Protocol E - Evaluation of sperm quality [Pratical guide of protocols: sperm quality]

in

Felip A. (ed.), Carrillo M. (ed.), Herráez M.P. (ed.), Zanuy S. (ed.), Basurco B. (ed.). Advances in fish reproduction and their application to broodstock management: a pratical manual for sea bass

Zaragoza : CIHEAM / CSIC-IATS
Options Méditerranéennes : Série B. Etudes et Recherches; n. 63

2009
pages 35-39

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I - Introduction

Like evaluation of egg quality, sperm quality is also crucial for aquaculture purposes in order to determine male gamete quality. Sperm quality defined as those traits of the sperm that determine its capacity to fertilize eggs needs to be monitored in fish farming to estimate male reproductive success (Billard *et al*., 1995). Studies of sperm motility of fish have been limited to species with a commercial interest in aquaculture or species involved in conservation programmes (Alavi and Cosson, 2005). However, in the last years studies on male reproduction have considerably increased (Trippel, 2003).

Many parameters have been used to study sperm biology, although motility parameters have usually been evaluated in order to estimate the quality and fertilizing ability of sperm of a male. These motility parameters include motility duration and the percentage of motile sperm observed visually (Chambeyron and Zohar, 1990). Accordingly, motility duration is determined to be the time period from sperm activation to cessation of cell displacement with only <5% of the sperm population beating the flagella. The percentage of motility is characterized using an arbitrary scale, based on five categories, in which category I represents 0% of the sperm population motile and category V represents 90-100% of the sperm population vigorously motile (Chambeyron and Zohar, 1990). In the sea bass, these procedures have been successfully applied in order to study sperm motility after sustained administration of gonadotropin-release hormone analogues (GnRHa) (Sorbera *et al*., 1996) or after irradiation treatments with UV-light (Felip *et al*., 1999). On the other hand, many studies include a quantitative approach to analyse sperm motility. This approach is based on a computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish (Kime *et al*., 2001). Assessment of sperm motility using a CASA approach facilities a rapid analysis of motility parameters such as curvilinear velocity (VCL), straight line velocity (VSL) and beat cross frequency (BCF) among other parameters. The motility analyser computes a complete track for any motile spermatozoa of the sperm population. Since fish sperm remains fully motile a short period of time, usually less than 2 min, after mixing with water, tracking data needs to be taped as quickly as possible after initiation of motility. This methodology has been used to evaluate sperm quality under various experimental conditions following short-term storage of sperm in different buffers, broodstock selection and monitoring the effects of heavy metal pollutants (Kime *et al*., 1996; Ravinder *et al*., 1997; Kime *et al*., 2001).

Under captivity conditions, sea bass males reach sexual maturation during the second year of life, although an important percentage of males reach puberty at 1-year-old (Carrillo *et al*., 1995). These males are called precocious or early-maturing males. In the present protocol, a CASA approach is used to determine the sperm count, the number of motile spermatozoa in different sperm samples, and motility parameters from 1-year-old early-maturing and 3-year-old maturing (i.e., adults) sea bass males for comparison during the spermiation period. This analysis is conducted to establish the reproductive potential of precocious sea bass males for viable sperm production of male stocks (Felip *et al*., 2006).

II - Purpose

The objective of this protocol is to show the application of the computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in sea bass. This procedure provides a simple and rapid quantitative assessment of the quality of fish sperm. The volume of expressible

sperm, the motility and their duration is estimated in order to determine sperm quality of male sea bass in the laboratory.

III - Procedure

(i) Anaesthetize males with MS-222 or 2-phenoxyethanol. Clove oil can be also used. Induction of anaesthesia is rapid (1-3 min) in small fish although it is slower in larger fish. The above doses may be lethal after about 20 min, thus animals must be carefully handled as soon as possible. The use of gloves is recommended during this process.

(ii) Clean the genital area with fresh water and dry.

(iii) Collect the total expressible milt by gentle abdominal massage pressure in a graduated conical tube supplied by a vacuum device. Sample contamination with faeces, urine and water must be avoided.

(iv) Keep samples on ice and note the sperm volume in millilitres.

(v) Dilute sperm samples 1/100 times in 0.5-ml eppendorf tubes with a non-activating medium used as extender for sea bass sperm (Fauvel *et al*., 1998) to assess sperm motility.

(vi) Resuspend samples and activate sperm using seawater (37-38‰ salinity) after a final dilution 1/1000 times.

(vii) Take 1 µl of each sample, drop it on a slide and cover up with a glass-cover before the analysis.

(viii) Record sperm motility with a camera (25 images sec^{-1}) attached to a computerized motility analyser (Sperm Class Analyzer 2000, Microptic, S.L., Barcelona, Spain) rapidly (5 sec after activation) and automatically recording motility parameters.

IV - Materials and equipment

- Computerized Sperm Class Analyser

- Graduated conical tubes

- Vacuum device

- 0.5-ml eppendorf tubes

- Pipettes

- Glass slides and glass covers

- Kitchen clothes

- Gloves, lab coat and boots

V - Reagents and solutions

- Anaesthetic: MS-222 or 2-phenoxyethanol (0.1 g l^{-1} and 0.5 ml l^{-1} of sea water, respectively). Alternatively, the induction of anaesthesia can be carried out using clove oil. Clove oil has been evaluated as an effective anaesthetic in sea bass and it can be used at almost 10-fold lower doses than 2-phenoxyethanol.

