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Reference values for sperm motility and morphology using computer-assisted sperm analysis; insight in four inbred mouse strains

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Abstract

Genetic background can influence the reproductive performance in laboratory mice, yet strain-specific reference values for sperm motility and morphology remain limited. In this study, we used computer-assisted sperm analysis (CASA) to examine epididymal sperm from 40 males of each of four inbred strains: C57BL/6J, C57BL/6N, BALB/c, and CBA/J, with the aim of establishing strain-specific reference ranges and cut-off values for key motility and morphometric parameters. Marked inter-strain differences were observed in all major motility categories and in most kinematic parameters, with C57BL/6N generally showing the highest motility values and CBA/J the lowest. Strain-specific differences were also evident in sperm head and midpiece morphometry, with CBA/J showing the largest heads and BALB/c the smallest. Reanalysis using strain-specific morphometric values allowed the definition of normality cut-offs for each background. These data provide standardized reference ranges for sperm motility and morphology in four widely used mouse strains and support the use of strain-adjusted thresholds for reproductive phenotyping, cryopreservation quality control, and the assessment of genetically altered mouse lines.

Key words

mouse sperm; sperm motility; sperm morphology; computer-assisted sperm analysis; genetic background; reference ranges; reproductive phenotyping

Introduction

Reference values for sperm motility and morphology have clear diagnostic and interpretive value because they provide the framework needed to distinguish expected biological variation from abnormal reproductive phenotypes. A similar principle is already well established in laboratory mice for clinical chemistry, where strain-, substrain-, sex-, and age-specific reference intervals have been calculated, and these values support the interpretation of clinical chemistry data from mouse mutants and corresponding wild-type controls[1]. Accordingly, applying background-specific reference ranges to sperm traits may improve the interpretation of sperm phenotypes in mice. In laboratory mice, strain-specific baseline values can improve sperm quality control, guide cryopreservation workflows, and reduce unnecessary reliance on IVF-based fertility testing. Sperm motility and morphology are key parameters in sperm evaluation, and their assessment can provide important information about the fertilizing potential of a sample[2,3].

In mouse models, the need for establishment of strain-specific reference ranges is particularly important because sperm traits are strongly influenced by genetic background. Earlier study has shown strain-dependent variation in epididymal sperm concentration, motility, morphology[4], while more recent works have highlighted the complexity of male reproductive phenotypes in genetically altered (GA) mice[5,6]. Despite extensive characterization of reproductive phenotypes in GA mice, systematic reference ranges for sperm motility and morphology in commonly used wild-type inbred strains remain limited. Establishing reference ranges and cut-off values enables quantitative comparisons that reveal whether sperm traits in GA lines deviate significantly from the corresponding wild-type values.

Previous studies have reported that sperm traits, including motility and morphology, differ markedly between mouse strains, reflecting underlying genetic and testicular histological variation[7,8]. These findings raise concern that applying uniform quality-control thresholds across strains may be inappropriate, particularly when evaluating GA lines maintained on different genetic backgrounds or on mixed backgrounds derived from common inbred strains[9].

Computer-assisted sperm analysis (CASA) provides an objective, standardized assessment of sperm motility and morphology, replacing the manual microscopic evaluation used in earlier studies[10,11]. Modern CASA platforms generate reproducible kinematic and morphometric data when pre-analytical handling, acquisition settings and analysis criteria are carefully controlled,

making them well suited for defining laboratory-specific baseline ranges[12,13]. Such standardized datasets are especially valuable for colony management, cryopreservation quality control and the evaluation of GA lines, where subtle but biologically meaningful deviations from the appropriate wild-type background must be detected with confidence.

The present study investigates sperm motility and morphology in four widely used wild-type inbred strains: C57BL/6J, BALB/c, CBA/J and C57BL/6N. These strains were primarily selected because they are well-established mouse backgrounds and have been used in a range of genetic and biomedical studies, including transgenic and gene-targeted mouse research[14,15]. Of central importance, however, is that they exhibit genetically based differences in sperm traits, including sperm production, morphology, and motility[16,17], as well as well-documented variation in reproductive performance, such as fertility rates, age at first mating, litter size, and overall fecundity[18,19].

Using a CASA system, specifically the Sperm Class Analyzer- SCA® software (Microptic SL, Hamilton Thorne, Barcelona, Spain), this study provides a comprehensive assessment of sperm motility parameters, including kinetic metrics and motion categories, together with morphometric analysis after SpermBlue® staining.

The primary aim is to establish strain-specific reference ranges and cut-off values for these semen parameters. Furthermore, we conducted a comprehensive comparative analysis across strains to identify differences in motility and morphology parameter values, thereby defining strain-specific distributions of sperm characteristics.

Results

In this study, we analyzed sperm motility and morphology in four widely used inbred mouse strains: C57BL/6J, C57BL/6N, BALB/c, and CBA/J, using a standardized CASA approach. Clear strain-specific differences were observed across both kinetic and morphometric parameters, allowing us to define reference ranges and cut-off values for each background.

Motility

All recorded motility parameters are significantly different in all four murine examined strains.

Forty male mice per strain (C57BL/6J, C57BL/6N, BALB/c, CBA/J), were assessed for motility parameters, to establish strain specific reference ranges. The SCA® motility software automatically recorded and analyzed more than 1000 spermatozoa per male donor. The minimum and maximum values for inter-strain motility parameters and motion types are presented in table 1. Minimum values of total motility (%) and progressive motility (%) can serve as diagnostic thresholds for establishing normal motility in both wild-type and genetically altered strains. Specifically, normal cut-off values (min) for total motility (%): C57BL/6J, 66.69; C57BL/6N, 77.85; BALB/c, 67.46; CBA/J, 59.72 and progressive motility (%) are C57BL/6J, 57.11; C57BL/6N, 65.45; BALB/c, 55.18; CBA/J, 49.41 (table 1).

