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ORIGINAL ARTICLE

Evaluation of domestic animal sperm head morphology via flow cytometric DNA labelling and pulse shape analysis using bull and stallion spermatozoa as model species

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Abstract

The aim of the present study was to test a rapid, robust flow cytometric technique for the detection of sperm head abnormalities of domestic bulls and stallions. The so-called PulSA approach detects the pulse profiles of propidium-iodide labelled spermatozoa. In the first experiment, species-specific threshold values were established on sperm samples that were tested for sperm head abnormalities with a classic visual morphology analysis. In the second experiment, serial mixtures of bull and stallion spermatozoa mimicking different percentages of sperm head abnormalities were analysed. Non-metric multidimensional scaling showed a clear separation between the normal and mixed samples. The PulSA approach may be a useful tool in identifying sub- or infertile breeding males as well as in studying the evolutionary aspects of sperm morphology and morphometry.

KEYWORDS
abnormalities, bull, PulSA, sperm quality, stallion

1 | INTRODUCTION

Semen doses for artificial insemination are usually evaluated based on semen volume, concentration, and motility (Rodriguez-Martinez, 2013). However, one of the most essential sperm attributes, sperm morphology is not always examined routinely, although several studies demonstrated a significant relationship between morphology and fertility (Al-Makhzoomi et al., 2008; Love, 2011; Nagy et al., 2013). Sperm morphology also indicates genital dysfunctions (Veeramachaneni & Sawyer, 1996). The classic subjective assessments of sperm morphology with light microscope have widely varying results because of the different techniques, smear preparation and the different classification systems (Baker & Clarke, 1987; Ombelet et al., 1997). The evaluation is poorly repeatable (Jequier & Ukombe, 1983; Zaini et al., 1985), and in some cases, lacks statistical power because counting too few spermatozoa (Kuster et al., 2004). Evaluating the sufficient number of spermatozoa is very time-consuming.

The need for quick, accurate, precise and repeatable methods has led to the development of automatized techniques. Computer-assisted sperm morphometry (ASMA) was used for human sperm morphology examination (Kruger et al., 1993) and later adapted to different mammalian species, for example, bull (Gravance et al., 1996), ram (Gravance et al., 1998), goat (Hidalgo et al., 2006) and rabbit (Gravance & Davis, 1995). The conventional ASMA technique combines a light microscope with image analysis software to detect the spermatozoa and measure the essential sperm head parameters such as length, width, area, and perimeter or other subdomains, like acrosome, midpiece or flagellum (Yániz et al., 2015). The ASMA method is a reliable, precise technique with good repeatability (Hidalgo et al., 2006). However, because of the different staining methods, it is difficult to compare the results of different studies (Gravance et al., 1998).

Another automated technique is flow cytometry, which has become an important analytical tool for animal andrology research and routine assessment at semen collection centers. The flow cytometer can evaluate multiple parameters at the same time for every cell (Hossain et al., 2011). This method is quick and precise, it can measure several thousands of cells within seconds (Martínez-Pastor et al., 2010). Due to the recent advances in hardware and fluorescent probes, flow cytometry is gradually replacing some other time-consuming techniques (Hossain et al., 2011).

However, one of the most important attributes, sperm morphology cannot be evaluated with the current cytometric assays. A quick, more precise, and automated technique would be beneficial for research and the breeding industry as well. Slit-scan flow cytometry (SSFCM) was successfully used to measure head shapes of sperm from several mammalian species (mouse, rabbit, bull, and hamster) detecting fluorescence profiles of sperm heads stained with DNA-specific fluorescent dye (Benaron et al., 1982; Gledhill, 1983). SSFCM could be used to detect abnormal sperm head shapes (Halamka et al., 1984). However, most of the recent models of benchtop flow cytometers lack the slit-scan detection option, therefore these instruments are not able to carry out such analyses.

An alternative flow cytometric approach was used to analyse particles of different sizes with conventional cytometers (Hoffman, 2009). This so-called PulSA (pulse shape analysis) method was also used for tracking intracellular protein trafficking in cells based on the use of pulse width and height measurements of fluorescently labelled proteins (Ramdzan et al., 2012).

