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RESEARCH ARTICLE
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Deciphering Storability of Lusitu Boar Semen in a Short-term Extender Based on Traditional Characteristics and CASA-motility Parameters as Predictive Biomarkers

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ABSTRACT

The quality of semen deteriorates as storage time progresses, which may compromise the fertilizing capacity of spermatozoa. This study investigated the storability of Lusitu boar semen (LBS) for 4 days. The study employed repeated measures factorial design that considered storage time and boar factors to generate the data. A total of 36 ejaculates, with six collected per boar, were preserved at 17°C in BTS extender, followed by analysis after 2 (D0), 48 (D2), and 96 (D4) hours of storage. The Data on traditional semen characteristics (TSCs), kinematics, and hyperactivity (HYP) of spermatozoa were analyzed using appropriate descriptive and inferential statistics. There was a reduction in mean semen pH, plasma membrane integrity, normal acrosomes (NMA), and total and progressive motility (TTM and PGM) across storage time points except for vitality, whose scores on D0 and D2 were similar. However, the observed values on D4 were within or above the recommended limits for all the TSCs. All kinematic parameters and HYP showed mean reduction between D0-D2 but not D2-D4, while that of VAP reduced significantly from D0-D2. The storage time but not boar factor influenced the means of all TSCs, while both factors affected those of TTM and PGM. All the kinematics and HYP were influenced by storage time and boar. In conclusion, the quality of LBS was found to be acceptable after storage for 4 days despite the level of deterioration observed. Additionally, some TSCs may be useful in assessment of LBS quality prior to storage, while kinematics like VCL, VAP, ALH, and HYP may be candidates for storability prediction.

Keywords: BTS-extender, Kinematic parameters, Lusitu boar, Semen storage, TSCs

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INTRODUCTION

The recent integration of biotechnology in animal reproduction is hinged on better reproductive biotechnologies, one of which is artificial insemination (AI) (Akandi et al., 2015; Hadgu & Fasseha, 2020). In Western and some developing countries, pig breeding is mainly carried out using AI (Yeste, 2017). More than 85% of inseminations are performed within 48 hours after semen collection, although variability exists in extent of AI-application among countries (Karunakaran et al., 2017; Singh et al., 2022). The prolonged semen storage has adverse effects on spermatozoa viability (Szymanowicz et al., 2019). Accordingly, only a portion of spermatozoa from original semen sample survives following preservation of any type (Johnson et al., 2000; Schulze et al., 2023). The survival of cells is much greater after liquid- than frozen-semen storage, hence the majority (99%) of AI in pigs are performed using liquid-stored semen (Yeste, 2017).

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Several attempts have been made to extend the duration of semen storage in liquid form beyond three days without any negative effects on spermatozoa fertility, however results remain inconsistent and unsatisfactory (Gadea, 2003; Singh et al., 2022). Use of extenders with long term effects can prolong the shelf-life of liquid-stored boar semen but such extenders are generally non-economical considering their prices (Akandi et al., 2015). It has been emphasized that AI would be economical for the swine industry if stored-semen is used within 4-5 days after collection (Waterhouse et al., 2004; Tremoen et al., 2018). This would allow for additional quality tests to be conducted and enable transportation of preserved semen over long distances, especially in areas where farmers are scattered (Tremoen et al., 2018). The Beltsville Thawing Solution (BTS) extender would be suitable since it is cheap, easy to prepare, and largely used for preservation of boar semen in liquid form. However, it confers protection to boar semen for about 3 days (Kadirvel et al., 2019; Thema et al., 2022).

There is evidence that variations between breeds and/or boars in terms of their spermatozoa motility, viability, DNA integrity, inter alia exist; these are attributed partly to the potential intrinsic resistance to deterioration during semen storage (Bielas et al., 2017; Yeste, 2017; Sevilla et al., 2024). It is not clear whether Lusitu pigs, that are adapted to the rearing conditions characterized by diseases, nutritional and heat stress (Abigaba et al., 2022), possess any advantage in terms of their semen stability to quality deterioration during storage. Noteworthy, the use of semen prepared locally from indigenous boars was recommended (Dotché et al., 2021; Pena Jr, 2023). However, successful utilization will require knowledge about its storability. Lusitu boars have not been characterized by their semen preservation ability, particularly using objective CASA-based parameters, hence their potential stability to storage deterioration during liquid preservation remains unknown. Understanding the liquid-preservation ability of boar spermatozoa is vital for efficient management and breeding of pigs (Johnson et al., 2000; Thema et al., 2022; Pena Jr, 2023). This study was conducted to: (1) assess the quality changes using selected traditional semen characteristics and CASA-motility parameters during LBS storage and (2) correlate selected TSCs with the CASA-motility parameters, and (3) explore the potential of TSCs and CASA-motility parameters for use in selection of ejaculates and/or boars for AI or breeding purposes.

MATERIALS & METHODS

Ethical Approval

This study was approved by Biomedical Research Ethics Committee, University of Zambia: No. 1595-2021.

