (continuação). Exemplos de painéis multicolor de citometria de fluxo já validados para análise de sêmen animal.

Referência (espécie)	Cores	Número de parâmetros	Parâmetros	Fluorocromos
ORTEGA-FERRUSOLA *et al.* (2017b) (equinos)	5	5	Viabilidade celular Atividade de caspase 3 Potencial de membrana Potencial mitocondrial Maturidade espermática	Live/dead Violet kit Caspase-3/7 green DiSBAC2 Mitotracker deep red CD44 APC
BUCHER *et al.* (2019) (bovinos)	5	5	Atividade de esterase Cálcio intracelular Integridade acrossomal Integridade de membrana Potencial mitocondrial	Calcein violet AM Fluo-4 AM PE-PNA PI $DiIC_1(5)$
GONZALEZ-CASTRO *et al.* (2022) (suínos)	4	3	Integridade de membrana Integridade acrossomal Atividade mitocondrial	PNA-Alexa Fluor 488 PI Mitotracker deep Red +Hoechst 33342

4.2 Quarto artigo científico

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A 5-color flow cytometry panel to assess plasma membrane integrity, acrosomal status, membrane lipid organization and mitochondrial activity of boar and stallion spermatozoa following liquid semen storage

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A 5-color flow cytometry panel to assess membrane integrity, acrosomal status, membrane lipid organization and mitochondrial activity of boar and stallion spermatozoa following liquid semen storage

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ABSTRACT

For a more practically applicable analysis of different sperm characteristics, this study aimed to develop a 5-color flow cytometry (FC) panel to concurrently analyze four sperm parameters in liquid boar and stallion semen, using also a DNA-marker for selecting sperm cell events. From each of thirty extended boar semen doses and twelve stallion semen doses, six aliquots were taken. For evaluating mitochondrial activity (A), degree of lipid disorder of plasma membrane (B), integrity of plasma membrane (C), acrosomal status (D) and marking DNA (E), five aliquots were individually stained with Rhodamine 123, Merocyanine 540, Propidium Iodide, PNA-Alexa Fluor 647, and Hoechst 33342, respectively. The sixth aliquot was stained with all the five fluorochromes simultaneously, correcting the spectral overlap through a compensation matrix. For all the sperm traits evaluated, strong correlations were found between the single and 5-color staining assays for boar sperm (0.99, 0.96, 0.93, 0.98, and 0.99 for A, B, C, D, and E; $P<0.01$). Furthermore, moderate and substantial Concordance Correlation Coefficients (CCC) were presented by all these parameters (0.99, 0.96, 0.92, 0.98, and 0.99, respectively). For stallion sperm, the correlation coefficients between the assays were also strong (A:0.99, B:0.98, C:0.99, D:0.99, E:0.95, $P<0.01$), and substantial CCC were observed for all of them (0.99, 0.97, 0.99, 0.99, and 0.90, respectively). For both species, the mean difference between the methods ($\bar{d}$) did not overtake 0.84. The results confirmed this 5-color panel can be

successfully implemented for analyzing boar and stallion sperm quality in a single, practical and quick FC assay.

Keywords: Boar semen; Compensation; Flow cytometry; Multiparametric assay; Stallion semen; Sperm quality.

INTRODUCTION

Flow cytometry (FC) technology, in addition to the computer assisted semen analysis system (CASA), has become the gold standard in specialized andrology laboratories because of its objectivity, sensitivity, and high accuracy. It has been used in human and veterinary andrology since the 1970s, allowing the assessment of different structures and functions of a great number of spermatozoa (>10,000) in a short time (Niżański et al., 2016).

This technology currently evaluates several sperm traits, including sperm plasma membrane measures, acrosome integrity, mitochondrial membrane potential, reactive oxygen species (ROS) and DNA damage (Boe-Hansen and Satake, 2019). Some of these parameters – such as membrane integrity and mitochondrial activity – were already regarded as putative predictors of sperm fertility in boars and stallions (Wilhelm et al., 1996; Schulze et al., 2013a; Battut et al., 2017; Schulze et al., 2021).

The availability of advanced FC instruments (multiple lasers, a broad range of detectors) as well as the variety of new fluorescent probes and tools for identifying equipment requirements and assessing spectral overlap, have opened new opportunities for developing and conducting multiparametric assays in the last years (Boe-Hansen and Satake, 2019). In fact, the simultaneous evaluation of multiple structures of a cell is one of the greatest advantages of FC. This approach can counteract one of the major historical drawbacks in sperm evaluation: the problem of not being able to test multiple attributes concurrently at a single cell level (Peña, 2015). In this sense, a simultaneous, multiparametric assessment of several sperm characteristics can more accurately estimate the sperm fertilizing potential (Torres et al., 2016).

Most of the FC assays, however, only focus on one or two parameters, which is a limitation for identifying the ideal sperm cells with all the required attributes for success in oocyte fertilization and embryo development (Boe-Hansen and Satake, 2019). Furthermore,

the use of non-multiparametric FC assays does not allow discovering possible associations between sperm attributes, which would be essential for better understanding the sperm function as well as for designing future multiparametric protocols. Moreover, it is a time and material-consuming practice.

In the light of all the aforementioned, this study aimed to develop a quick and practical multiparametric assay for simultaneously evaluating four different physiological characteristics, essential for superior quality of boar and stallion sperm, through a 5-color FC panel including the use of DNA-markers for excluding debris.

MATERIAL AND METHODS

Chemicals

All chemicals used in this study were of analytical grade unless otherwise stated. Rhodamine 123 (R123) and Merocyanine 540 (M540) were purchased from Sigma-Aldrich (Steinheim, Germany), whereas Propidium iodide (PI), *Arachis hypogaea* (peanut) agglutinin-Alexa Fluor 647 (PNA-647) and Hoechst 33342 (H342) were obtained from Life Technologies – Thermo Fisher Scientific (Eugene, USA). Semen extenders were acquired from Minitüb (Tiefenbach, Germany).

Semen processing

Semen collection was carried out in accordance with guidelines and regulations following the European Commission Directive for Animal Welfare. In addition, procedures were approved by the animal welfare committee of the Institute for Reproduction of Farm Animals Schönow (IFN-2021-V-08).

For developing the multicolor assay for boar semen, thirty semen doses, with ~ 30 × 10^6 sperm/mL in ~85 mL of BTS extender (Beltsville Thawing Solution, Minitüb) were used in this trial. Doses were produced from healthy and mature Pietrain boars in a single artificial insemination center (AIC) in Germany (52º58'31.224"N, 10º40'10.38"E), and stored at 17°C. The FC analyses were performed after 48 h of storage. Furthermore, twelve semen doses from healthy and mature stallions (Brandenburger warmblood), housed in a German AIC (52º50’58.41"N, 12º25’11.214"E), were used. The doses contained ~50 × 10^6 sperm/mL in ~20 mL of EquiPlus extender (Minitüb), and were stored at 5°C. The FC

analyses occurred after 72 hours of storage.

