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# Methodologies for spermatic evaluation in teleost

**Jesús Dámaso Bustamante-González[1]*, Mariela González-Rentería[1], Martha Rodríguez-Gutiérrez[1], Araceli Cortés-García[1] and Alejandro Ávalos-Rodríguez[2]**

1) Universidad Autónoma Metropolitana Unidad Xochimilco. Depto. El Hombre y su Ambiente, Laboratorio de Reproducción Genética y Sanidad Acuícola, México
2) Depto. Producción Agrícola y Animal, Laboratorio de Bioquímica de la Reproducción[2]. Calzada del Hueso N°1100, Col. Villa Quietud, C.P. 04960, Coyoacán, Ciudad de México

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**Abstract:** Seminal quality evaluation is essential in aquaculture to increase reproductive efficiency in commercial species or for the introduction and population of those species that have been affected by anthropogenic damages. Sperm quality is related to volume, concentration, viability, motility and morphology. Without viable spermatozoa, there would not be any egg fertilization and embryos production, so it is of great importance to have and develop methodologies for its evaluation. Therefore, the aim of this review is to analyze and compare different methodologies that help seminal quality evaluation in teleost.

**Keywords**: Quality sperm, concentration, viability, motility and morphology

## Introduction

In aquaculture, the quality of gametes is very important to guarantee the production and development of viable and good quality larvae (Bromage and Roberts, 1995). Also, the controlled production is related by biological events tightly linked with the reproductive success and, particularly, with the occyte fertilization (Rurangwa *et al*., 2004).

In addition, it must be regarded the low ability of sperm to succesfully fertilize an egg as the main factor affecting the fertilization rates. Such ability depends on qualitative and quantitative parameters i.e. volume, concentration, motility, viability and morphology sperm (Rurangwa *et al*., 2004; Hajirezaee *et al*., 2010), of which volume indicates the efficiency, and concentration along with motility determine the fertilization capability of spermatozoa. These parameters are often used to estimate semen quality (Cabrita *et al*., 2014), because of chemical properties of seminal fluid, fish spermatozoa are immotile in seminal fluid (Cosson, 2008; Coward *et al*., 2008).

During natural spawning, fish spermatozoa are rendered motile after the discharge into the aqueous environment (in oviparous species) or the female genital tract (in viviparous and ovoviviparous species) (Billard, 1986; Billard and Cosson, 1990). However, in both cases, motility is affected by temperature, pH, $Na^+$, $K^+$, $Ca^{2+}$ ions and osmolality, that affect fertilization rates (Alavi and Cosson, 2006; Alavi *et al*., 2007; Cosson, 2008; Coward *et al*., 2008). After activation of motility, spermatozoa move towards micropyles in the surface of eggs and then fertilization is done (Hajirezaee *et al*., 2010). Considering the previous facts, the quality of semen is an important and necessary parameter to optimize fertilization rates and therefore increase larvae production, through integrity and quality of DNA (Coward *et al*., 2002; Rurangwa *et al*., 2004).

The aim of this review is to analyze and compare used techniques in semen evaluation in teleost, according to particularities of their physiology.

## Sperm quality determinism

Factors affecting the quality of semen are diverse and depend on complex interactions. These factors have been split into four types: effects of biological characteristics of brooders (age, weight and length), rearing conditions of brooders (temperature, photoperiod, nourishment, undesirable, components and animal welfare and health), artificial induction of spawning, spawning season (repeated semen collection and spermiation time) and stress, which have been reported in detail in Hajirezaee, *et al*. (2010), and Bobe and Labbé, (2010).

(✉) jesusbustamantegonzalez@gmail.com

## Macroscopic characteristics

The macroscopic characteristics are those that can be assessed with naked eye (i.e. volume, color and viscosity of semen) and must be evaluated immediately after the collection of semen (Cruz, 2001).

### Collection and volume determination

In general, teleost have paired gonads to the sidewalls of the body, and during sexual maturation, gonads in males increase in size in such a way that, when the genital pore is under soft pressure, they release seminal liquid (Rodríguez *et al*., 1992).

Semen collection in most teleost is made under anesthesia, because in some cases organisms stress during handling, so that is necessary to sacrifice them to collect the sample. Semen is extracted through a slight pressure on the flanks of the body in an operculum-caudal direction, and the procedure ends when flow is null, or when there is presence of blood (Rodríguez *et al*., 1992).