Our aim was to test the applicability of the PulSA cytometry on sperm morphology by analysing mixed sperm samples from two model species, domestic bull and stallion, where different percentages of spermatozoa from another species imitating a real-life scenario of different percentages of sperm head abnormalities.

2 | MATERIALS AND METHODS

2.1 | Semen samples, experimental design

In experiment 1, we established the typical PulSA characteristics for bulls and stallions with normal sperm head morphology. To measure intra- and interspecific variation as suggested for genome size variation analysis by Dolezel and Bartos (2005), and adapted for animal sperm cytogenetic analysis by Nagy et al. (2016), three frozen–thawed semen batches were used from three bulls and three stallions, tested for sperm head abnormalities as described below. No ethical approval was required as all semen batches were collected and processed in commercial artificial insemination centers, according to the routine procedure of the center.

In experiment 2, we have done serial mixing of sperm from the two species, approximately 0%–100%; 25%–75%; 50%–50%; 75%–25%; 100%–0% bull and stallion sperm, imitating a real-life analysis with sperm samples containing different percentages of "abnormal" cells. The exact cell concentration of each semen batch was determined with a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) prior to mixing. These mixed samples were used in three repeats to test the repeatability of the PulSA approach.

2.2 | Sperm staining

Sperm was labelled with propidium iodide (PI) as part of the FXCycle PI/RNase kit (F10797, Invitrogen). Spermatozoa were fixed with 70% ethanol for 15 min, then washed from fixative (centrifuged at 300 × g for 10 min at room temperature). Five hundred µL of FXCycle PI/RNase staining solution was added to the cell pellets (approximately 1×10^6/mL spermatozoa). The samples were incubated for 60 min at room temperature in the dark before flow cytometric analyses.

2.3 | Flow cytometry measurement

The measurements were made using a BD FACSVerse flow cytometer (Becton Dickinson Biosciences) equipped with standard optics. Propidium iodide was excited with 20 mW blue laser at 488 nm. PI fluorescence pulse height (H), area (A) and width (W) values were detected on detector FL3 700/54 nm. The acquisitions were done using FACSSuite software (Becton Dickinson). Acquisitions were stopped after recording 100,000 events. Every sample contained Rainbow Calibration Particles (8 peaks), 3.0–3.4 µm (559,123, BD Biosciences) as internal control to monitor random instrument drift.

2.3.1 | Flow cytometric data analysis

To collect the pulse shape properties, the following gating method was applied (Figure 1.). First, a "Sperm" region was drawn, based on forward vs. side scatter properties (A). The sperm gate was applied to the FL3-A vs. FL3-H dot plots (B), where doublet discrimination was done. "Singlets" region contained the single sperm cells. This region was applied to the FL3-H histograms where the "Main Population H" region was drawn to identify sperm heads with similar orientation (C). This was applied as gate to the FL3-W histogram, where main peak identification was done (D). The FL3-W histogram coefficient of variation (CV) and median values were recorded.

In experiment 1, individual FL3-W histograms were used as described above. Histogram profiles were compared with Kolmogorov–Smirnov statistics using FlowJo™ v10.8 Software (BD Life Sciences) to reveal the maximum difference (D_{max}) between two histograms (Young, 1977). The D_{max} values were analysed for normality using the Shapiro–Wilks test. Descriptive statistics were made with R statistical software (version 3.6.1.). For the following experiments species-specific D_{max} threshold values were calculated as mean + 2SD (Indrayan, 2012).

