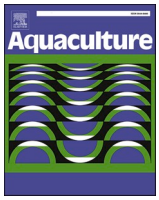
Aquaculture 612 (2026) 743197

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Standardizing CASA analysis to accurately assess sea urchin *Paracentrotus lividus* sperm motility

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ARTICLE INFO

Keywords:
Sea urchin
Sperm
CASA
Bovine serum albumin
Counting chamber

ABSTRACT

Although computer-assisted sperm analysis (CASA) system is widely applied for evaluating sperm motility in numerous vertebrate species, its application to sea urchin sperm remains comparatively underexplored, with limited data available on this invertebrate model. This study aimed to standardize the frame rate, type of counting chambers, extender, and dilution rate to use CASA system to evaluate sea urchin sperm motility. Semen samples were collected and diluted in artificial sea water alone (ASW) or supplemented with 0.3 % serum bovine albumin (ASW-BSA). The diluted semen was filled in 4 chamber types: drop displacement, Spermtrack® (SK) and R2D10 (R2); and capillary, Kubus® (KU); and SpermLide® (SL). Three dilutions ranging from low to high were studied. Sperm motility was evaluated using a commercial CASA system (AI Station v1.2; Sperm Analysis Technologies S.L., Buñol, Spain) that employs artificial intelligence for sperm recognition. Spermatozoa were immotile in drop displacement chambers when diluted in ASW; however total motility (TMOT) and progressivity (PMOT) did not differ significantly across chamber types when semen was diluted in ASW-BSA, independently of filling chamber type. KU chamber showed significantly higher velocity parameters compared to SL chamber when semen was diluted in ASW. However, these differences disappeared when in ASW-BSA medium, and straight line velocity (VSL) was even significantly higher in SL chamber. As dilution increased (i.e., lower cell concentration), sperm velocity increased, and linearity decreased. The optimal frame rate for accurate measurement of curvilinear velocity (VCL) was 510 fps, whereas lower frame rates (< 360 fps) were sufficient for evaluating VSL and average path velocity (VAP). In conclusion, to optimally assess sea urchin sperm motility using CASA requires a frame rate of 510 fps, dilution in ASW-BSA to concentrations below 75 × 10^6 sperm/mL and using capillary-filling chambers.

1. Introduction

The sea urchin (*Paracentrotus lividus*) is a marine invertebrate from the phylum Echinodermata, primarily found in the northeastern Atlantic Ocean and the Mediterranean Sea, where it populates rocky shores. It is the most commercially exploited echinoderm species in Europe (Boudouresque and Verlaque, 2020; Casal et al., 2020; Paredes et al., 2021). *P. lividus* plays a crucial role in marine ecosystems, especially in the Mediterranean where it helps maintain ecological balance (Hereu et al., 2013; Prado et al., 2012). Due high demands for its gonads as luxury seafood, *P. lividus* has been intensively harvested (Boudouresque and Verlaque, 2020). However, overexploitation of this species has led to early signs of local population disruptions (Parrondo et al., 2022; Piazzi and Ceccherelli, 2019), thus raising serious concerns about its population conservation in coastal waters. Moreover, *P. lividus* is widely recognized as a model invertebrate for ecotoxicological research due to its sensitivity to pollutants (Au et al., 2002; Hudson and Sewell, 2022), external fertilization mode, and ease of gamete collection. Its embryos and larvae are commonly used to assess the effects of contaminants, endocrine disruptors, and environmental stressors on development,

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[1] Retired

https://doi.org/10.1016/j.aquaculture.2025.743197
Received 27 May 2025; Received in revised form 27 August 2025; Accepted 15 September 2025
Available online 16 September 2025

making it a key indicator species in marine environmental monitoring (Prado et al., 2012). In this sense, assisted reproduction techniques in sea urchins are fundamental tools for studying developmental biology, preserving genetic diversity, and supporting ecological research (Mendes et al., 2019). These methods typically involve the collection and activation of gametes, with sperm typically diluted in seawater to stimulate motility. Advanced approaches, such as in vitro fertilization and sperm cryopreservation, are also used to support long-term research and conservation (Asahina and Takahashi, 1979; Epel, 2020; Fabbrocini et al., 2014; Paredes et al., 2022, 2019).

The sperm motility rates and their kinematic characteristics are key factors that strongly reflect fertilization potential. Particularly, sperm swimming velocity is a crucial indicator of sperm quality and fertilizing potential as it significantly influences fertilization success and hatching rate in broadcast spawners (Fabbrocini et al., 2016; Gallego and Asturiano, 2018; Gallo et al., 2022). In this context, the use of computer-assisted sperm analysis (CASA) has, for years, enhanced the evaluation of sperm motility (Gallagher et al., 2018; Gallego and Asturiano, 2018; Holt et al., 2018; Van Der Horst et al., 2018a), especially with the recent integration of artificial intelligence (AI) (Panner Selvam et al., 2024). Integrating machine learning and AI tools to analyse semen has allowed for a more refined evaluation of the heterogeneous spermatozoa population, which in turn enhances the identification of individual cells. (Bengio et al., 2013; Hicks et al., 2019; Hidayatullah et al., 2017; Ottl et al., 2022; Panner Selvam et al., 2024). Moreover, AI tools have demonstrated a good correlation between sperm kinematic and motile parameters reported automatically when compared to those obtained though manual evaluation (Battut et al., 2017; Dorado et al., 2013; Gallego and Asturiano, 2018; Gliozzi et al., 2017; Hicks et al., 2019).