Nevertheless, this procedure varies according to specie, size and type of organism. For example, in *Danio rerio* some authors recommend to sacrifice the organism to remove the testicles due to the limited volume that can be obtained with abdominal massage method (Yang *et al*., 2007; Wang *et al*., 2015).

On the other hand, in viviparous teleost like *Xiphophorus hilleri*, *Poecilia formosa* and *Poecilia* reticulate, it is necessary to stimulate the gonopodium with movements in an arc of 180° forward and backwards from 7 to 10 times, and hold the gonopodium forwards with a finger, while the sides of fish are simultaneously rubbed with the thumb and forefinger from behind the operculum until the base of gonopodium, Then, the sample is obtained (Aspbury and Gabor, 2004; Boschetto *et al*., 2011; Llanos and Scotto, 2014).

Diverse materials can be used for semen collection, such as graduated tubes (Asturiano *et al*., 2006), graduated cryovial (Agarwal and Raghuvanshi, 2009), capillarity tubes (Domínguez *et al*., 2015; Zadmajid *et al*., 2013), syringe (Alavi *et al*., 2007; Ceccon *et al*., 2010), plastic pipettes (Aguilar *et al*., 2011; Aguilar *et al*., 2014), or even a catheter (Cabrita *et al*., 2001), depending on quantity and specie. Volume can be measured in a direct way from the collection container or also, by the difference of weight between the collection container and semen. Volume is expressed in mL or µL depending on quantity.

### Color

Semen color is a qualitative characteristic that should also be considered. Depending on the species, it can vary from withe to light yellow, and it is commonly associated to consistency, which can be creamy or aqueous and it also depends on seminal liquid that each specie present, spermatic concentration and maturity stage (Navarro *et al*., 2004; Bastardo *et al*., 2004).

Semen can take the color of the contaminant. For example, if during the collection the organism ejects blood, semen will be reddish; similarly, urine and feces can change the semen color; any of these contaminants must be avoided because they activate spermatic motility and therefore it decrease the fertilization capacity (Nynca *et al*., 2014).

### Viscosity

Viscosity is subjectively qualified in a scale of 0–4, being 0 the lowest viscosity grade. This qualitative characteristic is related to spermatic concentration (number of spermatozoa$^{-1}$) (Cruz, 2001). Viscosity is evaluated by sucking the sample with a micropipette, and dropping the semen. Then, the filament is observed. Normally, semen must fall drop by drop or form a filament no longer than 2 cm of length; if the filament is longer than 2 cm is considered abnormal because this means that semen is not enough hydrated, so spermatozoids dispersion is more difficult and motility and fertilization capacity is reduced (Bastardo *et al*., 2004; Rasines, 2013).

## Microscopic characteristics

The microscopic characteristics, are those we cannot assess with the naked eye (i.e. concentration, motility, viability and morphology sperm). Therefore, it is necessary the use of the microscope to evaluate these parameters, which must be determined within 60 minutes after semen collection (Cruz, 2001).

### Spermatozoa concentration

Spermatozoa concentration is simple to quantify because there are methodologies that vary according to specie and available equipment. Nevertheless, in fishes like most chordates, concentration is high and it is easily activated by being in contact with an aqueous medium, so it is necessary to maintain them in a solution that does not activate it, as well as to maintain individual integrity so the counting is possible (Rodríguez *et al*., 1992).

On the matter, different diluent solutions have been used to develop a stock solution to evaluate the number of cells. In some cases, formalin is added to the solutions as preservative. Then, they are kept in refrigeration for further evaluation. The diluents and species that have been reported are described below (Tab.1).

