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SPERM MOTILITY ANALYSIS IN AQUATIC SPECIES: POTENTIAL AND APPLICATION OF CASA-MOT SYSTEMS IN AQUACULTURE

ANALISI DELLA MOTILITÀ SPERMATICA NELLE SPECIE ACQUATICHE: APPLICAZIONI E PROSPETTIVE DEI SISTEMI CASA-MOT IN ACQUACOLTURA

Abstract - *Sperm motility is a key factor in assessing semen quality, as it indicates the structural and physiological integrity of the sperm cells and their ability to fertilise eggs. Computer-assisted analysis of sperm motility (CASA-Mot) describes the movement kinetics of individual spermatozoa along their tracks. Studies carried out by our research group on fish, sea urchins and mussels have proven that CASA-Mot systems can evaluate sperm motility quickly and easily, and that they are also highly sensitive and effective at evaluating the effects of environmental parameters and the presence of pollutants, as well as the influence of sperm management and breeding conditions on functional sperm integrity. These factors are not always highlighted by a simple evaluation of total motile spermatozoa. Overall, CASA-Mot systems offer interesting prospects for wider use in aquaculture, a field which also relies on in-depth studies of sperm motility physiology in species of potential economic interest.*

Keywords: *Mussel, Sea urchin, Fish, Computer Assisted Analysis, Sperm Motility Pattern*

Introduction - Sperm motility is a key component in evaluating semen quality, as it indicates the structural and physiological integrity of spermatozoa and their ability to fertilise. Computer-assisted analysis of sperm motility (CASA-Mot) systems were initially developed for human fertility applications, then adopted for animal husbandry and, more recently, for aquatic species. In addition to determining the percentage of motile spermatozoa, CASA-Mot systems provide a quantitative and objective basis for evaluating sperm motility by measuring parameters such as sperm velocity, flagellar beating and amplitude of head ondulations. These parameters describe the kinetics of single spermatozoa movement along their tracks (Gallego and Asturiano, 2018). The combined analyses of these parameters, and their variations as a function of post-emission and post-activation time, allow the sperm motility pattern (SMP) to be defined, which provides a picture of physiological normality for each species considered (Gallego *et al.,* 2014; van der Horst *et al.,* 2018). The characterisation of species-specific SMPs in aquatic species, and the development of routine tools for their rapid and easy assessment, are key steps in establishing species-specific protocols for gamete management and embryo production for aquaculture and laboratory research purposes (Beirão *et al.,* 2019). In this framework, our research focused on using a CASA-Mot system to characterise the SMPs of several aquatic species, with the aim of developing a rapid assessment tool for studying sperm motility. The feasibility of using the SMP analysis by CASA-Mot as an evaluation tool was subsequently examined in a series of different experimental situations: rapid identification of gamete quality during controlled reproduction trials; optimisation of the gamete management protocols in the post-collection phase; as an evaluation parameter throughout the cryopreservation process and long-term cryostorage; as an end-point in ecotoxicological bioassays.

Materials and methods - The main species studied were of ecological and aquacultural interest: the bivalve mussel *Mytilus galloprovincialis* Lamarck, 1819, the echinoderms *Paracentrotus lividus* (Lamarck, 1816) and *Sphaerechinus granularis* (Lamarck, 1816), and the fish *Sparus aurata* Linnaeus, 1758 and *Pagellus erythrinus* (Linnaeus, 1758).

1) Semen collection and motility activation: Semen samples were collected from mussels after dissection, from sea urchins after spawning induction by 0.5 M KCl injection, and from fish by abdominal stripping. To activate sperm motility, 10 µL of each sample was diluted in ASW (artificial sea water; ASTM protocol) containing 0.05% BSA (bovine serum albumin, fraction V) as an anti-sticking agent. The dilution rate was optimised for each species studied, ranging from 1:100 to 1:1000. 2) Computer assisted sperm analysis: Sperm motility parameters were assessed by the Sperm Class Analyzer®, whose acquisition parameter settings for each species are described in detail by Fabbrocini *et al.* (2015; 2016; 2020; 2021; 2023). The main motion parameters assessed were: total motile sperm (TM, %), as the % of sperm with a curvilinear velocity > 10 µm/sec and straightness > 0%; rapid sperm (RAP, %), as the % of sperm with a curvilinear velocity > 100 µm/sec and straightness > 0%; curvilinear velocity (VCL, µm/sec); c) linearity (LIN, %); d) amplitude of lateral head displacement (ALH, µm); e) beat-cross frequency (BCF, Hz); f) DANCE (DNC, as VCL x ALH, μm^2/sec). 3) Motility duration assessment: activated samples were incubated at 18 °C in the dark, and motility parameters were recorded at regular intervals. 4) Motility longevity assessment: Motility longevity is defined as the retention of the capability of activation following collection or thawing; semen samples were stored undiluted in the dark at 4 °C and activated at regular intervals as previously described. For both motility duration and longevity assessments, the first significant difference in respect to the t0 records was evaluated by one-way ANOVA and Dunnett's post hoc test. 5) Evaluation of cryopreserved samples: the samples were thawed by immersion in a water bath and then activated as previously described at a rate ranging from 1:10 to 1:100, depending on the species. 6) Ecotoxicological bioassays: samples, either freshly collected or thawed, were diluted in the environmental matrices or in scaled concentrations of the reference toxicants to be tested. Following a 60-minute of incubation, the samples were activated, and motility parameters were recorded in accordance with the previously described methodology. ASW was utilised as a negative control.

