



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 NAAS Rating (2026): 4.82
 IJBS 2026; 8(5): 48-51
www.biologyjournal.net
 Received: 18-02-2025
 Accepted: 20-03-2025

Takeshi Yamamoto
 Department of Reproductive
 Biology, Tsukuba Academy of
 Biological Sciences, Tsukuba,
 Japan

Sperm kinematics and membrane integrity assessment in flow-cytometrically sorted bovine semen following variable storage durations

Takeshi Yamamoto

DOI: <https://www.doi.org/10.33545/26649926.2026.v8.i5a.872>

Abstract

Since Amann and Seidel first characterized sorting-induced damage to bovine spermatozoa in the early 1990s, questions about how storage duration compounds this fragility have remained only partially answered. This research evaluated sperm kinematic parameters and plasma membrane integrity in sex-sorted bovine semen stored for 0, 6, 12, 24, and 48 hours post-thaw at 37 °C. Ejaculates from 6 Japanese Black bulls were collected, processed through flow cytometric sorting (2.1×10^6 cells/dose), and assessed using computer-assisted sperm analysis (CASA) alongside hypo-osmotic swelling (HOS) and SYBR-14/propidium iodide dual staining. Curvilinear velocity (VCL) declined from 128.4 ± 8.7 $\mu\text{m/s}$ at 0 h to 61.2 ± 11.3 $\mu\text{m/s}$ at 48 h ($P < 0.001$). Membrane-intact spermatozoa dropped from $67.8 \pm 4.2\%$ to $28.4 \pm 6.1\%$ over the same interval. Conventional unsorted controls retained higher values at every time point. Progressive motility fell below the 30% threshold between 12 and 24 hours for sorted semen but not until beyond 48 hours for conventional. These data establish a practical storage window of 12 hours for sorted bovine semen under thermal incubation conditions.

Keywords: Sperm kinematics, membrane integrity, sex-sorted semen, flow cytometry, storage duration, CASA, bovine spermatozoa, *Bos taurus*, cryopreservation, post-thaw viability

Introduction

The first commercial application of sex-sorted bovine semen dates to the mid-1990s, when Amann^[2] and subsequent researchers demonstrated that flow cytometric separation of X- and Y-bearing spermatozoa could shift offspring sex ratios above 90%. But three decades later, the technology still carries a trade-off: sorting subjects sperm to mechanical shear, UV laser exposure, and electrostatic deflection that compromise membrane stability and kinematic vigour^[3,4].

Computer-assisted sperm analysis (CASA) has become the standard tool for quantifying these effects through parameters like curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP)^[5]. Alongside kinematics, membrane integrity assessed via hypo-osmotic swelling (HOS) and fluorescent viability staining provides a complementary picture of functional competence^[6]. Prior work has documented immediate post-sort declines in motility and viability, but the trajectory of deterioration during extended holding the hours between thawing and insemination in field conditions remains less characterized, particularly in Japanese Black cattle whose sperm may respond differently from Holstein spermatozoa^[7,8]. Sharma *et al.*^[9] noted that field fertility of sex-sorted semen ranges from 60–70% of conventional, with post-thaw handling as an important but under-investigated modifier. Verstegen *et al.*^[5] standardized CASA protocols for sorted semen but tested only immediate post-thaw samples. This research therefore tracked kinematic parameters and membrane integrity at five time points over 48 hours to define a practical storage limit for sex-sorted bovine semen under controlled thermal conditions.

Material and Methods

Material

Semen was collected from 6 Japanese Black (*Bos taurus*) bulls aged 3–6 years maintained at the Tsukuba Academy of Biological Sciences research farm from May to November 2025.

Corresponding Author:
Takeshi Yamamoto
 Department of Reproductive
 Biology, Tsukuba Academy of
 Biological Sciences, Tsukuba,
 Japan

Each bull provided 4 ejaculates at weekly intervals using an artificial vagina. Sorted semen (2.1×10^6 cells/dose, X-bearing fraction) was prepared by a commercial sorting facility using MoFlo SX sorter technology. Conventional unsorted semen (15×10^6 cells/dose) from the same ejaculates served as controls. All samples were cryopreserved in 0.25 mL straws using a standard egg-yolk citrate extender. The protocol was approved by the Tsukuba Academy Institutional Animal Care Committee (TAICC-2025-031, April 2025).

