Received: 31 January 2023 | Revised: 27 April 2023 | Accepted: 30 April 2023

DOI: 10.1111/rda.14374

Reproduction in Domestic Animals WILEY

ORIGINAL ARTICLE

Reaction time as a libido indicator and its relation to pre-freeze and post-thaw sperm quality in bulls

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Abstract

Libido and sperm quality output relationship is already not clear in farm animals. The present study compared reaction time (RT) as a libido indicator and the pre-freeze and post-thaw sperm quality of AI bulls. Before the collection of ejaculates ($n = 53$, from 22 AI bulls [4.2 ± 1 years of age]), RTs were collected using a chronometer as the interval between the bull's arrival at the semen collection area and his first false mount (FM) on another male. The ejaculates were examined for their volume, concentration and motility. Subsequently, all aliquots were diluted with a commercial semen extender and equilibrated for 3 h before freezing. Frozen semen samples were thawed and examined for sperm kinematics using CASA, plasma membrane and acrosome integrity of sperm (PMAI) by flow cytometry. Additionally, the temperature humidity index (THI) values were assessed during the study. Multiple linear regression analysis was used to analyse the data. The results indicated that THI had a significant effect on libido ($p < .001$). However, libido had no effect on either pre- or post-thaw sperm quality parameters except for the velocity of the average pathway (VAP) ($p < .05$). Therefore, relying solely on RT –libido- as an indicator of bull sperm quality at AI stations may not be reliable, as it is a complex behavioural assessment.

KEYWORDS
Bos taurus, ejaculate, heat stress, sexual behaviour, THI

1 | INTRODUCTION

Male fertility is one of the main factors that affect the livestock industry. In this context, bull sperm quality is crucial for successful breeding programmes and the genetic improvement of cattle populations. Currently, frozen semen from various bulls selected for this purpose is being transferred throughout the world with increasing demand. Their traits, such as milk and meat productivity, adaptability, disease resistance and improvement capacity, are essential factors for this trading. To achieve these traits, the optimum semen quality should be presented for every straw. Therefore, the selection of bulls and processes during collection and storage play a vital role in sustainable production. However, an accurate and reliable assessment of bull fertility in every straw is challenging for AI stations. There is a great need for accurate and reliable indicators of bull fertility that can help identify the best bulls for breeding programmes and ensure semen quality. The use of such indicators can not only improve the efficiency of breeding programmes but also reduce the

costs associated with suboptimal semen quality and fertility. In this regard, various studies have been conducted to explore different parameters that could serve as indicators of bull fertility at the beginning of their productive life and in subsequent years, including breeding soundness evaluation (BSE), reaction time, libido score and sperm quality (Chenoweth, 1983; Hoflack et al., 2006; Landaeta-Hernández et al., 2001; Singh et al., 2015).

Libido is defined for mammalian species as the likelihood of showing sexual behaviour at a given opportunity. This behaviour starts at puberty, with some particular expression due to various epigenetic and associated hormonal mechanisms, particularly testosterone, in males. It plays a significant role in farm animals as it affects reproduction, livestock sustainability and profitability (Wodzicka-Tomaszewska et al., 1981). Recent studies have revealed a strong correlation between male libido, semen quality, and fertilization rate (Bittar et al., 2015; Chacón et al., 2022; Jezierski et al., 1989; Kowalczyk et al., 2021; Singh et al., 2015, 2020). Although observing natural reproduction is the most reliable way to measure libido, the disease-free condition of AI bulls does not allow this type of evaluation (Chenoweth, 1983). Reaction time (RT) is a simple and reliable method for monitoring this industry (Singh et al., 2015). Nevertheless, there are several challenges to determining the relationship between bull libido and sperm quality. It is difficult to explain the relationship between sperm quality and libido because of factors such as age, breed differences, social effects and rearing types, as well as changes brought on by the physiological condition of the animals, including defects that cause pain or discomfort during movement, stress, and obesity (Chenoweth, 1983). Additionally, parameters such as temperature, humidity and location differences can affect libido within a group, and individual lameness, disability and aggressiveness can have permanent or temporary effects on this behaviour (Hürland et al., 2023; Jezierski et al., 1989; Petherick, 2005).