Table 1 Reference ranges for motility parameters across four inbred mouse strains (C57BL/6J, C57BL/6N, BALB/c, CBA/J) as measured and analyzed by the SCA® motility module. Total Motility, Progressive Motility, Rapid Progressive Motility, VCL, curvilinear; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; Hyperactivation

	C57BL/6J		C57BL/6N		BALB/c		CBA/J	
Parameter	Min	Max	Min	Max	Min	Max	Min	Max
Total Motility (%)	66.69	97.19	77.85	96.43	67.46	97.15	59.72	93.64
Progressive Motility (%)	57.11	95.72	65.45	93.75	55.18	95.12	49.41	91.15
Rapid Progressive Motility (%)	54.55	95.38	63.74	92.67	52.58	94.71	45.37	88.24
VCL (µm/s)	118.49	197.29	127.92	189.94	133.84	236.51	109.24	230.75
VSL (µm/s)	33.54	60.90	34.58	56.92	32.75	71.65	22.48	69.56
VAP (µm/s)	62.03	105.57	71.49	112.20	69.28	114.74	70.98	123.60
LIN (%)	21.98	36.68	20.16	34.48	21.78	32.16	17.24	30.08
STR (%)	42.76	63.72	38.71	58.07	42.62	61.68	34.61	55.04
WOB (%)	48.95	57.56	48.95	58.15	47.55	53.10	45.48	58.84
ALH (µm)	3.48	5.52	3.72	5.36	3.82	6.56	3.10	6.30
BCF (Hz)	6.43	10.77	6.94	9.39	6.57	10.99	5.94	9.46
Hyperactivation (%)	33.04	72.46	43.17	76.51	41.21	75.42	30.07	80.05

ANOVA analysis revealed significant differences among the four wild-type strains for all recorded motility parameters and specific motion types (Figure 1), as indicated by the corresponding *p*-values. Significant differences were also observed for the percentage of hyperactivated spermatozoa, although at a lower significance level ($p = 0.01$) compared to other parameters (Fig. 1l). The most highly significant differences ($p < 0.001$) were observed for % total motility (Fig. 1a), % progressive motility (Fig. 1b), % rapid progressive motility (Fig. 1c), VCL (Fig. 1d), VSL (Fig. 1e) LIN (Fig. 1g), STR (Fig. 1h), WOB (Fig. 1i), ALH (Fig. 1j) and BCF (Fig. 1k) indicating substantial inter-strain variation in these sperm quality parameters.

C57BL/6N exhibited the highest values for % total motility (Fig. 1a), % progressive motility (Fig. 1b), and % rapid progressive motility (Fig. 1c), with significant differences compared to the other three strains. Conversely, CBA/J displayed the lowest values for these motility parameters (Fig. 1a, b, c), with significant differences relative to the other strains. Interestingly, CBA/J also demonstrated the lowest BCF (Fig. 1k) values, differing significantly from the other strains, suggesting a distinct flagellar beating pattern in this strain.

C57BL/6J was characterized by the lowest values for individual kinematic parameters VCL (Fig. 1d), VSL (Fig. 1e), VAP (Fig. 1f), and ALH (Fig. 1j), but exhibited the highest linearity (LIN) (Fig. 1g). In all cases, significant differences were observed compared to the other strains, except for VSL ($p = 0.83$ with CBA/J; $p = 0.27$ with C57BL/6N) (Fig. 1e).

BALB/c and CBA/J displayed the highest values for VCL (Fig. 1d), VSL (Fig. 1e) and VAP (Fig. 1f), with no significant differences between these two strains except for VSL ($p = 0.0079$) (Fig. 1e). In contrast, BALB/c demonstrated the lowest WOB values (Fig. 1i), with significant differences compared to all other strains.

Comparison between the two C57BL/6 substrains (C57BL/6J and C57BL/6N) revealed significant differences (p<0,001) in most motility parameters. More specifically C57BL/6J exhibited higher values for % total motility (fig. 1a), % progressive motility (Fig. 1b), % rapid progressive motility (Fig. 1c), VAP (Fig. 1f) and hyperactivation (Fig. 1l), whereas C57BL/6N showed higher values for STR (Fig. 1h) and LIN (Fig. 1g). No significant differences between these substrains were detected for VCL (Fig. 1d), VSL (Fig. 1e), WOB (Fig. 1i), ALH (Fig. 1j) or BCF (Fig. 1k).