**Tab. 1: Diluent solutions to estimate spermatic concentration mL$^{-1}$ using Neubauer chamber.**

| Specie | Dilution | Diluent | Incubation time (minutes) | Ref. |
|---|---|---|---|---|
| *Barbus barbus* | 1:1000 | NaCl at 0.7% | 10 | Alavi *et al*., 2008; Alavi *et al*., 2009 |
| *Brycon amazonicus* | 1:1200 | NaCl at 0.9% | 10 | Cruz *et al*., 2006 |
| *Carassius auratus* | 1:1000 | NaCl at 0.7% | 10 | Zadmajid *et al*., 2013 |
| *Centropomus parallelus* | 1:2000 | Formalin, sodium bicarbonate and distilled water | - | Contreras *et al*., 2011 |
| *Cyprinus carpio* | 50:1500 | NaCl at 0.7% and formalin at 4% | 5 | Rodríguez *et al*., 2007 |
| *European eel* | 1:1000 1:10000 | Seawater | - | Asturiano *et al*., 2005, 2006 |
| *Oncorhynchus mykiss* | 10:2000 | NaCl | 2 | Nynca *et al*., 2016 |
| *Oncorhynchus mykiss* | 1:1000 | Hayem solution (5g Na2SO4, 1 g NaCl, 0.5 g HgCl2, 200 mL of bidistilled water) | - | Sahin *et al*., 2014 |
| *Oncorhynchus mykiss nelsoni* | 1:1000 | Lugol at 1% | | Aguilar *et al*., 2011 |
| *Paralichthys orbignyanus* | 1:2000 | Formalin at 4% and distilled water | 10 | Ceccon *et al*., 2010 |
| *Perca fluviatilis* | 1:1000 | NaCl | 10 | Alavi *et al*., 2007 |
| *Piaractus mesopotamicus* | 1:2000 | Citrate formalin (2.9 g of trisodium citrate dihydrate), formaldehyde at 35% and distilled water | 10 | Kuradomi *et al*., 2016 |
| *Pseudoplatystoma metaense* | 1:4000 | Formalin saline solution (0.9% of NaCl and formalin at 3%) | 10 | Ramírez *et al*., 2011 |

## Neubauer Chamber

Neubauer chamber is a precision instrument that is used to measure a number of particles per unit volume in a liquid. It is expressed in millions per mm$^3$. In aquaculture its use is very common to determine concentration of spermatozoa per mL$^{-1}$ with an acceptable variation. If concentration is high, then the counting turns difficult so it may be necessary to make a second dilution from the solution stock (Rodríguez *et al*. 1992).

Once the dilution is ready, the Neubauer chamber is prepared by moistening the edges where the slide will be settled with a micropipette. Two drops are spilled and then the chamber is charged by capillarity, being careful that there is not more quantity than needed or missing. It can be left from 5 to 10 minutes, which depends on specie, and then the sample is observed under microscope. The central grid, formed by 25 boxes, is focused. Some chambers are formed by 16; in that case, a correspondent adjustment must be made. If density is high, it is recommended to randomly select five boxes. If density is low, it is recommended to realize a counting in all 25 boxes. (Tab. 1; Rodríguez *et al*., 1992).

Concentration can be calculated with next formulas:

- Caille *et al*., 2006

$$\left[\frac{\left(\frac{1/a^2 \times b}{N}\right) x\ 100}{10^9}\right] \times \left[\frac{1}{V1/(V1+V2)}\right] \times \left[\frac{1}{V3/(V3+V4)}\right] \times [\sum(n \times N)]$$

Where: *a*= side of the box, mm$^2$; *b*= depth of the box, mm; *N*= number of boxes; *n*= number of spermatozoids per square meter; *V1*= volume of sperm, mm$^3$; *V2*= volume of saline physiological solution with formaldehyde mm$^3$; *V*3= total volume of spermatozoids previously diluted, mm$^3$; *V*4 = volume of additional diluent, mm$^3$.

- Contreras *et al*., 2011

Concentration of spermatozoids $mL^{-1}$ = ($\bar{x}$) (4) (2000) (2000).

Where: $\bar{x}$ = average number of spermatozoids per sample; 4= number of counted boxes in each counting trajectory; 2000 µL = total volume of sample; and 1:2000 = dilution of counting.

- Alavi *et al*., 2006

Spermatozoa concentrations ($mL^{-1}$) = 1000 x number of counted sperm/area ($mm^2$) x chamber depth (mm) x dilution ratio

## Spectrophotometer

Today, the most used methodology is the one proposed by Ciereszko and Dabrowski (1993). This technique allows to obtain in a quick way an estimation of sperm concentration through the use of absorbance registration of a spermatic suspension (nm), for which is necessary to first make a dilution. It is recommended to standardize a pattern curve (Tab. 2).