In order to differentiate the spermatological quality of bulls in AI centres, it is thought that determining the libido score, which has individual differences, and transforming it into a selection criterion will contribute to the profitability of the AI centre. Thus, the aim of the present study was to define the relationship between libido by measuring RT and sperm quality parameters in AI bulls.

2 | MATERIALS AND METHODS

2.1 | Animals

Semen samples were collected from 22 (Simmental ($n=10$), Brown Swiss ($n=10$), and Holstein Friesian ($n=2$)) AI bulls (4.2 ± 1 years of age) which maintained in the Lalahan International Center for Livestock Research and Training. The bulls were on a regular semen collection schedule, had passed a standard BSE, and had produced sperm with acceptable post-thaw characteristics (progressively motile sperm>75%), as well as requirements of routine disease control. Semen samples were collected twice weekly, with no apparent changes in animal health or semen quality throughout the semen collection interval. Additionally, THI values for AI station location were collected from the Turkish State Meteorological Service, and all data were evaluated for heat stress.

All bulls participated in the study while their semen was regularly collected at an artificial insemination station. Ejaculates that were collected for commercial use contained biological samples. For this experiment, there was no further handling of the animals; hence, an ethics committee's clearance was not deemed essential.

2.2 | RT and semen collection, evaluation and cryopreservation

RT of each bull was recorded for 13 weeks, and every collection of 53 ejaculates has optimum conditions for cryopreservation. RTs were recorded using a chronometer as the interval between the bull's arrival at the semen collection area and his first false mount (FM) on another male. After the first FM, semen was collected from each bull using an artificial vagina. Sperm motility was assessed subjectively by using a phase contrast microscope with×100 magnification. Sperm concentration was determined using an Accucell photometry device (IMV). Andromed (Minitüb GmbH) was used as an extender for diluting ejaculates with >75 progressive motile sperm to a final concentration of 20×10^6 million sperm/straw. Aliquots were cooled at +4°C for 3 h as an equilibration phase. Afterwards, they were packaged in 0.25 mL straws using an automatic straw filling and sealing machine (MX4; IMV). Straws were frozen to −140°C using an automatic freezing device (Digital cool 5300ZB 250; IMV), submerged in liquid nitrogen, and then kept at −196°C. For post-thaw sperm quality assessments, the straws were thawed in a water bath (37°C, 30 s) after 1 month of storage.

2.3 | Computer-assisted sperm analysis

The IVOS I CASA (Hamilton Thorne Inc.) system was used to assess the sperm motility characteristics of post-thawed bull semen samples. 12.2. software version was used to perform the analysis of motility. Sperm motility features were assessed at 30 frames/s and 60 Hz; spermatozoa with straight line velocity (VSL) 70% and average path velocity (VAP) 50 μm/s were classified as progressive by using an objective at 37°C and 10× magnification and using a 5 μL post-thawed semen sample on a prewarmed 20 μm-deep semen analysis Leja 4-chamber slide (Leja) (Şahi̇n et al., 2020).

2.4 | Flow cytometric analysis

Cytoflex Flow Cytometer was used to perform the flow cytometry analysis (Beckman Coulter). Semen samples were examined using a laser beam at 488 nm (50 mW laser output) with emission filters at 525 nm, 585 nm and 610 nm. Data were collected from 10,000 events after gating correctly.

The FITC-PNA/PI double staining method was used to assess the percentage of sperm acrosome and plasma membrane integrity. For this purpose, 2.5 μL of FITC-PNA (100 μg/mL) and 1.5 μL PI (2.99 mM) were added to 245 μL of buffer solution (PBS), prepared with 10 μL of semen previously diluted in phosphate-buffered saline solution, to obtain a final concentration of 5×10^6 sperm/mL.

All samples were incubated for 15 min at 37°C. CytExpert 2.2 software was used to assess the analyses (Beckman Coulter).

