Advances in Sole Broodstock Management

Instituto Andaluz de Investigación
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Consejería de Agricultura,
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Advances in Sole Broodstock Management

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1.- Introduction

Figure 1. Senegalese sole breeder

The aquaculture of Senegalese sole (Figure 1) in Southern Europe is expanding rapidly to meet the high market demand. Currently, sole production has reached 1,700 metric tons, mainly in the Iberian peninsula. However, the industrial exploitation of this species is limited by several constraints in reproduction and fry production (Manchado et al., 2019). To overcome these limitations, there is a need for more knowledge on reproduction control, gamete production, and handling.

One major challenge in the *in vitro* fertilization techniques of Senegalese sole is to obtain sufficient volume of high-quality sperm, primarily due to low production and difficulty to avoid urine contamination. Sperm production in this species is typically in the range 40-100 µl and wild fish with reproductive success produce higher volumes than fish produced in captivity (F1). Additionally, it is extremely challenging to obtain urine-free samples because the spermatic ducts and urinary structures share the urogenital pore. A possible solution to urine contamination was recently suggested with the use of modified Leibovitz and Marine Freeze® extenders (González-López et al., 2020).

The BestBrood project is an international consortium funded by the ERA-NET Cofund BlueBio initiative. Significant achievements in sperm conservation methods, specifically in the areas of chilling and cryopreservation, and the use of antioxidants to preserve sperm quality have been done. These advancements, combined with the upscaling of cryopreservation methods, is a step forward for the industry to produce larvae and manage sperm banks. This guide will present the advances in broodstock management, sperm collection, evaluation and cryopreservation.

References

González-López et al (2020). Aquaculture 516, 734649.

Manchado M. et al. (2019) In: The Biology of Sole. pp. 375-94

2. Housing and handling procedures

Figure 2. Broodstock housing. Animals are kept in dark and the opertators use torchs to handle

To ensure the optimal health and welfare of sole broodstock, it is recommended to follow the following guidelines:

- **Photoperiod.** It should be 8:16 Light:Darkness with low light intensity (~200 lux). Operators should avoid to disrupt light regimes using torchs during handling (Figure 2)
- **Feeding.** It should be based on squids and mussels supplemented with polychaetes. Up to now, this dietary regime was the most effective to produce high-quality gametes
- **Stocking density**. It should be ~1-1.5 kg m^{-2} in circular or rectangular tanks (at least 1m height to make feasible the courtship behavior).
- **Salinity**. It should be kept constant around 33-35 ppt to make feasible vitelogenesis and egg collection by buoyancy.
- **Temperature**. Tanks should be equipped by **temperature control systems** to induce spawning by thermocycling. Gonad maturation is activated by weekly increasing 0.5°C temperature from 13°C to 18°C. After ten weeks, 2°C-intervals cycles, from 16 to 18 °C (four days raising and three days decreasing), should be done.
- **Tagging.** All animals should be tagged for traceability by using PIT-Tags in dorsal muscle or abdominal cavity (Figure 3).

Figure 3. Sampling. Soles are left in a soft and wet surface with a rule and the PIT-Tag is read using specific devices.

2. Housing and handling procedures

Broodstock should be handled on a monthly basis for weighing and measurement, including total length, and to assess the gonad developmental stage. It is also important to monitor any lesions on the blind side of the fish carefully, as this area is in permanent contact with the tank surface

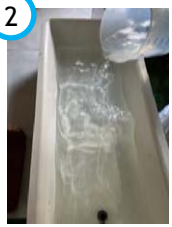
Anesthesia and handling procedures

Before any fish handling, animals should be sedated and anesthetized as follows:

1

Clove oil preparation (Stock 1:10 ratio in 95% ethanol). Sedation: 50-100 ppm. Anesthesia: 200 ppm

2

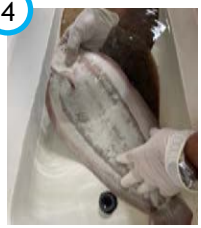


Sedation and anaesthesia tank preparation. Pour clove oil in the tank and roughly mix water

3

Take sole in the head and carefully release in the tank. Avoid abdominal cavity

4



Monitor anaesthesia deep. Optimal point is when animal stay bottom-up without agitation

Sampling procedure

1

Prepare scale and rule before fish handling and close to the anaesthesia tank avoiding long distances

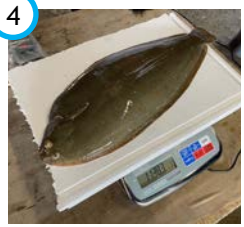
2

Take sole in the head and avoid abdominal cavity

3

Register PIT-Tag and length until caudal fin peduncle.