Results - The SPM provides an indication of the physiological normality for each species. Table 1 shows the main parameters recorded by CASA-mot for some of the species studied.

Table 1 - Range of values recorded for the main CASA-Mot parameters describing the sperm motility pattern in some of the species studied.
Intervalli dei valori registrati per i principali parametri CASA-mot che definiscono il pattern di motilità in alcune delle specie studiate.

	TM (%)	**VCL (µm/sec)**	**ALH (µm)**	**BCF (Hz)**	**DNC (μm^2/sec)**	**Motility duration**	**Motility longevity**
M. galloprovincialis	35÷90	25÷100	0.6÷1.9	2÷18	15÷150	≥ 60 min	≥ 6 hr
P. lividus	85÷99	140÷400	1.4÷5.1	30÷50	230÷1700	≥ 48 hr	≥ 48 hr
S. granularis	70÷90	50÷190	1.1÷2.3	5÷12	60÷380	≥ 60 min	≥ 24 hr
S. aurata	90÷99	140÷400	-	-	-	< 30 min	≥ 24 hr

The percentage of motile spermatozoa was consistently above 70%, whereas the VCL showed values below 100 µm/sec in *M. galloprovincialis* and *S. granularis*, and above 350 µm/s *in P. lividus* and *S. aurata*. ALH and BCF are descriptive parameters of the

flagellar beat, and thus of how motile cells progress along their tracks (Gallego *et al.,* 2014). Their values also differ between species, albeit less markedly. The DNC, calculated as VCL × ALH, represents the estimated space covered by sperm per second (van den Horst *et al*., 2018) and naturally shows the highest values, even exceeding 1000 μm^2/sec, in the species in which the VCL is greatest. The most marked differences are found in the duration of motility and its post-emission activation capacity, which, as known, can vary from a few minutes to a few hours or even days, reflecting, together with the other descriptive parameters of motility, the different reproductive strategies adopted by the individual species (Gallego *et al.,* 2014).
Fig. 1 shows the values of some of the main representative motility parameters recorded in the five species under the different experimental conditions tested.