2.5 | Statistical analysis

The variables were primarily evaluated in terms of parametric test assumptions. The relationships between the RT (the dependent variable) and semen quality characteristics and THI (independent variables) were examined using multiple linear regression analysis. Backwards stepwise method was used in the regression analysis. The autocorrelation of the residuals was evaluated using the Durbin-Watson test, and the multicollinearity of the independent variables was evaluated using the Tolerance and VIF values. Descriptive statistics were calculated (mean, standard deviation). Data were analysed for correlations by using Spearman's rho. SPSS® 22.0 (IBM) for Windows was used for all analyses, and figures were generated using GraphPad Prism software version 9.02 for Windows (GraphPad Software). Differences with $p < .05$ were considered statistically significant.

3 | RESULTS

The average time to ejaculation was 86.26 ± 104.42 s (s) with a range of 6–300 s. Figure 1 shows descriptive statistics. Before performing to the multiple linear regression analysis, the assumptions of the regression analysis were checked. For this purpose, the autocorrelation of the residuals was examined by Durbin-Watson test, and the multicollinearity problem was examined by VIF and tolerance values. As a result of the Durbin-Watson test, it was determined that the residuals were not related to each other. As a result of the analysis that performed to determine the multicollinearity problem, VSL, ALH and STR were not included in the multiple linear regression model since their VIF values were > 10. The regression model created with the remaining sperm motility characteristics and THI was shown in Table 1. In addition, as a result of the multiple linear regression analysis, the variables that were not statistically significant were shown in Table 2.

VAP ($p = .033$) and THI ($p < .001$) were found to be statistically significant on RT. It was revealed that the variables given in Table 2 had an effect of 36% on RT and the established model was statistically significant ($R^2 = .362$) ($p < .001$). The model created as a result of the regression analysis was shown in the equation below.

$$RT = -312.065 + (1.894 \times VAP) + (4.373 \times THI).$$

According to this model, one-unit increase in THI and VAP increased RT by 4.373 and 1.894 units, respectively.

There was also no correlation between RT and sperm quality parameters except beat cross frequency (BCF) ($r = -.323$, $p < .05$), STR