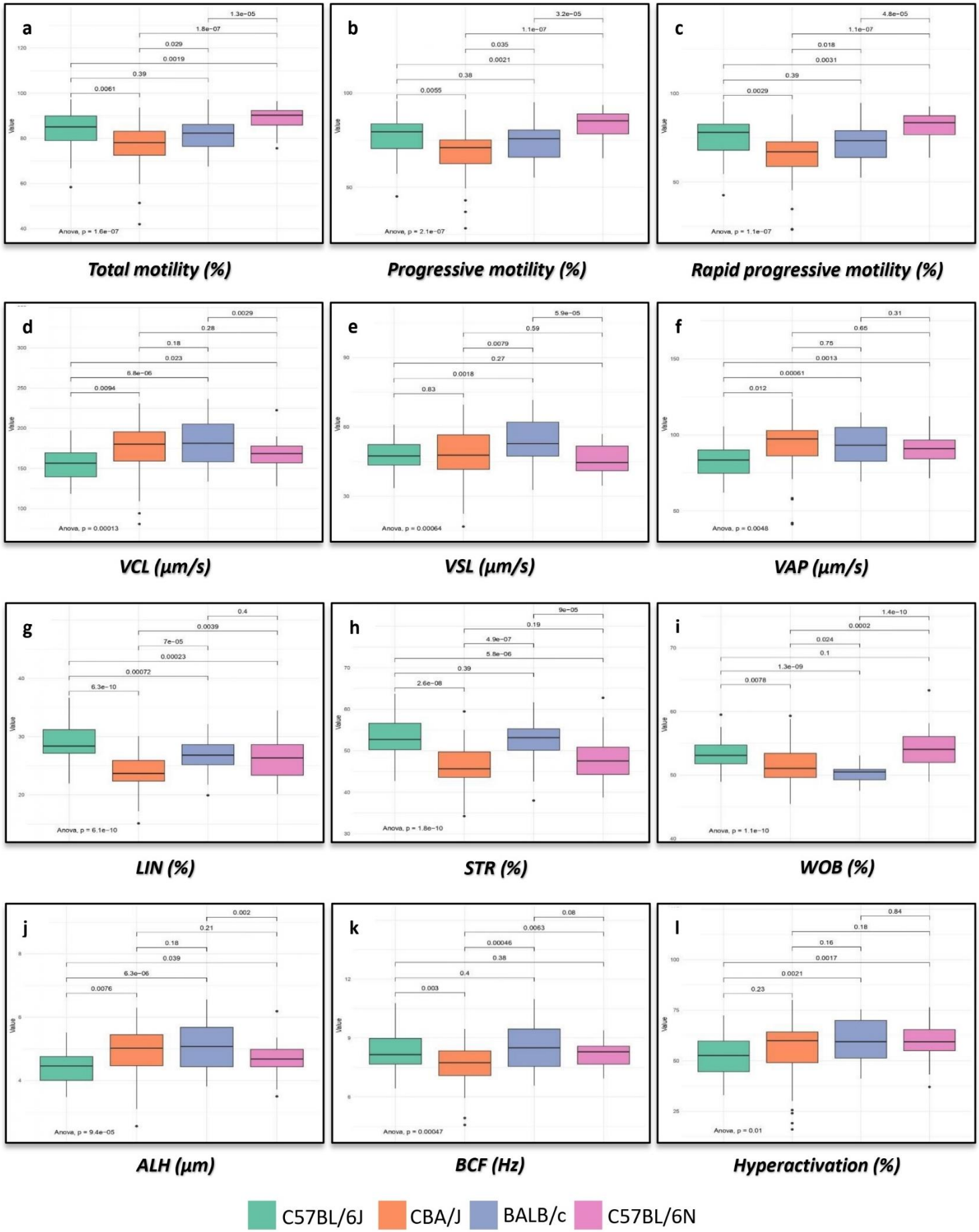


Figure 1 Box plots of the percentages of sperm motility parameters (a. total motility, b. progressive motility, c. rapid progressive motility) and individual velocity types (d. VCL, e. VSL, f. VAP, g. LIN, h. STR, i. WOB, j. ALH, k. BCF, l. Hyperactivation), as determined by the SCA® motility module. P-value for each parameter, as calculated upon ANOVA analysis, revealed significant differences among the strains C57BL/6J, CBA/J, BALB/c, C57BL/6N

Morphology

All morphometric characteristics evaluated by the SCA® morphology software for both head and midpiece were significantly different.

Reference ranges for morphometric parameters of the sperm head (arc, width, area, perimeter, angle, linearity, roughness, chord) and midpiece (width, angle) were determined following analysis of 40 male mice per strain using the SCA® morphology module (Table 2). The new morphometric values were integrated into the SCA® software, creating strain-specific morphometry programs for each strain. These programs enable assessment of morphological normality in mice through comparisons with males of the same genetic background.

Table 2 Reference ranges for morphometric parameters of sperm head and midpiece across four inbred mouse strains (C57BL/6J, C57BL/6N, BALB/c, and CBA/J), as measured and analyzed by the SCA® morphology module

	C57BL/6J		C57BL/6N		BALB/c		CBA/J	
Parameter	Min	Max	Min	Max	Min	Max	Min	Max
Head arc (µm)	9.9	14	10.19	13.36	9.43	13.61	11.43	15.62
Head width (µm)	1.92	3.28	1.88	3.25	1.66	3.28	1.72	3.23
Head area(μm^2)	15.9	23.2	16.12	22.26	15.04	21.88	16.95	24.04
Head perimeter (µm)	23	30.3	23.46	28.97	21.77	29.67	25.69	33.26
Head Angle (°)	46.6	99.4	49.91	98.46	28.5	102.3	40.37	97.86
Roughness	0.27	0.43	0.28	0.42	0.26	0.44	0.23	0.37
Linearity	48.2	72.9	50.8	73.48	40.92	75.17	48.55	72.38
Chord (µm)	5.97	8.55	6.29	8.36	5.13	8.21	7.19	9.14
Midpiece width (µm)	0.58	1.06	0.55	1.03	0.54	1.03	0.58	1.06
Midpiece angle (°)	-0.24	60.9	2.25	61.08	2.13	66.96	-4.13	57.62

The 40 males from each strain were reanalyzed using their novel corresponding strain-specific morphometric reference values, establishing updated normality percentages (%). Strain-specific morphology normality (%) cut-off values, determined through statistical analysis, were as follows: C57BL/6J ≥ 58.00%; C57BL/6N ≥ 59.33%; BALB/c ≥ 56.00%; CBA/J ≥ 54.67%.

The analysis outlined above evaluated the morphometric characteristics of the sperm head and the anterior segment of the tail, primarily the midpiece, for spermatozoa of each strain (Figure 2). Measuring the total tail length proved challenging in spermatozoa where the entire tail appeared

within the microscopic field, as this could interfere with accurate assessment of the head and midpiece. Visual inspection revealed few abnormalities in tail morphology. When feasible, tails were also subjectively assessed, and the SCA® system allowed their classification as either normal or abnormal.