Animals

The study was conducted in the School of Veterinary Medicine, University of Zambia, during November 2023-March 2024. A total of six Lusitu boars were used. They were physically healthy and sexually mature with an average weight and age of 69.56±2.92kg and 19.60 months, respectively.

Experimental Design

The study employed a repeated measures factorial design that incorporated two factors, including storage time and source of ejaculate (boar), to generate data on the storability of spermatozoa in LBS preserved at 17℃. The storage time factor had three levels, namely D0, D2, and D4, while the boar factor had six levels, viz. A22, A11, A3, A15, A21, and A2. Semen samples were considered as subjects; these were obtained from boars by the gloved hand method. This study used a total of 36 ejaculates, with six samples collected per boar and a collection interval of approximately two weeks. Additionally, only those samples whose volume and initial motility values were ≥50mL and ≥70%, respectively, were considered.

Semen Processing and Storage

Suitable samples were processed and preserved in liquid state based on the procedures described by Karunakaran et al. (2017). A commercial short-term extender (BTS, Minitüb, Tiefenbach, Germany) was used for semen dilution. Pre-dilution was done by mixing pre-warmed (35℃) extender and semen in a 1:1 (v/v) ratio, followed by holding at 35℃ in a water bath for 30min. The final extension resulted in samples with approximately 30×10^6 spermatozoa/mL. The samples were introduced into pre-warmed semen bottles (100mL), followed by holding at 25℃ in a fluid thermostatic box for 2 hours, before storage at 17℃ in a biochemistry incubator (Biobase, BJPX-I-80, Biobase Scientific (Shandong) co. Ltd, Shandong, China).

Semen Evaluation

Each sample was assessed for quality changes at three storage time points, viz. after 2, 48, and 96 hours of storage, which is D0, D2, and D4, respectively. The storage at 2 hours determined the initial point (D0). Prior to the quality examination, approximately 200µL were drawn into Eppendorf tubes and warmed at 37℃ in a dry bath incubator (HiTec IS-61 Yamato Scientific Co. Ltd, Tokyo, Japan) for 25min to reactivate the spermatozoa. The TSCs evaluated included semen pH, spermatozoa vitality (VIT), plasma membrane integrity (HOST), acrosome integrity (normal acrosomes; NMA), total motility (TTM), and progressive motility (PGM), while the kinematics considered were straightness (STR; %), linearity (LIN; %), curvilinear velocity (VCL; µm/s), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), average lateral displacement of the spermatozoa head (ALH; µm), wobble (WOB; %), beat cross frequency (BCF; Hz), as well as spermatozoa hyperactivity (hyperactivity; HYP %).

Semen pH was measured at each storage point immediately after drawing off an aliquot meant for other tests. Samples were measured for pH using a hand-held pH meter. The measurement of semen pH was done according to the procedure described in an earlier study (Ratchamak et al., 2019). Calibration of the pH meter followed the manufacturer's directions. The measurement

range of the pH meter was 0-14, while its accuracy and resolution were ±0.02 and 0.01, respectively.

The evaluation of VIT was carried out using a semi-automatic counter software (Vitality; SCA®, version 6 (Microptic S.L., Barcelona, Spain)). To do this, an aliquot of a warmed semen sample was mixed with the stain (BrightVit®, a nigrosin-eosin stain (Microptic SL, Barcelona, Spain)) in a ratio of 1:1 (v/v), followed by incubation at 37℃ for approximately 3min. The semen-mixture was then used to prepare a dry smear according to the procedures adopted from the manufacturer. The prepared smear was then placed onto the microscope for image analysis. Spermatozoa viewing was done with a 20xobjective in an A-mode. A minimum of 200 spermatozoa were counted for each slide, and an average of two counts from the replicate smears were considered as a single datum.

The plasma membrane integrity was analyzed using a hypo-osmotic swelling test (HOST). The preparation of HOST solution and incubation with the diluted semen sample was based on the previous procedure (Jeyendran et al., 1984). The HOST solution and liquid-stored semen, both pre-warmed at 37℃, were mixed in a 1.5mL Eppendorf tube in proportions of 1000µL and 100µL, respectively. The semen-HOST mixture was incubated at 37℃ for 45min. Then, a wet smear was prepared using an aliquot of the incubated mixture on a microscopic slide with a cover slide. Image analysis was performed at 20×objective under a phase-contrast microscope (Nikon Eclipse E200, microscope), with a total of 200 spermatozoa analyzed per slide. The HOST-reactive/positive or spermatozoa with coiled tails were counted as viable cells, while those with uncoiled tails were considered non-viable. The average of the two counts from replicate smears was calculated and recorded as a single datum.