4



Register weight and monitor growth gain in the last period

3. Sperm collection

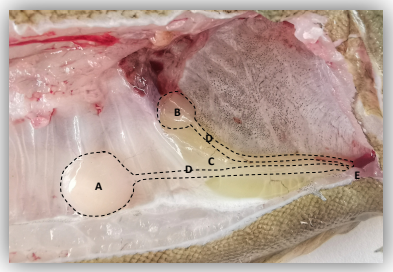


Figure 4. Anatomy of male reproductive system showing ocular testicular lobe (A), blind side testicular lobe (B), urinary bladder (C), spermatic ducts (D) and urogenital pore (E).

3.1 General considerations

Testis Anatomy

Sole testis is semi-cystic with an asynchronic spermatogenesis that occurs almost all-year. The testis lobe on the ocular side is typically larger than the one on the blind side. In terms of spermatogonial type, Senegalese sole testis belongs to the unrestricted type, with spermatogonia distributed throughout the seminiferous lobules. Following spermatogenesis, spermatozoa are collected and stored in the efferent duct system.

Urine contamination

Spermatic ducts are situated over the urine bladder (**Figure 4**) and they join in the urogenital pore, hence, it is extremely difficult to avoid urine contamination during sperm collection. This contamination will lead to the unintentional activation of sperm, which can negatively impact the quality and viability of the collected sperm. Operators should be trained to empty urine bladder before collecting sperm.

Methods to neutralize urine contamination

The average volume of sperm collected normally does not exceeds 100 µl. Modified Leibovitz and Marine Freeze® extenders were shown to preserve the percentage motility up to 24 h after collection. Other solutions are feasible although with lower effectiveness such as the solutions Ringer, NAM and Stor Fish®. In all cases, sperm should be directly collected in prefilled syringes with extenders in an one-step procedure. Two-step procedures, which involve collecting pure sperm followed by addition of the extender, usually result in less effective preservation with higher losses of sperm motility and velocity.

3. Sperm collection

3.2 Procedure

Sperm collection requires specific training to: 1) detect testis lobes in both sides, ocular and blind, 2) remove urine from bladder, 3) collect sperm by gently pressing testes and efferent ducts in one-step procedure. Steps are indicated below.

1

Before handling, fish must be **deeply anesthetized** as indicated in section 2. Always manage soles from the head to avoid pressing any soft areas, such as the abdominal cavity.

To accurately see the sperm inside the urogenital pore, animals should be placed on a damp surface with ideal illumination.

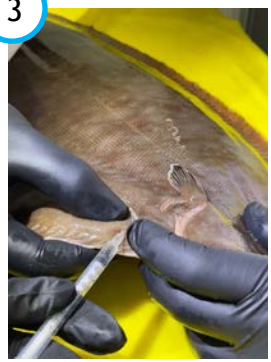
2

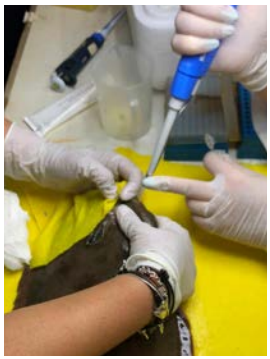
The testis should be located by simultaneously pressing with two fingers on the blind and ocular sides.

Remove urine from the bladder with caution, being careful not to disrupt the testes, and use a paper towel to dry the urogenital pore.

Examine the white tone and density of the fluid by gently pressing the testes toward the urogenital pore.

3





Collect the sperm with prefilled syringes or tips.

Initial prefilled volume of Marine Freeze should be set at 60 µl.