However, the accuracy of CASA system output is still affected by several factors, including frame rate (fps) and the type of counting chamber used (Bompart et al., 2019; Caldeira et al., 2019; Castellini et al., 2011; Gacem et al., 2020b, 2020a; Valverde et al., 2019a, 2019b), as well as the extender (Araya-Zúñiga et al., 2023; Cejko et al., 2023; Kowalski et al., 2014; Peñaranda et al., 2010) and the dilution rate (Hadi Alavi et al., 2004; Hayden et al., 2015; León et al., 2024; Wu et al., 2024). These factors lead to discrepancies between results, thus prompting numerous studies across vertebrate species to pursue a standardization of CASA system protocols for sperm analysis (Del Gallego et al., 2017; Ibănescu et al., 2016; Palacín et al., 2013; Spiropoulos, 2001; Valverde et al., 2019a; Van Der Horst et al., 2018a; Yang et al., 2019). However, limited information is available regarding sperm motility assessment by CASA system in aquatic invertebrates (Van Der Horst et al., 2018a) and in particular in sea urchin sperm (Fabbrocini et al., 2016; Fabbrocini and D'Adamo, 2017). In *P. lividus*, Fabbrocini et al. (2016) linked highest motility to the spawning stage, while Fabbrocini and D'Adamo (2017) showed diluted semen remains viable for 24 h with only a slight velocity decline after 3 h.

In this context, establishing a standardized and consistent protocol to analyze sea urchin sperm motility is essential, and, to the best of our knowledge, no such study has been conducted to date. Therefore, the present study aims to develop a standardized methodology to evaluate sea urchin sperm motility using the latest generation CASA system equipped with a high-frame-rate camera to assess the effect of different factors such as counting chamber model types, dilution rate, and frame rate on sperm motility parameters.

2. Material and methods

2.1. Semen collection and media preparation

Sea urchin individuals were collected from two locations: the first was nearby the Cies islands at the Illas Atlanticas (42° 14′ 00″ N, 8° 54′ 00″ W) from the Galicia National Park (Vigo, Spain) in the Atlantic Ocean and the second in Cala Blanca (38° 47′ 21" N, 0° 9′ 48" E) in the Valencian region in the Mediterranean Sea (Xàbia, Spain) with all the required permits from local authorities. *P. lividus* were covered with wet paper to keep them humid during the trip from the sea to the laboratory. On the day of semen collection, a sea urchin was collected from the tank and manipulated within the first 24 hours. To obtain semen from a sea urchin, 1 mL KCl 0.5% (0.5M KCL dissolved in water) was injected through the peristomal membrane surrounding the mouth five minutes prior to dissection. Semen was aspirated with a micropipette from each male. A total of twelve male sea urchins were used for all studies of the present work (see 2.2. Experimental design). Aliquots of sperm were first diluted at 1:200 in sea water at room temperature. Later they were diluted a second time in two different extenders, artificial sea water (ASW) 3.4% (Sea-salt, Tropic Marin®, Germany) dissolved in milli-Q purified water) or ASW supplemented with 0.3% bovine serum albumin (ASW-BSA) (BSA, Sigma Aldrich, Spain).

2.2. Experimental design

In order to assess the effects of counting chamber type, dilution rate, and frame rate on sperm motility parameters three studies were carried out. In this study 1, effects of type of counting chamber depending on whether filling was by capillarity or drop and presence of BSA (0.3%) in the medium on sperm motility were studied. Droplet displacement chambers included the Spermtrack® 10 μm depth (SK), and ISAS® R2D 20 μm depth (R2) (Proiser R + D S.L., Paterna, Spain). Capillary filling chambers comprised the Kubus® (KU), and Spermlide® (SL) (Sperm analysis Technologies, Buñol, Spain) both with a depth of 20 μm (Fig. 1). In the study 2, effect of dilution rate on sperm motility assessment was studied. The samples were categorized retrospectively based on their recorded sperm concentrations recorded by CASA system from a first dilution (1:200), as described above, and two further successive dilutions. Three dilution groups were defined: group 1 for low dilution (more than 150×10^6 spermatozoa/mL), group 2 for medium dilution (between 75 and 150×10^6 spermatozoa/mL) and the last group 3 with a high dilution (less than 75×10^6 spermatozoa/mL). Sperm concentration was determined using the data generated by the CASA system, which calculates concentration based on counts performed by its integrated software. In the study 3, the effect of frame rate on sperm kinematic parameters and definition of the optimum frame rate were studied. Videos were recorded with a high-performance camera at 500 fps as described earlier. These videos were then segmented into frame rates of 50, 100, 200, 250, 300, and 400 up to 500 fps. All studies were conducted using samples from the same 12 collected sea urchins.

2.3. Motility assessment

For the studies 1 and 2, a new technology CASA system using AI for sperm recognition was used to assess sperm motility (AI Station v1.2; Sperm Analysis Technologies S.L, Buñol, Spain). The module used was designed for fish sperm recognition. The system includes a digital camera (Blackfly S bfs-u3, Sperm Analysis Technologies S.L, AI station R+D, Buñol, Spain) that captures images at 100 frames during one second. The setting set for sea urchin was at cellular size, between 6 μm^2 and 69 μm^2, Progressive sperm when STR > 50%, speed classification were VAP: 15 μm/s < slow < 30 < medium < 50 < rapid. Immotile sperm were defined as sperm swimming below threshold values of 15 μm/s VAP. Connectivity setting was fixed at 12 μm.