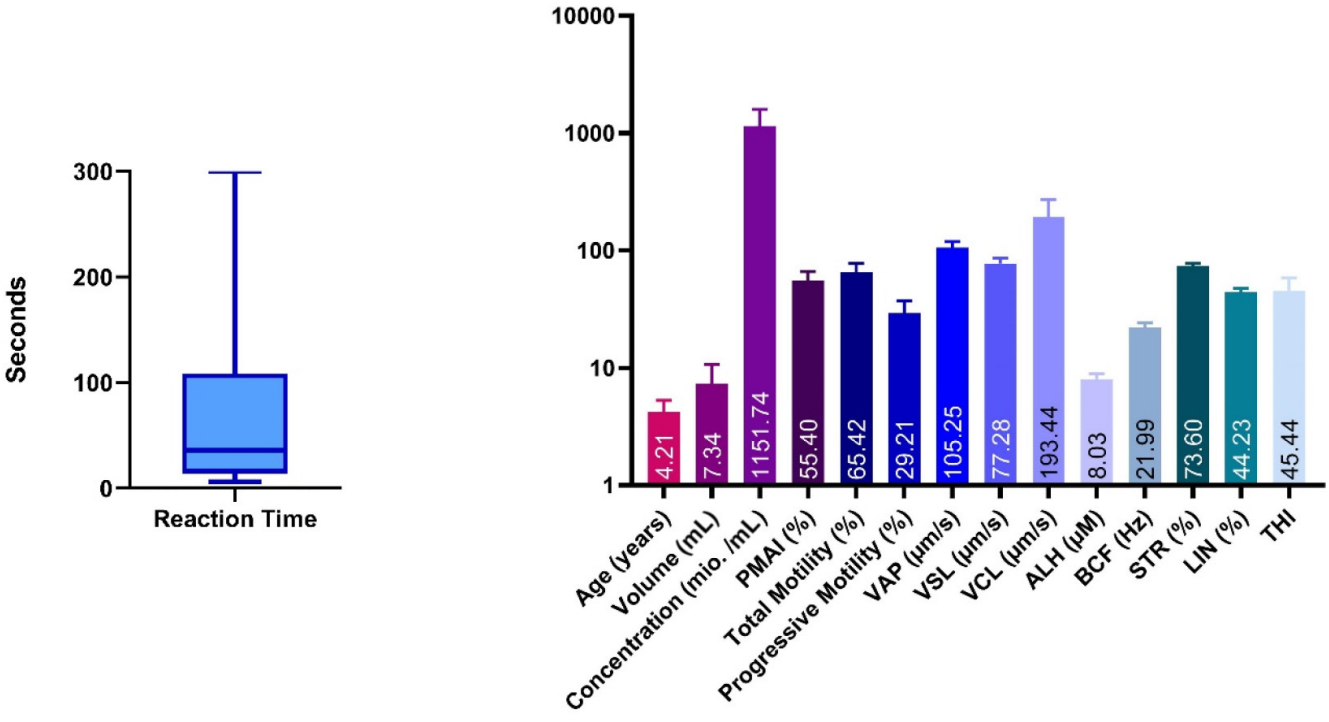

FIGURE 1 Descriptive statistics of RT, sperm quality parameters and THI. (Reaction time (RT), Plasma membrane and acrosome integrity (PMAI), Velocity Average Path (VAP), Velocity Straight Line (VSL), Amplitude of Lateral Head Displacement (ALH), Beat Cross Frequency (BCF), Velocity Curve Linear (VCL), Straightness (STR), Linearity (LIN) and Temperature Humidity Index (THI)).

TABLE 1 Result of multiple linear regression model (Velocity Average Path (VAP)) and the environmental variable (Temperature Humidity Index (THI)).

Variables	β	SE	t	p
Constant	−312.065	100.548	−3.104	.003
VAP (μm/s)	1.894	0.863	2.196	.033
THI	4.373	0.897	4.876	<.001

Note: $R^2 = 0.362$ ($F = 14.202$; $p < .001$).

Abbreviations: β, regression coefficient; SE, standard error; t, t statistic.

TABLE 2 The variables removed from regression model (Pre-freeze sperm traits; Volume, Concentration, and post-thaw sperm quality parameters; Total motility, Progressive motility, Plasma membrane and acrosome integrity (PMAI), Beat Cross Frequency (BCF), Velocity Curve Linear (VCL), Linearity (LIN)).

Variables	β	t	p
Age	−0.120	−1.061	.294
Volume (mL)	−0.041	−0.360	.721
Concentration (M /mL)	−0.082	−0.722	.474
Total motility (%)	0.025	0.192	.849
Progressive motility (%)	−0.038	−0.302	.764
PMAI (%)	−0.111	−0.942	.351
VCL (μm/s)	0.012	0.101	.920
LIN (%)	0.013	0.082	.935
BCF (Hz)	−0.120	−1.006	.319

Abbreviations: β, regression coefficient; t, t statistic.

($r = -.379$, $p < .01$), linearity (LIN) ($r = -.277$, $p < .01$), and THI effect ($r = .528$, $p < .01$) (Table 3).

4 | DISCUSSION

The relationship between behaviour and biological parameters has attracted the attention of researchers for many years. In particular, the effect of sexual behaviour on reproductive outcomes is a comprehensive issue. In a couple of studies, libido was chosen as an indicator in conducting a correlation between animal sexual behaviour and sperm quality (Hoflack et al., 2006; Silva-Mena et al., 2000). However, it may be misleading to evaluate the short-term effects of behavioural effects on the organism only from quantitative outputs. In this study, we evaluated the relationship between libido and first-stage tests for both pre-freezing and post-thaw (EV and concentration, post-thaw motility parameters, and PMAI) to determine the quality of sperm production stations.