Once collected the sperm, the final volume should be corrected to match the expected ratio 1:3

4. Sperm evaluation

Figure 5. Chambers to evaluate sperm. Top, Makler chamber; Bottom, leja counting chamber.

4.1 General considerations

Computer-aided sperm analysis (CASA)

The CASA methodology is a video-micrography technique used to track the movement of spermatozoa. There are several machines and software that provide sensitive and accurate measurements of sperm kinetic features such as IVOS-II or ISAS. These instruments have become essential tools to measure spermatozoa concentration with the right kinematics to penetrate oocyte, washed sperm preparation analysis and evaluation of sperm hyperactivation.

Chambers for sperm evaluation

Two main chambers (Figure 5) are used to evaluate sperm quality. A) The Makler sperm counting chamber with a depth of 10 microns. It is reusable, the grid is on the cover glass and the sample is observed in one focal plane. B) Leja counting chamber with a a thickness of 20 µm allowing the spermatozoa to move in one focal plane. It is dispensable and useful for measuring cell concentration.

CASA parameters

CASA provide several parameters grouped in cell concentration, motilities and velocities: a) Total motility indicates the percentage of motile spermatozoa; b) Progressive motility indicates the percentage of motile spermatozoa that have a progressive movement or that swim in a mostly straight line or large circles; c) Curvilinear velocity (VCL) is the actual velocity along the trajectory and the d) Straight line velocity (VSL) is the straight line distance between the start and end points of the track divided by the time of the track.

4. Sperm evaluation

4.2 Settings and visualization

Laboratories require robust meaningful data from CASA systems, showing relationships between sperm velocities, motility, and in vitro fertilization rates.

The IVOS-II CASA is an automated system that produces accurate data regarding total cells and concentration, motilities, velocities, sperm morphology, and six measurements of sperm head morphometry. It has better integrated phase contrast optics (Mortimer et al., 2015)

Figure 6 is an illustration of how the IVOS-II measures velocities and motilities.

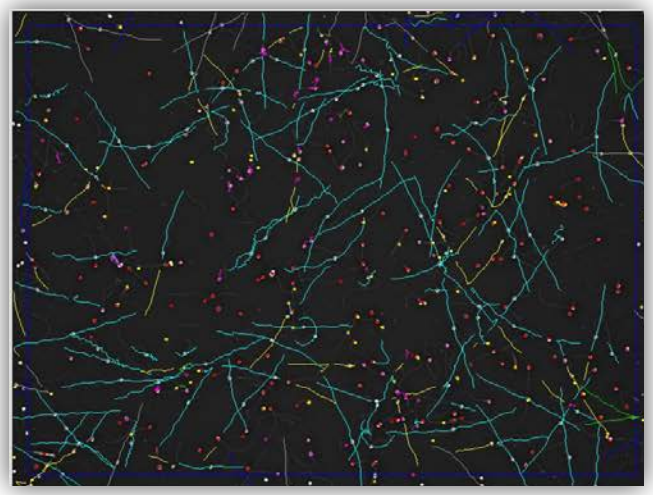


Figure 6. Sperm image acquired by IVOS-II.

Motile Progressive Slow Static Late Track

Table 1. Settings used in IVOS-II to evaluate fish sperm

Video capture	
Exposition	16
Gain	300
Frames count (fps)	25
Frame rate (Hz)	60

Kinematics	
Progressive STR (%)	45
Static VAP (μm s^{-1})	4
Slow VAP	20
Progressive VAP (μm s^{-1})	45
Static VSL (μm s^{-1})	1
Slow VSL (μm s^{-1})	30

Cell detection	
Max. elongation (%)	100
Min. elongation (%)	3
Min. tail brightness	255
Max. head size (μm^2)	90
Min. head size (μm^2)	1

Analysis limits	
Min total count	200

Reference

Mortimer et al (2015). Asian J Androl 17(4): 545-553.