**Tab. 2: Diluent solution to estimate spermatic concentration $mL^{-1}$ using a spectrophotometer.**

| Specie | Nanometers | Dilution | Diluent | Ref. |
|---|---|---|---|---|
| *Clarias gariepinus* | 505 | 1:100 | NaCl at 0.9% | Viveiros *et al*., 2003 |
| *Oncorhynchus mykiss* | 600 | 1:2000 | NaCl at 0.7% | Nynca *et al*., 2016 |
| *Osnerus eperlanus* | 505 | 1:1000 | NaCl at 0.7% | Kowalski *et al*., 2006 |
| *Salvelinus fontinalis* | 505 | 1:1000 | NaCl at 0.7% | Nynca y Ciereszko, 2009 |
| *Salmo trutta f. fario* | 405 | 1:100 | Formaldehyde at 4% | Lahnsteiner *et al*., 2005 |

## Fluorescent microscopy

Fluorescent microscopy is an interaction process between radiation and matter, in which a material absorbs radiation from a specific source so that it emits light very quickly, and the energy released is lower (higher wave length) than the absorbed radiation. To apply this method is used the NucleoCounter SP-100, developed by Chemometec, Denmark, which offers the possibility to determine spermatozoa concentration and even spermatic viability (Nynca and Ciereszko, 2009). This equipment is based in computerized analysis of numerous cells stained with propidium iodide (PI) (Nynca and Ciereszko, 2009). NucleoCounter SP-100 is an innovative instrument that have a compact fluorescence microscope, CCD (charge coupled device) camera and an advanced software for image processing. Semen solution have to be diluted with lysis buffer (Reagent S-100 from Chemometec) and loaded in a disposable cassette that contains PI. According with Nynca and Ciereszko (2009), this equipment is appropriated for seminal analysis in salmonids (Tab. 3).

**Tab. 3: Estimation of spermatic concentration $mL^{-1}$ through the use of NucleoCounter SP-100.**

| Specie | Dilution | Diluent | Staining | Data processing | Ref. |
|---|---|---|---|---|---|
| *Oncorhynchus mykiss* | 1:100 | PBS and then 51 times with Reagent 100 | Propidium iodide | Software Semen View (Chemometec, Denmark) | Nynca *et al*., 2016 |
| *Salmo trutta* | 1:100 | Sperm immobilizing solution (100 mM NaCl, 40 mM KCl, 3 mM $CaCl_2$, 1.5 mM, $MgCl_2$ and 50 mM Tris, pH 8.5) and then 51 times with Reagent S100 | Propidium iodide | Software Semen View (Chemometec, Denmark) | Nynca *et al*., 2014 |

## Flow cytometry

This methodology allows to determine previously stained cells with a specific colorant, which fluoresces when is appropriately excited with light of a laser. Therefore, quantity of fluorescence is directly proportional to cell number (Pineda *et al*., 2004).

Hossain *et al*. (2011) reports that flow cytometry for cell count has been restricted to laboratory conditions. Nevertheless, a portable flow cytometry has been developed recently (Muse Cell Analyzer), enabling analysis by cytometry under field conditions for the first time. (Tab. 4; Nynca *et al*., 2016).

**Tab. 4: Estimation of sperm concentration mL$^{-1}$ through flow cytometry.**

| Specie | Methodology | Ref. |
|---|---|---|
| *Oncorhynchus mykiss* | Sperm concentration is estimated by using a Muse Cell Analyzer (Millipore, Billerica, MA, USA), following manufacturer's instructions. Semen samples were diluted first a 100 times with PBS, then 300 times with PBS. 20 µL of diluted sample were taken and mixed with 380 µL of Muse Count and Viability Assay Reagent (Millipore, USA) in tubes for micro centrifuge of 1.5 mL, and incubated during 5 minutes in darkness at room temperature. Samples are introduced to system by capillarity. Data is generated with Muse™ Count and Viability Software Module (Millipore, USA). | Nynca *et al*., 2016 |

According to different spermatic quantification methods, this one can be quickly evaluated, in a precise and low cost way, through simple tools like Neubauer chamber, although the main disadvantage of this method is that it requires more time than spectrophotometry, fluorescent microscopy and flow cytometry to get an estimation, the las last one being the most expensive and not commonly available in laboratories (Fauvel *et al*., 2010).