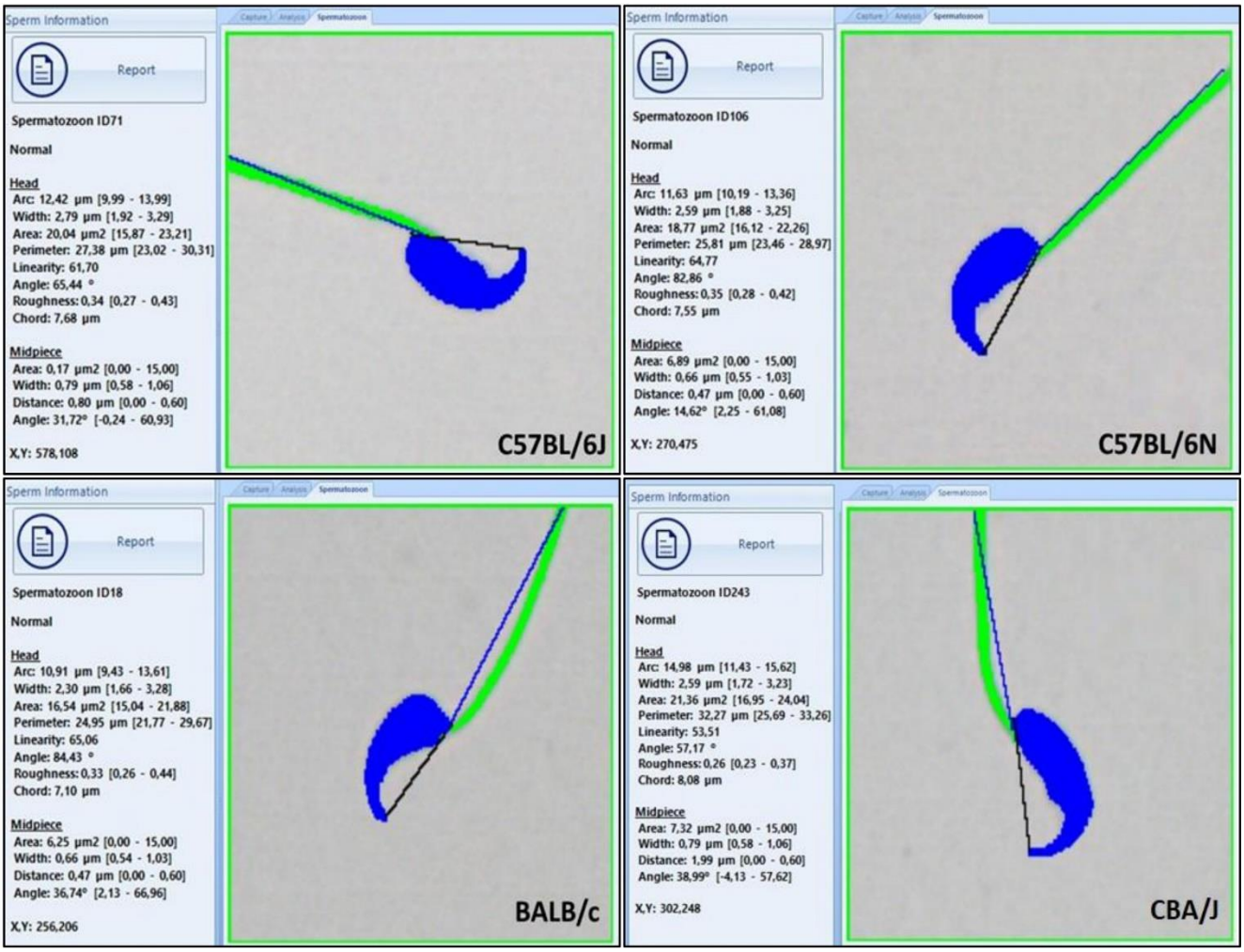


Figure 2 Representative images of individual spermatozoa from each strain as recorded by the SCA® morphology module. The boundaries of the head (blue) and midpiece (green) are clearly delineated by the system, and the measurements of each parameter are displayed on the left side of the image

Following establishment of inter-strain reference values for morphometric parameters of the sperm head and midpiece, ANOVA analysis was performed to compare the four strains. Statistical analysis revealed highly significant differences ($p < 0.001$) in all morphometric characteristics evaluated by the SCA® morphology software for both head (Fig. 3a-h) and midpiece (Fig. 3i, j). In most cases, highly significant differences were observed when comparing two, three, or all four strains.

CBA/J exhibited the largest sperm head among all strains, with highly significant differences compared to other strains in head arc (Fig. 3a), area (Fig. 3c), perimeter (Fig. 3d), and chord (Fig.

3h), as indicated by the corresponding p-values. In contrast, CBA/J demonstrated the lowest roughness values (Fig. 3f) compared to the other strains.

Conversely, BALB/c displayed the smallest sperm head compared to other strains, with minimum values recorded for multiple head dimensions, particularly arc (Fig. 3a) width (Fig. 3b), area (Fig. 3c), perimeter (Fig. 3d), angle (Fig. 3e), linearity (Fig. 3g) and chord (Fig. 3h). In all cases, highly significant differences were observed, except for head width ($p = 0.3$ between CBA/J and BALB/c Fig. 3b).

Higher head width values (Fig. 3b) were recorded in C57BL/6J, whereas C57BL/6N exhibited higher head angle values (Fig. 3e). Significant differences were observed in both cases compared to other strains.

Regarding midpiece dimensions, BALB/c demonstrated the highest angle values (Fig. 3j) but the lowest width values (Fig. 3i), with highly significant differences compared to other strains. The lowest midpiece angle values (Fig. 3j) were observed in CBA/J. However, C57BL/6J and CBA/J exhibited higher midpiece width values (Fig. 3i) with no differences between them, but significant differences compared to the other strains.

Notably, highly significant differences were detected in all morphometric parameters of both head (Fig. 3a-h) and midpiece (Fig. 3i. j) when comparing C57BL/6J and C57BL/6N substrains.