5. Sperm storage

5.1. Chilled Short-term storage

Chilled sperm refers to the method in which sperm is preserved at temperatures close to 4ºC. For this method, it is required the use of extenders to protect cell membrane integrity. Since the volume is normally small, the Eppendorf tubes should be closed and maintained in 1.5 ml Eppendorf tubes in a styrofoam rack over ice or in the fridge at 4°C for 24 h (Figure 7). Those samples suspicious of contamination with water, urine, or feces —which can be identified by color and consistency— must be thrown away. Chilled sperm obtained by the one-step method losses as average just 27.6±6.4% of its total motility (TM). Two-step sperm collection resulted in an average TM reduction of 88.7±9.0% (Figure 8). Therefore, when sperm is intended to be employed in ordinary hatchery operations for the generation of larvae, the one-step approach is favored.

Figure 7. Storage of chilled sperm. Tubes containing approx 100—200 µl of sperm in Marine Freeze should avoid direct contact with cold surfaces but keeping temperature in the range 0-4ºC.

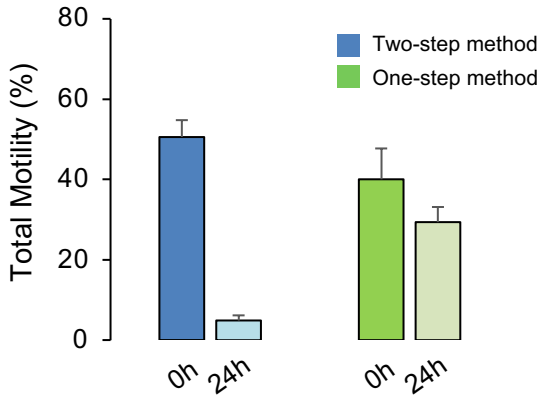


Figure 8. Effect of storage time on chilled sperm as function of the method collection.

5. Sperm storage

5.2. Reference sperm indicators in chilled sperm

Wild soles produce more sperm than F1 animals. Reference values (Table 2) regarding sperm parameters in both sole groups are required fro in vitro fertilization. Hence, IFAPA Broodstock was monitored for two reproductive seasons (spring 2021 and 2022). Wild fish (n=17) was from the Bay of Cadiz and F1 (n=60) was produced by CUPIMAR from a wild broodstock. All animals were fed natural feed (squids, mussels and worms.

Table 2. Reference values in wild (left) and F1 (right) males. Range of maximum and minimum values obtained in monthly samplings and the average values are indicated for volume collected, cell concentration, total number of cell, total motility, progressive motility, curvilinear velocity (VCL), Straight line velocity (VSL), % live cells, viable cells with reactive oxygen species (ROS), DNA fragmentation and malondialdehyde levels (MDA).

Wild soles

	Maximum	Minimum	Average
Volume (µl)	95-250	5-20	80.5±67.3
Concentration (M/ml)	7722-13033	1497-2378	6244±3080
Total number of cells (M)	353.4-2196.2	31.6-68.6	525.8±568.7
Total motility (%)	42.9-45.9	2.7-9.7	21.5±12.4
Progressive motility (%)	28.1-33	0-4.1	10.7±8.9
VCL (µm/s)	193.6-205.5	59-104.9	142.4±35.8
VSL (µm/s)	103.8-160.3	7.9-74.1	64.5±35.6
Live cells (%)	78-92.9	62.7-67.4	77.1±7.7
ROS	85.4-97	65.5-86.9	87.1±6.7
DNA Fragmentation (%)	14.6-31.3	9.7-15	15.3±5
MDA	361.2-402.6	128.9-154.5	289.9±80.3

F1 soles

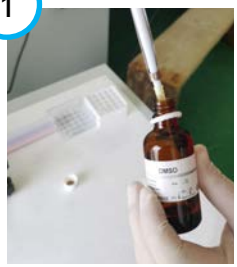
Maximum	Minimum	Average
86-130	5-20	33.1±28.8
7582-12823	2546-5693	6596±2798
170.8-662.5	30.7-151.6	211.6±186.4
41.2-58.3	5.6-17	25.2±13.1
16.9-29.5	0.1-3.2	12.2±8.7
11.8-214.1	54.8-105.5	139.7±41.3
79.1-118.2	12.8-23	57.8±25.3
81.9-88.2	68.2-76.2	77.6±7
88.7-96.2	59.7-85.9	84.6±9.5
12.2-21.4	8.9-11.6	13.9±3.5
288.9-443.6	154.5-300.6	287±91.8

