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Evaluation of sperm and hormonal assessments in Wagyu, Nellore, and Angus bulls

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Research Article

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Summary

Wagyu bulls are known to have a highly exacerbated libido, as shown by the intense sexual interest of young calves. Therefore we believe that Wagyu male animals have specialized Sertoli and Leydig cells that are directly involved with the sexual precocity in this breed as mature bulls have a small scrotal circumference. This study aimed to evaluate whether there were differences in the hormone and sperm characteristics of Wagyu bulls compared with the same characteristics of subspecies *Bos indicus* and *Bos taurus* sires. Frozen–thawed semen from Wagyu, Nellore, and Angus sires were analyzed for sperm kinetics (computer-assisted sperm analysis), plasma membrane integrity, chromatin integrity, acrosome status, mitochondrial activity, lipid peroxidation and hormone [luteinizing hormone (LH) and testosterone] serum concentration. The results showed that Wagyu had lower total motility and an increased number of sperm with no motility when compared with Nellore and Angus bulls. Wagyu breed did not differ from those breeds when considering plasma and acrosome membranes integrity, mitochondrial potential, chromatin resistance, sperm lipid peroxidation or hormone (LH and testosterone) concentrations. We concluded that Wagyu sires had lower total motility when compared with Nellore and Angus bulls. Wagyu breed did not differ from these breeds when considering plasma and acrosome membranes integrity, mitochondrial potential, chromatin resistance, sperm lipid peroxidation, or hormone (LH and testosterone) concentrations.

Introduction

Brazil is the world's second-largest producer of beef with a herd of 218.2 million animals. Notably, Brazil has become the world's largest beef exporter, exporting 2.69 million tons of bovine carcass weight, a trade worth more than US$8.4 billion in 2021 (IBGE, 2017; De Nadai Fernandes *et al.*, 2020; Zu Ermgassen *et al.*, 2020; ABIEC, 2021). Roughly 80% of the Brazilian herd is composed of Zebu cattle (*Bos taurus indicus*), mainly the Nellore breed. The other 20% are European breeds (*Bos taurus taurus*), especially the Angus breed and more recently the Wagyu breed (Rodrigues *et al.*, 2017; Rezende-de-Souza *et al.*, 2021).

Consumers have become more careful with the quality of food in general, especially with the quality of meat and how it can directly influence health. In this sense, in Brazil, the high demand for special meats has directed enterprises, such as slaughterhouses, meat shops, or restaurants, to produce high-quality meat. The increased intramuscular or marbling fat content is correlated to juiciness, tenderness, and meat flavour. Wagyu adds value from the marbling process as Wagyu has a high propensity to accumulate intramuscular fat. Furthermore, the fatty acid composition of lipids in meat is also important for human health and, therefore, this subject has been extensively studied in recent years (Radunz *et al.*, 2009; Ladeira *et al.*, 2018; Connolly *et al.*, 2020; Facioli *et al.*, 2020).

Animal reproduction is fundamental for addressing the growing demand for access to animal proteins. Therefore, this leads farmers to be as efficient as possible in the breeding and management of livestock (Yánez-Ortiz *et al.*, 2022). To increase reproductive capacity, bulls must produce morphologically normal and large numbers of sperm (Teixeira *et al.*, 2019). In this context, a detailed breeding soundness evaluation (BSE) of the bull should be carried out to assess the sire's reproductive health (Silva *et al.*, 2011; Romanello *et al.*, 2018; Rodrigues *et al.*, 2020; Leite *et al.*, 2021).

Nonetheless, Wagyu bulls have a higher libido, higher levels of testosterone and sperm production when compared with other taurine and indicine breeds (Smith-Thomas, pers. commun.; Sosa *et al.*, 2002; Tatman *et al.*, 2022). The purpose of this study was to evaluate whether there were differences in the hormone and sperm characteristics of Wagyu bulls

compared with the same characteristics of subspecies *Bos indicus* and *Bos taurus* sires. Wagyu bulls are known to have a highly exacerbated libido, as shown by the intense sexual interest of young calves. Therefore we believe that Wagyu male animals have specialized Sertoli and Leydig cells that are directly involved with the sexual precocity in this breed as mature animals have a small scrotal circumference that can affect sperm production (Sosa *et al.*, 2002).

Materials and methods

Experimental procedures adopted by this study are in agreement with the Principles of Ethics in Animal Research adopted by the Commission of Bioethics, Federal University of ABC (UFABC, protocol # 8993100516). This study was partially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Experimental design

Before starting the experiment, we performed a power analysis using PROC POWER (SAS System for Windows 9.3) to determine the minimal number of experimental units for each group. We considered only the variable motility. A result above 0.8 indicated that five experimental units per group would be adequate for the experiment, ensuring a high probability of observing the effect of treatments. Therefore, the experimental design was performed with five animals per group; each breed was considered as an experimental group: Angus, Nellore, and Wagyu.