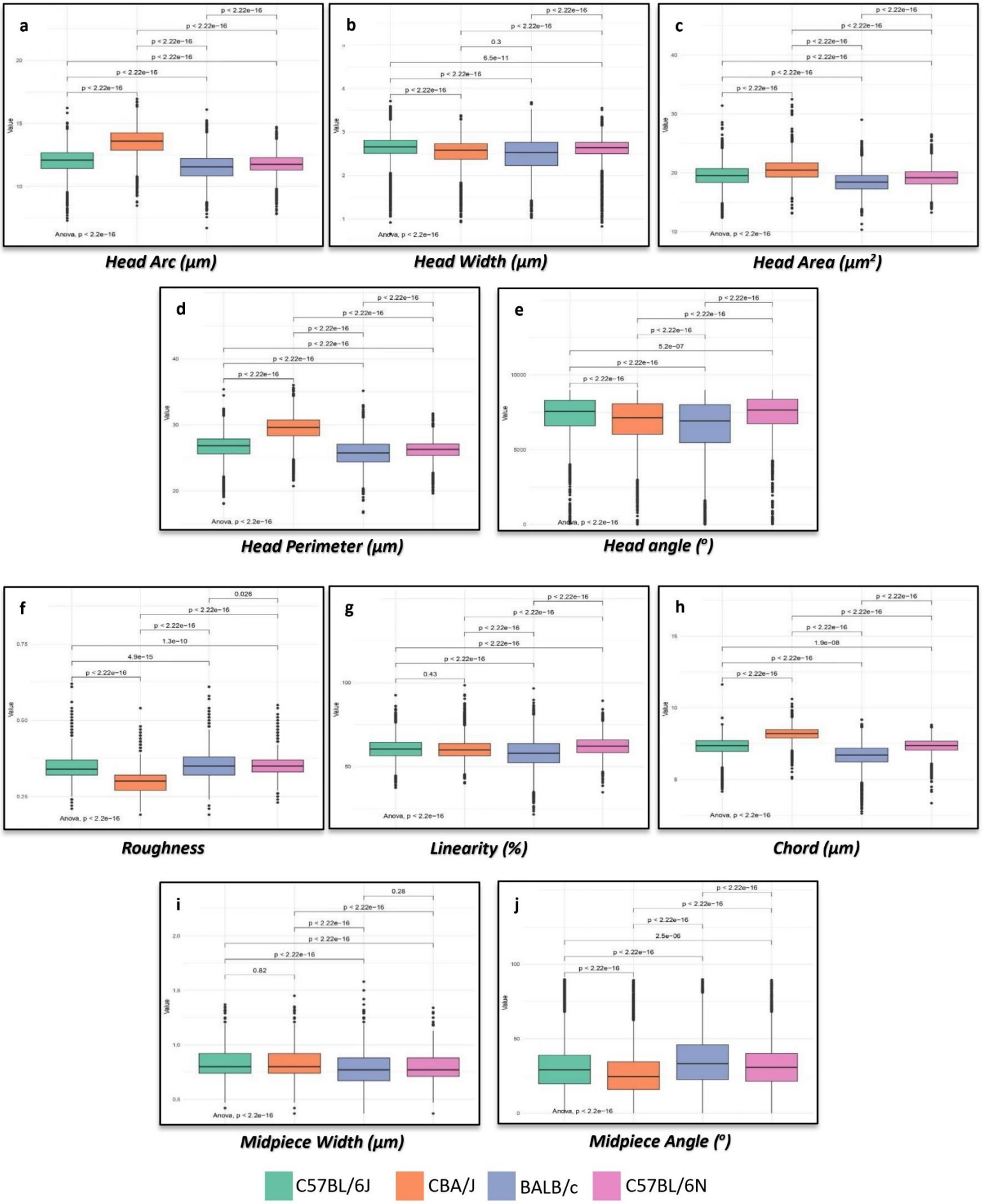


Figure 3. Box plots of the morphometric parameters for sperm head (a. arc, b. width, c. surface area, d. perimeter, e. angle, f. roughness, g. linearity, h. chord) and midpiece (i. width, j. angle), as measured and analyzed by the SCA® morphology module. P-values for each parameter, as calculated upon ANOVA analysis, revealed highly significant differences among the strains C57BL/6J, C57BL/6N, BALB/c, CBA/J

Discussion

Genetic background determines sperm traits in mice

The present study is the first to perform a comprehensive, standardized characterization of sperm motility and morphology parameters across four widely used inbred mouse strains (C57BL/6J, C57BL/6N, BALB/c, and CBA/J) using computer-assisted sperm analysis (CASA). Our findings establish strain-specific reference ranges and cut-off values for key sperm quality parameters. Additionally, we provide a detailed description of normal variability within and between strains, thus extending earlier work that showed strain effects on epididymal sperm concentration, motility and morphology, using manual or semi-quantitative approaches[4,7,20,21]. The inter-strain differences we observed, confirm that sperm motility, as well as head and midpiece dimensions are strongly modulated by the genetic background, highlighting the necessity for strain-specific reference standards in reproductive assessment. Similar breed- or strain-specific differences in sperm kinematic parameters have been documented across domestic species, including dogs[22], chickens[23] and bulls[24,25], which means that the genetic background determines sperm functional traits in mammals and birds.

A stable, heritable factor determines baseline motility characteristics in four inbred strains

Our motility analysis revealed highly significant differences among the four inbred strains for all major motility categories (total, progressive, rapid progressive) and most kinematic parameters (VCL, VSL, VAP, LIN, STR, ALH, BCF, hyperactivation). C57BL/6N exhibited the highest total and progressive motility, whereas CBA/J conversely showed consistently lower values, with BALB/c and C57BL/6J occupying intermediate positions depending on the parameter examined. This pattern is similar to strain-dependent motility profiles described in studies using advanced CASA-based classifiers, such as CASAnova, where sperm from different inbred strains (e.g. C57BL6, 129, PWK) displayed distinct distributions of motility classes[13]. Our results are also consistent with earlier studies, showing epididymal sperm motility vary between inbred strains such as C57BL, BALB and CBA[4,26].

However, those studies examined a broader age range and relied primarily on optical assessment of motility and morphology. In contrast, the marked differences observed here in adult animals maintained under standardized conditions and analyzed using objective, automated methods

suggest that stable, heritable factors rather than temporary developmental effects, determines baseline motility characteristics in these genetic backgrounds.

Kinematic signatures observed among mouse strains likely reflect functional adaptations

The kinematic profiles also differed qualitatively among strains. C57BL/6J males showed lower VCL, VSL, VAP and ALH but higher LIN, indicative of a more linear, less curved swimming pattern. On the other hand, BALB/c and CBA/J displayed higher curvilinear velocities with lower linearity indices. The functional significance of individual kinematic parameters has been extensively studied across mammalian species, providing broader context for our strain-specific findings. In boars, sperm with higher velocities (VCL, VSL, VAP) and more linear trajectories (LIN, STR) are positively associated with fertility, whereas slower and less directional sperm correlate with poorer reproductive success[27]. Similar positive relationships between progressive motility, VSL, VAP and fertility have been reported in bulls[24,25]. Moreover, in humans, studies have reported that higher values of VAP, VCL and VSL are associated with lower DNA damage, which supports their value to indicate fertility potential[28]. Together, these cross-species data support our interpretation that the distinct kinematic signatures observed among mouse strains likely reflect functional adaptations in flagellar control with potential consequences for fertilization efficiency.

Variable hyperactivation percentages among strains support strain-specific regulation of motility changes

Our discovery that CBA/J exhibited the lowest beat cross-frequency (BCF) values among all strains, while showing high curvilinear velocities, is something that deserves particular attention. BCF is a measure of the sperm head's lateral displacement frequency and reflects the underlying flagellar beat frequency[29]. In mammals, hyperactivation characterized by high-amplitude, asymmetric flagellar bending and often reduced beat frequency, which is essential for penetration of the cumulus matrix and zona pellucida[30,31]. In humans, hyperactivation involves a reduction in beat frequency in most cells, accompanied with an increase in flagellar bending amplitude[30–32]. The relatively low BCF but high VCL and ALH in CBA/J sperm may indicate a tendency toward a hyperactivation-like motility pattern, even in the absence of capacitating conditions. In contrast, the higher BCF but more linear trajectories in C57BL/6J may reflect a different balance between flagellar beat frequency and waveform amplitude. The variable hyperactivation percentages we

observed among strains (ranging from ~30% in CBA/J to ~43-76% in C57BL/6N) further support strain-specific regulation of motility changes that are associated with capacitation.

Significant motility differences among C57BL/6J and C57BL/6N underlie the need for substrain-specific thresholds.

The comparison between C57BL/6J and C57BL/6N is particularly informative, as these substrains are widely used as similar backgrounds for transgenic and knockout lines. Despite the close genetic relationship of these substrains, we identified significant variations in most motility and kinematic parameters. Previous work has demonstrated that even subtle genetic differences among C57BL/6 substrains can affect reproductive performance and sperm-freezing response. C57BL/6N often shows better performance in cryopreservation or IVF protocols[33]. Our data support the need for substrain-specific reference ranges and the setting of cut-off values in a particular C57BL/6 background.