The acrosome integrity was determined by assessing the percentage of spermatozoa with intact acrosomes (NMA) according to a previously described procedure (Daramola & Adekunle, 2015). Briefly, 50µL of a pre-warmed stored semen sample was added to 500µL formalin citrate solution (96ml 2.94% sodium citrate added to 4mL 37% stock formaldehyde) in a 1.5mL Eppendorf tube and mixed carefully. An aliquot of this mixture was placed on a microscope slide, covered with a cover slip, and then placed on a microscope (Nikon eclipse E200, Nikon cooperation, Tokyo, Japan) for image analysis at 400×magnification. A total of 200 spermatozoa were counted in more than three different microscopic fields for each sample and a proportion of the NMA obtained. The average of two counts from the replicate smears was calculated and recorded as a single datum.

The motility parameters, including the TTM, PGM, and selected kinematics of Lusitu boar spermatozoa were determined using the CASA-Mot module (SCA, version 6 (Microptic S.L., Barcelona, Spain)). The procedures used to analyze these parameters followed the previously described steps (Masenya et al., 2011; Karageorgiou et al., 2016). The settings used to analyze these motility characteristics were adopted from the manufacturer, sperm class analyser® settings for boar/swine species. Briefly, an aliquot (3µL) was loaded into a pre-heated (37°C) chamber of Leja-4 chamber slide followed by parameter analysis using the CASA-Mot module and recording of the data.

Statistical Analysis

The data were analyzed in SPSS IBM® (SPSS IBM 26 version, USA). The Levene's test was used to ascertain homogeneity of the error of variance, while data normality was checked using Mauchly's test, Greenhouse-Geisser and Huynh-Feldt epsilon values. Means and standard deviations (SD) were used for descriptive analysis, while inferential statistics included ANOVA and correlation coefficients. The effect of time and boar on motility characteristics and their interaction were tested using a Two-Way repeated ANOVA. In the generalized linear model used, storage time and boar were treated as the within- and between-subject factors, respectively. The sphericity assumed, Greenhouse-Geisser, and Huynh-Feldt methods were used to detect the differences, while Bonferroni-pairwise comparisons were conducted to establish pairs with significant differences. The trending over time across boars was also ascertained for selected kinematics. In all the tests, significance was taken at $P<0.05$.

RESULTS

The Changes in Selected TSCs of LBS on D0, D2, and D4 of Storage at 17℃.

The mean values and standard deviations (Mean±SD) of selected TSCs and spermatozoa kinematics for LBS on D0, D2, and D4 of storage are presented in Table 1. The mean values for semen pH, HOST-positive, TTM, PGM, and NMA differed significantly ($P<0.05$) between D0 and D2 as well as the case of D2 and D4 ($P<0.05$). Those of VIT on D0 and D2 were similar ($P>0.04$) but not the case of D2 and D4 ($P<0.05$). In terms of the general trend, the mean values for semen pH, HOST-positive, VIT, and NMA decreased over time ($P<0.05$). There was no significant effect of the semen source (boar) ($P>0.04$) and the interaction between storage time and boar was also not significant ($P>0.05$) for all parameters except for TTM and PGM whose mean values were influenced by the boar ($P<0.05$).

Table 1: The scores for mean and standard deviations of selected TSCs of LBS on D0, D2, and D4 of storage at 17℃.

	Time			Source of variation (P values)		
VAR	D0	D2	D4	Time	Boar	T*B
pH	7.59^{a}	7.35^{b}	7.21^{c}	0.001	0.411	0.510
HOST	71.89 ± 8.30^{a}	68.79 ± 8.72^{b}	60.61 ± 8.50^{c}	0.001	0.121	0.378
VIT	90.96 ± 2.80^{a}	90.21 ± 2.70^{a}	88.45 ± 2.86^{b}	0.001	0.078	0.125
NMA	80.96 ± 6.78^{a}	72.33 ± 8.46^{b}	61.83 ± 9.50^{c}	0.001	0.492	0.438
TTM	93.62 ± 1.89^{a}	91.34 ± 2.02^{b}	88.80 ± 1.91^{c}	0.001	0.001	0.098
PGM	82.94 ± 3.54^{a}	74.63 ± 4.78^{b}	72.22 ± 5.18^{c}	0.001	0.001	0.338

Means with different superscripts a, b, c in a row differ significantly at $P<0.05$; VAR=Variable; T*B=interaction between storage time and individual boar; HOST=HOST-Positive spermatozoa; VIT=Vitality; TTM=Total motility; NMA=Normal acrosome; PGM=Progressive motility

The Changes in Selected Spermatozoa Kinematics on D0, D2 and D4 of Storage at 17℃.

The observed mean values for STR, LIN, VCL, VSL, ALH, BCF, and HYP differed between D0 and D2 ($P<0.05$) but did not vary significantly between D2 and D4 ($P>0.05$) (Table 2). Additionally VAP scores did not differ between

D0 and D2 (P>0.05) as well as the case of D2 and D4. The observed reduction in mean values of VSL and WOB was significantly (P<0.05) attributed to both storage time and boar effects (P<0.05) and not the interaction effect (P>0.05). The mean values for STR, LIN, and BCF also reduced from D0-D2 as well as D2-D4 with storage time, boar, and interaction effects contributing to the observed mean variations. The mean VCL, HYP, and ALH increased as storage time advanced; these parameters were influenced by the storage time, boar, and interaction effects (P<0.05).