Semen batches from Nellore and Angus bulls were donated from a commercial artificial insemination centre in Brazil (Seleon Biotechnology, Itatinga, SP, Brazil). Semen batches from Wagyu bulls were donated from a breeding herd located in the state of Rio Grande do Sul, Brazil. Data regarding sperm analysis before cryopreservation were not available for this study. However, all samples from all three breeds were diluted to a concentration of 25 million sperm cells/straw for cryopreservation. The cryopreservation procedure was performed according to local standard protocols and both used egg yolk-based extenders.

All semen samples were submitted to evaluate sperm motility using computer-assisted sperm analysis (CASA), acrosome integrity (*Pisum sativum* agglutinin; FITC-PSA), plasma membrane integrity (propidium iodide; PI), mitochondrial membrane potential (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide; JC-1), and chromatin resistance using flow cytometry and oxidative stress by thiobarbituric acid reactive substances assay (TBARS). Blood samples were used to evaluate testosterone and luteinizing hormone (LH).

Semen preparation

Semen straws were thawed at 37°C for 30 s. To remove the extender and recover sperm cells, 250 µl of semen were centrifuged at room temperature for 6 min at 9000 *g* in FertTALP warmed at 38.5°C (Parrish *et al.*, 1988) with no capacitation inductors. Sperm cells were recovered and washed again in FertTALP medium for 3 min at 9000 *g*. The sperm samples were then evaluated for acrosome integrity, membrane integrity, mitochondrial membrane potential, sperm chromatin status, and lipid peroxidation.

Assessment of sperm kinetics

Sperm motility was evaluated using CASA (Hamilton-Thorne, HTR Ceros 12.3, USA) as described by Leite *et al.* (2022) elsewhere. Briefly, 7 µl of each sample were placed between a Leja counting chamber (Orange Medical, Brussels, Belgium) previously heated to 37°C. A minimum of five randomly selected microscopic fields per sample was analyzed. The following variables were considered: total motility (MOT; %), progressive motility (PROG; %), average path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH; µm), beat cross-frequency (BCF; Hz), straightness (STR, VSL/VAP; %), and linearity (LIN; %). Sperm cells were also classified based on velocity: (1) rapid (RAP, VAP > 50 µm/s, %); (2) medium (MED, 30 µm/s < VAP < 50 µm/s, %); slow (SLOW, VAP < 30 µm/s or VSL < 15 µm/s, %); or (4) static (STATIC, %). The setup for sperm kinetic analysis was 60 Hz frames/s for image acquisition, with a minimum of 80 cells per field, minimum cell size of 5 pixels, 70 for cell intensity, path velocity of 50 µm/s and STR of 70% for progressive cells; and VAP and VSL cutoff at 30 and 15 µm/s for slow cells.

Acrosome integrity, membrane integrity, mitochondrial membrane potential, and sperm chromatin status evaluation

Acrosome integrity, membrane integrity, mitochondrial membrane potential, and sperm chromatin status were evaluated as described by Siqueira *et al.* (2018). Evaluations were performed using flow cytometry (Guava EasyCyte™, Guava® Technologies, Hayward, CA, USA) equipped with a 20 mW 488 nm argon excitation laser. Probes were purchased from Sigma and Molecular Probes.

Cytometry data were analyzed using the FlowJo software (version 10.2 Flow Cytometry Analysis Software–Tree Star Inc., Ashland, Oregon, USA). In total, 20,000 events per sample were analyzed. Cells were identified and selected excluding debris, probes particles and non-single sperm events, applying a gate on forward scatter (FSC) versus green fluorescence dot plot (log mode) around the single sperm events to acrosome, membrane, and mitochondrial analysis. For chromatin analysis, the single sperm events were selected by applying a gate on forward scatter (FSC) versus red fluorescence dot plot (log mode).

Simultaneously, a staining protocol was performed by incubating 187,500 cells for 5 min with 6 µM of propidium iodide (PI) associated with 5 µg of fluorescein-conjugated *Pisum sativum* (FITC-PSA). PI emits red fluorescence for damaged plasma membrane, while FITC-PSA emits green fluorescence for damaged acrosome. Mitochondrial membrane potential was analyzed by incubating 187,500 cells for 5 min with 1 µM of tetraethylbenzimidazolycarbocyanine iodide (JC-1) that emits green fluorescence for low mitochondrial potential and red/orange fluorescence for high mitochondrial potential.

As a positive control for damaged acrosome and membrane, and lack of mitochondrial membrane potential, a sample was prepared as described by Siqueira *et al.* (2018). Briefly, a sperm sample was submitted to freezing/thawing cycles (1 min in liquid nitrogen/1 min in a water bath at 60°C, five times). As a negative control, one sample was prepared as described above for experimental samples.