## Sperm motility

Spermatozoids motility is given by its correct morphology and viability; this test is important because fecundity success depends in its vigor. Metabolism for sperm motility depends the number of mitochondria and the available energy reserves (Snook, 2005). In teleost fish, spermatozoids are immobile in vas deferens and at ejaculation (Perchec *et al*., 1993; Müller *et al*., 1994; Darszon *et al*., 1999; Rurangwa *et al*., 2004) and metabolic activation is acquired at contact with an aqueous medium, either fresh, brackish water or ovarian fluid, losing it few seconds later (Billard *et al*., 1995; Kime *et al*., 2001; Rurangwa *et al*., 2004).

Sperm motility has been described in different ways according to study objective. Use of phase contrast or microscopy of dark field have been considerably improved the possibilities to observe both the head and flagella of spermatozoa. Application of different types of high speed video tapping or application of stroboscopic light sources proportionate static images of high quality of flagellar movement in different successive positions (Fauvel *et al*., 2010).

## Categorical subjective method

Motility is classified subjectively according to percentages of cells in movement, like: 0, when there is no movement; 1, when 25% of cells are in movement; 2, when 50% of cells are in movement; 3, when 75% of cells are in movement and 4, when more than 75% of cells are in movement (Borges *et al*., 2005; Viveiros *et al*., 2003), or also it can be only expressed with percentages (%) of mobile cells (Tab. 5).

**Tab. 5: Activator solutions to activate sperm motility according to percentages of cells in movement.**

| Specie | Dilution | Activator solution | Ref. |
|---|---|---|---|
| *Clarias gariepinus* | 15:45 | Water | Viveiros *et al*., 2003 |
| *Paralichthys orbignyanus* | 1:50 | Seawater | Ceccon *et al*., 2010 |
| *Piaractus mesopotamicus* | 1:5 | Water | Kuradomi *et al*., 2016 |
| *Rhamdia quelen* | 1:1000 | Water | Borges *et al*., 2005 |
| *Barbus barbus* | 1:1000 | Tris-HCl 30 mM, pH 8.0 and add BSA at 0.1% to avoid that spermatozoa to stick in the microscope slide | Alavi *et al*., 2008 |
| *Catla catla*, *Labeo rohita* *Labeo calbasu* *Cirrhinus mrigala* *Hypophthalmichthys molitrix* *Ctenopharyngodon idella* | 1:100 | Sterile water | Verma *et al*., 2009 |
| *Cyprinus carpio* | 1:2000 | NaCl at 0.7% | Bastami *et al*., 2010 |
| *Osmerus eperlanus* | 2:200 2:400 | Sodium bicarbonate $NaHCO_3$ | Kowalski *et al*., 2006 |
| *Oncorhynchus mykiss* | 1:100 | NaCl at 0.3% | Sahin *et al*., 2014 |
| *Oncorhynchus mykiss* | 1:40 | Ovarian fluid | Wojtczak *et al*., 2007 |

**Quantitative method Computer Assisted Sperm Analysis (CASA)**

In the last decade it has been reported the use of CASA software as tool for sperm motility evaluation in fish (Fauvel *et al*., 2010). This program allows to compare movement and speed parameters of spermatozoa under different physiological conditions (Ramírez *et al*., 2011), and integrate successive positions of spermatozoa heads in movement in consecutive video recording frames to calculate trajectories and descriptors Tab. 6 (Fauvel *et al*., 2010).

**Tab. 6: Movement and speed parameters reported by using CASA.**

| Descriptors | Unit | Ref. |
|---|---|---|
| Percentage of motile sperm | % | Nynca *et al*., 2014<br>Ciereszko *et al*., 2014<br>Alavi *et al*., 2013<br>Verma *et al*., 2009<br>Kowalski *et al*., 2006 |
| Linearity | % | |
| Straight line velocity | $\mu m^{-1}$ | |
| Curvilinear velocity | $\mu m^{-1}$ | |
| Average path velocity | $\mu m^{-1}$ | |
| Amplitude of lateral head displacement | µm | |
| Beat cross frequency | Hz | |
| Duration | S | |

Regarding to comparison of methods to evaluate motility, both are affected by parameters as: temperature, pH, $Na^+$, $K^+$ and $Ca^{2+}$ ions, from the used activator solution (Alavi and Cosson, 2005, 2006; Alavi *et al*., 2007). The categorical method it can be affects by the experience of observer, evaluation criteria that are chosen and mode of interpretation in contrast with CASA software that implicates higher cost but quantifies parameters, in a more precise, fast, able to provide information that is are not observable in categorical method.