For both species, additional information regarding ejaculates and semen doses, such as ejaculate volume, sperm concentration, sperm morphology and total motility, was recorded (**Table 1**). The volume of ejaculate and semen doses were obtained weighting the samples (1 g = 1 mL), whereas sperm concentration in semen doses was assessed using a NucleoCounter SP-100^{TM}, as specified by Revision 1.5 of the User Guide Manual No. 991-0100 (ChemoMetec A/S, Lillerød, Denmark). For the analysis of sperm morphology, spermatozoa were fixed by 1% formalin in phosphate-buffered saline (PBS) at a concentration of 50 – 100 × 10^6 sperm/mL. Per sample, 200 sperm cells were evaluated using phase-contrast microscopy (800 × magnification, Jenaval, Carl Zeiss Jena, Germany).

Sperm motility was determined with CASA system (AndroVision®, Minitüb). Preheated Leja chambers (Leja Products B.V., Nieuw Vennep, The Netherlands) were filled with 3 µL of a sample taken from each semen dose (1 mL), which was previously incubated for 10 min at 38°C; four fields were recorded at a rate of 30 frames per 0.5 s and under 100 × magnification. Following the manufactures' defaults setting, boar spermatozoa were considered as motile (total motility) when the velocity curved line (VCL) was ≥24 µm/s and the amplitude of lateral head displacement (ALH) was ≥1.0 µm. Progressive motility was defined when boar sperm cell exhibited VCL ≥24 µm/s and velocity straight line (VSL) ≥48 µm/s. Still following the manufacture's recommendation, stallion spermatozoa were classified as motile when the ALH and the beat cross frequency (BCF) were ≥4 µm and ≥4 Hz, respectively. Progressive motility was defined when these cells exhibited VCL ≥40 µm/s and VSL ≥10 µm/s.

Staining of samples

From each boar semen dose, six 250 µL-aliquots (A-F) were taken (final concentration ~ 30 × 10^6 sperm/mL). Aliquot A was stained with R123 (final concentration: 0.2 µg/mL) for evaluating the mitochondrial activity, whereas aliquot B was stained with M540 (4.0 µM) for assessing the degree of lipid disorder of plasma membrane. Aliquot C was stained with PI (10.0 µg/mL) and aliquot D with PNA-647 (9.0 µM) for evaluating plasma membrane integrity and acrosomal status, respectively. Aliquot E was stained with H342 (3.2 µM), for marking DNA and selecting sperm cells events, and, finally, aliquot F

was stained with all five fluorochromes simultaneously (keeping the final concentration of each fluorochrome achieved in the single stained samples). The same procedure was followed for stallion semen doses, but using 100 µL-aliquots (final concentration ~ 50 × 10^6 sperm/mL).

The concentrations used for R123, M540, and PI were based on protocols already applied in our laboratory (Schulze et al., 2013a), whereas for PNA-647 and H342 the concentrations were based on Jäkel et al. (2021) and Torres et al. (2016), respectively. Stock solutions of R123, PI, PNA-67 and H342 were prepared with double distilled water, whereas M540 stock solutions were elaborated using PBS.

Analysis of samples

After 10 minutes of incubation at 37°C, samples were briefly vortexed, and a volume of 20 µL (for boar semen) or 10 µL (for stallion semen) was diluted in 1 mL of isotherm phosphate-buffered NaCl solution, achieving a final concentration of approximately 0.6 (boar) and 0.5 (stallion) million cells/mL. Then, the analysis was performed using the CytoFLEX S device (Beckman Coulter, Indianapolis, USA) equipped with four lasers: violet: 405 nm (80 mW), blue: 488 nm (50 mW), yellow-green: 561 nm (30 mW) and red: 638 nm (50 mW). Sample were analyzed in simplicate and, for each one, 10,000 events into the sperm gate were obtained, referring to the expected Forward- and Side-scatter signals (FSC and SSC) plotted on logarithmic scales. The sperm gate was previously defined on the basis of H342 staining of a pool from semen doses and agglutinations were excluded by FSC gating (**Figure 1a-b** and **Figure 1h-i**).

Fluorochromes were excited by four different lasers: blue (R123), yellow (M540 and PI), red (PNA-647), and violet (H342). All the signals were plotted on logarithmic scales and fluorescence signals of R123, M540, and PI were detected by 525/40 nm, 585/42 nm, 610/20 nm band-pass filters, respectively. Fluorochromes PNA-647 and H342 had their fluorescence signals detected by 660/10 and 450/10 nm band-pass filters, respectively. All data were recorded with CytExpert software (version 2.3.) and the instrument was calibrated previously to each routine to verify the cytometer's optical alignments and fluidic system. For this, specific calibration beads provided by the manufacturer were used. Events positive for R123 were considered sperm cells showing

mitochondrial activity, whereas events exhibiting low fluorescence for M540 were classified as cells with low degree of lipid disorder of membrane. Events positive for PI were registered as sperm cells with non-intact plasma membrane. Similarly, events positive for PNA-647 were considered as cells presenting damaged/reacted acrosome. The events positive for H342 were determined as events with DNA content.

The gates for the fluorochromes (**Figure 1c-g** and **Figure 1j-n**) were stablished previously to the assays. A pool from semen doses was obtained and used for preparing unstained, positive and negative controls for all fluorochromes, except for H342. With respect to fluorochromes M540, PI and PNA-647, the negative control was the original pool stained with each fluorochrome individually, while the positive control was the pool submitted to three rounds of flash-freezing process in liquid nitrogen, followed by rapid thawing at 37°C and subsequent staining. For R123, the flash-frozen sample was used as a negative control and the original pool as a positive one. The strategy for the H342 gate was based on an unstained sample.

Statistical analysis

Using the SPSS software version 28.0 (IBM, Armonk, NY, USA), the parameters were tested for normal distribution, by the Shapiro-Wilk test, and homoscedasticity, by the Levene's test. Then, the Pearson correlation coefficient (r_p) between the parameters obtained by the single and the 5-color staining methods was calculated. To further evaluate the agreement between the two methods, the differences between paired measurements ($\bar{d}$) were plotted against the means of paired measurements, according to the Bland and Altman approach (Bland and Altman, 1999). The $\bar{d}$ and the expected value for this difference (zero) were compared using the one-sample *t*-test (differences normally distributed) or Wilcoxon test (differences non normally distributed) after testing for normal distribution through the Shapiro-Wilk test. In addition, the Concordance Correlation Coefficient (CCC) was calculated as established by Lin (1989) and classified into poor (<0.90), moderate (0.90 to 0.95), substantial (0.95 to 0.99) or almost perfect (>0.99), according to Akoglu et al. (2018). Statistical significance was set at $P \leq 0.05$.