For the third study, ISAS®v1.2 CASA-Mot system (Proiser R+D S.L., Paterna, Spain) was used as the CASA system with a MQ003MG- CM digital camera (Proiser R+D S.L., Paterna, Spain) can capture 500 fps. The settings for CASA were: 10 < Particles Area (in μm^2) < 90; VCL: 10 < slow < 45 < medium < 100 < rapid (μm/s); progressivity: 80% of the STR; connectivity: 14. The same microscope UB203 (UOP; Proiser R+D S.L., Paterna, Spain) was used for both experiments. The microscope had a 10x negative phase contrast objective (AN 0.25). Multiple captures were made for each sample to have a final count of 1,000 spermatozoa per sample. The videos were recorded during 3 s for each field. Sperm

Fig. 1. The different types of chambers used to analyze sea urchin sperm motility. A, B: capillary filling chambers (A: Kubus®, B: Spermlide®); C, D: drop displacement chambers (C: Spermtrack®, D: ISAS® R2D20).

total motility (TMOT, %) and progressivity (PMOT, %) were assessed, along with key kinematic variables: curvilinear velocity (VCL, µm/s), measuring speed along the actual path of the sperm; straight line velocity (VSL, µm/s): the speed along the straight line connecting the first and last points of the track; average path velocity (VAP, µm/s), and the speed along a smoothed trajectory. Additionally, the amplitude of lateral head displacement (ALH, µm) was recorded, indicating the maximum width of head oscillation as the sperm swam. Three progression ratios, expressed as percentages, were also derived from these velocities: linearity (LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100), and wobble (WOB = VAP/VCL·100).

2.4. Type of counting chamber: Capillary vs droplet displacement filling

Two types of chambers were used based on the filling mechanism: droplet displacement or capillary. Droplet displacement chambers included the Spermtrack® 10µm depth and ISAS® R2D 20 µm depth (Proiser R+D S.L., Paterna, Spain). Capillary filling chambers comprised the Kubus® and Spermlide® (Sperm analysis Technologies, Buñol, Spain), both with a depth of 20µm (Figure 1). Prior to analysis, diluted semen samples were thoroughly homogenized in Eppendorf tubes. Sample volumes of 2 µL and 3 µL were loaded into the chambers with 10 µm and 20 µm depths, respectively. For droplet displacement chambers, the cover was gently placed on the top of the droplet to ensure uniform distribution across the chamber. In capillary chambers, semen was applied to the filling space, allowing capillary action to distribute the sample throughout the chamber. The sequence of chambers used was randomized to minimize the effect of incubation time.

2.5. Video segmentation

Videos were recorded with a high-performance camera at 500 fps. These videos were then segmented into frame rates of 50, 100, 200, 250, 300, 400 and up to 500 fps. This approach allowed us to minimize variations that might occur between different replicates. The command used was: [echo off: set fps= 50, 100, 200, 250, 300, 400 and 500: for %%i in (.*.avi) do (set fname=%%~ni) & call: encodeVideo; goto eof : encodeVideo: ffmpeg.exe -i %fname%.avi -r %fps% -clibx264 -preset slow -qp 0 %fname%_(%fps%fps).avi"; goto eof] (Gacem et al., 2020b, 2020a). The resulting files were saved in .avi format, labeled with the animal ID, frame rate, and replicate number. Subsequent analyses were performed using the ISAS®v1.2 CASA-Mot system (Proiser R+D S.L., Paterna, Spain), following the specifications previously cited for the ISAS®v1.2 system. Only the frame rate setting was adjusted for each analysis. After each analysis in the CASA system, the corresponding data cleaning and export process was conducted according to the analyzed frame. The optimal frame rates (OFR) were determined based on the curvilinear velocity of the sperm, which were calculated by reconstructing their trajectories point-by-point for all the frame rates until 500. Exponential regression analysis was applied to the results using the model:$y = \beta.\alpha \exp.(-\beta/x)$where y is the VCL, x is the FR, α is the asymptotic level, β is the rate of increase to the asymptote, and exp. based on the natural logarithm. No significant increase in VCL was observed with higher FRs once the asymptotic value α was reached, thus indicating that at least 95 % of the maximum VCL was achieved. The rate at which the curve approaches the asymptote reflects its dependency on FR, where a high β value suggests a stronger increase in VCL with higher FR, and a low β indicates a lesser dependency.

2.6. Statistical analysis

The assumptions of normality and homoscedasticity were tested with the Shapiro-Wilks and Levene tests, and normal probability plots were used to evaluate normal distribution for all sperm variables analyzed. The effect of extender (ASW or ASW-BSA), dilution rate, chamber type (capillary or droplet dispersion) and frame rate, including their interactions on motility and kinematic variables, were analyzed using generalized linear models and analysis of variance (ANOVA). Post-hoc comparisons were conducted using the Bonferroni test ($P < 0.05$). The influence of optimal frame rate was further examined through exponential regression models. Results were expressed as least square means ± standard error of the mean. Data were analyzed using the IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, US).

3. Results

3.1. Effects of filling chamber type (droplet vs. capillary) on sperm motility

Sea urchin spermatozoa remained immotile in droplet-filling chambers (SK and R2) when it was only diluted in ASW. In contrast, over 30% TMOT was observed when samples were assessed using capillary-filling chambers, with no differences found between these chambers (KU and SL; Table 1). Among these, sperm assessed in the KU chamber with ASW exhibited significantly higher PMOT, as well as all kinematic parameters (VCL, VSL, VAP, and LIN), except for STR, since it was higher in the SL

Table 1
Sea urchin sperm motility and kinematic parameters (means ± SEM) analyzed in capillarity filling chamber diluted in artificial sea water (ASW) medium.

Motility sperm variables	Capillarity filling chamber	
	KU	SL
TMOT	34.13 ± 4.65	33.96 ± 5.17
PMOT	54.21 ± 2.53	57.99 ± 3.00*
VCL	287.99 ± 0.60	257.25 ± 0.62*
VSL	105.10 ± 0.36	92.70 ± 0.38*
VAP	189.07 ± 0.44	165.34 ± 0.46*
LIN	35.86 ± 0.07	34.41 ± 0.08*
STR	57.76 ± 0.09	59.05 ± 0.10*

TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); Capillarity filling chambers (Kubus® (KU), Spermlide® (SL)). * indicate significant differences between different capilary filling chambers $P < 0.05$; SEM = standard error of the mean.

chamber ($P < 0.05$; Table 1).