**Sperm viability measurement**

Sperm viability is referred to membrane integrity. Plasmatic membrane controls ion and water exchanges between intra and extracellular medium which trigger the bendings of the axoneme (Fauvel *et al*., 2010). This type of test is based on double staining protocols with the purpose to differ live spermatozoa to dead ones (Rurangwa *et al*., 2004). Below it is presented some methodologies that varies according to available equipment, solution, kit and specie.

**Eosin-nigrosine staining**

This staining evaluates viability according to eosin colorant absorption by the spermatozoid head and is based under next criteria: dead spermatozoa are stained of red or pink color which indicates permeability to eosin due to partial or complete catchment of colorant and non-stained spermatozoa are cataloged as live Tab. 7 (Maria *et al*., 2010; Kuradomi *et al*., 2016).

**Tab. 7. Determination of spermatic viability through eosin-nigrosine staining.**

| Specie | Dilution | Solution | Smear | Sperm sample$^{-1}$ | Ref. |
|---|---|---|---|---|---|
| *Brycon henni* | 1:1000 | Isosmotic solution and 5 µL of Eosin-Nigrosine | + | - | Tabares *et al*., 2006 |
| *Brycon opalinus* | 1:100 | Eosin-Nigrosine (5% Eosin B and 10% Nigrosine; pH = 6.9) | + | 300 | Viveiros *et al*., 2012 |
| *Colossoma macropomum* | 1:10 | Eosin-Nigrosine (5% Eosin Y and 10% Nigrosine; pH 6.9) | + | 300 | Maria *et al*., 2010 |
| *Danio rerio* | | Eosin-Nigrosine (5 g Eosin Y and 10 g Nigrosine) | + | - | Gerber *et al*., 2016 |
| *Piaractus mesopotamicus* | 10:100 | Eosin-Nigrosine (5% Eosin Y and 10 % Nigrosine) | + | 200 | Kuradomi *et al*., 2016 |
| *Pangasianodon gigas* | 1:100 | Eosin-Nigrosine (0.2 g Eosin, 1 g de Nigrosine, 0.3 g of dehydrated sodium citrate and 20 mL of distilled water) | + | 40 | Kriangsak *et al*., 2010 |
| *Prochilodus lineatus* | 30:120 | Eosin-Nigrosine | + | 400 | Romagosa *et al*., 2010 |
| *Lutjanus argentimaculatus* | 10:40 | Eosin-Nigrosine (5% Eosin and 10% Nigrosine) | + | - | Vuthiphandchai *et al*., 2009 |

The results using this staining (Eosin-Nigrosine) vary between species which is attributed to the integrity and permeability of the plasma membrane so that the staining does not penetrate the sperm that have their plasma membrane intact (bright white head). Therefore, it is important that the analyst standardize this type of staining according to the specie of study.

## Fluorescent microscopy

Recently, semen quality has been evaluated through use of DNA fluorescent markers such as Hoechst 33258, propidium iodide (PI) o better, through the use of kits that contain specific markers (SYBR 14/PI) that allows a simultaneous observation of live and dead spermatozoa (Fauvel *et al*., 2010).

## Hoechst 33258

Spermatozoa are classified by: dead when nucleus show bright blue fluorescence on the head of spermatozoa and live when fluoresce is not strong Tab. 8 (Asturiano *et al*., 2006).

**Tab. 8: Spermatic viability determination using Hoechst 33258.**

| Specie | Methodology | Ref. |
|---|---|---|
| *European eel* | Add 1 µL of Hoechst 33258 in 2 µL of semen and 997 µL of PBS, leave incubating during 5 minutes in darkness and at room temperature, after that time observe under epifluorescence microscope using a UV-2A (Ex: 330-380 nm, DM: 400, BA: 420) filter at 100X, evaluate at least 100 spermatozoa per sample. | Asturiano *et al*., 2006 |

## Propidium iodide (PI)

Spermatozoa are classified as: dead those who stain and live those who were not stained Tab. 9 (Nynca *et al*., 2016).