RESULTS

The percentage of events positive for the five parameters evaluated by FC, using the single or the multicolor assay, are presented in the **Table 1**, for both species (mean ± standard deviation).

Comparison of single and 5-color panel flow cytometric assays – rp

Strong correlations (r_p; P<0.01) were found between the sperm parameters evaluated by the single and the 5-color staining assays for boar and stallion sperm. For active mitochondria (R123 positive), the r_p was 0.99 to both species (**Figures 2a** and **3a**). For low degree of lipid disorder (low M540 fluorescence) r_p of 0.96 and 0.98, respectively, were observed (**Figures 2b** and **3b**); whereas for non-intact plasma membrane (PI negative), the r_p found were 0.93 and 0.99, in this order (**Figures 2c** and **3c**). Regarding the events with damaged/reacted acrosome (PNA-647 negative), r_p = 0.98 for boar and 0.99 for stallion were registered (**Figures 2d** and **3d**). The r_p observed for the DNA-marker (H342 positive) were 0.99 and 0.95, respectively. (**Figures 2e** and **3e**).

Comparison of single and 5-color panel flow cytometric assays – CCC and $\bar{d}$

Regarding the CCC between the methods for analyzing boar semen, values considered as substantial were observed for active mitochondria, low degree of lipid disorder, damaged/reacted acrosome and DNA-marker (0.99, 0.96, 0.98, and 0.99; **Figures 4a, b, d, and e,** respectively). For non-intact plasma membrane, the agreement was moderate (0.92, **Figure 4c**). The $\bar{d}$ value for boar semen was significantly different from zero for non-intact plasma membrane (PI positive) and damaged/reacted acrosome (PNA-647 positive): $\bar{d}$ = -0.74 and -0.54, respectively (P<0.01 and = 0.02, in this order, **Table 3**).

Considering the stallion semen, the CCC between the methods was observed to be substantial for the following parameters: active mitochondria, low degree of lipid disorder, non-intact plasma membrane and damaged/reacted acrosome (0.99, 0.97 0.99, 0.99; **Figures 5a, b, c,** and **d,** respectively). For the DNA-marker the CCC was 0.90 (**Figure 5e**). The $\bar{d}$ for damaged/reacted acrosome in stallion semen (-0.69) was statistically different from zero (P=0.04; **Table 3**).

DISCUSSION

In this study, we developed a quick and practical 5-color multiparametric panel for simultaneously evaluating four sperm parameters of extended boar and stallion semen routinely used in a spermatological reference laboratory. We compared the values obtained from single or multicolor stained samples, after compensating the spectral overlap for the multicolor assay. The high correlation and concordance coefficients found between the methods showed that it is feasible to simultaneously evaluate mitochondrial status (R123), degree of lipid disorder of the plasma membrane (M540), plasma membrane integrity (PI), acrosomal status (PNA-647), also excluding cell debris (H342) in a single sperm sample.

To further explore the agreement between the methods, we statistically studied the $\bar{d}$ between the single and the multicolor assay patterns. Although the traits evaluated by the fluorochromes PI (for boar semen) and PNA-647 (for boar and stallion semen) showed a $\bar{d}$ statistically different from zero, it is important to consider that the biological interpretation of this discrepancy and the mean difference for these parameters did not overtake 0.74 (PI). Moreover, it is widely known that any variable measurement always implies some degree of error (Giavarina, 2015).

Few other studies reported the development of multiparametric FC assays. Peña (2015) set a 4-color panel to evaluate three parameters: plasma membrane permeability, membrane integrity and mitochondrial potential or plasma membrane permeability, membrane integrity and oxidative stress. The author also applied panels for concurrent analysis of apoptotic-like changes, plasma membrane integrity and mitochondrial potential or oxidative stress, always using a DNA-maker. In one study, Torres et al. (2016) introduced a 4-color panel for boar sperm evaluation, simultaneously assessing the status of three structures: plasma, acrosome, and mitochondrial membranes while using a DNA-marker to exclude cell debris. Jäkel et al. (2021) tested two 4-color panels for evaluating three boar sperm parameters simultaneously: plasma membrane permeability, fluidity and acrosomal status or viability, calcium influx, and mitochondrial membrane potential, also using a DNA-marker in both panels. More recently, a 4-panel was validated to assess plasma membrane integrity, acrosomal status and mitochondrial activity applying the protocol to cooled and frozen boar sperm (Gonzalez-Castro et al., 2022).

A 5-color panel for stallion semen was developed in Peña's laboratory,

simultaneously determining five structures/functions: cell viability, caspase 3 activity, plasma membrane polarization, mitochondrial membrane potential and sperm maturity (Ortega Ferrusola et al., 2017). Although our 5-color panel provides information about only four traits, compared to Ortega Ferrusola et al. (2017), these traits can still offer valuable information regarding sperm quality. Our test allows evaluating the status of structures related to fundamental attributes to the fertilization route, such as sperm motility (mitochondrial activity) and function (plasma membrane lipid ordering and acrosomal status).

In recent years, FC systems with two or three lasers have become more affordable for laboratories. The fact that multicolor analytical approaches are still quite limited in veterinary andrology, compared to cell biology and medicine fields, seems to be mainly related to avoiding compensation due to spectral overlap (Petrunkina and Harrison, 2013). Correcting overlap is one of the most important steps when developing multicolor assays. In our case, the overlap between the M540 and PI emissions represented the biggest challenge for developing this panel. Both are excited by the same laser (yellow = 561 nm), emitting fluorescence signals detected by the band-pass filters 585/42 nm and 610/20 nm, respectively. Our results, however, support that the compensation matrix was appropriately defined and applied as the relationship between the staining presented strong correlations and almost perfect concordance for all fluorochromes, including M540 and PI.

As far as we know, this is the first panel associating M540 and PI, which could be related to the apprehension of the users on applying higher compensation values. According to Maciorowski et al. (2017), however, this should not be a concern, because using compensation mathematical formulae corrects fluorescence spillover equally for all values, no matter if it is 2% or 200%. In any case, the maximum compensation values applied in our study were 45.7% for boar semen and 51.9% for stallion semen. A real concern about developing multicolor assays should be to carry out previous tests associating all the fluorochromes chosen for the panel, mainly when it is designed with fluorochromes which have never been associated in the laboratory routine analysis before. This step is important for assessing any possible cytotoxicity occurrence, which could influence the sperm viability. In addition, this approach will also allow detecting any influence on fluorescence intensity caused by molecular interactions among the fluorochromes: the quenching

phenomenon (Song et al., 1997; Schmid et al., 2000; Vives-Rego et al., 2000). These previous tests were performed for this study, and none of these phenomena was observed.