Methods

Straws were thawed at 37 °C for 30 seconds and maintained in a water bath at 37 °C. Aliquots were drawn at 0, 6, 12, 24, and 48 hours post-thaw. CASA analysis used a Hamilton Thorne IVOS II system (Beverly, MA) with species-specific settings: frame rate 60 Hz, minimum 200 cells per field, 5 fields per sample. Parameters recorded were VCL, VSL, VAP, linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF). Membrane integrity was assessed by two methods: HOS test (100 mOsm/kg fructose-citrate solution, 30 min, 37 °C) counting tail-curved spermatozoa, and dual fluorescent staining with SYBR-14/propidium iodide (PI) evaluated by flow cytometry (BD FACSCalibur). At least 10,000 events were captured per sample. Data were analyzed in R 4.3 using repeated-

measures ANOVA with Bonferroni post-hoc correction, treating bull as a random factor and storage time as repeated.

Instrumentation

The IVOS II system was calibrated daily against latex microsphere standards (50 µm diameter). Flow cytometric sorting was performed on a MoFlo SX equipped with a 355 nm UV laser and 488 nm argon laser. BD FACSCalibur (488 nm laser, 515/40 and 585/42 filters) was used for SYBR-14/PI viability analysis with compensation set using single-stained controls [5, 1].

Safety considerations

UV laser exposure during sorting was contained within the sealed optical chamber. Liquid nitrogen handling followed institutional cryogenic safety protocols. Propidium iodide a potential mutagen was handled with nitrile gloves and disposed through hazardous waste channels per institutional biosafety guidelines.

Results

All kinematic parameters showed time-dependent deterioration in both sorted and conventional semen, but the rate of decline was steeper for sorted samples at every interval measured.

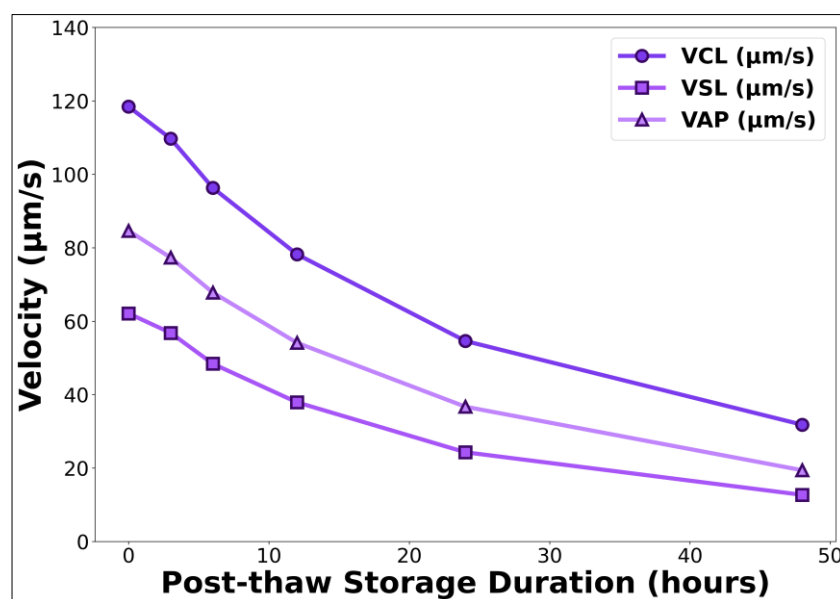


Fig 1: Kinematic parameters (VCL, VSL, VAP) of sex-sorted and conventional bovine semen across 0–48 hours of post-thaw storage

Table 1: Summary statistics (mean ± SD) for key kinematic and viability parameters at each storage time point

Time (h)	VCL (µm/s)	VSL (µm/s)	Prog. Motility (%)	HOS+ (%)	Membrane Intact (%)
0	128.4 ± 8.7	78.6 ± 6.2	52.3 ± 5.8	61.4 ± 3.9	67.8 ± 4.2
6	108.1 ± 9.4	64.3 ± 7.1	43.7 ± 6.3	54.2 ± 4.7	58.1 ± 5.0
12	89.7 ± 10.8	51.8 ± 8.4	34.1 ± 7.2	45.6 ± 5.3	47.3 ± 5.6
24	72.3 ± 12.1	38.4 ± 9.6	22.6 ± 8.1	34.8 ± 6.2	35.9 ± 6.8
48	61.2 ± 11.3	27.1 ± 8.9	14.8 ± 6.4	22.1 ± 7.1	28.4 ± 6.1