Table 2: The scores for mean and standard deviations of spermatozoa kinematics in LBS on D0, D2, and D4 of storage at 17°C.

	Time			Source of variation (P values)		
VAR	D0	D2	D4	Time	Boar	T*B
STR	74.66±5.02^{a}	65.91±5.76^{b}	65.40±5.18^{b}	0.001	0.001	0.007
LIN	46.00±5.68^{a}	36.92±5.46^{b}	37.27±4.64^{b}	0.001	0.001	0.040
WOB	60.14±4.00^{a}	54.65±3.55^{b}	55.59±3.08^{b}	0.001	0.001	0.211
VCL	128.63±14.97^{a}	143.11±13.71^{b}	144.64±13.00^{b}	0.001	0.001	0.004
VAP	76.24±7.35^{a}	77.69±7.34^{ab}	80.15±7.65^{b}	0.003	0.003	0.395
VSL	58.12±5.75^{a}	52.13±6.31^{b}	53.54±6.34^{b}	0.001	0.001	0.963
ALH	2.49±0.39^{a}	3.13±0.42^{b}	3.12±0.37^{b}	0.001	0.001	0.001
BCF	23.41±1.95^{a}	20.11±2.59^{b}	20.38±2.11^{b}	0.001	0.001	0.002
HYP	7.81±5.88^{a}	20.88±10.52^{b}	19.50±9.50^{b}	0.001	0.001	0.001

Means with different superscripts a, b, c in a row differ significantly at P<0.05; VAR=Variable; T*B=Interaction between storage time and individual boar; STR=Straightness; LIN=Linearity; WOB=Wobble; VCL=Curvilinear velocity; VAP=Average path velocity; VSL=Straight line velocity; ALH=Amplitude of lateral head displacement; BCF=Beat cross frequency; HYP=Hyperactivity

Differential Trending over Storage Time for Speed Parameters of Spermatozoa across Lusitu boars

The trends over storage time for the VCL, VAP, and VSL parameters of spermatozoa from individual boars are presented in Fig. 1. There was a general linear increase in the mean VCL values of all boars, as storage time progressed, except for A15 and A21 whose scores declined considerably between D2 and D4. The observed mean changes for boars A15 and A21 revealed similar trends; their means presented a pronounced curvilinear trend. All boars except A15 and A21 showed a gradual increase in the VCL values from D2-D4. The mean VAP values for spermatozoa in boars A22, A11, and A2 gradually increased from D0 through D2 until D4, while A3 and A21 revealed a gradual reduction between D0 and D2 before a sharp increase and/or levelling from D2-D4 for A3 and A21, respectively. The mean VAP values for A15 generally levelled at all storage time points. The mean VSL values of spermatozoa in all boars revealed a similar trend, with a gradual reduction between D0-D2 and an increase and/or levelling between D2-D4. All the graphs were generally parallel to each other, but the mean VSL values particularly the case of A21 including initial VSL scores were way higher than those of other boars.