Chromatin sperm analysis was performed according to Simões *et al.* (2013) with some modifications. Briefly, 375,000 sperm cells were added to 50 µl TNE buffer (10 mM Tris−HCl, 0.15 M NaCl, 1 mM EDTA disodium pH 7.4) and 100 µl of acid detergent solution [0.08 M HCl, 0.15 M NaCl, 0.1% (v/v) Triton X-100, pH 1.4]. After 30 s, 300 µl of acridine orange solution (6 µg/ml in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM de EDTA, 0.15 M NaCl, pH 6.0) were

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added, and an evaluation was performed using flow cytometry at 3–5 min following acridine orange solution addition. As a control of chromatin damage, a sample was incubated with HCl (1.2 M in acid detergent solution, pH 0.1) for 1 min. As a control of chromatin resistance, a sample was prepared as described above for the experimental samples.

To calculate the determination coefficient of cytometry analyses, a positive (100%) and a negative control (0%) for each analysis were evaluated individually and mixed in different proportions: 1:3 (25%); 1:1 (50%); 3:1 (75%). Negative and positive thresholds and gates for each analysis were set up to achieve the highest determination coefficient on controls and proportions analysis. All traits analysis reached coefficients higher than 0.94. Compensation parameters and gates applied were held for all samples. Dot plots showing rectangular gates applied to select single sperm events and histograms showing bisector gates applied to separate negative and positive events are presented as Supplementary Material (Figures S1–S4). We presented graphs of negative and positive controls, along with their respective mixtures from each probe analyzed.

Sperm lipid peroxidation assessment

Sperm susceptibility to lipid peroxidation was evaluated by the TBARS assay. The TBARS reaction evaluates malondialdehyde (MDA) concentrations as products of lipid peroxidation. Thiobarbituric acid reacts with MDA to produce a pink-coloured complex.

This analysis was performed as described by Ohkawa *et al.* (1979) and adapted by Nichi *et al.* (2006). In summary, lipid peroxidation was induced by adding ferrous sulfate (100 µl, 4 mM) and sodium ascorbate (100 µl, 20 mM) to 400 µl of the sperm suspension (2.5×10^6 sperms/ml). The mixture was then incubated for 90 min at 37°C. Subsequently, ice-cold 10% trichloroacetic acid (TCA; ratio 2:1) were added. Samples were then centrifuged (18000 *g*, 10 min, 5°C) to precipitate the protein. The supernatant was mixed with 1% thiobarbituric acid (TBA, ratio 1:1) in a cryotube vial. Samples were placed into a boiling water bath (100°C) for 15 min and then immediately cooled in an ice bath (0°C) to stop the chemical reaction. The TBARS were then quantified using a spectrophotometer (UV–vis spectrophotometer, Ultrospec 3300 Pro, Biochrom Ltd, Cambridge, UK) at a wavelength of 532 nm. The lipid peroxidation index was described as nanograms of TBARS/10^6 sperm.

Blood samples and hormonal assay

Blood samples (10 ml) were collected from a jugular vein into centrifuge vacuum tubes containing heparin. Blood tubes were centrifuged at 3000 *g* for 20 min. Plasma samples were stored individually at −20°C until further analysis.

Plasma LH was evaluated as previously described by Bolt and Rollins (1983) and Bolt *et al.* (1990). Plasma LH concentrations were measured by double-antibody radioimmunoassay (RIA) using purified bovine LH standards. A highly purified LH (AFP8614B; National Hormone and Pituitary Programme) was used for both the iodinated tracer and reference standard preparation. The intra-assay coefficients of variation (high and low) and the sensitivity for LH were 9.15%, 5.97% and 0.0373 ng/ml, respectively. Testosterone concentration was determined by an RIA using a commercial kit RIA Testosterone, direct (Beckman Coulter, Prague, Czech Republic) according to the manufacturer's instructions.

Statistical analysis

Statistical Analysis System 9.3 (SAS Institute, Cary, NC) was used to analyze the dependent variables. All data were tested for normality of residues and homogeneity of variances. Variables that did not comply with these statistical premises were transformed (logarithmic transformation was used to evaluate the chromatin susceptibility; testosterone quantification; percentage of sperm with damaged acrosome and intact membrane; percentage of sperm with static movement; and intracellular lipid peroxidation; square root transformation was used to evaluate the percentage of sperm with damaged plasma and acrosome membranes; and the inverse of the data was used to evaluate the percentage of sperm with medium movement). The general linear models (GLM) procedure was used to evaluate the breed effect (Angus, Nellore, and Wagyu). Comparisons of means were performed using the Duncan Test. A 5% significance level was used to reject the null hypothesis. The results are presented as mean ± standard error of the mean (SEM).

Results

Assessment of sperm kinetics

Unfortunately, two Wagyu bulls presented difficulties during semen collection compromising the sperm processing, therefore the Wagyu group was formed with only three experimental units.

The results corresponding to sperm kinetics from Angus, Nellore, and Wagyu bulls are shown in Figure 1. Sperm from Nellore bulls showed greater amplitude of lateral head displacement (ALH) when compared with Wagyu and Angus bulls ($P = 0.016$; Figure 1a). However, Wagyu bulls showed no significant difference for the same parameter when compared with Angus bulls. Considering the variable STR, sperm from Wagyu bulls showed higher straightness (STR) cells when compared with Nellore bulls ($P = 0.032$; Figure 1b). However, Angus bulls showed no significant difference for the same parameter when compared with Nellore and Wagyu bulls.