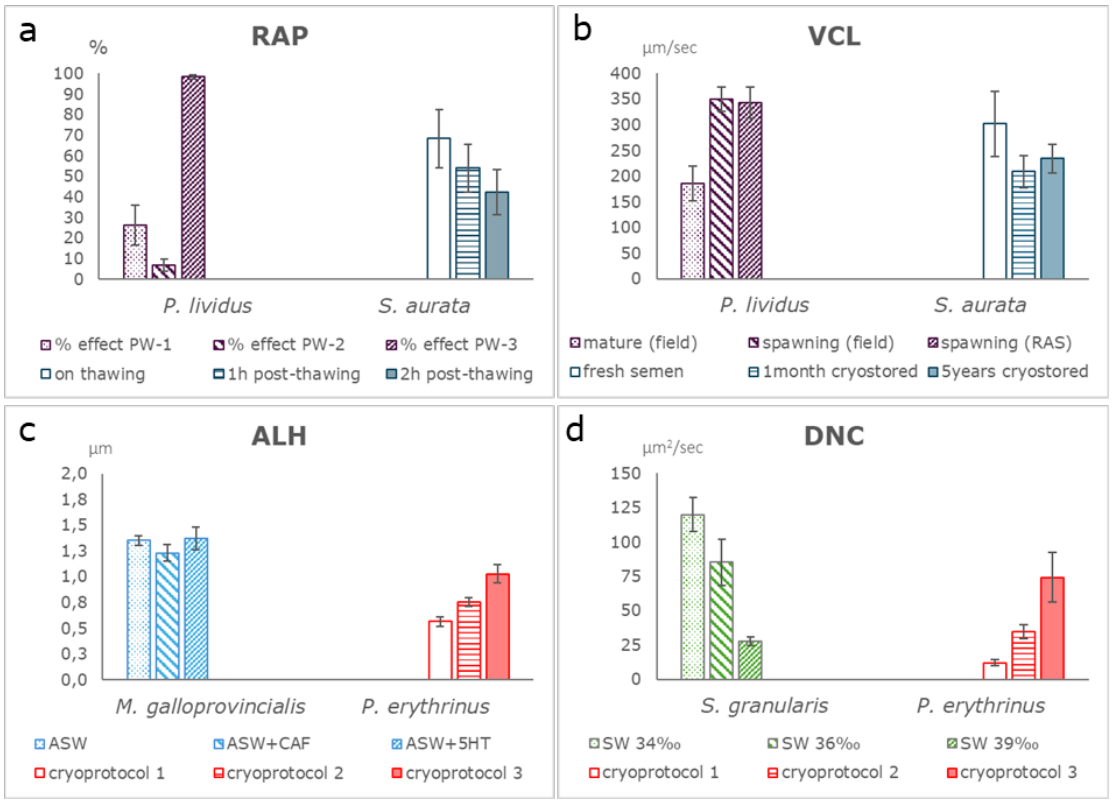

Fig. 1 – (a) RAP sperm subpopulation as endpoint in ecotoxicity test (PW=pore water) and for the evaluation of cryopreserved samples; (b) VCL levels recorded in different gonad stages and after different cryostorage times; (c) effects of different activating media and different cryopreservation protocols on ALH values; (d) effects of salinity and of different cryopreservation protocols on DNC levels.
(a) subpopolazione degli spermatozoi rapidi (RAP) come endpoint in test ecotossicologici (PW=acqua interstiziale) e per la valutazione di campioni criopreservati; (b) livelli di VCL registrati in differenti stadi di maturazione gonadica e dopo differenti tempi di criostoccaggio; (c) effetti di differenti mezzi di attivazione e differenti protocolli di criopreservazioni sui livelli di ALH; (d) effetti di differenti salinità e differenti protocolli di criopreservazione sui livelli di DNC.

As seen in Fig. 1, the results highlight the sensitivity and discriminative ability of this tool in relation to the specific analysis being performed. The parameters showing the highest sensitivity were VCL (Fig. 1a) and RAP (Fig. 1b), the latter representing the consistency of the sperm sub-population characterised by the highest VCL values (Beirão *et al.,* 2019). These parameters also have promising practical applications in ecotoxicology and in the development of reproductive technologies for aquaculture, such as reproductive cycle control in RAS systems, assisted reproduction and

cryopreservation. Also, ALH (Fig. 1c) and DNC (Fig. 1d) showed good discrimination ability, such as damage due to different cryopreservation methodologies or the effect of diluent composition on motility activation ability.

Conclusions - The SMP evaluation by CASA-Mot systems has been shown to be a highly sensitive and discriminating tool in studies investigating the interaction between sperm and the environment by evaluating the effects of pH, temperature, osmotic pressure on motility inhibition and activation. Also, the interaction between broodstock and the environment were studied by examining variations in SPM as a function of the reproductive cycle and the environmental conditions in which the specimens matured. Moreover it proved to be a rapid and effective evaluation tool in routine semen quality assessments, such as during cryopreservation or in ecotoxicology. Summarizing our results on various species and under different experimental conditions, and also considering the current literature, we can draw some final conclusions. Firstly, SPM analysis using CASA-Mot systems is an objective tool that, unlike traditional microscopic analysis, is less dependent on operator bias and is more replicable (Gallego and Asturiano, 2018). The CASA-Mot analysis protocols are fast and relatively simple, and can be easily transferred from one species to another; they require small sample volumes and simple reagents and equipment, which is important for routine analysis (Beirão *et al*., 2018). Among CASA-Mot parameters, VCL and RAP in particular are positively correlated with the fertilisation rate (Gallego *et al.,* 2014; van der Horst *et al.*, 2018), thus providing indirect information on the fertilising capacity of the analysed sample without the need to find eggs simultaneously, which is not possible through a simple evaluation of the total number of motile spermatozoa. Overall, analysing SMP using CASA-Mot systems offers interesting prospects for wider use in aquaculture, as the development of technology in support of sustainable fish and shellfish productions also relies on in-depth studies of sperm motility physiology in a broader range of species of potential interest to aquaculture.

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