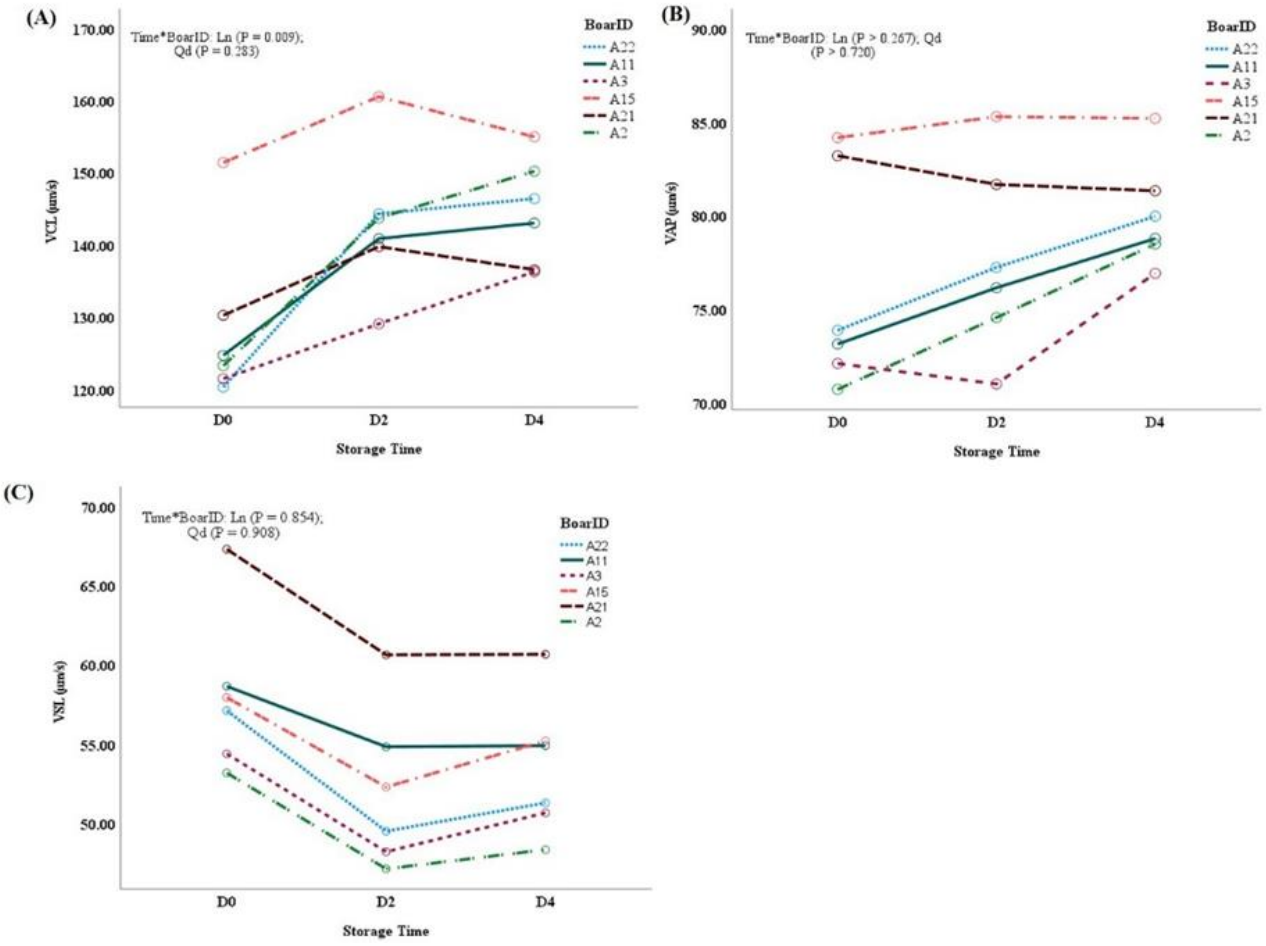

Fig. 1: Differential trending over time for the VCL, VAP, and VSL of Lusitu boar spermatozoa in semen stored at 17°C for 4 days. The graphs A, B, and C represent the estimated marginal means of VCL, VAP, and VSL, respectively, as measured on Day 0, Day 2, and Day 4. VCL=Curvilinear velocity; VAP=Average path velocity; VSL=Straight line velocity; BoarID=Boar identification; Ln=Linear; Qd=Quadratic; P=P value.

Differential Trending over Storage Time for Parameters of Spermatozoa-head Patterns across the Study Boars

The trending over time for BCF, ALH, and HYP of spermatozoa are presented in Fig. 2. Boars A22, A11, and A2 had a similar sharp reduction in BCF scores between D0-D2, followed by the gradual levelling from D2-D4, while boars A15 and A3 showed a sharp reduction and increment between D0-D2 and D2-D4, respectively. The gradual downward trend observed was generally linear between all storage time points for the case of A21. The mean values for ALH across boars revealed an increasing trend from D0-D2 with A22 having a sharper increase compared to the rest, while the scores gradually levelled between D2 and D4 except for boars A15 and A21 with the downward mean scores. Boar A15 had the highest initial ALH value and continued to increase until D2 before a sharp reduction from D2-D4, with a pronounced curvilinear trend. The mean scores for spermatozoa hyperactivity indicated an upward trend between D0 and D2 with boars A2 and A22 revealing sharper increase compared to the rest. Between D2 and D4 their mean values generally levelled for all boars except A15 whose spermatozoa hyperactivity reduced sharply with a pronounced curvilinear trend. Moreover, boar A15 had the highest initial hyperactivity proportions compared to other boars.

Correlation between Selected TSCs and Kinematics of Spermatozoa in LBS on D0 of Storage at 17℃.

The correlation coefficient results obtained through bivariate comparisons of each TSC with individual parameters of spermatozoa speed as well as those of head patterns are presented in Table 3. The VCL, ALH, and HA of spermatozoa were associated ($P<0.05$) negatively with vitality. The VCL, VAP, and ALH of spermatozoa were found to be correlated ($P<0.05$) negatively with PGM, while PGM relationship with the BCF of spermatozoa was positive ($P<0.05$). It was also observed that VAP and VSL had a positive association ($P<0.05$) with NMA, while only VSL was correlated positively with HOST. Additionally, TTM and pH were not associated ($P>0.05$) with any of the parameters of spermatozoa speed and those of the head patterns.

Table 3: Correlation between the TSCs and kinematics of spermatozoa in LBS on D0 of storage at 17℃.