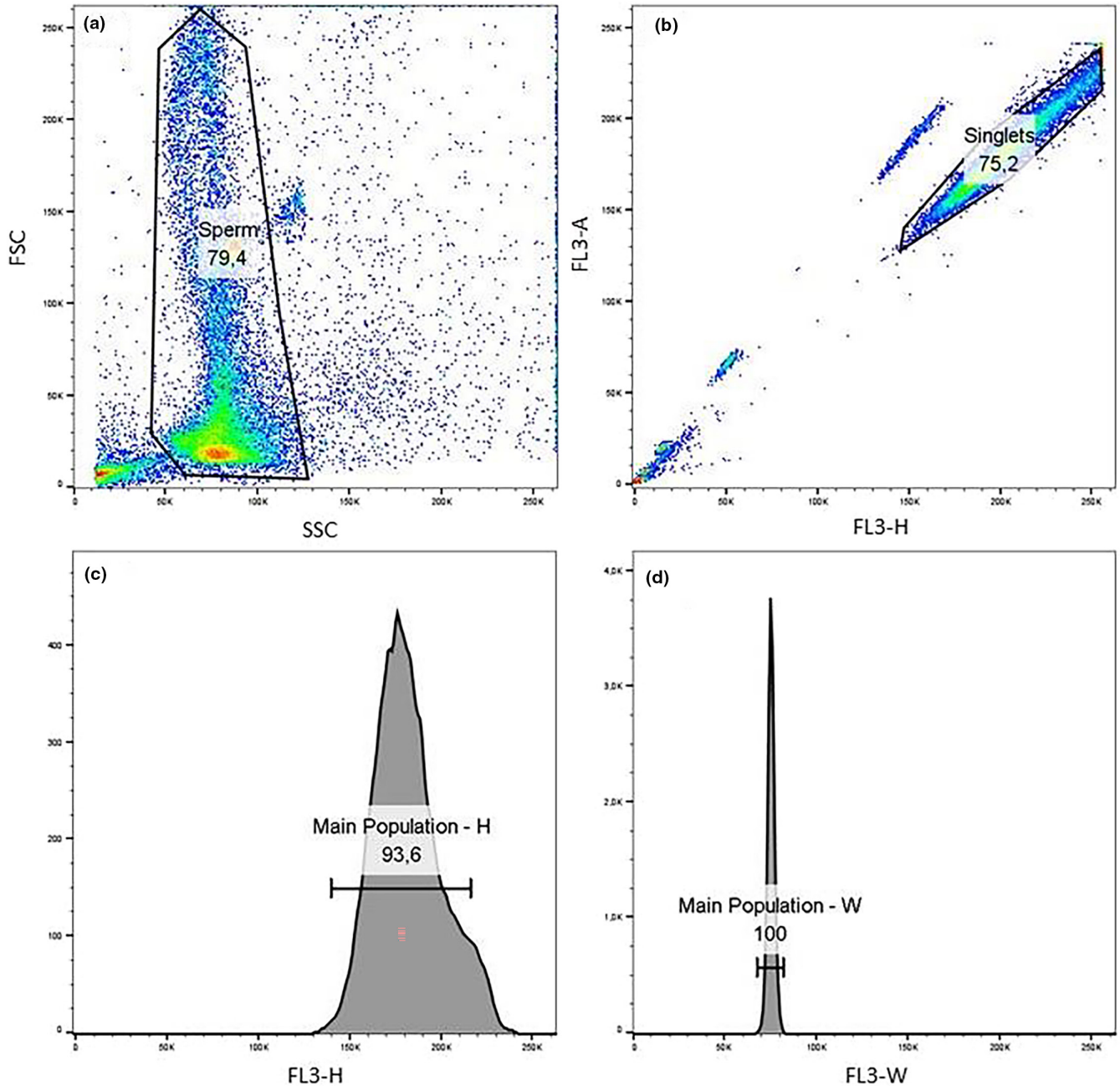

FIGURE 1 Dot-plots and histograms showing the successive steps of gating.

Alternatively, non-metric multidimensional scaling (NMDS, Hout et al., 2013) was done in R statistical software (3.6.1 version, FlowCyBar package 1.22.0) on individual histograms to see whether the two species form two separate populations based on the CV and median values of the histograms (Schumann et al., 2023).

In experiment 2, the histogram profiles of the mixed bull-stallion samples were compared to the 100% bull and 100% stallion samples using the Kolmogorov–Smirnov test as described above. The threshold D_{max} value to differentiate abnormal (mixed samples) and normal morphology was derived from descriptive statistics in experiment 1 as described above. Non-metric multidimensional scaling was also used to differentiate between normal and abnormal sperm samples.

2.4 | Morphology evaluation

In order to exclude sperm samples that contain large percentage of sperm head abnormalities, microscopic morphology evaluations were done. Smears of thawed sperm samples were made on clean glass slides. Williams-stained slides were examined by light microscopy by skilled technicians for the following morphological defects in 500 spermatozoa: pear-shaped heads, heads narrow at the base,

TABLE 1 Sperm morphology analysis outcomes.