5. Sperm storage

5.3. Cryopreserved long-term storage

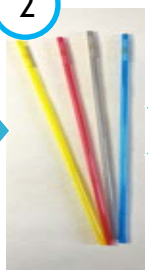
Long-term sperm storage can be carried out in straws (150 µl) or cryovials (500 µl) depending on the volume collected and cryobank characteristics. The cryopreservation methodology remains largely similar in both cases (see figure below). Cryovials are preferred for *in vitro* fertilization in hatcheries since sperms are pooled reducing handling procedures (usually between 5-10 straws vs 1-2 cryovials were required to fertilize 100,000 viable eggs). Straws are preferred to preserve individual high-quality sperms in genetic breeding programs.

1



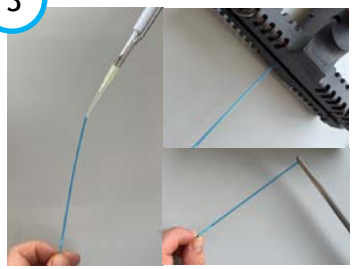
Before cryopreservation, add DMSO to the sperm sample in a final proportion of 10% (v:v)

2



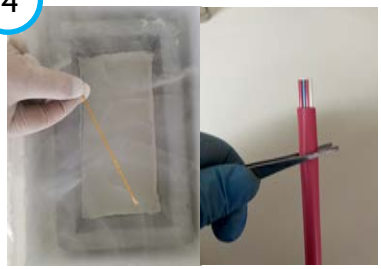
Straw

3



Load straw with a micropipette. Close the straw with heat and tweezers

4



Expose straws to the N_2 gas phase during 10 min in a floating catamaran. Then, transfer to liquid phase and storage in the cryobank

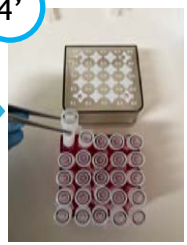
2'

cryovials

3'

Transfer to the gas phase of N_2 liquid during 15 min in a floating catamaran. Then, transfer to the liquid phase.

4'



Storage in the cryobank

6. Use of antioxidants

Numerous antioxidants have been proven to safeguard spermatozoa from oxidative stress and maintain membrane integrity, as reactive oxygen species (ROS) generated during the cooling, freezing, and thawing stages of chilling or cryopreservation storage can reduce significantly sperm viability and quality.

In sole, vitamin C acts as a protector of chilled and cryopreserved sperm maintaining initial total motilities (Figure 9). Values were from 10 to 21% higher than non-supplemented control after 2h incubation. Moreover, percentages of spermatozoa alive at 60s after activation were higher when added Vitamin C (Figure 10).

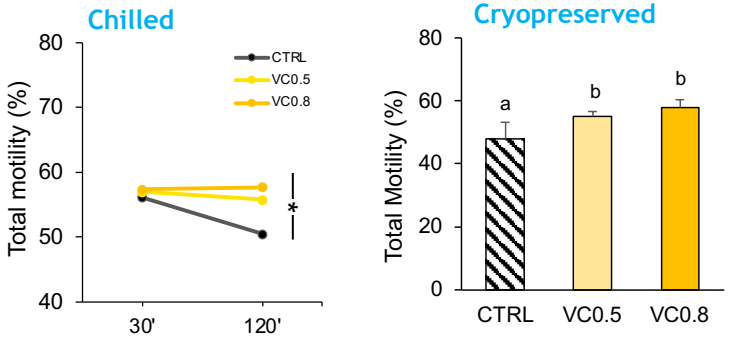


Figure 9. Effect of vitamin C (0.5 and 0.8 mM) on total Motility (TM) in chilled (after 30 and 120 min) and cryopreserved sperm (after24h). Loss of TM in chilled sperm was almost negligible after 120 min. In cryopreserved sperm TM was also higher than in the non-supplemented control (CTRL).