VAR	pH	HOST	VIT	NMA	TTM	PGM
NMA	-0.414*	0.829**	0.098	1.000	0.063	-0.340*
STR	0.156	0.020	0.371*	-0.205	-0.242	0.588**
LIN	-0.071	0.265	0.432**	0.048	-0.196	0.407*
WOB	-0.197	0.365*	0.434**	0.208	-0.081	0.231
VCL	0.084	0.049	-0.416*	0.286	0.118	-0.496**
VAP	-0.121	0.252	-0.153	0.452**	0.114	-0.448**
VSL	-0.069	0.408*	0.082	0.435**	-0.120	-0.136
ALH	0.133	-0.151	-0.420*	0.116	0.154	-0.512**
BCF	0.106	0.010	0.051	-0.201	0.274	0.782**
HYP	0.209	-0.229	-0.432**	0.002	0.244	-0.294

** and *=Significance at $P<0.01$ and $P<0.05$, respectively; VAR=Variable; HOST=Host-positive spermatozoa; VIT=Vitality; NMA=Normal acrosome; TTM=Total motility; PGM=Progressive motility; STR=Straightness; LIN=Linearity; WOB=Wobble; VCL=Curvilinear velocity; VAP=Average path velocity; VSL=Straight line velocity; ALH=Amplitude of lateral head displacement; BCF=Beat cross frequency HYP=Hyperactivity.

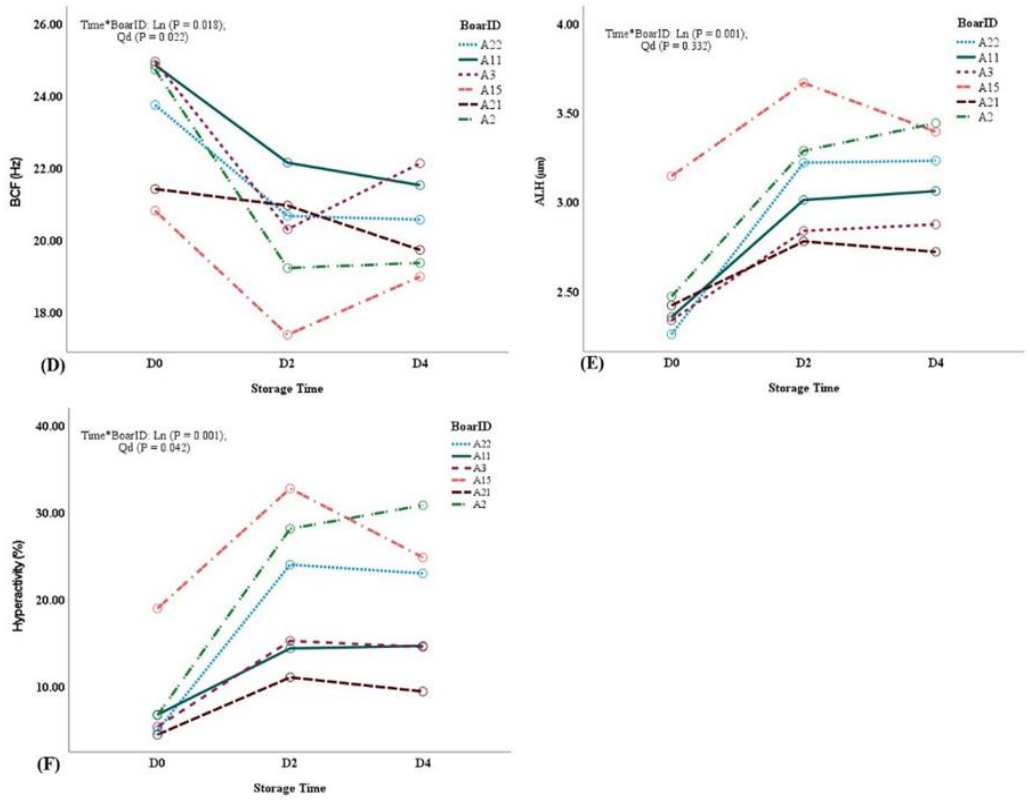

Fig. 2: Differential trending over time for BCF, ALH, and HA of Lusitu boar spermatozoa in semen stored at 17℃ for 4 days. The graphs D, E, and F represent the estimated marginal means of BCF, ALH, and HA, respectively, as measured on Day 0, Day 2, and Day 4. BCF=Beat cross frequency; ALH=Average lateral displacement of the head; HA=Hyperactivity; BoarID=Boar identification; Ln= Linear; Qd=Quadratic; P=P value

DISCUSSION

This study evaluated the stability to storage deterioration of LBS preserved in BTS, a short-term extender. Using the *in vitro* semen characteristics to explore the changes over a period of 4 days, this study has confirmed that the quality of LBS generally reduced as storage time advanced which agreed with earlier findings (Tremoen et al., 2018; Kadirvel et al., 2019). The deterioration in the quality of semen, in turn, leads to compromised fertilizing capacity of spermatozoa (Karunakaran et al., 2017; Hallberg et al., 2024). Notwithstanding the quality changes observed, the current study found LBS and/or spermatozoa with acceptable quality after 4 days of storage in BTS. This points to the relative stability of LBS to storage deterioration for the aforementioned period. Stability for 4 days is important because semen is rarely used for AI on the day of collection, its evaluation for quality as well as transportation to the farm affect availability for use until the day after collection. According to Karunakaran et al. (2017) and Tremoen et al. (2018), semen storage in liquid state for at least 96 hours is preferred for economic reasons. The current study also observed boar influence on most kinematic parameters, which supported the previous findings (De Ambrogi et al., 2006; Mircu et al., 2008; Bielas et al., 2017) and suggested their potential as biomarkers for storability of LBS.