When semen was diluted in ASW-BSA medium, no significant differences in TMOT and PMOT were observed across the different chamber types, including the droplet displacement chambers (Table 2). However, significant differences were found in kinematic parameters. Capillary chambers (KU and SL) yielded significantly higher values for VSL, LIN, and STR than the droplet displacement chambers ($P < 0.05$; Table 2). The SL chamber showed higher values of VSL, LIN and STR than KU chamber ($P < 0.05$; Table 2). The SK chamber showed the lowest value for all kinematic parameters. While some kinematic variables differed notably among the R2, KU, and SL cambers, the numerical differences were minor.

3.2. Effects of extender on sperm motility in capillary-filling chamber

Motility parameters of sperm diluted in ASW or ASW-BSA, assessed using capillary-filling chambers are presented in Table 3. Total motility doubled when sperm were diluted in ASW-BSA compared to ASW alone. However, PMOT did not differ significantly between the two extenders for either chamber. Notably, in the SL chamber, all the kinematic parameters were significantly higher in ASW-BSA medium compared to ASW. In contrast, in the KU chamber, only VCL and VAP were significantly higher in ASW compared to ASW-BSA.

Table 2
Sea urchin sperm motility and kinematic parameters (means ± SEM) analyzed in drop displacement and capillary chamber diluted in sea water medium supplemented with BSA (ASW-BSA).

Motility sperm variables	Drop displacement chambers		Capillarity chambers	
	SK	R2	KU	SL
TMOT	58.4 ± 4.45	57.54 ± 4.78	62.37 ± 4.19	60.78 ± 5.34
PMOT	55.7 ± 2.78	55.39 ± 2.97	55.62 ± 2.81	54.73 ± 2.78
VCL	268.93 ± 0.39^{a}	273.79 ± 0.43^{bc}	272.35 ± 0.36^{b}	271.82 ± 0.43^{b}
VSL	109.35 ± 0.28^{a}	120.91 ± 0.31^{c}	116.62 ± 0.26^{b}	123.14 ± 0.31^{d}
VAP	177.63 ± 0.29^{a}	179.97 ± 0.32^{b}	179.03 ± 0.27^{b}	179.61 ± 0.32^{b}
LIN	38.12 ± 0.05^{a}	41.15 ± 0.06^{c}	39.95 ± 0.05^{b}	42.16 ± 0.06^{d}
STR	59.86 ± 0.06^{a}	63.00 ± 0.07^{c}	61.79 ± 0.06^{b}	64.46 ± 0.07^{d}

TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%). Drop displacement chambers (Spermtrack® (SK), R2D10® (R2)); Capilary filling chambers (Kubus® (KU), Spermlide® (SL)).$^{a\text{-}d}$ indicate significant differences between different chambers types $P < 0.05$; SEM = standard error of the mean.

Table 3
Effect of medium type on sea urchin sperm kinematic parameters (means ± SEM) in the capillary filling chambers.

Motility sperm variables	KU		SL	
	ASW	ASW-BSA	ASW	ASW-BSA
TMOT	34.13 ± 4.65	62.37 ± 4.19*	33.96 ± 5.17	60.78 ± 5.34*
PMOT	54.21 ± 2.53	55.62 ± 2.81	57.99 ± 2.99	54.73 ± 2.78
VCL	287.99 ± 0.55	272.35 ± 0.35*	257.25 ± 0.57	271.82 ± 0.42*
VSL	105.10 ± 0.38	116.61 ± 0.24*	92.69 ± 0.40	123.13 ± 0.29*
VAP	189.07 ± 0.41	179.02 ± 0.26*	165.33 ± 0.42	179.61 ± 0.31*
LIN	35.86 ± 0.80	39.95 ± 0.05*	34.40 ± 0.08	42.16 ± 0.06*
STR	57.76 ± 0.09	61.79 ± 0.06*	59.05 ± 0.09	64.46 ± 0.06*

TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); KU: Kubus®, SL: Spermlide®, ASW (artificial sea water), ASW-BSA (artificial sea water supplemented with 0.3 % bovine serum albumine; * indicate significant differences between diluted in ASW or ASW-BSA for each capilary filling chambers $P < 0.05$; SEM = standard error of the mean.

3.3. Effects of sperm dilution rate on sperm motility assessment

The effect of sperm concentration at the time of motility assessment is summarized in Table 4. TMOT, PMOT, VCL, and VAP were significantly higher in highly diluted samples (lowest sperm concentration; group 3). However, these samples exhibited the lowest values for LIN and STR when compared to more concentrated groups.

3.4. Effects of frame rate on sperm motility

The OFR for the estimation of VCL was determined to be 510 fps. As expected, the rest of the variables, which are less sensitive to frame rate variation, exhibited lower OFRs. For example, the OFR estimation of LIN and STR was achieved at frame rates below 80 fps (Table 5, Figure 2). As shown in Table 6, sperm velocity parameters (VCL, VSL, and VAP) exhibited an approximate 25% increase in variability for every 100-fps increment. In contrast, LIN and STR remained stable across different frame rates when compared to the baseline at 50 fps. Figure 3 illustrates

Table 4
Effect of sperm dilution on sea urchin motility and kinematic parameters (means ± SEM) analyzed in ASW-BSA and ASW medium for all chambers.

