NOTE: Please save this file locally before filling in the table, DO NOT work on the file within your internet browser as changes will not be saved. Adobe Acrobat Reader (available free here) is recommended for completion.

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	This was an observational study using sexually mature wild type male mice from four inbred strains (C57BL/6J, C57BL/6N, BALB/c and CBA/J). For each strain, sperm motility and morphology were evaluated once per male using standardized CASA protocols, and the four strains served as comparison groups for inter-strain analyses and for The experimental unit was one single male mouse.
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Forty males per strain were analyzed, in line with minimum cohort sizes recommended for defining laboratory specific reference ranges in systemic phenotyping frameworks (INFRAFRONTIER quality principles).This corresponds to a total of 160 animals The sample size was based on existing recommendations and literature indicating that a minimum of 40 observations per group is required to define robust reference ranges for quantitative phenotypes.
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	Clinically healthy male mice without visible symptoms or lesions and aged 13–15 weeks were included. These criteria were defined before the start of the study. For data analysis, CASA flagged artefacts and obvious tracking errors were excluded No animals were excluded after enrollment. Only CASA flagged artefacts and statistical outliers were excluded at the data level. For all main analyses, n = 40 males per strain (C57BL/6J, C57BL/6N, BALB/c, CBA/J).
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Within each strain, males were selected from different littermate cages using an online random number generator. Allocation to groups was determined by genetic background (strain). All mice were bred and housed in the same room of the animal facility under identical environmental conditions. Sperm analyses were performed using the same equipment, settings and operators throughout the study to minimize technical confounders.
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	CASA motility and morphology measurements were generated automatically using predefined settings. Personnel performing sperm collection and sample preparation were aware of the strain identity for correct labeling, but both trained operators performing CASA analysis and data extraction, did not use strain information
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Outcome measures included CASA derived sperm motility parameters (total, progressive and rapid progressive motility; VCL, VSL, VAP; LIN, STR, WOB; ALH; BCF; and percentage of hyperactivated sperm) and The primary outcome measures for defining sample size and reference ranges were the CASA derived sperm motility parameters and head-midpiece morphometric parameters for each strain
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Percentile analysis was used to determine strain specific reference ranges (2.5–97.5 percentiles) for all kinematic and morphometric parameters after removal of outliers (values outside 1.5× the inter quartile range). Inter strain comparisons employed Normality was assessed using the Anderson–Darling test. When data did not meet the assumptions for ANOVA, the non parametric Kruskal–Wallis test was used instead. Significance thresholds were set at
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Wild type male mice from four inbred strains (C57BL/6J, C57BL/6N, CBA/J, BALB/c) were used. At sampling, mice were 13–15 weeks old. Median body weights were: C57BL/6J 28.58 g, C57BL/6N 27.78 g, CBA/J 29.04 g and BALB/c 27.18 g. All mice were wild type, bred and maintained under specific pathogen free conditions at the BSRC Alexander Fleming animal facility. Health monitoring followed FELASA recommendations. Animals had not
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	Male mice were euthanized by gradual CO_2 exposure in a chamber followed by cervical dislocation. Bilateral cauda epididymides were dissected, minced in a cryoprotective Each male was sampled and analyzed once, upon availability. All procedures were performed in the animal and laboratory facilities of BSRC Alexander Fleming, where animals were housed under standard specific pathogen free conditions. Animals were bred and maintained in the The procedures were performed to establish strain specific reference ranges for sperm motility and morphology in common inbred laboratory mouse strains and to enable a comparative analysis of sperm parameters
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	For each strain, summary statistics for all motility and morphometric parameters are reported in Tables 1 and 2, with inter strain comparisons illustrated by boxplots in Figures 3 and 4. Formal effect sizes with confidence intervals were not calculated, as the primary aim was to define reference ranges and demonstrate the presence of statistically significant inter strain differences rather than to estimate treatment effects

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	The objective of this study was to define refere
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	Using the SCA® system, we accurately and objectively determined the kinetic and morphometric parameters of mouse sperm. Some of the most relevant scientific publications include: 1. Van Der Horst, G., Skosana, B., Legendre, Sexually mature male mice from the inbred strains C57BL/6J, C57BL/6N, CBA/J and BALB/c were used, because they are among the most common backgrounds in reproductive and transgenic research. By defining reference ranges in these strains
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	This study asks what normal values for sperm
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	No ethical approval was required because the animals were not subjected to any procedures while alive. Epididymides were collected only after euthanasia, and all tissue collection was performed post-mortem in accordance with institutional and national animal welfare regulations.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
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	The mice were bred and maintained under spe
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	No specific interventions to reduce pain or distress were required because all experimental procedures took place after euthanasia. No expected or unexpected adverse events occurred The study did not involve live experimental procedures and therefore did not include humane endpoints
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	The study achieved its objectives by establishing CASA-based reference ranges for sperm motility and morphology in four widely used inbred mouse strains and showing that these parameters differ significantly between strains, confirming the The main limitations of this work are the restricted range of mouse strains studied, the narrow age window, and the single-center design. Only four inbred strains were examined, so additional commonly used backgrounds still need to be characterized
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	These reference ranges and strain differences are specific to the four inbred mouse lines, age range and experimental conditions used, so they should not be directly transferred to other strains, species or settings. However, the clear effect of genetic background on sperm motility and morphology is likely to be relevant for other mouse strains and mammals, and the same SCA system is
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	The study protocol was prepared before the work began and was submitted to the Veterinary School of the Aristotle University of Thessaloniki, where it was registered and received the relevant license.
Data access	20	Provide a statement describing if and where study data are available.	The datasets generated and analyzed during t
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	The authors declare no conflicts of interest related to this study There were no external funding sources for this study