Sample	Pyriform (%)	Narrow at base (%)	Abnormal contour (%)	Underdeveloped (%)	Detached head(%)	Narrow (%)	Heads of variable size (%)	Abaxial (%)	Normal (%)
Stallion 11.	2.8	2.8	0.2	0.4	0.0	2.4	2.2	–	89.2
Stallion 12.	0.8	3.0	0.0	1.0	0.0	1.2	3.0	–	91.0
Stallion 13.	1.4	3.0	0.2	0.2	0.2	1.4	2.8	–	90.8
Stallion 21.	1.0	2.0	0.0	0.0	0.2	1.2	2.6	–	93.0
Stallion 22.	1.2	2.4	0.0	0.2	0.0	1.2	3.2	–	91.8
Stallion 23.	1.2	2.4	0.0	0.0	0.2	1.0	2.6	–	92.6
Stallion 31.	1.6	3.6	0.2	0.4	1.2	1.2	3.2	–	89.2
Stallion 32.	2.6	4.6	0.2	1.2	1.4	1.6	3.6	–	84.8
Stallion 33.	1.4	5.6	0.0	0.4	1.0	1.4	2.6	–	87.6
Bull 11.	1.0	0.0	0.0	0.4	0.4	0.0	1.6	0.2	96.4
Bull 12.	1.0	0.2	0.2	0.6	0.0	0.0	1.0	0.0	97.0
Bull 13.	0.8	0.6	0.0	0.4	0.0	0.0	1.0	0.2	97.0
Bull 21.	1.2	2.4	0.2	0.2	0.4	0.4	0.8	0.0	94.4
Bull 22.	2.4	2.4	0.0	0.6	0.2	0.4	1.2	0.2	92.6
Bull 23.	2.0	2.0	0.0	1.0	0.4	0.0	1.2	0.2	93.2
Bull 31.	2.0	1.0	0.2	0.6	0.2	0.2	1.6	0.2	94.0
Bull 32.	0.8	0.8	0.0	0.8	0.0	0.6	1.6	0.2	95.2
Bull 33.	1.6	1.0	0.0	1.2	0.0	0.0	1.8	0.4	94.0

Note: Smple codes: indiviual – batch i.e. Bull 11: Bull 1 – batch 1.

heads with abnormal contour, undeveloped heads, detached heads, narrow (tapering) heads, heads of variable size and abaxial attachment of tail for bulls (Table 1.). The method used was the Williams method (Williams & Utica, 1920) modified by Lagerlöf (1934).

3 | RESULTS

3.1 | Intra-and interspecific variation

The results of the morphology evaluation and the median and CV values of the individual histograms from Experiment 1 are shown in Table 2. The species-specific D_{max} values of the paired histograms are shown in Table 3 and Table 4. The Kolmogorov–Smirnov test revealed significant differences between individuals and even between straws from the same individuals ($p < .05$). D_{max} values showed normal distribution for stallions and for bulls ($p = .07$ and $p = .17$, resp., Shapiro-Wilks test). The descriptive statistics are shown in Table 5. The estimated species-specific threshold value was 0,289 for bulls and 0,423 for stallions. The Non-metric multidimensional scaling revealed that the stallion and bull samples form two different populations (Figure 2.).

TABLE 2 Individual PI fluorescence histogram median and CV values of the cytometric evaluation.

Sample	Median	CV
Stallion 11.	75,008	2.33
Stallion 12.	75,648	2.41
Stallion 13.	75,264	2.37
Stallion 21.	73,792	3.14
Stallion 22.	75,264	2.13
Stallion 23.	74,240	3.17
Stallion 31.	75,008	2.5
Stallion 32.	76,032	2.3
Stallion 33.	75,136	2.55
Bull 11.	93,376	3.75
Bull 12.	94,976	3.67
Bull 13.	93,248	3.37
Bull 21.	92,672	4.61
Bull 22.	94,528	3.95
Bull 23.	92,736	4.72
Bull 31.	93,696	4.13
Bull 32.	92,864	3.95
Bull 33.	92,352	4.04

Note: Sample codes: individual – batch (i.e. Bull 11: Bull 1 – batch 1).