RT has been used as a direct indicator of libido (Moghaddam et al., 2012; S. Singh et al., 2015; Solanki et al., 2019). However, various researchers have defined RT differently. Some of them have defined it as the interval between the arrival of the bull at the semen collection area and his first false mount (Singh et al., 2015); others part of them have defined it as a time lapse between the exposures of the bull to the phantom bull till successful ejaculation (Solanki et al., 2019). Apart from them, another group used animals of different sexes to define reaction time (Silva-Mena et al., 2000). Regarding our results, we could not find any RT effect on either EV or spermatozoa concentration ($p > .05$). Furthermore, we could not find any relationship between post-thaw total motility parameters, except VAP ($p < .05$). Silva-Mena et al. (2000) have found a relationship between libido and serving capacity in Bos Indicus. The libido was evaluated with a female teaser prior to the mating activity. The presence of a female by the bull would affect the libido and output of the study. However, our study was conducted with AI bulls and a phantom bull. Therefore, sexual behaviours can be presented in different dimensions during hetero- and homosexual impressions. Various studies on AI bulls have been exposed to females during the collection period, and their effectiveness has been well presented (Chenoweth, 1983; Mondal et al., 2019). In addition to the female influence, numerous variables, like age, breed, ejaculate collection interval and frequency, and accessory gland development, can impact EV (Snoj et al., 2013). In this context, Savic and Petrovic (2015) have shown that boars' EV depends on multiple parameters, including the breed, season and collector. In another study, Singh et al. (2015) could not find any statistically significant effect of RT on EV. Therefore, it is difficult to conduct any relation between two parameters, such as ejaculate volume and libido.

Sperm concentration has a vital role with EV for AI stations to keep production at high levels. In this study, concentration values showed no significant correlation with RT ($p < .05$). However, we also know that concentration has a strong relationship with age, breed, collection frequency, and feed regime (Islam et al., 2018; Rehman et al., 2016; S. Singh et al., 2015). Therefore, it can be concluded, like EV values, that libido is not one of these multiple factors, such as genetic and paragenetic (age, collector, collection interval, etc.) traits that affect the ejaculation output.

In our study, sperm motility parameters were assessed in pre-freezing and post-thaw samples. Total sperm motility values were not affected by RT ($p > .05$). Various studies have shown that libido affects sperm motility as well as other sperm quality parameters (Islam et al., 2018; S. Singh et al., 2015). In these studies, motility increased with an increase in libido, EV, and concentration. According to our results, motility showed the same stability as other parameters. It can be speculated that stability depends on other parameters, such as EV and concentration. This relation was shown by Singh et al. (2015) by using the "sperm motility index (SMI)" which includes the parameters of sperm analyses and integrates them into a numerical value. It considers parameters, such as normality, motility and progressivity. The study presented the correlation between concentration and SMI and, subsequently, motility. In our study, there is an effectiveness of libido on VAP values ($p < .05$). VAP, or average path velocity, is one of the several parameters used to evaluate sperm motility. It measures the average velocity of a sperm cell along its actual curvilinear trajectory. It is reported that VAP value with other sperm kinematics are highly positively correlated with pregnancy rates and sperm

TABLE 3 Correlations among Volume, Concentration, Total motility, Progressive Motility, Plasma membrane and acrosome integrity (PMAI), Velocity Average Path (VAP), Velocity Straight Line (VSL), Amplitude of Lateral Head Displacement (ALH), Beat Cross Frequency (BCF), Velocity Curve Linear (VCL), Straightness (STR), Linearity (LIN) and Temperature Humidity Index (THI).