When evaluating linearity (LIN), Nellore bulls showed cells with lower LIN than Wagyu and Angus bulls ($P = 0.016$; Figure 1c). Angus and Wagyu sires did not show significant differences for LIN. Still, Wagyu bulls showed sperm with lower total motility (MOT) when compared with Nellore and Angus bulls ($P = 0.012$; Figure 1d). Conversely, Angus and Nellore bulls showed no significant difference for MOT. Wagyu sires showed sperm cells with lower medium movement (MEDIUM) than Angus and Nellore bulls ($P = 0.004$; Figure 1e). However, Angus bulls showed no significant difference for the same parameter when compared with Nellore bulls. Finally, sperm from Wagyu bulls had a higher percentage of static sperm (STATIC) when compared with Nellore and Angus bulls ($P = 0.022$; Figure 1f). Angus bulls showed no significant difference for the same parameter when compared with Nellore bulls.

Acrosome integrity, membrane integrity, mitochondrial membrane potential, and sperm chromatin status evaluation

The results corresponding to acrosome integrity, plasma membrane integrity, mitochondrial membrane potential, and sperm chromatin status evaluations from Wagyu, Nellore, and Angus bulls are shown on Table 1. Spermatozoa from the three bull breeds (Nellore, Wagyu and Angus) showed no significant difference in terms of sperm chromatin structure assay (SCSA) quality ($P = 0.903$), high mitochondrial membrane potential (HIGH JC-1;

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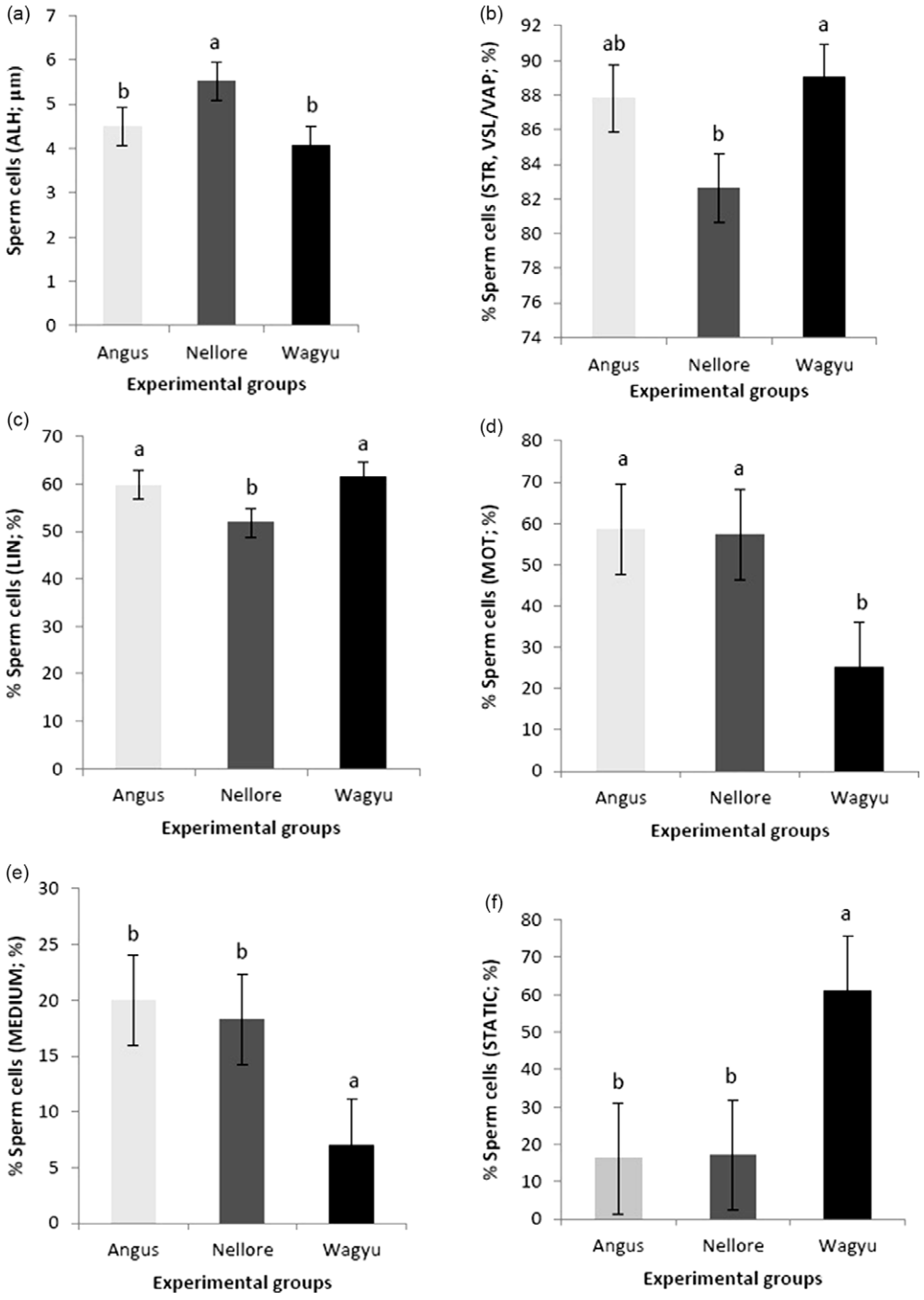