Subtle genetic background variations are able to produce distinct, strain-specific morphometry profiles

The morphometric analyses revealed equally robust strain effects on sperm head and midpiece dimensions. CBA/J sperm had the largest heads (higher arc, area, perimeter and chord) combined with low roughness values, whereas BALB/c showed the smallest heads across multiple parameters, and C57BL/6J and C57BL/6N differed significantly in head width and head angle, respectively.

Our results agree with earlier biophysical work showing that sperm nuclei from CBA and C57BL/6 differ in length, width and area, with CBA nuclei being longer and slightly larger in projected area despite smaller reconstructed volumes[34]. More recent high-throughput nuclear morphometric studies confirm that automated quantitative methods detect subtle sperm shape differences more precisely than manual scoring, and clearly distinguish between CBA and C57BL/6 sperm; specifically, CBA spermatozoa have larger head areas, greater length and slightly shorter hooks[35] The marked morphometric differences we observed among all four strains, including between the closely related C57BL/6J and C57BL/6N substrains, fit well with this picture.

Our results extend classical work by Krzanowska and colleagues showing that mouse inbred strains differ in both the proportion and the types of sperm head abnormalities, and that these traits are

genetically controlled[36]. Genetic studies with recombinant inbred lines show that the Y chromosome accounts for most of the variation in the total percent of head abnormalities, while the autosomal and X linked genes have an impact on head shape indices and specific abnormal form[20,37]. Additional genetic mapping research has shown that genes located in particular chromosomal regions are also involved in the regulation of sperm head morphology[38]. Together with our demonstration of marked strain differences in sperm morphology, these data support the view that subtle genetic background variations are able to produce distinct, strain-specific morphometry profiles.

Personalized reference values are necessary for correct interpretation of reproductive phenotypes

Integrating our new morphometric values into strain-specific SCA® analysis programs and conducting re-analysis of 40 males per strain, allowed us to define quantitative cut-off values for morphology normality (for example, ≥58% normal forms for C57BL/6J and ≥54.67% for CBA/J). Such cut off values are directly comparable to species specific thresholds reported in other mammals. In human andrology, strict Kruger-type criteria typically use ≥4% normal forms as the threshold[39], even though detailed automated sperm morphometry analysis (ASMA) has shown that the staining method strongly influences head dimension measurements[40]. Previous study comparing two rat strains using the SCA® system proposed strain-adjusted morphometric thresholds and recommended normality cut offs that were specific to the different abnormal head types for toxicological and fertility assessments[41]. In livestock, bulls suitable for artificial insemination typically have <30% abnormal sperm, a threshold widely adopted in Australia and other countries, while limits for individual head defect types are set according to their known effects on fertility[42]. These inter-species variations support our strain-specific thresholds and emphasize the necessity of having personalized reference values for correct interpretation of reproductive phenotypes.

Strain morphological differences and Implications for Reproductive Phenotyping

The highly significant morphometric differences we detected between C57BL/6J and C57BL/6N, indicate that even closely related substrains exhibit distinct head and midpiece profiles. This agrees with work demonstrating that C57BL/6 substrains differ in behavior, metabolism and physiology[43]. Therefore, they should not be considered as completely identical in reproductive trait

characterization or morphology standard setting. For example, a higher rate of sperm head abnormalities (mainly bent heads) has been reported in C57BL/6JBomTac compared to C57BL/6NTac, linked to a Y-chromosome deletion[44]. This implies that mutations arising spontaneously during the generations may cause phenotypic differences in the inbred lines.

In addition, many genetically altered mouse lines show characteristic sperm defects caused by specific genetic modification[5,45]. For instance, the *azh* mutation in *Hook1* gene leads to severe head and tail abnormalities[46], while *Actl7a* mutations are associated with distinct acrosomal defects[47,48]. Careful phenotyping of sperm morphology and function is essential for correct evaluation of such models. Moreover, the strain-specific morphometric profiles established here, provide a useful baseline for distinguishing mutation-induced defects from variations related to genetic background.

Limitations, standardization and future directions

This work provides detailed reference data for four widely used inbred strains, however some limitations suggest the next steps quite clearly. Only a small subset of the many available mouse strains was examined, while other important backgrounds including FVB/N[49], NOD[50] and 129[51] remain to be systematically characterized using comparable methodology. Our study focused exclusively on adult males aged 13-15 weeks, however establishing age-specific reference ranges across the lifespan, would allow assessment of reproductive ageing by comparison with the motility and morphometric values reported here, since spermatogenesis efficiency and sperm parameters decline with age in mice[52]. Multi-site studies with independent replication in different facilities would also be useful to test whether the strain differences observed are robust across laboratories and to more clearly distinguish environmental from genetic effects. In addition, the functional impact of the morphometric differences still needs to be defined, particularly in relation to chromatin packaging, DNA integrity and fertilization outcomes, which are known to influence male fertility and Assisted Reproductive Techniques (ART) success[53].

Conclusions and broader implications

This study provides standardized CASA reference ranges for sperm motility and morphometry across four key mouse strains, establishing strain-specific cut-offs that account for genetic background variation. These reference standards will improve the precision of reproductive

phenotyping, enhance cryopreservation success, and support more reliable fertility predictions and colony management for mouse mutant lines.

Beyond immediate applications, this work aligns with 3Rs animal welfare principles in terms of reducing the number of animals used. The standardization of protocols and establishment of objective, quantitative assessment criteria also represent refinements that enhance data quality while minimizing procedural variability and associated animal use. In our recent work with partners in the INFRAFRONTIER consortium, we defined quality control (QC) principles to ensure the reliable and efficient operation of the European Mouse Mutant Archive (EMMA)[54]. The findings of the present study may further support the development of more robust QC testing strategies for GA cryopreserved sperm within this framework.

Materials and Methods

Animals

Wild-type male mice from inbred strains C57BL/6J, C57BL/6N, BALB/c, CBA/J were used as sperm donors. At the time of collection, the animals were 13-15 weeks old, clinically healthy, without any symptoms or lesions. The selection of male age was based on studies indicating that the typically used age range for setting up fertility studies in mice is 10–16 weeks[55]. The mice were bred and maintained under specific pathogen-free conditions, at the animal facilities of the BSRC "Alexander Fleming", housed at 21–24 ^{o}C, 50 +/- 5% humidity, and a 12 h light-dark cycle (light off 19.00-7. 00). Food (4RF21, Mucedola) and water (pre-filtered, UV-treated) were provided ad libitum. Littermate mice were housed together in filter-top cages. All breeding and experimental procedures conformed to the Directive 2010/63/EU, as well as to national legislation and institutional guidelines for the care and use of laboratory animals. Animals were health monitored according to FELASA recommendations[56].