## SYBR 14/PI

Spermatic viability is evaluated according to staining in spermatozoa head. Viable spermatozoa (lives) are stained with SYBR 14 (green fluorescence) while no viable (dead) are stained with PI (red fluorescence) Tab. 10 (Berríos *et al*., 2010; Aguilar *et al*., 2014).

## Morphology

Spermatic quality is determined by its morphology because abnormal spermatozoa tend to have low fertility (Tuset *et al*., 2008). Morphology is easy to characterize as it can be used commercial kits or solutions that allow to detect abnormalities. Between more common measurements there are: size of head, length (*L*, in µm), width (W, in µm), area (A, in $\mu m^2$), perimeter (P, in µm); and shape variables: ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation ((L-W)/(L+W)), regularity (πLW/4A), tail length (Tuset *et al*., 2008), flagella diameter (nm) flagella diameter with lateral extensions (nm), microtubule diameter (nm) (Kowalski *et al*., 2006), intermediate piece (µm), front width of intermediate piece (µm), back width (µm) and nucleus vesicles (µm) (Alavi *et al*., 2008). In Tab. 11 is shown some stain techniques for spermatic morphology evaluation.

**Tab. 9: Spermatic viability determination using PI.**

| Specie | Methodology | Ref. |
|---|---|---|
| *Oncorhynchus mykiss* | Dilute semen 100 times in PBS, 52 times in S100 (total count) or in immobilizing solution 100 mM NaCl, 40 mM KCL, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 50 mM Tris, pH 8.5 (no viable count). When immobilizing solution is used as diluent of semen, only no viable semen are count. Samples are loaded in cassettes that have PI and then it proceeds its observation under fluorescence microscope. | Nynca *et al*., 2016 |

From the methodologies mentioned before for spermatic viability evaluation, in a practical way, Eosine-Nigrosine satin is simple and cheap; nevertheless, it must be considered that colorant concentration varies according to specie due to different compounds in plasmatic membrane, even though it is less precise than other methods with fluorescent and specific DNA markers, this ones require more economic investment because measuring equipment are expensive and sophisticated like confocal and fluorescence microscopy and flow cytometry.

**Tab. 10: Spermatic viability determination using SYBR 14/PI.**

| Specie | Methodology | Ref. |
|---|---|---|
| *Oncorhynchus mykiss* | Suspend 3 x $10^6$ spermatozoa in 1 mL of spermatic diluent, centrifuge at 470 g per 5 minutes eliminating supernatant, dilute *pellet* in 1 mL of solution made of SYBR 14-IP at a final concentration of 1 µM and 5 µM respectively, and incubate for 15 minutes at 4 °C away from light. Subsequently, centrifuge at 470 g per 5 minutes the suspension made of spermatozoa, diluent and colorant, eliminate supernatant. Finally, dilute resulting *pellet* in 400 µL of diluent and evaluate in cytometer. | Berríos *et al*., 2010 |
| *Oncorhynchus mykiss nelsoni* | Take 10 µL of semen and mix with 0.05 µL of SYBR-14 (20 nM final concentration), it is incubated in darkness during 10 minutes at 19 ºC. Subsequently add 0.50 µL of propidium iodide (PI, 12 mM final concentration), samples are incubated for 10 minutes. Membrane integrity is evaluated by triplicate counting a total of 10 fields (100 cell per field) using a fluorescence microscope at 20 or 40X, with blue filter of excitation wavelength (490 nm). | Aguilar *et al*., 2014 |
| *Salmo salar* | Suspend 4 x $10^6$ spermatozoa $mL^{-1}$ in 250 µL PBS + 1.25 µL SYBR-14 + 1.25 µL of propidium iodide during 6 minutes at 10 °C, add again 250 mL of PBS and analyze through flow cytometry and confocal microscopy. | Figueroa *et al*., 2015 |
| *Silurus glanis* | Make a mix between SYBR-14 and propidium iodide (PI), add 10 µL in 1 mL of semen, mix with a vortex equipment and add 40 µL in a microscope slide, put the coverslips, incubate during 10 minutes and proceed to visualize in epifluorescence microscope. | Linhart *et al*., 2005 |
| *Sparus aurata* | Add 5 µL of SYBR-14 (1:50) to 1 mL of spermatic suspension previously diluted (1:100 in NaCl al 1.1 %), and is incubated at 36 °C during 10 minutes. Subsequently, add 5 µL of propidium iodide in 1 mL of diluted semen simple and incubate during 5 minutes, after that time, put the sample in a microscope slide, put the coverslips and watch immediately under fluorescence microscope equipped with appropriated filters. | Zilli *et al*., 2011 |
| *Dicentrarchus labrax* | Use SYBR-14/PI and fluorescence microscopy, incubate for 5 minutes and proceed to visualization, counting 100 cells per sample and by duplicate. | Cabrita *et al*., 2011 |
| *Tinca tinca* | Add 5 µl of SYBR-14 (2 µM) and 10 µl of propidium iodide (PI 5 mM) in 500 µL of diluted semen in isotonic buffered diluent, incubate during 20 minutes in darkness at room temperature, after that time the analysis though flow cytometry is done. | Oropesa *et al*., 2016 |