Figure 1. Mean ± standard error of the mean (SEM) of sperm kinetic analysis by computer-assisted sperm analysis (CASA). (a) Amplitude of lateral head displacement (ALH; µm). (b) Straightness (STR, VSL/VAP; %). (c) Linearity (LIN; %). (d) Total motility (MOT; %). (e) Medium movement (MEDIUM; %). (f) Static sperm (STATIC; %) in semen samples of Angus ($n = 5$), Nellore ($n = 5$) and Wagyu ($n = 3$) bull. Different superscript letters represent significant statistical differences ($P < 0.05$).

$P = 0.753$). Nellore, Wagyu, and Angus bulls did not show significant differences in terms of sperm cells with damaged acrosome and intact plasma membrane (FITC–PI+/–; $P = 0.900$); number of sperm cells with damaged acrosome and plasma membrane (FITC–PI+/+ ; $P = 0.146$); number of sperm cells with intact acrosome and damaged plasma membrane (FITC–PI–/+; $P = 0.860$); and amount of sperm cells with intact acrosome and plasma membrane (FITC–PI–/–; $P = 0.625$).

Sperm lipid peroxidation assessment

The results corresponding to sperm cell lipid peroxidation status from Wagyu, Nellore, and Angus sires are shown in Table 1. Spermatozoa from the three bull breeds (Nellore, Wagyu, and Angus) showed no significant difference ($P = 0.646$) in terms of sperm lipid peroxidation status.

Hormonal assay

The results corresponding to serum testosterone and LH concentrations from Wagyu, Nellore, and Angus sires are shown in Table 2. Spermatozoa from the three bull breeds (Nellore, Wagyu and Angus) showed no significant difference ($P = 0.384$) in terms of serum testosterone and LH concentrations.

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Table 1. Mean ± standard error of the mean (SEM) and probability (*P*) of sperm chromatin structure (SCSA), mitochondrial membrane potential (JC-1), acrosome and plasma membrane integrities (FITC–PI) and sperm lipid peroxidation status evaluated by induced thiobarbituric acid reactive substances (TBARS) assay among Angus ($n = 5$), Nellore ($n = 5$) and Wagyu ($n = 3$) bulls

	Bull breeds (experimental groups; Mean ± SEM)			
Variables	Angus	Nellore	Wagyu	*P*-value
SCSA +	0.027 ± 0.007	0.039 ± 0.021	0.029 ± 0.005	0.903
JC-1 HIGH	38.764 ± 9.519	30.366 ± 8.019	37.067 ± 5.263	0.753
FITC–PI+/–	12.682 ± 3.114	12.762 ± 5.684	9.587 ± 2.857	0.900
FITC–PI+/+	33.462 ± 6.289	46.040 ± 3.477	39.800 ± 2.730	0.146
FITC–PI–/+	19.900 ± 4.277	17.880 ± 2.768	21.000 ± 5.001	0.860
FITC–PI–/–	34.022 ± 10.643	23.326 ± 5.661	29.667 ± 1.842	0.625
TBARS	650.453 ± 119.439	920.289 ± 152,809	1220.29 ± 763,067	0.646

SCSA+ = intact sperm chromatin; JC-1 HIGH = high mitochondrial membrane potential; FITC–PI (+/–) = damaged acrosome and intact plasma membrane; FITC–PI (+/+) = damaged plasma and acrosome membranes; FITC–PI (–/+) = intact acrosome and damaged plasma membrane; FITC–PI (–/–) = intact plasma and acrosome membranes; TBARS = induced thiobarbituric acid reactive substances (ng TBARS/10^6 sperm).

Table 2. Mean ± standard error of the mean (SEM) and probability (*P*) of serum testosterone and luteinizing hormone (LH) concentrations among Angus ($n = 5$), Nellore ($n = 5$) and Wagyu ($n = 3$) bulls

	Bull breeds (experimental groups; mean ± SEM)			
Variables (ng/ml)	Angus	Nellore	Wagyu	*P*-value
LH	1.592 ± 0.271	1.457 ± 0.367	0.922 ± 0.058	0.384
Testosterone	4.700 ± 2.647	9.380 ± 3.616	7.500 ± 0.961	0.297