Motility sperm variables	Dilutions		
	1	2	3
TMOT	44.51 ± 4.3^{b}	45.32 ± 5.76^{b}	54.78 ± 4.29^{a}
PMOT	47.02 ± 2.29^{c}	57.59 ± 2.24^{b}	65.02 ± 1.09^{a}
VCL	270.22 ± 0.3^{b}	267.88 ± 0.3c	277.19 ± 0.46^{a}
VSL	112.94 ± 0.21^{b}	117.08 ± 0.21^{a}	100.27 ± 0.32^{c}
VAP	176.64 ± 0.22^{b}	177.01 ± 0.22^{c}	182.18 ± 0.34^{a}
LIN	38.89 ± 0.04^{b}	41.06 ± 0.04^{a}	34.43 ± 0.06^{c}
STR	61.64 ± 0.05^{a}	62.97 ± 0.05^{b}	56.14 ± 0.07^{c}

1: low diluted (more than 150 × 10^{6} sperm/ml); 2: Medium diluted (between 75 and 150 × 10^{6} sperm/ml); 3: high dilution (less than 75 × 10^{6} sperm/ml). TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); SEM = standard error of the mean. $^{a\text{-}c}$ Different superscripts indicate significant differences between the different sample concentration. $P < 0.05$.

Table 5
Asymptotic level (α). rate of increase (β). correlation α/β ($\rho^{\alpha,\beta}$) and estimate (at a frame rate of 500 fps) of sea urchin sperm kinematic variables needed to obtain the threshold level (95 % of the maximum).

Sperm kinematic variables						Estimated values at different fps						
	α	SEM	β	SE	$\rho^{\alpha,\beta}$	50	100	200	250	300	400	500
VCL	510.1	0.7	59.1	0.3	0.80	156.4	282.5	379.6	402.7	418.9	440.0	453.2
VSL	268.0	0. 5	58.3	0.4	0.80	83.5	149.6	200.2	212.3	220.7	231.7	238.5
VAP	360.2	0.5	62.4	0.3	0.81	103.4	193.0	263.7	280.6	292.6	308.2	317.9
LIN	54.4	0.1	2.1	0.2	0.75	52.2	53.3	53.8	53.9	54.0	54.1	54.2
STR	77.7	0.1	5.5	0.2	0.75	69.6	73.5	75.6	76.0	76.3	76.6	76.8
WOB	68.9	0.05	−3.5	0.1	0.76	73.9	71.4	70.1	69.9	69.7	69.5	69.4
ALH	157.0	0.01	−27.2	0.1	0.78	2.7	2.1	1.8	1.8	1.7	1.7	1.7
BCF	83.8	0.2	80.6	0.5	0.83	16.7	37.4	56.0	60.7	64.1	68.5	71.3

VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); SEM = standard error of the mean); fps = frame rate;

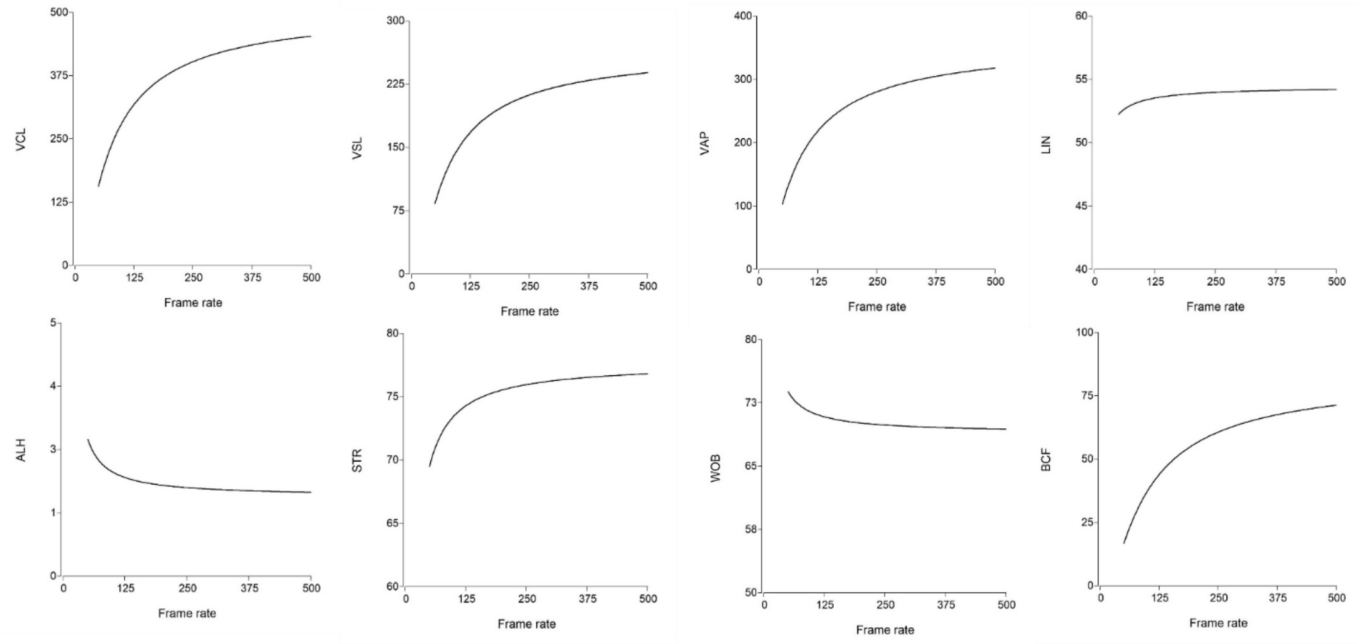

Fig. 2. Effect of frame rate on sperm kinematic parameters of sea urchin sperm. VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); ALH = amplitude of lateral head displacement (μm); STR = straightness (%); WOB = wobble (%); BCF = beat-cross frequency (Hz).