Media and Reagents

All media were prepared in-house. For sperm collection, a cryoprotective agent (CPA), containing 18% raffinose pentahydrate (Sigma-Aldrich, USA), 3% skim milk (Difco™, BD, USA) and 100 mM L-glutamine (Sigma-Aldrich, Brazil) was used, as previously described[57]. Sperm suspensions were diluted for analysis in an HTF (Human Tubal Fluid) medium, which has been widely used for embryo culture and IVF experiments in humans and mice[58]. HTF complete medium consists of 101.6 mM

NaCl, 4.7 mM KCl, 0.37 mM KH2PO4, 0.2 mM MgSO4_7H2O, 2mM CaCl2, 25 mM NaHCO3, 2.78 mM glucose, 0.33 mM Na pyruvate, 21.4 mM Na lactate, 4mg/ml of bovine serum albumin, 100U/ml of penicillin (Gibko™), 0,1 mg/ml of streptomycin (Gibco™). All ingredients were purchased from Sigma-Aldrich, USA.

Sperm Collection

Male mice were euthanized by gradual CO_2 chamber exposure followed by cervical dislocation. The gas was introduced at a rate that displaces 30% of the chamber's total volume per minute. Bilateral caudal epididymides were dissected from each male. Aliquots of 60 µL CPA were pipetted into 35 mm culture dishes and overlaid with paraffin oil (Sigma-Aldrich, Germany). A second 60 µL CPA aliquot was added to each drop (final volume: 120 µL), forming a tall semispherical droplet. The epididymides were transferred to the CPA droplets, and 5–6 portions per epididymis were cut by mincing with micro-spring scissors. Dishes were gently rotated manually for ten seconds every minute, for 3 min total time, to release and disperse sperm. Sperm collection was performed at 37°C using a thermoplate[57].

Assessment of sperm motility

Sperm motility parameters were determined using the Sperm Class Analyzer® (SCA® v6.6; Microptic SL, Hamilton Thorne, Barcelona, Spain), consisting of a Zeiss phase-contrast microscope (4× and 10× negative phase contrast objectives, green filter), a high-speed digital camera (Basler 780-75 Gigabit ethernet camera) and dedicated analysis software (version 6.6- motility program). Sperm suspensions were diluted 1:25 in HTF medium to optimal density, and 3 µL aliquots were loaded onto prewarmed (37°C) Leja® slides (20 µm chamber depth; Leja Products B.V.) on a stage warmer. Ten random fields per objective (more than 1000 analyzed spermatozoa) for each semen sample were captured (frame rate 50 fps; 50 images per field) and analyzed automatically according to manufacturer settings. The SCA® system tracked individual spermatozoa, classifying them into motility categories (rapid, medium, non-progressive, immotile) (Figure 4), while simultaneously measuring key kinematic parameters.

The system recorded measurements of total motility (%), progressive motility (%) and rapid progressive motility (%), as well as individual kinematic parameters including: **VCL**-curvilinear velocity (µm/s), **VSL**-straight line velocity (µm/s), **VAP**-average path velocity (µm/s), **ALH**-amplitude of lateral head displacement (µm), **BCF**-beat/cross-frequency (Hz), VSL/VCL x100 ratio

(LIN-linearity), VSL/VAP x100 ratio (**STR**-straightness), VAP/VCL x100 ratio (**WOB**-wobble), and the percentage of hyperactivated spermatozoa.

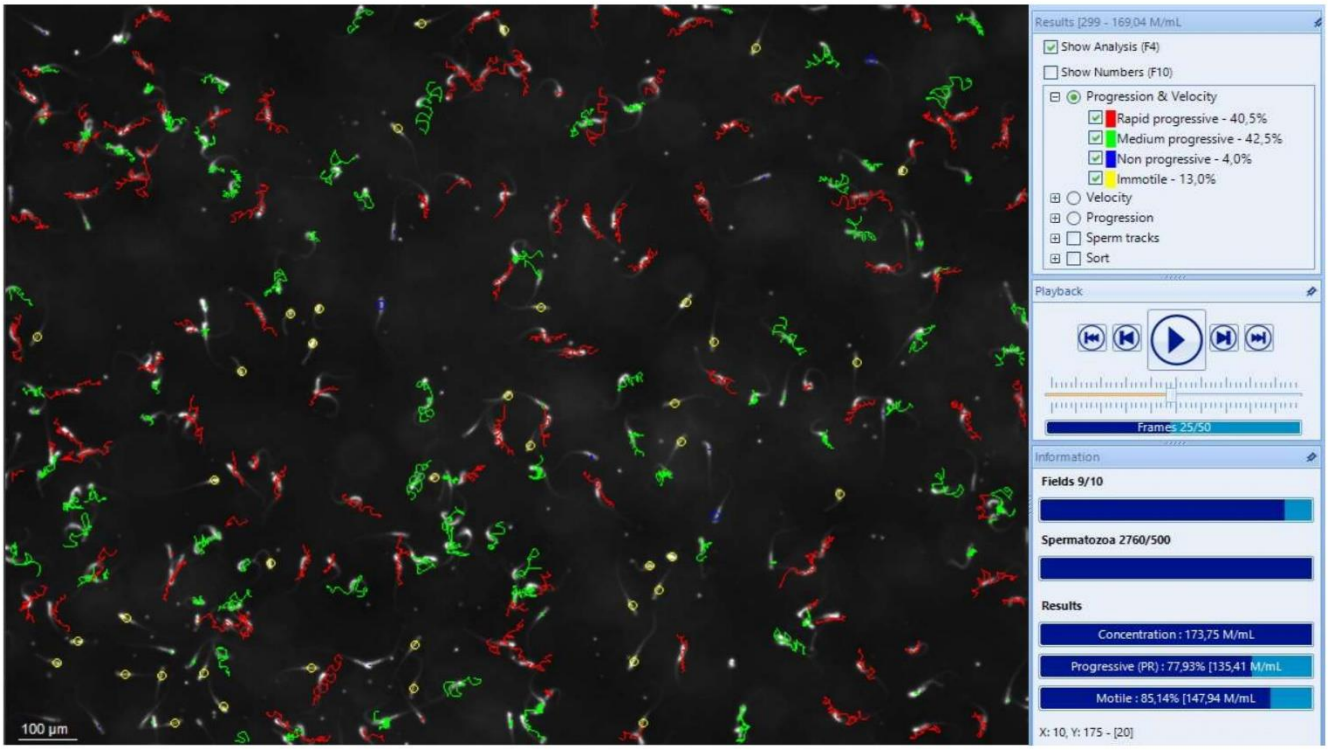


Figure 4 Snapshot of the motility analysis field of a sample. The trajectories of the different spermatozoa are shown in different colors, indicating the category to which each one belongs. The SCA® system provides the percentages of the different motility category to which each one belongs. The SCA® system provides the percentages of the different motility categories within the field