3.2 | Analysis of mixed sperm samples

The mixed ("abnormal") samples compared to the 100% bull sample showed higher D_{max} values than the threshold value of the bull samples in all three series (Table 6). Compared to the 100% stallion samples, however, the mixed samples showed lower D_{max} values than the threshold limit for stallions except for the 75%–25% bull-stallion mixtures (Table 7). On the other hand, non-metric multidimensional

TABLE 3 Matrix of D_{max} values of the three stallions and three batches measured.

Sample	11	12	13	21	22	23	31	32	33
11	0.000	0.156	0.058	0.286	0.068	0.195	0.028	0.239	0.038
12		0.000	0.099	0.399	0.109	0.314	0.164	0.082	0.124
13			0.000	0.335	0.021	0.243	0.070	0.182	0.045
21				0.000	0.354	0.091	0.265	*0.474*	0.290
22					0.000	0.262	0.089	0.187	0.063
23						0.000	0.173	0.262	0.199
31							0.000	0.246	0.045
32								0.000	0.206
33									0.000

Note: Sample codes: individual – batch (i.e. 11: Stallion 1 – batch 1). D_{max} values in italics exceed the species-specific threshold.

TABLE 4 Matrix of D_{max} values of the three bulls and three batches measured.

Sample	11	12	13	21	22	23	31	32	33
11	0.000	0.201	0.022[a]	0.113	0.141	0.103	0.054	0.065	0.128
12		0.000	0.219	0.271	0.066	0.260	0.161	0.252	*0.322*
13			0.000	0.128	0.162	0.120	0.076	0.078	0.133
21				0.000	0.213	0.011[a]	0.118	0.055	0.052
22					0.000	0.207	0.095	0.186	0.256
23						0.000	0.112	0.047	0.120
31							0.000	0.092	0.161
32								0.000	0.070
33									0.000

Note: Samples codes: individual – batch (i.e. 11: Bull 1 – batch 1). D_{max} values in italics exceed the species-specific threshold.
[a]It means not significant at $P < 0{,}05$.

TABLE 5 Descriptive statistics of the measured D_{max} values.

Stallions	D_{max}	Bulls	D_{max}
Mean	0.183	Mean	0.135
SD	0.120	SD	0.077
Minimum	0.021	Minimum	0.011
Q1	0.079	Q1	0.074
Median	0.178	Median	0.120
Q3	0.263	Q3	0.190
Maximum	0.474	Maximum	0.322

scaling showed a clear separation between the normal and mixed ("abnormal") samples and also between the different mixtures (Figure 3).

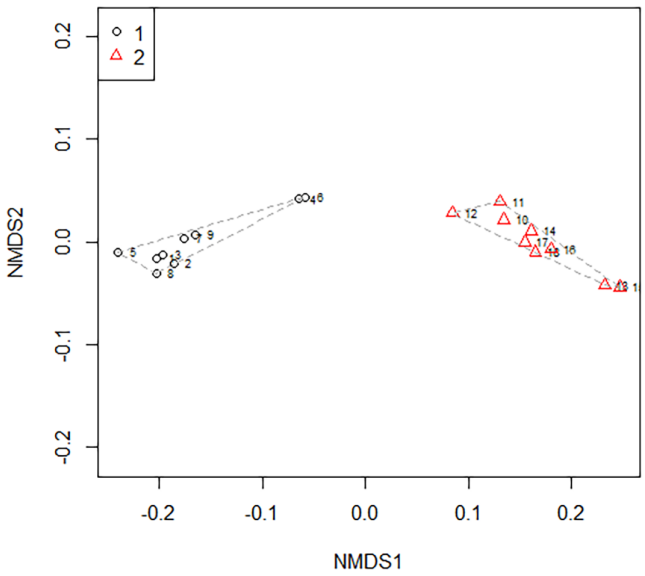

FIGURE 2 NMDS plot of stallion and bull samples (o: stallions, Δ: bulls).

4 | DISCUSSION

The PulSA technique offers an easy-to-use approach to evaluate semen morphology. This method is quick, repeatable, and eliminates the human subjectivity in classical morphology evaluations. The fluorescent labelling kit can be used with standard laser and optics, making this test suitable for most benchtop flow cytometers. The test fits in the laboratory routine semen quality control where flow cytometry is available.

TABLE 6 D_{max} values of paired histograms of mixed bull-stallion sperm samples as compared to the 100% bull sperm samples.