Table 6
Effect of frame rate (fps) on sea urchin sperm kinematic measurements (means ± SEM, and percentage of variation with respect to 50 fps in brackets). $n = 120{,}037$.

Sperm kinematic variables	Frame rates (fps)						
	50	100	200	250	300	400	500
VCL	190.7 ± 0.9^{a}	267.7 ± 0.9^{b} (40.3)	354.3 ± 1.0^{c} (85.7)	391.8 ± 1.0^{d} (105.4)	406.2 ± 1.0^{e} (113.0)	447.1 ± 1.1^{f} (134.4)	489.4 ± 1.1^{g} (156.6)
VSL	98.9 ± 0.6^{a}	145.4 ± 0.7^{b} (47.1)	187.0 ± 0.7^{c} (89.1)	206.9 ± 0.7^{d} (109.2)	213.8 ± 0.8^{e} (116.3)	235.6 ± 0.8^{f} (138.3)	256.3 ± 0.8^{g} (159.3)
VAP	151.0 ± 0.6^{a}	183.5 ± 0.6^{b} (21.6)	225.3 ± 0.6^{c} (49.2)	250.2 ± 0.7^{d} (65.7)	269.0 ± 0.7^{e} (78.2)	324.6 ± 0.7^{f} (115.0)	381.5 ± 0.7^{g} (152.7)
LIN	51.8 ± 0.1^{a}	54.5 ± 0.2^{b} (5.2)	53.7 ± 0.2^{c} (3.7)	54.1 ± 0.2^{bc} (4.4)	54.1 ± 0.2^{bc} (4.5)	54.3 ± 0.2^{b} (4.8)	53.3 ± 0.2^{c} (2.9)
STR	65.5 ± 0.2^{a}	77.0 ± 0.2^{b} (17.6)	80.7 ± 0.2^{c} (23.3)	81.1 ± 0.2^{c} (23.8)	78.3 ± 0.2^{d} (19.5)	72.4 ± 0.2^{e} (10.6)	67.4 ± 0.2^{f} (2.9)
WOB	77.3 ± 0.1^{a}	68.6 ± 0.1^{b} (−11.2)	64.7 ± 0.1^{c} (−16.2)	65.2 ± 0.1^{d} (−15.6.)	67.8 ± 0.1^{e} (−12.3)	74.0 ± 0.1^{f} (−4.2)	78.7 ± 0.1^{g} (1.8)
ALH	2.6 ± 0.01^{a}	2.2 ± 0.01^{b} (−15.8)	2.1 ± 0.01^{c} (−20.5)	1.9 ± 0.01^{d} (−26.9)	1.7 ± 0.01^{e} (−35.1)	1.5 ± 0.01^{f} (−42.1)	1.3 ± 0.01^{g} (−49.4)
BCF	11.5 ± 0.2^{a}	39.3 ± 0.2^{b} (242.6)	59.0 ± 0.2^{c} (413.9)	61.9 ± 0.2^{d} (438.3)	59.2 ± 0.2^{c} (415.8)	63.0 ± 0.2^{e} (448.9)	78.1 ± 0.3^{f} (580.6)

n = total number of spermatozoids analyzed. VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz); SEM = standard error of the mean. [a-f] Different superscripts indicate significant differences among frame rates. $P < 0.05$.