Another challenge in developing multicolor assays is designing a suitable fluorochrome panel to investigate different important sperm attributes according to the characteristics of the particular equipment. For this panel, we chose PNA-647 for acrosomal analysis in replacement to FITC-PNA (fluorescein isothiocyanate-conjugated peanut agglutinin). FITC-PNA is one of the most used fluorochromes for acrosome assessment (Boe-Hansen and Satake, 2019), and it is routinely utilized in our laboratory, in association with PI, to assess membrane integrity (Schulze et al., 2013b). However, FITC-PNA is excited and detected by the same laser (blue: 488 nm), and channel (FITC-channel: 525/40 nm) as R123; whereas PNA-647 is excited by the red laser (638 nm) with emissions being detected by the APC-channel (660/10 nm). Other studies previously replaced FITC-PNA by PNA-647 for evaluation of boar (Jäkel et al., 2021) and stallion semen (Silva et al., 2017). It, therefore, was important for this panel to free the FITC-channel for detecting the R123 signal when determining mitochondrial and acrosomal status.

In our study, the analysis of plasma membrane integrity, lipid ordering, acrosomal status and mitochondrial activity were performed through three different assays (panels). With the multiparametric panel proposed in this study, the time, labor and material are reduced by two-fold. Moreover, this panels would act as a basis for designing future multiparametric assays. Based on the general concept that acrosomes should be intact in sperm populations presenting fertilizing ability and that acrosomal labelling cannot occur in intact plasma membrane cells, Petrunkina and Harrison (2013) highlighted that plasma membrane integrity analysis would be sufficient to identify a non-functional cell. Analyzing these two parameters would, therefore, be considered a redundant procedure. In this sense, the correlation between several parameters of a multicolor panel could allow us to critically analyze whether a fluorochrome would bring additional and useful information for the investigation, or it could rather be replaced by a more suitable one.

CONCLUSIONS

Our results confirm that this 5-color panel could be successfully implemented to analyze boar and stallion sperm simultaneously for mitochondrial activity, lipid ordering of

plasma membrane as well as plasma membrane integrity and acrosomal status. This single, practical, and quick FC assay can be used to optimize sperm analysis and served as a tool to assist prediction of male fertility. Furthermore, it can act as a feedback tool for designing new multiparametric assays in the future.

DECLARATION OF COMPETING INTERESTS

The authors declare that there is no conflict of interest.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

MQ: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **UJ:** Supervision, Review & editing **APGM:** Supervision, Review & editing **FPB:** Supervision, Review & editing, Funding acquisition, Project administration. **MJ:** Supervision, Review & editing. **MS:** Supervision, Review & editing, Funding acquisition, Project administration.

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Table 1. Descriptive statistics (means ± standard deviation) for boar and stallion semen quality, and flow cytometry analysis.

Sperm quality characteristics	**Boar**		**Stallion**	
Number of animals, *n*	30		12	
Age of animals, year	1.4 ± 0.6		8.9 ± 4.4	
Ejaculate volume, mL	250 ± 63		31.3 ± 14.7	
Sperm concentration, 10^9/mL	0.324 ± 0.128		0.363 ± 0.087	
Dose volume, mL	~ 85		~ 20	
Sperm morphology, %	69.2 ± 17.0		80.5 ± 8.9	
Total sperm motility, %	72.7 ± 9.8		72.5 ± 6.8	
Flow cytometry analysis	**Single**	**Multicolor**	**Single**	**Multicolor**
Mitochondrial activity (R123+, %)	78.5 ± 5.0	78.4 ± 4.9	55.0 ± 11.9	54.8 ± 11.7
Low degree of lipid disorder (Low M540, %)	59.7 ± 12.3	60.6 ± 12.3	67.2 ± 11.4	68.0 ± 11.0
Non-intact plasma membrane (PI+, %)	13.1 ± 4.0	13.9 ± 4.0	43.3 ± 11.0	43.1 ± 11.0
Damaged/reacted (PNA-647+, %)	16.2 ± 6.3	16.7 ± 6.4	33.3 ± 8.4	34.0 ± 8.5
DNA content (H342+, %)	91.1 ± 4.3	91.0 ± 4.2	98.4 ± 1.1	98.6 ± 0.9

Single: single staining assay, Multicolor: 5-color staining assay.

R123: Rhodamine 123, M540: Merocyanine 540, PI: Propidium Iodide, PNA-647: PNA-Alexa Fluor 647, H342: Hoechst 33342.

Table 2. Compensation matrix applied for boar and stallion semen analysis using the 5-color flow cytometry panel.

Species	Fluorochrome	R123	M540	PI	PNA-647	H342
Boar	R123	.	0.00	**0.16**	**0.02**	0.00
	M540	0.00	.	**30.55**	**1.85**	0.00
	PI	0.00	**45.68**	.	**0.37**	0.00
	PNA-647	0.00	**0.14**	**0.23**	.	0.00
	H342	0.00	**0.02**	**0.25**	**0.04**	.
Stallion	R123	.	**0.04**	**0.05**	0.00	0.00
	M540	**0.06**	.	**51.92**	**0.50**	0.00
	PI	**0.05**	**44.87**	.	**1.00**	0.00
	PNA-647	0.00	**0.13**	**0.13**	.	0.00
	H342	0.00	0.00	0.00	**0.04**	.

R123: Rhodamine 123; M540: Merocyanine 540; PI: Propidium Iodide; PNA-647: PNA-Alexa Fluor 647; H342: Hoechst 33342. Corresponding channels: FITC detecting R123 (525/40 nm), PE detecting M540 (585/42 nm), ECD detecting PI (610/20 nm), APC detecting PNA- 647 (660/10 nm) and PB540nm detecting H342 (450/10 nm).

Table 3. Bland and Altman plot statistics (estimated mean difference: $\bar{d}$, lower and the upper limits of agreement) for comparing the percentage of positive sperm cells for each fluorochrome determined by two flow cytometry methods: single and multicolor staining assay for the evaluation of boar and stallion semen.

Semen	**Estimate**				
	R123+	**Low M540**	**PI+**	**PNA-647+**	**H342+**
Boar (*n* = 30)					
Mean difference ($\bar{d}$)	0.11	-0.84	-0.74	-0.54	-0.10
P-value*	0.55	0.17	< 0.01	0.02	0.41
Lower limit of agreement	-1.40	-7.42	-3.60	-2.91	-1.17
Upper limit of agreement	1.63	5.73	2.10	1.82	1.36
Stallion (*n* = 12)					
Mean difference ($\bar{d}$)	0.19	-0.79	0.29	-0.69	-0.19
P-value*	0.74	0.22	0.41	0.04	0.14
Lower limit of agreement	-3.67	-4.92	-2.02	-2.70	-1.00
Upper limit of agreement	4.05	3.34	2.60	1.39	0.63

R123+: mitochondrial activity (Rhodamine 123), Low M540: low degree of lipid disorder (Merocyanine 540), PI+: non-intact plasma membrane (Propidium Iodide), PNA-647+: damaged/reacted acrosome (PNA-Alexa Fluor 647), H342+: DNA-marker positive (Hoechst 33342). *For retain or reject the hypothesis that the mean difference is equal to 0.