Bull	1. 25%	2. 25%	3. 25%	1. 50%	2. 50%	3. 50%	1. 75%	2. 75%	3. 75%
1. 100%	*0.792*	*0.762*	*0.804*	*0.660*	*0.628*	*0.604*	*0.356*	*0.479*	*0.311*
2.100%	*0.798*	*0.769*	*0.809*	*0.665*	*0.634*	*0.609*	*0.362*	*0.485*	*0.316*
3. 100%	*0.786*	*0.755*	*0.798*	*0.654*	*0.622*	*0.599*	*0.351*	*0.472*	*0.304*

Note: Three repeats are shown. D_{max} values in italics indicate larger value than the species-specific threshold.

TABLE 7 D_{max} values of paired histograms of mixed bull-stallion sperm samples as compared to the 100% stallion sperm samples.

Stallion	1. 25%	2. 25%	3. 25%	1. 50%	2. 50%	3. 50%	1. 75%	2. 75%	3. 75%
1. 100%	*0.648*	*0.530*	*0.699*	0.348	*0.427*	0.401	0.300	0.262	0.298
2.100%	*0.643*	*0.524*	*0.692*	0.340	0.406	0.394	0.278	0.250	0.276
3. 100%	*0.643*	*0.526*	*0.694*	0.342	0.420	0.396	0.297	0.255	0.296

Note: Three repeats are shown. D_{max} values in italics indicate larger value than the species-specific threshold.

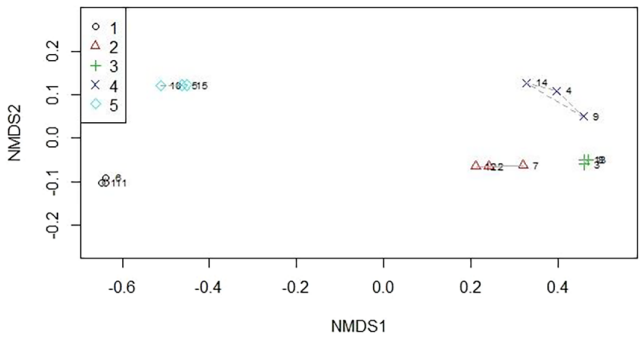

FIGURE 3 NMDS plot of normal and mixed sperm samples (o:100% stallion; Δ: 25%–75% bull-stallion; +: 50%–50% bull-stallion; ×: 75%–25% bull-stallion; ◇: 100% bull samples).

Although pulse profiles cannot be interpreted directly as classic, visual sperm head morphology evaluations, the rapid and robust flow cytometric assay can reveal abnormal males or semen batches and a time-consuming thorough visual morphology analysis can be done only on such semen samples. We noticed species-specific differences though, as bull sperm samples containing even 25% stallion spermatozoa were clearly identified with the PulSA approach, in case of stallions, the test was less sensitive. On the other hand, non-metric multidimensional scaling showed clear separation of samples containing even the lowest % of "abnormal" sperm cells from either the 100% bull or 100% stallion sperm cells.

Besides the importance of evaluation of sperm abnormalities in the quality control of breeding animals used either for artificial insemination or natural breeding, there is a growing interest in sperm morphology from the points of view of evolutionary aspects (Kahrl et al., 2021, 2022) or taxonomy (Jamieson, 1991). A comprehensive database on sperm morphology and morphometry of animal taxa is continuously developed (Fitzpatrick et al., 2022). In our opinion, the PulSA approach could be a useful addition to such database, but further validation studies will be needed on several taxa.

The PulSA technique could be a valuable tool for a quick morphology screening with a flow cytometer. However, further research is needed on different species with normal sperm head morphology to establish valid, species-specific D_{max} threshold values as well as the diagnostic value of the PulSA approach on the sub- and infertility detection of breeding males with sperm head abnormalities.

AUTHOR CONTRIBUTIONS

SN contributed to the initial hypothesis, experimental planning, data analysis, manuscript writing and submission. BK contributed to the data acquisition, laboratory measurements, data analysis and initial manuscript preparation. AJ contributed to the experimental planning, data acquisition and manuscript writing.

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CONFLICT OF INTEREST STATEMENT

None. There is no financial/personal interest or belief that could affect our objectivity.

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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