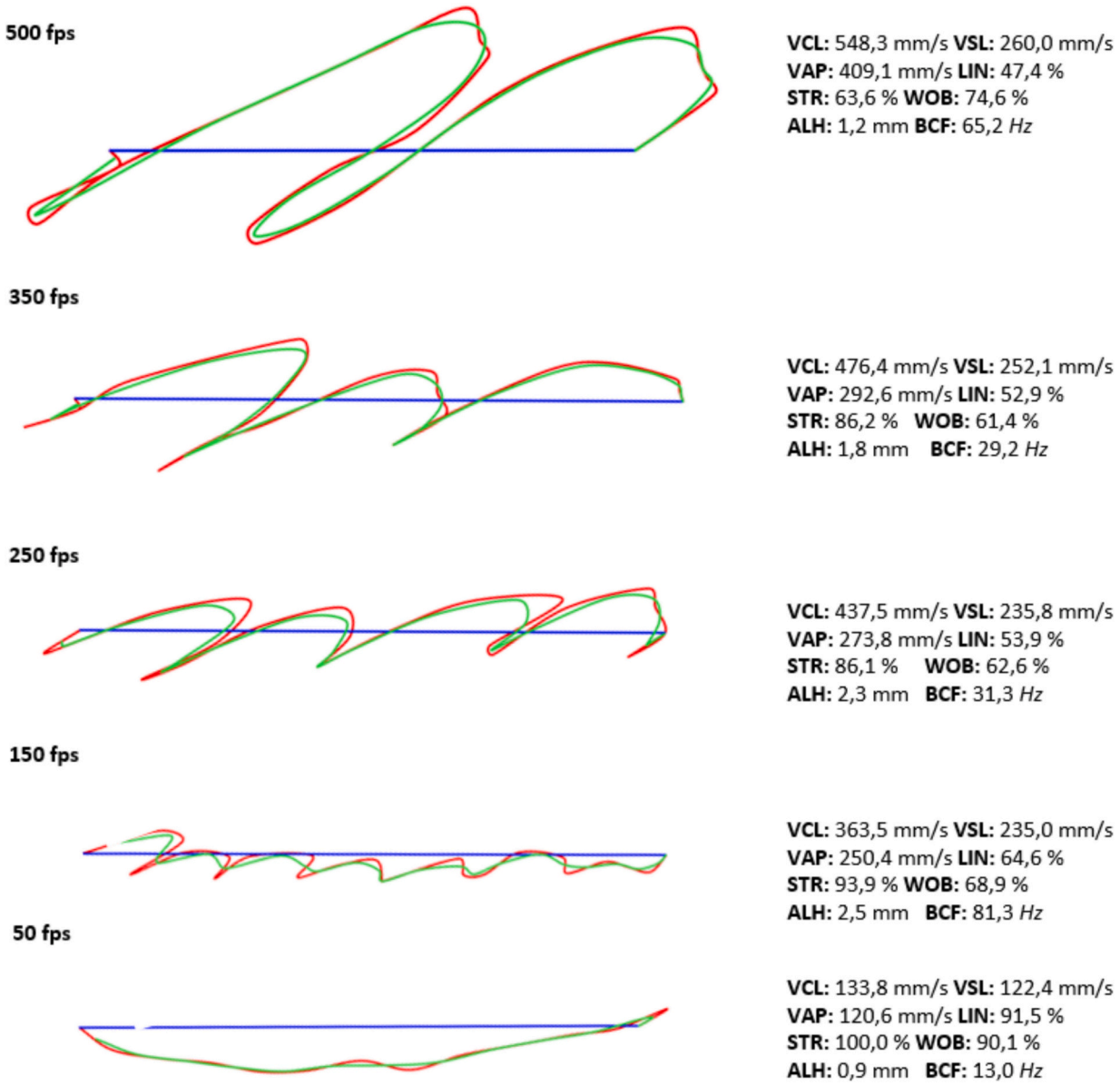

Fig. 3. Example of *Sea urchin* sperm trajectory at different frame rates. Fps: frames per second, VCL = curvilinear velocity (μm/s); VSL = straight line velocity (μm/s); VAP = average path velocity (μm/s); LIN = linearity of forward progression (%); STR = straightness (%); WOB = wobble (%); ALH = amplitude of lateral head displacement (μm); BCF = beat-cross frequency (Hz).

an example of sperm trajectory recorded using the different frame rates and highlights the changes in trajectory as a function of frame rate.

4. Discussion

In the last decades, CASA systems have gained widespread popularity for their practical assessments of sperm motility across various species as they offer important advancements in the standardization of protocols, mainly in vertebrate species and particularly in mammals (Gacem et al., 2020b; Gallego et al., 2013; Palacín et al., 2013; Yang et al., 2019). However, little information has been found in the literature on the use of CASA-mot systems in aquatic invertebrates (Gallego et al., 2014), and even less specifically on sea urchins; information is especially scarce regarding optimal frame rates, sperm density per field, or the influence of chamber design on results. To the best of our knowledge, the present study is the first comprehensive attempt to standardize CASA-based sperm motility assessment in sea urchins.

One of the main challenges of using CASA for sea urchin sperm is their tendency to adhere to glass surfaces, leaving the sperm immotile and compromising the assessment of sperm motility when sea water, their natural fertilization medium, is used as extender. Thus, incorporating substances that reduce sperm adhesion is essential when preparing aliquots for sea urchin sperm motility assessment (Bracho et al., 1997; Fabbrocini et al., 2016). However, to the best of our knowledge, the effect of BSA supplementation on fresh sea urchin sperm motility parameters was not studied. BSA has a relatively high molecular weight (approximately 66-69 kDa), which can help reduce surface tension in experimental chambers (Rodríguez Niño and Rodríguez Patino, 1998). This property prevents sperm cells or other particles from adhering to glass surfaces. Furthermore, BSA is commonly added as an activating fluid to the sperm of many fish species (Cejko et al., 2024; Kowalski et al., 2014). However, the addition of BSA could increase the viscosity of the medium (Yadav et al., 2011), potentially altering sperm kinematics, especially in species like sea urchins whose sperm are adapted to swim in the low-viscosity environment of sea water.

In the current study, sperm mounted on droplet displacement

chambers showed no motility, like boar sperm findings, which also adhere to chamber surfaces (Hackerova et al., 2025). However, by adding 0.3% BSA to the ASW extender, the cells doubled TMOT with a good progressivity. The concentration of BSA used for CASA analysis of sea urchin by the different authors ranged from 0.01 to 1% (Bracho et al., 1997; Fabbrocini et al., 2023; Fabbrocini and D'Adamo, 2017; Ohmuro et al., 2004). BSA has also been shown to increase sperm velocity and motility in ide (*Leuciscus idus*) and common carp at 0.5–1% concentrations (Cejko et al., 2023; Kowalski et al., 2014), although no effect was found in whitefish sperm (Cejko et al., 2024), indicating potential species-specific responses to BSA. When an SL capillary filling chamber is used, adding BSA to ASW resulted in increased TMOT and kinematic parameters. However, in the KU chamber, higher values for VCL and VAP were observed with ASW alone. Capillary chambers, which are pre-assembled with glued coverslips and surfactant-coated glass, allow samples to fill by capillary action, offering more consistent conditions than manually assembled droplet chambers. This design likely minimizes glass surface boundary effects, as previously reported for sperm of mammals (Bompart et al., 2018) and fishes (Gee and Zimmer-Faust, 1997), as well as for terrestrial invertebrates such as honeybees (Yániz et al., 2019).

In the present study, significant differences in sperm kinetics were observed between capillary-filling chambers. This difference can be attributed to the chamber design, which has been reported in many studies involving mammalian sperms to have a direct effect on sperm movement (Bompart et al., 2018; Del Gallego et al., 2017; Gacem et al., 2020a; Gloria et al., 2013; Hoogewijs et al., 2012; Ibănescu et al., 2016; Peng et al., 2015). In fact, the variations in the width and shape of the space where the sample is distributed create differences in capillary forces as the semen moves, leading to a non-uniform distribution of spermatozoa (Bompart et al., 2018). When the chamber is filled with the sample, it migrates by capillary action between the slide and cover slide, driven by surface tension between the liquid and the surface. This migration creates a meniscus at the leading edge known as laminar Poiseuille flow (Douglas-Hamilton et al., 2005). This flow generates a transverse lifting force making sperm floating to the surface and affecting their velocity.