	Reaction time (sec)	Age (months)	Volume (mL)	PMAI (%)	Concentration (M/mL)	Total motility (%)	Progressive motility (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LİN (%)	THI
Reaction Time (sec)	1	−0.091	0.145	−0.003	0.014	0.095	−0.087	0.251	0.118	0.214	0.128	−0.323[a]	−0.379[b]	−0.277[a]	0.528[b]
Age (months)		1	−0.094	−0.387[b]	0.116	−0.216	−0.195	−0.017	−0.127	0.043	0.045	−0.168	−0.139	−0.121	0.118
Volume (mL)			1	0.281[a]	0.039	0.115	0.125	0.051	0.116	−0.013	−0.030	−0.040	0.024	0.090	0.158
PMAI (%)				1	−0.021	0.684[c]	0.737[c]	0.244	0.333[a]	0.154	0.049	0.279[a]	0.025	0.018	−0.138
Concentration (mio. /mL)					1	0.325[a]	0.079	−0.010	−0.183	−0.031	0.036	−0.297[a]	−0.388[b]	−0.252	−0.179
Total Motility (%)						1	0.807[c]	0.402[b]	0.371[b]	0.267	0.219	0.074	−0.305[a]	−0.218	−0.132
Progressive Motility (%)							1	0.315[a]	0.467[c]	0.188	0.091	0.347[a]	0.115	0.092	−0.276[a]
VAP (μm/s)								1	0.913[c]	0.937[c]	0.898[c]	−0.368[b]	−0.624[c]	−0.659[c]	0.030
VSL (μm/s)									1	0.828[c]	0.775[c]	−0.187	−0.297[a]	−0.370[b]	−0.045
VCL (μm/s)										1	0.881[c]	−0.390[b]	−0.649[c]	−0.712[c]	0.010
ALH (μm)											1	−0.398[b]	−0.672[c]	−0.777[c]	−0.035
BCF (Hz)												1	0.665[c]	0.518[c]	−0.180
STR (%)													1	0.907[c]	−0.194
LIN (%)														1	−0.131
THI															1

[a]Correlation is significant at the 0.05 level (2-tailed).

[b]Correlation is significant at the 0.01 level (2-tailed).

[c]Correlation is significant at the 0.001 level (2-tailed).

migration ability in the female genital tract (Cox et al., 2002; Tekin & Daşkın, 2016). However, there is no direct research evidence linking sperm VAP values and fertility alone. Therefore, further research is needed to determine the correlation among libido, sperm kinetic parameters such VAP and fertility.

The effect of climate on reproductive output and sexual behaviour of mammalian species has been well documented before (Abdelnour et al., 2021). According to our results, low THI values correlated with low RT. It can be explained by heat stress's effectiveness on the reproductive system and hormonal mechanisms. THI is a vital heat stress metric, and high THI values can affect sperm quality. In our study, parallel with Llamas-Luceño et al. (2020), there is a correlation between progressive motility and THI. We have also shown the THI effect on libido like Hürland et al. (2023). It can be explained by heat stress effect on hormonal levels, especially "testosterone" in bulls (Ferrer et al., 2022). High levels of this stress impact sperm quality and quantity as described before (Al-Kanaan et al., 2015; Llamas-Luceño et al., 2020) Therefore, low levels of THI are suitable for presenting a short time of RT as a libido indicator in bulls.

In conclusion, the results of our study showed that libido does not affect either sperm productivity output, post-thaw sperm motility, or PMAI. Additionally, a negative impact of heat stress on sperm productivity of AI bulls was observed. However, a negative correlation was detected between the reaction time and sperm kinetic values of the BCF, STR and LIN. Therefore, it is important to assess the contribution of libido to sperm production and post-thaw quality using additional sperm quality indicators such as in vitro conception rates and field outcomes such as non-return rates.

AUTHOR CONTRIBUTIONS

FK: Designed the study, collected data and semen, performed sperm quality analyses, wrote the original draft, and reviewed and edited the paper; İB: Collected data, semen and performed sperm quality analyses, reviewed and edited the paper; DŞ: Collected data and performed sperm quality analyses; SŞ: Collected data and performed sperm quality analyses; UK: performed statistical analyses, prepared tables and reviewed and edited the paper, and; MS: Conducted the study and reviewed and edited the paper.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Korkmaz, F., Baştan, İ., Şahin, D., Şimşek, S., Kaya, U., & Satılmış, M. (2023). Reaction time as a libido indicator and its relation to pre-freeze and post-thaw sperm quality in bulls. *Reproduction in Domestic Animals*, *58*, 965–971. https://doi.org/10.1111/rda.14374