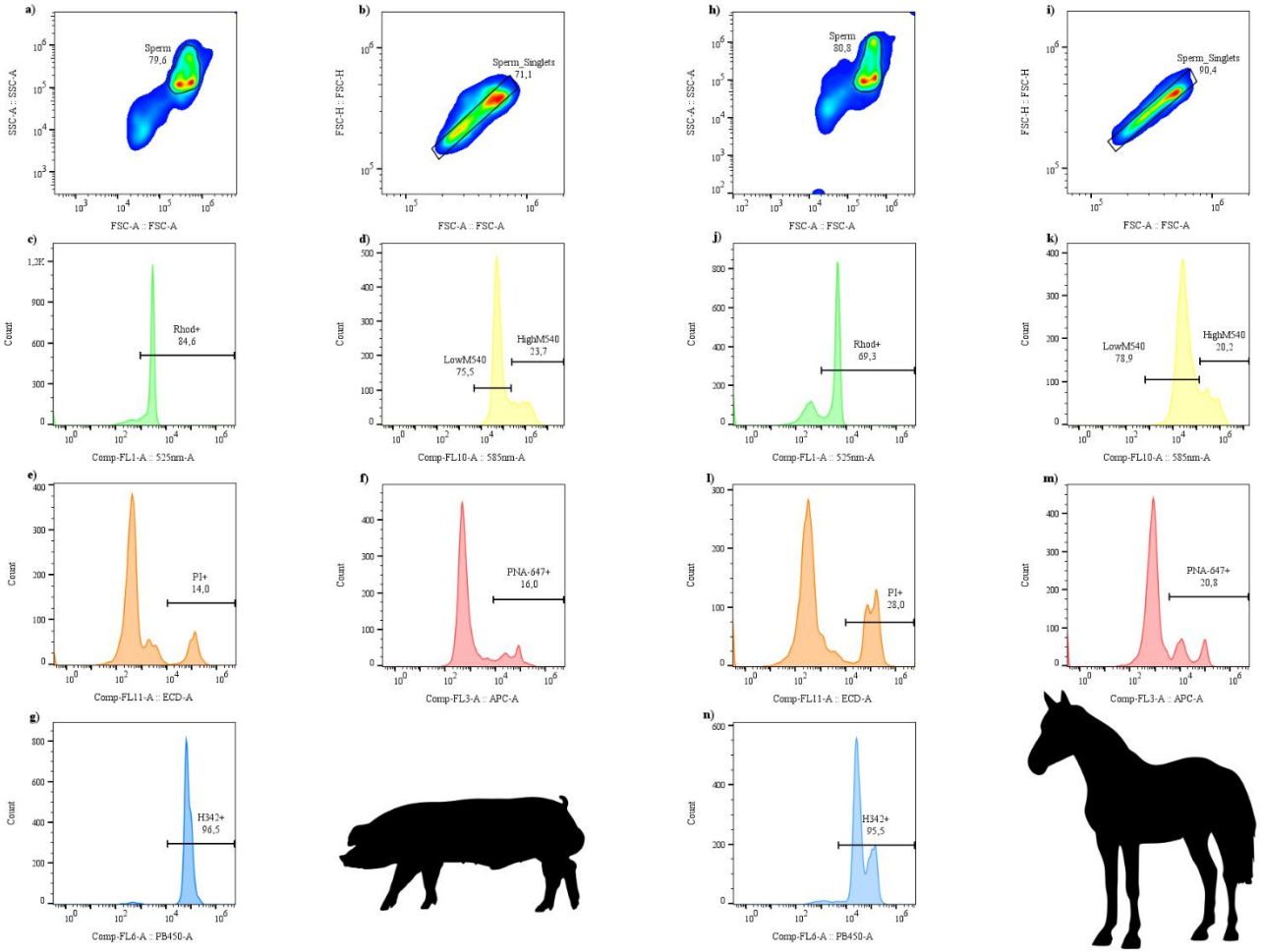

Fig. 1. Representative plots and gating strategy of multicolor protocol (5 color-panel) for boar (**a-g**) and stallion semen (**h-n**) evaluation. For both species, the sperm gate was set in the pseudocolor plot (**a** and **h**), referring to the expected Forward- and Side-Scatter signals (FSC

and SSC), and based in a previous H342 staining using a pool of semen doses. The sperm population was plotted in a new pseudocolor plot, and agglutinations were excluded by FSC gating (Sperm Singlets; **b** and **i**). Based on positive and negative controls, gates of fluorochromes were stablished to evaluate the percentage of events exhibiting mitochondrial activity (R123+; **c** and **j**), low degree of lipid disorder (LowM540; **d** and **k**); non-intact plasma membrane (PI+; **e** and **l**), damaged/reacted acrosome (PNA- 647+; **f** and **m**) and positive staining for the DNA-marker (H342+; **g** and **n**). R123: Rhodamine 123, M540: Merocyanine 540, PI: Propidium Iodide, PNA-647: PNA-Alexa Fluor 647, H342: Hoechst 33342.