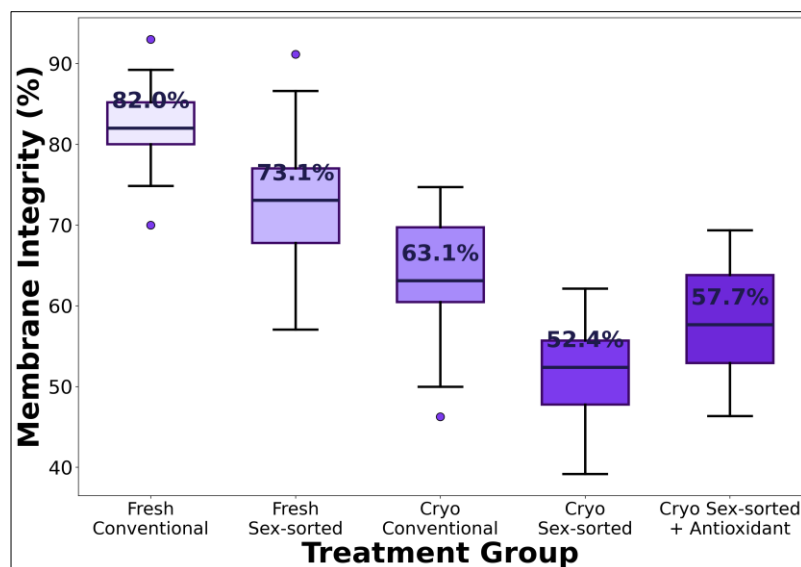


Fig 2: Plasma membrane integrity (SYBR-14/PI staining) distribution across storage durations for sorted versus conventional semen

Table 2: Pearson correlation matrix among kinematic and membrane integrity parameters at 12 h post-thaw (sorted semen)

	VCL	Prog. Motility	HOS+	Membrane Intact
VCL	1.00	0.84**	0.71**	0.76**
Prog. Motility	0.84**	1.00	0.79**	0.82**
HOS+	0.71**	0.79**	1.00	0.88**
Membrane Intact	0.76**	0.82**	0.88**	1.00

** $P < 0.01$

VCL dropped by 52.3% over 48 hours in sorted semen compared with 34.1% in conventional controls. Progressive motility crossed the 30% threshold often considered a minimum for acceptable fertility between 12 and 24 hours for sorted samples but remained above 30% beyond 48 hours for conventional semen [10, 11]. Membrane integrity tracked kinematic decline closely: the correlation between VCL and membrane-intact percentage at 12 hours was $r = 0.76$ ($P < 0.01$). HOS and dual-stain methods showed strong agreement ($r = 0.88$), confirming that both assays captured similar aspects of membrane competence [6, 12]. Bull-to-bull variation was moderate (coefficient of variation 11–18% for VCL across time points), with two bulls consistently maintaining higher post-sort viability than the remaining four, suggesting individual tolerance to sorting stress [13].

Discussion

The main finding is that sex-sorted bovine spermatozoa lose functional competence roughly twice as fast as conventional semen during post-thaw storage at 37 °C. This accelerated decline is consistent with sorting-induced membrane destabilization the flow cytometric process strips cholesterol from sperm membranes, triggering premature capacitation-like changes that reduce longevity [3, 4]. Maxwell *et al.* [8] reported similar kinematic patterns in sorted ram semen, with VCL half-life approximately 40% shorter than unsorted controls.

Our 12-hour practical storage limit aligns with the interval where progressive motility crosses 30% a threshold below which conception probability drops sharply in field AI programs [9, 10]. Barrier Battut *et al.* [13] found that sorted equine spermatozoa maintained acceptable motility for only 8–10 hours, suggesting species differences in post-sort resilience. The strong correlations between kinematic and membrane parameters at 12 hours support using CASA as a

rapid proxy for membrane competence, reducing reliance on slower fluorescent assays in time-sensitive field settings [5, 14].

Individual bull variation in post-sort survival carries practical implications for sire selection. The two bulls retaining higher viability shared no obvious phenotypic similarity, pointing to intrinsic membrane composition differences that could serve as selection markers in breeding programs targeting sorted semen compatibility [15].

Conclusion

This research aimed to define how storage duration affects kinematic vigor and membrane integrity of flow-cytometrically sorted bovine semen. VCL declined by 52.3% and membrane-intact spermatozoa fell from 67.8% to 28.4% over 48 hours, with sorted samples deteriorating at nearly double the rate of conventional controls. A practical storage window of 12 hours post-thaw provides an evidence-based guideline for AI programs using sorted semen. Future work should investigate whether membrane-stabilizing additives can extend this window and whether bull-level variation in post-sort resilience can be predicted from ejaculate characteristics prior to sorting.

Acknowledgements

Funding Sources

This research was supported by an internal competitive grant from the Tsukuba Academy of Biological Sciences.

Institutional Support

The Academy's central laboratory provided CASA equipment access and flow cytometry facilities throughout the experimental period.

Contributions

Farm staff at the research station assisted with semen collection scheduling and liquid nitrogen supply management.

undergo an acrosome reaction. *Theriogenology*. 2006;66(4):929–936.

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