When comparing sperm movement in ASW-BSA across chamber types (capillary vs. droplet), only slight differences in kinematic parameters were observed among R2, KU, and SL chambers. Similar results were observed when using Makler® or ISAS DC420® counting chambers to analyze European eel sperm motility (Gallego et al., 2013). This supports the hypothesis that adding BSA standardizes conditions within the chamber and neutralizes the effects caused by a filling method or design. Similar results were found in honeybee spermatozoa (Yániz et al., 2019). Finally, the SL chamber showed the highest kinematic parameters for VSL and LIN in BSA supplemented medium, suggesting it offers optimal conditions for assessing sea urchin sperm motility. Besides the counting chamber type or the shape of the chamber, sperm dilution significantly influences the CASA-mot results (WU et al., 2024; Yeste et al., 2018). In the current study, increased dilution (i.e., lower sperm concentration) improved sperm motility and velocity parameters but reduced LIN and STR. This agrees with studies in fish species (Hadi Alavi et al., 2004; Muchlisin et al., 2024), although the opposite trend is often observed in mammals (Gacem et al., 2020b; Iguer-ouada and Verstegen, 2019), highlighting the role of fertilization environment (external vs. internal). In sea urchins, high dilutions stimulate motility and respiration, a phenomenon known as the 'sperm dilution effect' (Rothschild, 1948), which is regulated by environmental pH (Potter et al., 2024). However, the low motility behavior of sea urchin sperm as sperm concentration increased can be explained by condensation and proximity, in other words, each sperm movement is restricted by its proximity to others. This high-density environment can reduce the overall motility and agility of individual spermatozoa, a concept referred to as allostatic load (Rothschild, 1948). Moreover, dense sperm suspensions in a small space may accumulate carbon dioxide in the medium and that can inhibit or reduce their motility (Rothschild, 1948), elevate reactive oxygen species (ROS), and thus compromise viability (Kazama et al., 2014; Rahman et al., 2001).

CASA systems rely on short video captures to analyze sperm motion. Advances in video technology have led to the development of high-capacity cameras, capable of recording at 500 fps. However, most of the published studies on sea urchin motility analysis have used fps values below 100 fps (Au et al., 2002; Bracho et al., 1997; Fabbrocini and D'Adamo, 2017). To the best of our knowledge, this study is the first to examine sea urchin sperm motility at such high frame rates, showing that VCL increases with fps and reaches an optimal at 510 fps. Even if other sperm kinematic parameters are less sensitive to FR, all motility characteristics differed notably between 100 and 500 fps recordings. These results agree with those previously described in spermatozoa of different fish species (Caldeira et al., 2019) and suggest that higher fps is necessary to study sperm motility in sea urchins compared to mammals (Bompart et al., 2019; Gacem et al., 2020a, 2020b; Valverde et al., 2019b). Sea urchins are broadcast spawners that fertilize in sea water; therefore, their flagella beats at high frequencies and this aids efficient movement towards the eggs (Guerrero et al., 2020). Unlike mammalian sperm, which fertilizes internally and must swim through high-viscosity fluids, sea urchin sperm operates in the low-viscosity environment of sea water. In fact, in the low viscosity of ASW, the flagellum can beat at higher frequencies, a capability enabled by the specific structure of their axoneme which has a mechanotransducer function (Guerrero et al., 2020). Besides, as sperm speed increases, so does the size of its helical swimming path, and this enhances its prospect of reaching an oocyte (Van der Horst et al., 2018b). Our findings confirm that only at high frame rates can the full complexity of sea urchin sperm motility be captured, including the three-dimensional helical paths of movement (Elgeti et al., 2010; Gong et al., 2021).

In conclusion, ASW-BSA extender is effective to evaluate the sperm motility of sea urchins, capillary-filling chambers, particularly the SL chamber in ASW-BSA medium, offer optimal conditions for consistent CASA analysis in sea urchin spermatozoa. A dilution level below 75×10^6 spermatozoa/mL is recommended for accurate motility measurements. Lastly, the use of high-frame-rate cameras, preferably at 510 fps, is needed to accurately analyze sperm VCL in the sea urchin.

CRediT authorship contribution statement

Sabrina Gacem: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Estefanía Paredes:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sara Campos:** Writing – original draft, Methodology, Data curation. **Francisco Sevilla:** Methodology, Investigation, Formal analysis. **Carles Soler:** Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Jesús Yániz:** Writing – original draft, Project administration, Methodology, Funding acquisition. **Anthony Valverde:** Project administration, Methodology, Funding acquisition, Formal analysis, Data curation. **Miguel A. Silvestre:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding sources

This study forms part of the AGROALNEXT program and was supported by Ministerio de Ciencia e Innovación with funding from the European Union NextGenerationEU (PRTR-C17.I1), by Generalitat Valenciana (AGROALNEXT/2022/063). The authors thank the Costa Rica Institute of Technology (ITCR) and Postgraduate Office of ITCR. This work was part of the research project VIE-2151083. E.P. holds a Ramon y Cajal grant by MICIU/AEI/10.13039/501100011033 and by

"European Union NextGenerationEU/PRTR.

Declaration of competing interest

Miguel A. Silvestre reports financial support was provided by University of Valencia. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We thank Conselleria de Agricultura, Agua, Ganadería y Pesca (Generalitat Valenciana) to permit to collect sea urchin specimens. Funding for open access charge: Universidade de Vigo/ CRUE-CISUG.

Data availability

Data will be made available on request.

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