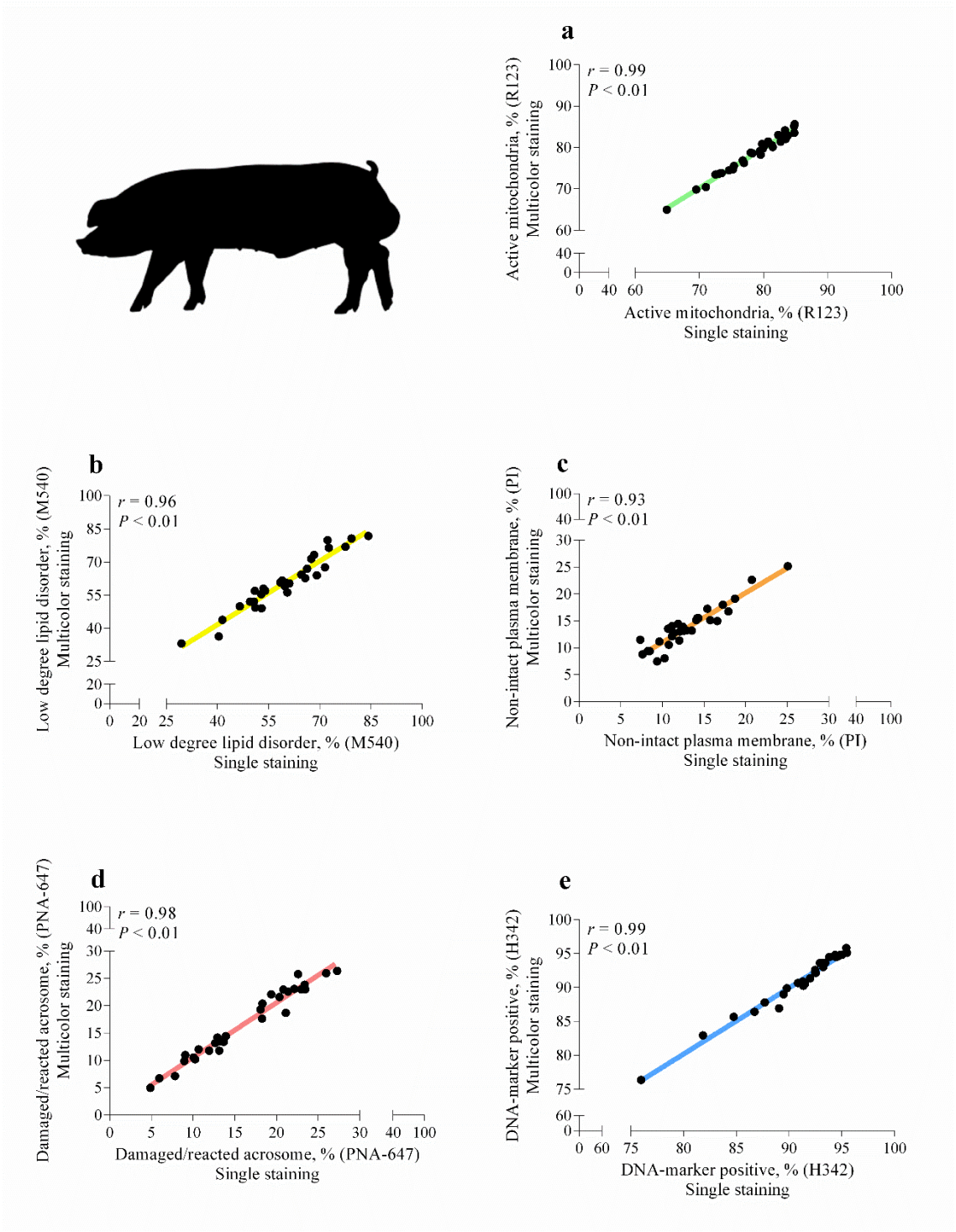

Fig. 2. Scatter plots showing the correlation between the parameters obtained by the single and multicolor flow cytometry staining for boar semen evaluation. R123: Rhodamine 123 (A), M540: Merocyanine 540 (B), PI: Propidium Iodide (C), PNA-647: PNA-Alexa Fluor 647 (D), H342: Hoechst 33342 (E).

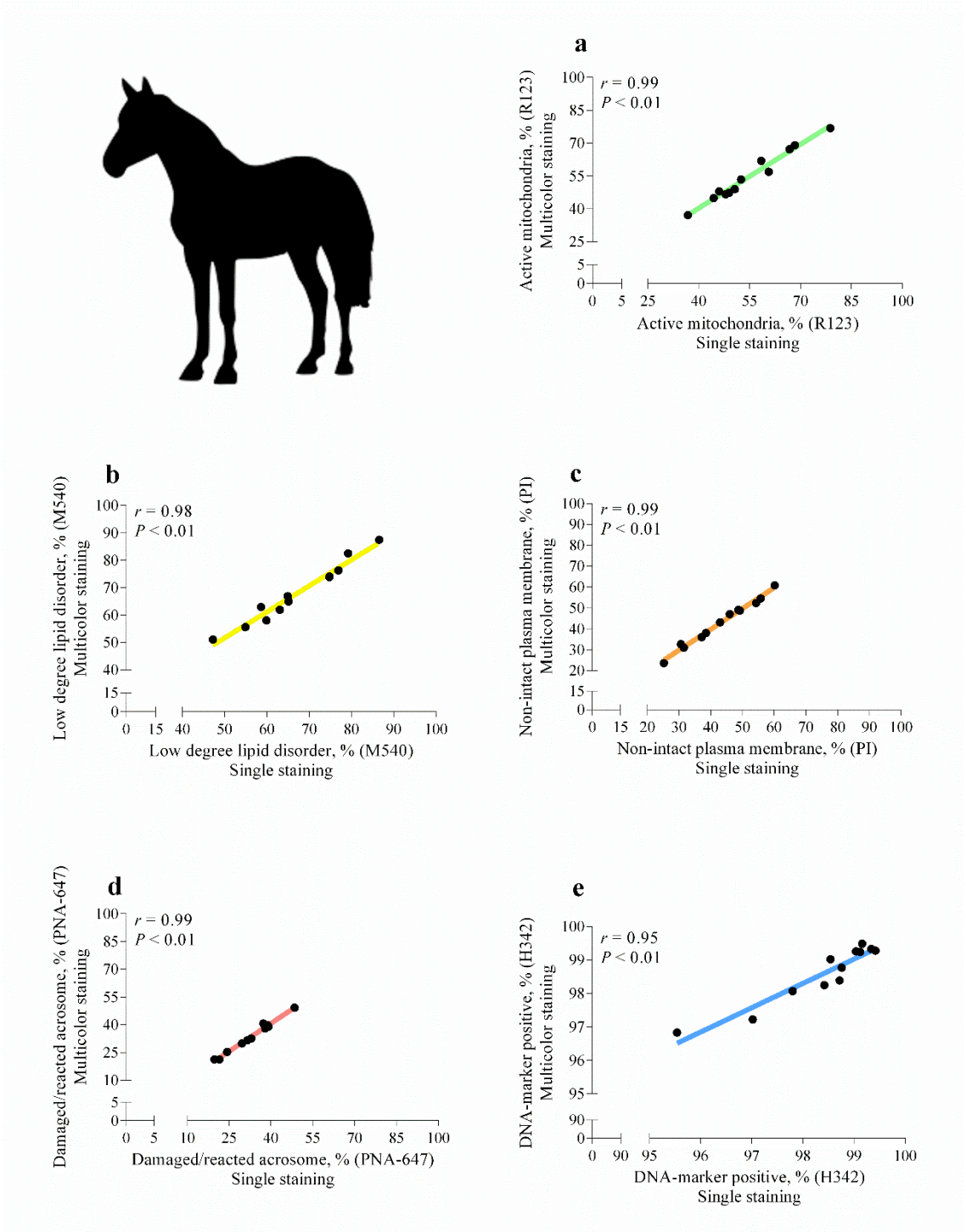

Fig. 3. Scatter plots showing the correlation between the parameters obtained by the single and multicolor flow cytometry staining for stallion semen evaluation. R123: Rhodamine 123 (A), M540: Merocyanine 540 (B), PI: Propidium Iodide (C), PNA-647: PNA-Alexa Fluor 647 (D), H342: Hoechst 33342 (E).