- Non-activating medium: 100 mM NaCl, 13.4 mM KCl, 26.2 mM $NaHCO_3$ and 83.2 mM glycine, pH 7.35.

VI - Results and discussion

The evaluation of sperm quality in order to assess the potential reproductive performance of males is crucial in fish stocks. Sperm quality is usually assessed based on motility, duration of the movement, sperm count and volume (Table E.1). The evaluation of sperm motility can be carried out automatically using a computer-assisted sperm analysis (CASA system) (Fig. E.1). Although sperm motility and duration can also be monitored microscopically (Chambeyron and Zohar, 1990).

Table E.1. Sperm quality measurements in 1-yr-old precocious and 3-yr-old adult male sea bass

Quality trait	Precocious	Adult
Sperm volume (ml)	0.1*	1-2
Concentration (spz ml^{-1})	9.7 ± 0.37x10^9	15.9 ± 0.40x10^9
VCL (μm s^{-1})	10.70 – 117.77*	17.66 – 131.41
VSL(μm s^{-1})	4.81 – 69.82*	6.23 – 45.73
BCF (Hz)	4.15 – 5.13	4.23 – 4.96

VCL, curvilinear velocity; VSL, straight line velocity; BCF, beat cross frequency. Significant differences $P < 0.05$ (*). Modified from Felip *et al*. (2006)

In general terms, in fish, sperm is usually thick and it needs to be diluted in order to evaluate its performance. Additionally, in teleost fish, sperm is immotile on ejaculation and it is activated on contact with water. Accordingly, appropriate extender solutions need to be used for diluting the sperm (e.g. a non-activating medium). After dilution, the sperm is activated and its movement recorded rapidly because it varies from ≤2 min to 1 h, depending on the species (Billard *et al*., 1995).

The sea bass is a marine species in which the sperm is activated upon contact with seawater. It immediately requires an observation under the microscope to estimate the duration of the motility that varies from 2.5 to 3.5 min. On the other hand, those males showing >80% of motile spermatozoa are considered potential reproductive breeders. Since sperm fish has no acrosome, it must rapidly find the micropyle on the surface of the egg to enter. Consequently, motility parameters may be correlated with the fertilizing capacity of spermatozoa in fish.

The computerized motility analysers can record several motility parameters including: (i) velocity parameters (μm/s) (curvilinear velocity, VCL; straight line velocity, VSL and average path velocity, VAP); (ii) motility track or trajectory patterns (%) (linearity index, LIN=VSL/VCL; straightness index, STR=VSL/VAP and wobble, WOB=VAP/VCL); (iii) other motility parameters of spermatozoa, the amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF; Hz). Several studies suggest that the curvilinear velocity (VCL), the straight line velocity (VSL) and the beat cross frequency (BCF) are some reliable indicators of the quality of sperm and the fertility ability in fish. It is of importance for the availability of stockable fish, animal management and breeding programmes, as well as the maintenance of the broodstock fitness.

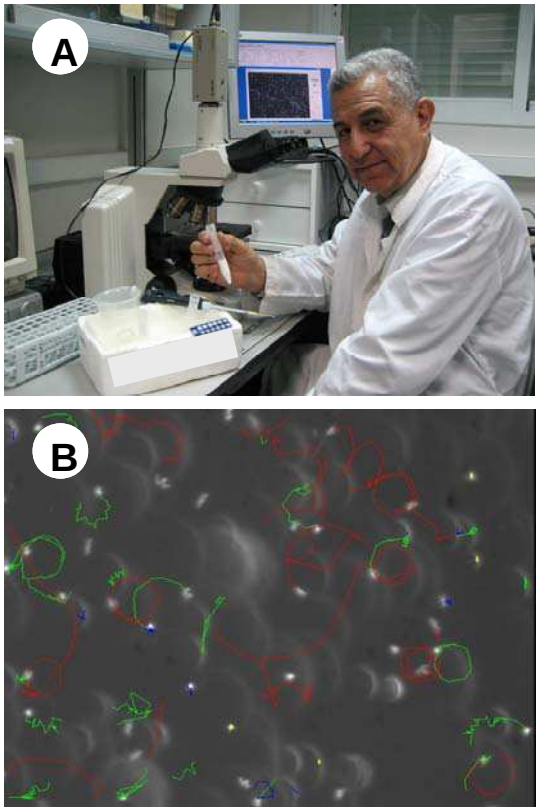

Fig. E.1. **Computerized image from a Sperm Class Analyzer for recording motility parameters in the sea bass. (A) Sperm collection and dilution for motility analysis using an Integrated Semen Analysis System (ISAS programme, Proiser R+D S.L.). (B) A sperm sample for motility analysis after a final sperm dilution 1/1000 (pre-dilution with sperm extender and further dilution with activation media, sea water). Magnification: x 200; Dark field.**

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