Discussion

Sire fertility is extremely important for reproduction in several species, including cattle. After the introduction of artificial insemination (AI) practice and increasing worldwide export of semen straws, sperm analysis became even more substantial (Amann and Waberski, 2014; Nagy *et al.*, 2015; Leite *et al.*, 2022; O'Meara *et al.*, 2022). Motility is one of the most important seminal parameters associated with the sperm fertilizing ability and it may indicate spermatozoa viability and structural integrity (Kathiravan *et al.*, 2011; Nagy *et al.*, 2015; Singh *et al.*, 2021; Leite *et al.*, 2022). Conventional semen analysis (semen volume, colour, density, viscosity, and sperm motility, vigour, concentration and morphology) is an important and also a practical and low-cost evaluation to identify sperm quality. However, as sperm cells are highly specialized cells, conventional analysis is not sufficient to evaluate sperm function and fertilization ability (Perumal *et al.*, 2014; Nagy *et al.*, 2015; Leite *et al.*, 2022; O'Meara *et al.*, 2022). There is a large variation among laboratories and even within each or between technicians. According to Brazil *et al.* (2004) the variation coefficient among laboratories is ~23–73% for sperm concentration, 9–37% for sperm motility and 25–87% for sperm morphology evaluation.

To overcome the operator dependency of conventional semen analysis, technologies have been developed. CASA is a computer system equipped with a high-resolution camera and microscope that allows a more accurate sperm motion evaluation. Moreover, CASA allows the reduction in bias compared with visual evaluations (Kathiravan *et al.*, 2011; Amann and Waberski, 2014; Nagy *et al.*, 2015; Valverde *et al.*, 2020; Fernandez-Novo *et al.*, 2021). The results for sperm kinetics analyses in the present study indicated that the Nellore bulls showed the highest head displacement amplitude (ALH) compared with Angus and Wagyu sires. Still, Wagyu and Angus bulls, despite showing lower ALH values, did not show a significant difference between them. The sperm cells with higher ALH indicated a higher ability to penetrate cervical mucus and fuse with the zona pellucida of the oocyte. It is believed that the higher the ALH, the greater the chance of fertilizing the female gamete (Perumal *et al.*, 2014).

The STR and LIN parameters are related to the straightness of the sperm's movement and ability to migrate in cervical mucus (Goovaerts *et al.*, 2006; Robayo *et al.*, 2008; Kathiravan *et al.*, 2011; Perumal *et al.*, 2014). The results of sperm kinetics analysis in the present study indicated that the Wagyu sires showed higher values for both parameters when compared with Angus and Nellore bulls. However, the STR from Wagyu bulls sperm cells was not significantly different from Angus bulls. Conversely, the STR of Angus bulls was not significantly different from Nellore bulls. Regarding linearity (LIN), the Angus bulls had the best results when compared with Nellore bulls, but did not differ significantly when compared with the LIN of the Wagyu bulls. High ALH and low STR values are characteristic of hyperactive spermatozoa. Conversely, high values of LIN and STR indicated a progressive movement pattern of sperm cells (Ratnawati and Luthfi, 2020). Considering all this together, it seems that sperm cells from Nellore sires that showed higher ALH and lower STR and LIN had started the capacitation process before Angus and Wagyu. Still, Wagyu spermatozoa are more capable of swimming in a straight line.

Considering total motility (MOT) and not motile (STATIC) cells, it was observed that Wagyu sires showed lower MOT than Angus and Nellore bulls and a higher number of STATIC cells. Despite having a straighter pattern than Nellore and Angus bulls, Wagyu sperm were slower. Some might think that these characteristics of sperm kinetics in Wagyu sires may be worrying, as the bovine oocyte after being ovulated remains viable in the

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fallopian tube for an average of 6–8 h (Miller, 2018). In other words, fertilization of aged oocytes produces poorer quality embryos. Notwithstanding, another possible hypothesis that could explain lower motility is the sperm freezing procedure. According to Siqueira *et al.* (2018) and Kathiravan *et al.* (2011), sperm cells that survive suboptimal conditions during the cryopreservation process are more resistant to *in vitro* conditions, showing higher longevity after thawing, indicating that those spermatozoa may be more prone to fertilize the oocyte, even though they were less motile. Yet, sperm motion characteristics seem to be of minor importance, as the uterine contractions may be of greater consequence (Kunz *et al.*, 1997) or when considering IVP conditions in which samples are submitted to motile sperm selection techniques as density gradient centrifugation overcoming lower initial motility.

Differences in sperm kinetics evaluation among sires may be due to differences in the genetic group of animals (Hoflack *et al.*, 2007; Kathiravan *et al.*, 2008; Morrell *et al.*, 2018). Detailed studies, however, must be conducted to more accurately determine the associations among values for sperm kinetics and those for fertility variables. Conversely, a higher ALH and lower STR and LIN as observed in Nellore bulls may not be good sperm traits to be considered as biomarkers. Sperm hyperactivation alters sperm swimming patterns, increasing ALH and decreasing LIN values (Shi and Roldan, 1995; Sansegundo *et al.*, 2022). It seems reasonable to hypothesize that higher hyperactivated sperm cells as seen in Nellore bulls in the present study may be related to a premature event that leads to higher energy consumption and ATP decrease more rapidly. This situation could diminish fertilization rates in Nellore sires when compared with Angus and Wagyu. However, more studies are needed to confirm this hypothesis.