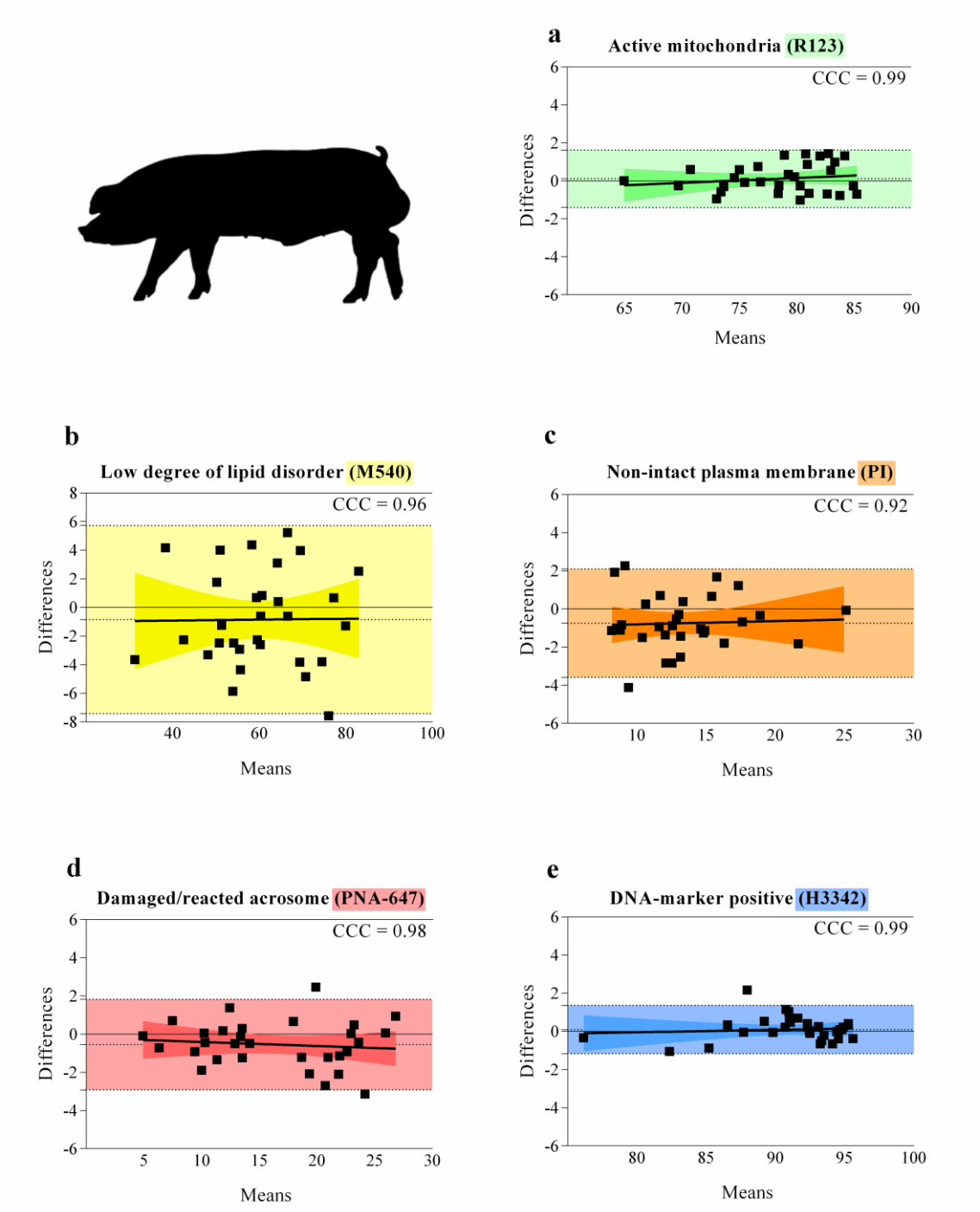

Fig. 4. Bland and Altman plot for the percentage of positive cells for each fluorochrome assessed by the single and multiple stained flow cytometry assay for boar semen evaluation. The differences between the both methods are plotted against their means. From top to bottom, the horizontal dot-dashed lines represent the estimated upper limit of agreement, the mean difference between the two methods and the lower limit of agreement. The lower and upper limits of agreement were calculated as the mean differences minus or plus 1.96 standard-deviation, respectively, considering a

confidence interval of 95% (lighter shaded area). The regression line (thicker line) describes the relation between the differences and the means with 95% CI (darker shaded area). CCC: Concordance Correlation Coefficient. R123: Rhodamine 123 (A), M540: Merocyanine 540 (B), PI: Propidium Iodide (C), PNA-647: PNA-Alexa Fluor 647 (D), H342: Hoechst 33342 (E).