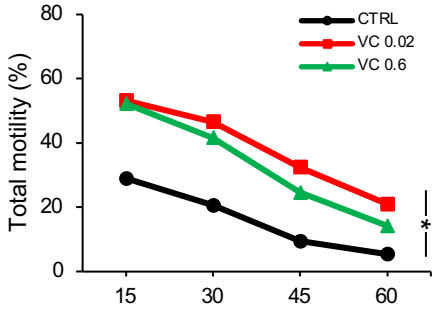


Figure 10. Effect of vitamin C (0.02 and 0.6mM) on spermatozoa life span in chilled sperm. TTM of spermatoza after 60s was still higher than the non-supplemented control(CTRL)

7. *In vitro* fertilization

Sole is a species that requires a low spermatozoa—to-egg ratio for sucessful fertilization. It has been estimated that the ratio of motile spermatozoa to viable egg to get a 99% of fecundation were in the range 1,819-2,691 (**Figure 11**)

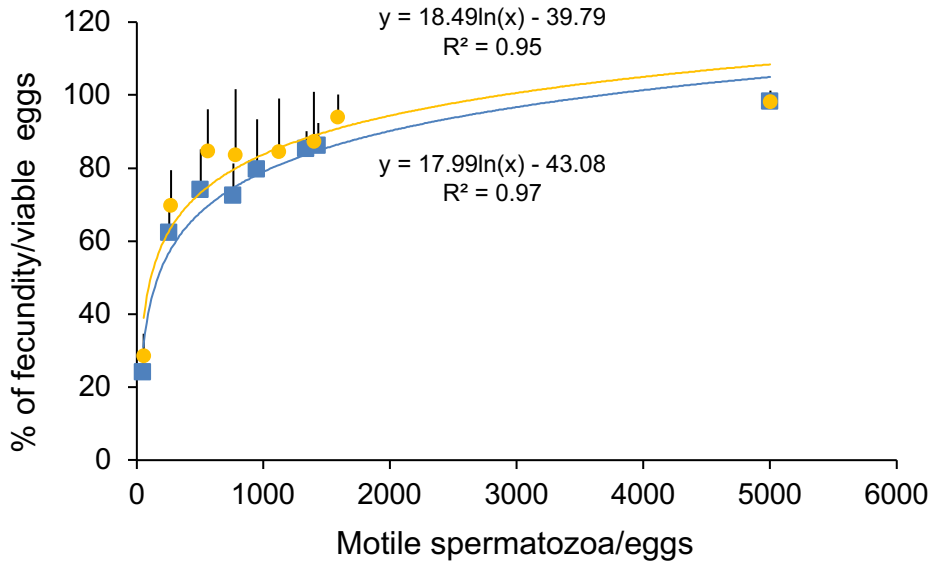
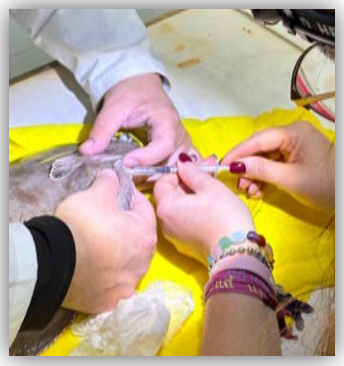


Figure 11. Percentage of viable eggs fertilized in relation to the number of motile spermatozoa per viable egg. Two independent trials were carried out in orange and blue. The dashed line shows an exponential regresión. Determination coeeficient, slope and intercept are shown.

8. Conclusions



- Optimal housing and handling procedures for sole broodstocks were reported
- To counteract the unfavorable effects of urine contamination, sperm should al ways be collected using the one-step approach and directly buffered in a suitable extender, such as Marine Free ze (MF).
- Methodology to collect, evaluate and cryopreserve sperm was described.
- Sperm can be cryopreserved and stored in cryovials, which increases storage capacity without compromising safety. Additionally, the amount of volume used in these devices for upscaling can approach 500 l without adversely compromising total motility.
- Reference values for sperm collected from wild and F1 sole were reported.
- Vitamin C supplementation increases the total motility and extends the life of chilled and cryopreserved sperms, making its use recommended in all up-scaling techniques.
- To achieve 99 percent fecundity rates, a low ratio of motile spermatozoa to viable eggs is needed, and it ranges between 1,819 and 2,691.

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