As fertilization is a complex and multistep process, it is unlikely that only one sperm attribute could be considered a fertility biomarker (Oliveira *et al.*, 2013). Therefore, it is important to evaluate a wide range of sperm features to identify potential biomarkers for bull fertility (Utt, 2016). In addition to sperm motility, the evaluation of sperm function attributes such as acrosomal and plasma membrane integrity, chromatin status and mitochondrial activity remains one of the keystones in semen laboratory analysis (Simões *et al.*, 2013; Malama *et al.*, 2017; Bernecic *et al.*, 2021). IVP is a useful tool to compare *in vitro* fertility of samples from bulls and breeds under certain conditions. Nevertheless, until now, no single assay has been able to accurately predict bull fertility.

In Angus sires it was shown that 82% of *in vitro* fertilization rates may vary according to sperm features such as sperm morphology, acrosome and plasma membrane integrities (Tartaglione and Ritta, 2004). In Holstein Friesian bulls, Bernecic *et al.* (2021) developed a linear and logistic predictive model to evaluate bull fertility. According to the authors, 47% of bull fertility variation is due to plasma membrane changes and 90% to acrosome damage. Oliveira *et al.* (2014) showed that semen of Nellore sires with intact plasma and acrosome membranes and high mitochondrial potential resulted in increased pregnancy rates when compared with spermatozoa with membrane damage and diminished mitochondrial potential. The role of mitochondria in several intracellular processes related to sperm metabolism, cell signalling and sperm apoptosis has made the assessment of mitochondrial function a promising tool for the assessment of sperm fertilization capacity (Malama *et al.*, 2017; Leite *et al.*, 2022).

With respect to sperm chromatin integrity, several studies have already shown that bull fertility and embryo development can be negatively affected when sperm DNA is damaged (Simões *et al.*, 2013; Dogan *et al.*, 2015; Kumaresan *et al.*, 2017; Narud *et al.*, 2020). Nonetheless, in the present study, semen functional parameters were not different among sires. Even with no apparent impairment of sperm function, Wagyu bulls may have lower fertility as they have slower sperm cells and therefore may produce poorer quality embryos. This fact can decrease the pregnancy rate, which increases the cost of breeding. Given the considerable proportion of variation in bull fertility that yet remains not explained, it would be of interest to carry out a further study focusing on sperm molecular-based characterization and also explore other sperm features to identify possible cellular structures that may be related to the decline in bull fertility.

Reactive oxygen species (ROS) mediate essential intracellular signalling cascades needed for sperm physiological function, namely sperm flagellar hyperactivation, capacitation, and acrosome reaction, sperm oocyte interactions, signalling pathways involved in the fertilization, implantation and early embryo development (Agarwal and SenGupta, 2020; Dutta *et al.*, 2020; Alyethodi *et al.*, 2021; Upadhyay *et al.*, 2022). However, oxidative stress (imbalance of ROS not mitigated by the antioxidant mechanisms) may reduce sperm fertilizing ability due to toxicity (Ugur *et al.*, 2019; Ribas-Maynou *et al.*, 2020; Saraf *et al.*, 2021). Mitochondrial impairment and sperm DNA fragmentation are positively correlated with oxidative stress (Simões *et al.*, 2013; Leite *et al.*, 2022). It is known that frozen–thawed semen has an increased lipid peroxidation rate, once sperm cells are rich in polyunsaturated fatty acids and lack antioxidant enzyme defences (Amaral *et al.*, 2013; Castiglioni *et al.*, 2021; Pintus and Ros-Santaella, 2021). Lipid peroxidation is considered a specific indicator of oxidative stress in sperm cells (Gallo *et al.*, 2021). Indeed, there is damage caused by oxidative stress to the nature and amount of ROS involved and also on the duration of ROS exposure and extracellular factors, namely temperature, oxygen tension and the surrounding environment (Agarwal *et al.*, 2008; Pintus and Ros-Santaella, 2021).

In the present study, semen lipid peroxidation was not different among sires. Taken together with the results discussed above (sperm kinetics and sperm function analysis) it was possible to infer that the amount of ROS was not able (or sufficient) to trigger mitochondrial dysfunction, leading to altered respiratory chain activity, plasma membrane degradation, and thereby decreasing cell motility. The same was true for sperm DNA damage, as chromatin is the last cell structure to be affected by oxidative stress. It has been demonstrated that when sperm are exposed to low levels of oxidative stress, blastocyst formation is decreased, but not the cleavage rate. Conversely, when exposure to oxidative stress is severe, it is able to reduce cleavage and blastocyst rates, embryo quality and also compromise sperm DNA integrity (de Assis *et al.*, 2015; de Castro *et al.*, 2016; Simões *et al.*, 2013).