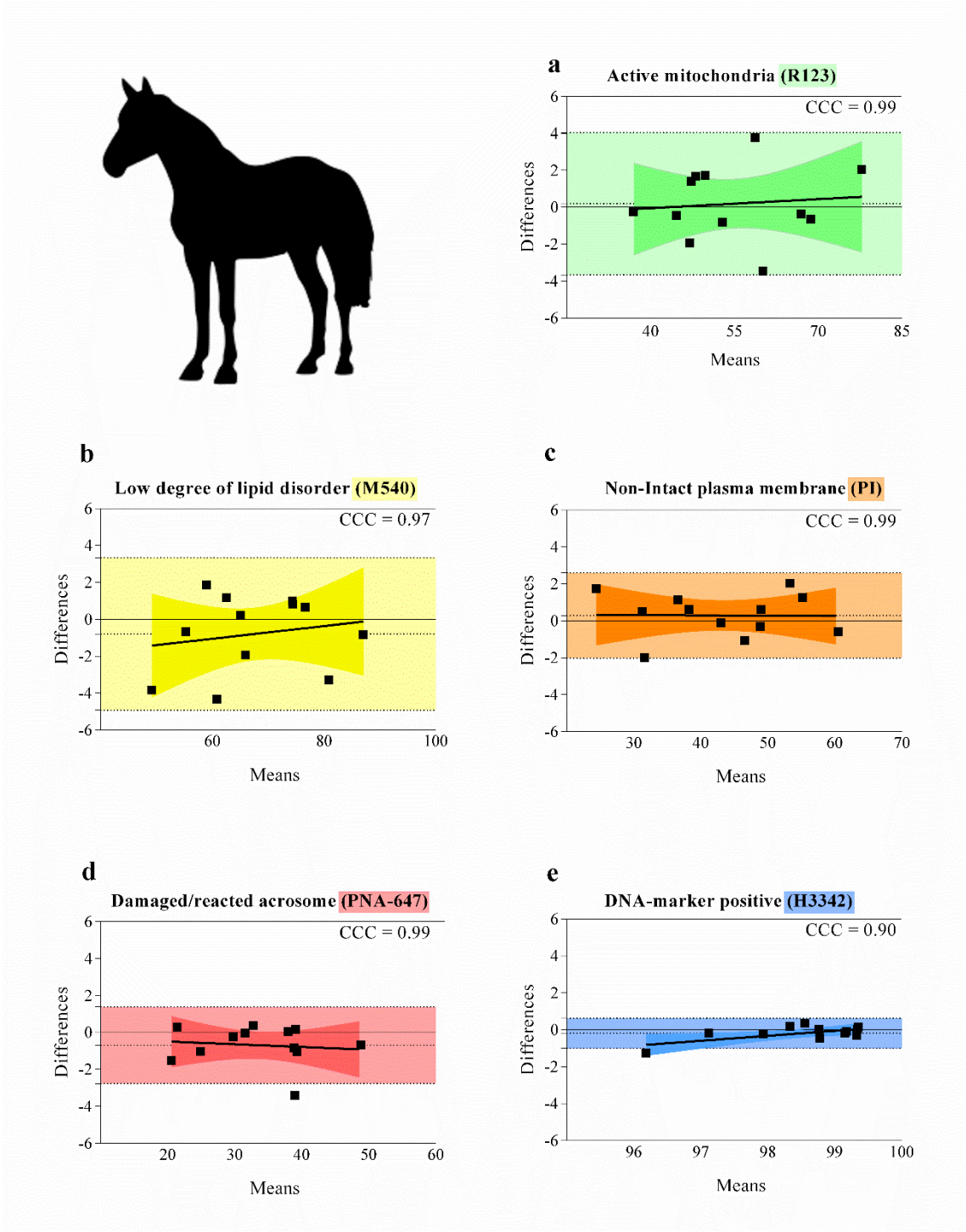

Fig. 5. Bland and Altman plot for the percentage of positive cells for each fluorochrome assessed by the single and multiple stained flow cytometry assay for stallion semen evaluation. The differences between the both methods are plotted against their means. From top to bottom, the horizontal dot-dashed lines represent the estimated upper limit of agreement, the mean difference between the two methods and the lower limit of agreement. The lower and upper limits of agreement were calculated as the mean differences minus or plus 1.96 standard-deviation, respectively, considering a

confidence interval of 95% (lighter shaded area). The regression line (thicker line) describes the relation between the differences and the means with 95% CI (darker shaded area). CCC: Concordance Correlation Coefficient. R123: Rhodamine 123 (A), M540: Merocyanine 540 (B), PI: Propidium Iodide (C), PNA-647: PNA-Alexa Fluor 647 (D), H342: Hoechst 33342 (E).

5. CONSIDERAÇÕES FINAIS

O controle de qualidade de doses de sêmen suíno é um processo complexo, envolvendo diversas etapas, pontos críticos e fatores que ainda precisam ser melhor identificados ou explorados, como o efeito da concentração espermática e a presença de células mortas em doses inseminantes suínas sobre a qualidade espermática. Em relação à concentração espermática das doses inseminantes suínas, os resultados obtidos neste projeto indicaram que doses altamente concentradas ($\geq 60 \times 10^6$ células/mL) prejudicam a qualidade espermática em termos de motilidade, obtendo-se motilidade inferior a 70% após 120 h de armazenamento a 17°C. Dessa forma, recomenda-se que as centrais de IA sejam cautelosas e considerem esses limites em situações nas quais é necessário aumentar o número de células na dose (sem ajustes em seu volume final), como quando se deseja compensar a baixa qualidade de alguns ejaculados. Os dados também destacam que o uso de diluentes de longa-ação não foi capaz de minimizar os efeitos causados pelo aumento da concentração espermática. Logo, ainda não há estratégias práticas e viáveis para contornar o efeito negativo que altas concentrações espermáticas exercem sobre a qualidade da dose.

Em contrapartida, a baixa concentração espermática nas doses não parece ser um fator de grande relevância para as centrais no que diz respeito à qualidade das doses. Aparentemente, baixas concentrações espermáticas ($\sim 16.7 – 20.0 \times 10^6$ células/mL) podem, inclusive, propiciar menor perda de motilidade ao longo do teste de termorresistência (TTR), indicando uma maior resiliência espermática *in vitro*. Esse resultado provavelmente está associado a menor disputa por nutrientes e melhor controle do meio e metabolismo espermático, contudo, mais estudos são necessários para compreender as reais razões para esse resultado, e também avaliar as consequências in vivo deste cenário.

Investigações adicionais também são necessárias para melhor entender os possíveis efeitos das células mortas presentes nas doses inseminantes suínas sobre a sua qualidade. Os dados preliminares obtidos nesse projeto mostraram que as células viáveis não tiveram sua motilidade ou estrutura afetada pelo contato com células mortas ao longo de um TTR. Contudo, ainda é necessário verificar como ocorreria essa relação entre células mortas e viáveis ao longo do armazenamento das doses. Portanto, recomenda-se que as centrais de IA não desconsiderem o possível efeito negativo das células mortas sobre a qualidade das doses, e também ponderem esse fator ao realizar a compensação espermática ou produzir doses com base no número de células viáveis.

A aplicação e o desenvolvimento de tecnologias, como a citometria de fluxo, e novos ensaios também são fundamentais para otimizar a aprimorar a avaliação da qualidade espermática em doses de sêmen suíno. Nesse sentido, os resultados desse projeto confirmaram que a tecnologia de citometria de fluxo tem muito a contribuir para a avaliação espermática quanti e qualitativa em programas de controle de qualidade de doses de sêmen suíno. Considerando a aplicação qualitativa dessa tecnologia, um ensaio simples, rápido e inédito foi desenvolvido com sucesso neste projeto, permitindo a análise prática e simultânea de quatro parâmetros espermáticos: atividade mitocondrial, organização lipídica da membrana plasmática, integridade de membrana plasmática e *status* acrossomal; com identificação das células a partir de um marcador celular. Esse painel multicolor de cinco cores pode contribuir significativamente para otimizar a análise espermática, promover novos *insights* sobre a fisiologia espermática, auxiliar na identificação de parâmetros relacionados à fertilidade e, ainda, fomentar o desenvolvimento de novos ensaios multiparamétricos para a citometria de fluxo aplicada à andrologia.

Em compensação, o uso da citometria de fluxo para avalições quantitativas de doses de sêmen, como da concentração espermática, ainda precisa ser melhor explorado. Os resultados obtidos com os protocolos alternativos abordados neste projeto não alcançaram a concordância esperada em relação aos métodos convencionais para mensuração da concentração espermática. Nesse sentido, diversos pontos ainda devem ser melhor controlados e ajustados para tornar a técnica uma prática na rotina de controle de qualidade, como configurações do equipamento e de aquisição da amostra, condições da amostra em termos de presença de partículas, custo da análise, etc.

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