Sperm staining

Sperm morphology was assessed using the SpermBlue® staining protocol, which provides clear color differentiation of head, midpiece, and tail regions for accurate boundary identification. From each semen sample, a 20 µl aliquot of concentrated sperm suspension was mixed with 40 µl Phosphate Buffered Saline-PBS 10x (Gibko™, UK) using a positive-displacement pipette. Samples were centrifuged (Thermo Scientific Fresco17 Microcentrifuge; 300 × g, 10 min), the supernatant was discarded, and the pellet resuspended in PBS (volume adjusted per sperm concentration). A 10 µl aliquot of the resuspended sperm was placed on a slide and a sperm smear in duplicate was made, using the feathering technique (dragging at a 45° angle- Figure 5). Slides were air-dried at room temperature, then fixed by immersing them vertically into a staining tray containing SpermBlue® fixative (Microptic SL, Barcelona, Spain) for 10 min. Excess fixative was drained at a 60–80° angle and slides were stained by immersion in SpermBlue® stain (Microptic SL, Hamilton Thorne, Barcelona, Spain) for 15–20 min, as previously described[59]. Slides were briefly rinsed in

distilled water for 3 sec to remove excess stain, then air-dried at a 60–80° angle before mounting in DPX medium. Stained slides were stored in the dark prior to morphological analysis.

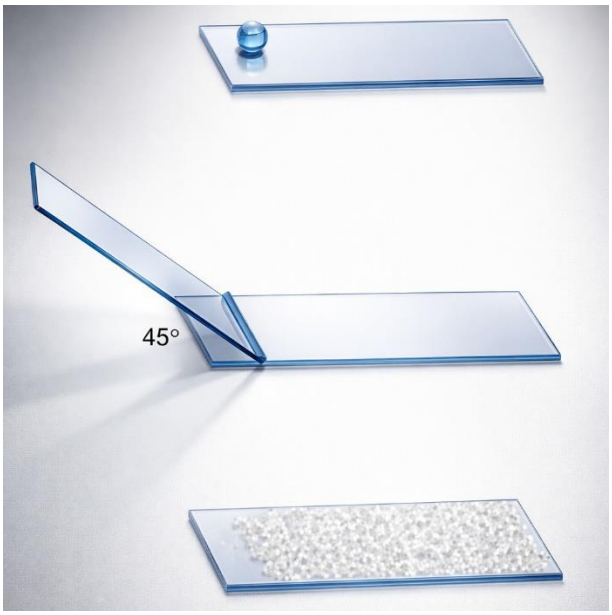


Figure 5 Feathering technique for preparing a sperm smear. A drop of semen is placed near the edge of a slide, and a second slide is then used to draw the drop along the surface, creating a thin layer of spermatozoa.

Assessment of sperm morphology

Sperm morphology and morphometry were evaluated using the SCA® Morphology module (version 6.6; Microptic SL, Hamilton Thorne, Barcelona, Spain) with a Zeiss brightfield microscope (63× objective, blue filter). Contrast and brightness were optimized for complete thresholding of sperm head and midpiece (MP). Images were captured using a Basler 780-75 Gigabit Ethernet camera, with 150 spermatozoa analyzed per male donor from randomly selected fields. Visual quality control was performed, and images with acquisition errors were excluded.

The system provided us with automated measurements of head morphometry, such as length-arc (µm), width (µm), perimeter (µm), area (µm^2), roughness and angle (degrees). Measurements of the MP included width (µm) and angle of insertion of the flagellum to the head (degrees). Additionally, there were recorded the chord length (µm), corresponding to distance from upper point of acrosome to base of head and the linearity expressed as percentage (%) value referring to ARC/chord x 100.

Data Storage and Analysis Records

For each sample, a dedicated record folder was created prior to analysis, capturing all sample details including genetic background, collection date, and processing information. Motility analysis files (mrf format) and corresponding video captures were stored in this folder. During motility analysis, fields with aberrant values flagged by the CASA system could be excluded as needed.

Morphology analysis data were similarly archived as mrf files within each male donor's record folder. Each morphology file contained 150 processed spermatozoon images with head and midpiece morphometry measurements, plus overall percentages of normal/abnormal sperm and specific head/midpiece abnormality rates relative to predefined normal thresholds in the analysis system.

Statistical analysis

Kinematic parameters were calculated as mean values derived from more than 1000 spermatozoa per male across a total of 40 individuals. Morphometric parameters were assessed using 150 spermatozoa per male from the same cohort. The number of animals was deemed sufficient on the basis of a power analysis assuming moderate Cohen's d (0.65) and standard thresholds for significance (α=0.05) and power (1-β=0.8). Furthermore, the number of animals per strain is consistent with the minimum cohort sizes recommended for establishing laboratory-specific reference ranges within systemic phenotyping frameworks, as outlined in the INFRAFRONTIER quality principles[60]

We used a percentile analysis to determine strain-specific reference ranges for kinematic (total motility, progressive motility, rapid progressive motility, VCL, VSL, VAP, ALH, BCF, LIN, STR, WOB, Hyperactivation) and morphometric (head arc, width, perimeter, area, roughness, angle; midpiece width, angle; chord length; linearity) parameters. For each strain and parameter, we first verified that a sufficient number of measurements were available to allow for the necessary statistical power (at α=0.001). We then identified outliers as values lying outside the +/-1.5 inter-quantile range (IQR) and removed them. For the remaining measurements we obtained the 2.5%-97.5% percentile range as reference.

Morphology normality thresholds were derived by reanalyzing the original 40 males per strain using strain-specific morphometric reference values integrated into the SCA® system, yielding

normality percentages (%) for each male. These were statistically summarized (median, 5th–95th percentiles) to define strain-specific cut-offs for normal morphology.

Inter-strain comparisons of all kinematic and morphometric parameters employed one-way ANOVA (or Kruskal–Wallis for the few cases were data did not pass an Anderson-Darling normality test). Significant differences ($p < 0.001$) were further explored using post-hoc tests (Tukey HSD or Dunn's), with results visualized as boxplots.

All data analysis was performed in R with custom scripts that are available upon request.

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