The reduction in mean values of pH, HOST, VIT, and NMA observed as storage time progressed supported the findings of earlier studies on various exotic boars (Apić et al., 2015; Lima et al., 2015; Haque et al., 2018; Khoi et al., 2023). The mean pH reduction was probably attributed to accumulation of metabolic products, such as lactic acid, that resulted from the intracellular glycolytic metabolism taking place during storage at 17℃ and/or spermatozoa demise caused by bacterial proliferation (Paulenz et al., 2000; Gadea, 2003; Khoi et al., 2023). The change in semen pH could have influenced the acrosome status by way of its effect on capacitation (Mishra et al., 2018). Additionally, inducers of premature capacitation and acrosome reaction, such as ROS, also seal apoptotic fate or cell death (Wysocki et al., 2013; Aitken et al., 2015; Karunakaran et al., 2017). It is likely that such processes contributed to the reduction in vitality scores in the current study. As for the reduction in HOST-positive spermatozoa, the decrease in membrane fluidity, ATP content, motility loss particularly the progressive motility pattern, and initiation of oxidative apoptosis resulting from oxidative stress (Bucak et al., 2010; Karunakaran et al., 2017) may be responsible. The adverse spermatozoa membrane damage occurs mostly in boars due to abundance of PUFAs that are susceptible to free radical attack during semen storage (Karunakaran et al., 2017; Gautier & Aurich, 2022).

This study found that the scores for TSCs after 4 days of storage were suggestive of acceptability for use despite the quality reduction with increasing storage time. The reason is that pH, NMA, vitality, and HOST scores recorded after 4 days were within and/or above the limits, viz. 6.8-7.9, 51.0%, 60-90%, and more than 60%, respectively, acceptable for fresh boar semen (Johnson et al., 2000; Paulenz et al., 2000; Rozeboom, 2000; Haque et al., 2018). The current results support Karunakaran et al. (2017) who reported suitability of stored large white Yorkshire semen in BTS for 4 days, probably because of the hybrid status of boars used. However, the current results contradict those of Khoi et al. (2023) who confirmed stability of Yorkshire semen in BTS for 3 days. Notably, this study did not find significant influence of boars on any TSC scores at each storage point, except for the case of TTM and PGM, perhaps due to the protocol, boar fertility status, and sample size used. Accordingly, the methods used to evaluate TSCs may serve to identify poor ejaculates (Singh et al. 2020) but not necessarily predict Lusitu boars with better semen storability. Future studies should explore more reliable basic techniques or improve the existing ones for quality, storability and in vivo fertility prediction.

The motility-related TSCs, including TTM and PGM, also demonstrated significant reduction between storage points and was in agreement with the earlier study findings (Haque et al., 2018; Lucca et al., 2022). The reduction in mean TTM was probably attributed to a progressive decline of nutrients in the extender, pH, loss of ATP, as well as uptake of the Ca^{2+} (Yeste, 2017). Preservation of semen for a longer time results in spermatozoa spending more energy to reactivate their motility (Henning et al., 2022). Despite the observed reduction, mean TTM scores at D4 was still above the 60.0 and 50.0% minimum limit acceptable for liquid-stored and the 3-day stored boar semen, respectively (Johnson et al., 2000; Lima et al., 2015; Singh et al. 2020). The scores for PGM on D2 and D4 were also above the 70% value that was previously found to maintain acceptable in vivo fertility (Gottardi, 2020). In terms of boar influence, significance was observed on both TTM and PGM which agreed with Bielas et al. (2017) even though the storage time and boar interaction was not observed. The current study suggests PGM parameter as a potential indicator for storability, however, reliable deductions will require additional studies (Lucca et al., 2021).

The VCL, VAP, VSL ALH, BCF, and HYP of spermatozoa were the main motility parameters considered for storability evaluation because of their correlation with spermatozoa morphometric characteristics in another study on Lusitu boars (Abigaba et al., 2024). Moreover, Johnson et al. (2000) recommended the study of different spermatozoa movement forms, especially for boars because their spermatozoa exhibit a higher percentage of circular movement. The general increase in mean VCL and VAP with storage time supported the findings of Tremoen et al. (2018) except for the current VSL whose values reduced. The increase in VCL scores could be related to the growing number of spermatozoa that acquired a 'hyperactive motility' status. Coincidentally, this study confirmed a general raise in the proportion of HYP in most boars as storage time increased. The current findings support those of Schmidt and Kamp (2004) and Sansegundo et al. (2022) who found higher mean values of

VCL and VAP in hyperactive compared with the case of non-hyperactive spermatozoa. Similarly, observations on ALH and BCF are in line with those of Schmidt & Kamp (2004) and Bielas et al. (2017). This study has demonstrated potential of these parameters as storability biomarkers for LBS considering the observed influence of storage time and boar factors.