The relationship between testosterone and LH can be used to measure Leydig cell development (Teerds and Huhtaniemi, 2015). Both are released in response to GnRH during puberty. Luteinizing hormone together with FSH is able to modulate testosterone synthesis in Leydig cells (Anand-Ivell *et al.*, 2019; Kowalczyk *et al.*, 2021). Conversely, testosterone is an important hormone for spermatogenesis and is responsible for secondary sexual traits and libido (Kowalczyk *et al.*, 2021). Also, testosterone is an important hormone in male fertility as its concentration in seminal plasma shows a positive correlation with sperm concentration, sperm motility, and other sperm traits (Souza *et al.*, 2011).

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In the present study, testosterone and LH serum concentration were not different among sires. Nonetheless, our results showed a higher testosterone serum concentration in Nellore bulls than found in a previous studies. Santos *et al.* (2004) observed an average of 4.04 ng/ml for the same bull breed. A similar result was shown in a study conducted by Amorim *et al.* (2015) that found a serum testosterone concentration with an average of 5.41 ng/ml. In another study, Chacur *et al.* (2013) evaluated seasonal effects on semen and testosterone concentration in Zebu (Nellore; *Bos indicus*) and taurine (Simental; *Bos taurus*) bulls. They showed that there was a difference between breeds when considering testosterone serum concentrations, demonstrating a higher adaptability of Nellore sires to the field conditions. Their results showed that Nellore had 2.34–4.3 ng/ml of serum testosterone and Simental had 5.84–9.01 ng/ml of serum testosterone. Interestingly, in the present study the mean testosterone serum concentration for *Bos taurus* (Angus bulls) was lower than for the data found by Chacur *et al.* (2013). Despite differences between testosterone serum concentration in the present study and in previous literature, we showed that there was no difference for other sperm traits, except for sperm kinetics as discussed above.

One possible reason for the results present in this study is that the bulls were not grouped according to fertility or other traits, as the goal was to evaluate whether there were differences in the hormone and sperm characteristics of Wagyu bulls compared with the same characteristics of subspecies *Bos indicus* and *Bos taurus* sires. Moreover, the age of the bull could be an aspect that influenced the results as the mean ages of the Angus, Nellore, and Wagyu sires were 44.8, 39.0 and 12 months, respectively.

Bos indicus cattle are more resistant to higher temperature and humidity than *Bos taurus*. Indeed, as a consequence, its productivity and precocity are diminished. In general, Nellore bulls have slower testicular development and reach puberty later than *Bos taurus* (Nogueira, 2004). According to Reis *et al.* (2016), Nellore puberty lasts from 18 to 26 months of age. In a study conducted by Brito *et al.* (2004), age at puberty ranged from 17.6 to 22.4 months in Zebu (*B. indicus*) cattle. The seminal traits at this age were slightly poor, as mentioned above. Only after 26 months of age, with an increasing level of LH and testosterone, Nellore bulls reached sexual maturation. At this time, bulls exhibit an increased seminal quality (Reis *et al.*, 2016). Sexual maturity in *B. taurus* is achieved earlier than in *B. indicus* (Nogueira, 2004; Brito, 2021). Fields *et al.* (1982) found an average age at puberty of 15.7 months for the Angus breed. In agreement with these results, Brito *et al.* (2012) reported that Angus bulls reached puberty at 12.6 months of age and sexual maturity at 15.4 months old. Also, Tatman and co-workers (2022) observed that Angus bulls reached puberty at 12 months old. Considering Wagyu sires, to the best of our knowledge, there are scarce data available from Wagyu sire puberty. Tatman *et al.* (2022) found that Wagyu cattle were the youngest breed at puberty (10.7 months old). Casas *et al.* (2007) showed that the age at puberty was 10.06 months for Wagyu sires.

It is possible that we were not able to show significant differences between sires from different breeds considering sperm traits and sexual hormone levels because Nellore and Angus were older and probably more sexually mature than Wagyu sires. It is noteworthy that the presented data were based on a limited number of bulls. Perhaps, increasing the number of bulls and selecting animals with similar ages might allow additional significant relationships to be revealed. Once the present study was not able to find differences among Wagyu, Nellore, and Angus sires and some studies had pointed out that even Wagyu sires had a smaller scrotal circumference, had a higher libido and higher sperm production, therefore more studies are needed to determine what makes Wagyu different from other sires considering reproductive traits.

In conclusion, the results from the present study indicated that Wagyu sires had lower total motility when compared with Nellore and Angus bulls. This difference could be an effect of the high percentage of sperm with no motility observed in Wagyu semen. However, even failing the breeding soundness exams, because scrotal circumferences were too small, the Wagyu breed did not differ from those breeds when considering plasma and acrosome membranes integrity, mitochondrial potential, chromatin resistance, sperm lipid peroxidation or hormone (LH and testosterone) concentrations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0967199423000278

Data availability. The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Competing interests. The authors declare that they have no competing interests.

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