It is noteworthy that no significant differences were observed between the D2 and D4 scores for these kinematics, including VCL, VAP, VSL, ALH, BCF, and HYP, which probably suggested a considerable similarity in terms of the potential fertility when a 2- and 4-days' stored LBS dose is used. According to Karunakaran et al. (2017), most inseminations are performed using boar semen stored for 2 days, hence the basis for the current deduction on the mean values after D2 and D4 of storage. It remains to be established whether this non-significant difference between the two storage points was attributed to the boar genotype, extender type, test method or nature of specific parameter used. This notwithstanding, the findings of this study suggested suitability of LBS for use post-storage in BTS extender within 4 days. The observations on these kinematic parameters also supported the findings and/or deductions on the TSCs on Day 4 of storage. Consideration of some kinematics like VCL, VAP, ALH, and HYP during LBS evaluation is necessary because of their correlation with *in vivo* fertility (Mircu et al., 2008; Vyt et al., 2008; Broekhuijse et al., 2012).

Spermatozoa HYP was suggested for use during boar semen quality evaluation (Mircu et al., 2008; Akthar et al., 2024), hence the adequate attention given to this parameter in the current study. The initial sharp increase in the mean HYP observed for each boar was perhaps attributed to temperature and pH changes; these induce Ca^{2+} influx, capacitation, and finally acrosome reaction and HYP (Mircu et al., 2008; Martin-Hidalgo et al., 2018). However, the mean HYP generally waned between D2-D4 except for A11 that gradually increased. This reduction could be attributed to a decline in the nutrients, ATP levels, pH, as well as the uptake of Ca^{2+} that occurs with increasing storage time (Mishra et al., 2018; Sansegundo et al., 2022; Akthar et al., 2024). It is probable that the trend for VCL and ALH evaluations relate to the foregoing since they are descriptors of HYP. The boar (A15) with the highest initial mean HYP score showed a corresponding mean reduction between D2-D4 probably because of the poor quality spermatozoa produced. Notably, premature hyperactivation results in a smaller number of intact spermatozoa available for interaction with the oocyte (Holt et al., 1997). Hence HYP and its descriptors may be used to predict a boar with poor ejaculate quality and/or storability on the basis of observed initial mean scores and trend over time.

The correlation between the TSCs with kinematics and/or HYP was explored in relation to ejaculate quality rather than storability evaluation. Therefore, the current study confirmed that LBS with more hyperactive spermatozoa had a lower number of live cells. Additionally, the relationship between the VIT with VCL and ALH was also negative, and supported Schmidt and Kamp (2004) who used VCL and ALH to describe HYP in boar spermatozoa. In view of this, LBS with low VIT scores is undesirable since premature spermatozoa hyperactivation is associated with reduced fertility (Holt et al., 1997). Moreover, this study confirmed a positive correlation between HYP with the ALH and VCL of spermatozoa in LBS. As expected, a negative association between the PGM with HYP relates to the variation in spermatozoa movements, which is more round in the case of HYP due to asymmetric tail movement. The correlations between NMA with VSL, pH, and HOST suggest preference for higher NMA scores of spermatozoa during evaluation of LBS despite the observed contradiction with Holt et al. (1997) in terms of its association with PGM and VAP. The observed findings on parameter correlations suggest preference for higher VIT and PGM as well as lower VCL, ALH, and HYP values of spermatozoa in LBS during quality evaluation prior to storage.

Conclusion

The quality of liquid-stored LBS was investigated for 4 days. It has been established that semen quality reduced with increasing storage time but final scores of the TSCs after 4 days revealed suitability for use. This study also confirmed a non-significant difference between D2 and D4 scores of the kinematic and HYP parameters evaluated. The TSCs viz. vitality, NMA, and PGM were found to be correlated with a number of kinematic parameters. The current findings revealed potential of some TSCs for use in deciphering the LBS quality prior to storage, while HYP and many kinematic parameters may serve as candidates for storability prediction. However, further studies must be conducted to establish the cut-off limits of each parameter for efficient utilization and standardize their methods of determination for reliable results. Additionally, the identification of potential protein or lipid biomarkers that may be associated with the observed relative stability of LBS to storage conditions is suggested.

Authors' Contribution: RA conceived the idea and designed the study, collected and analyzed data, and wrote the manuscript draft; PCS and ESM designed and supervised the study and reviewed the manuscript; PHN and WNMM supervised the study. All authors read the final draft and approved the manuscript submission for publication.

Conflict of interest: All authors declare that there is no conflict of interest in the work presented in this manuscript.

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