**Tab. 11: Fixation and staining methods for determination spermatic morphology.**

| Specie | Methodology | Ref. |
|---|---|---|
| *Catostomus macrocheilus* | Dilute 2.5 µL of semen in HBSS 1:100 and mix with 2.5 µL of eosin-nigrosine, make a smear and observe under optic microscope at 100X, count more than 500 cell per sample. | Jenkins *et al*., 2014 |
| *Pseudoplatystoma metaense* | Dilute semen in formaldehyde solution at 35% (4%) - sodium citrate [2.9%, $Na_2HA$ ($C_3H_5O(COO)_3$] and distilled water and observe under microscope. | Ramírez *et al*., 2011 |
| *Barbus barbus* | Mix semen with glutaraldehyde at 2.5 % and in 0,1 M phosphate buffer (pH 7.6) for 2 days at 4 °C (dilution relation: 1 µL of semen: 49 µL of fixer), post-fixation, wash reputedly during 2 h at 4 °C in osmium tetroxide and dehydrate in acetone series (30, 50, 70, 90, 95 y 100%). | Alavi *et al*., 2008 |
| *Prochilodus lineatus* | Dilute semen in citrate formaldehyde$^{-1}$ (2.9 g sodium citrate, 4 mL formaldehyde at 35% and 100 mL of distilled water) 10:1000 (semen: entire solution), make a smear, dye with bengal rose, proceed to visualization under microscope at 100X and do observation of 100 cells per sample. | Felizardo *et al*., 2010 |
| *Misgurnus fossilis* | Mix semen in glutaraldehyde at 2.5% and in 0.1 M of phosphate buffer, store at 4 °C for 2 days. Subsequently, fix the samples by adding phosphate buffer (0.5 mL), wash constantly during 2 h at 4 °C in osmium tetroxide and dehydrate in acetone series. | Alavi *et al*., 2013 |
| *Oncorhynchus mykiss* | Dilute the semen 1:100 in sodium citrate at 3%, put it in Eppendorf tubes and centrifuge during 15 s at 300 x g. After that, make a smear with 5 µL of dilution, let it air dry during 20-30 s and stain through commercial kits Diff-Quick®, Hemacolor® or Spermac®. | Tuset *et al*., 2008 |

Morphological variables of spermatozoa are generally established through stain techniques, where authors like Tuset *et al*. (2008) mention that there can be morphological variations due to stain kit, drying time and fixation, which proves the necessity to design and standardize efficient protocols related to the specie, that also include corresponding modifications, considering if studied semen is fresh or cryopreserved.

## Conclusion

The knowledge of techniques for evaluating semen quality has proven to be a fundamental tool in the increase of reproductive efficiency. Thus, the current

review allowed the compilation, analysis and description of the main prevailing methodologies that implement a full evaluation of the semen quality in some teleost fish, providing a very valuable and useful data to optimize the reproductive process in aquaculture sector.

The most commonly used parameters in the evaluation of semen quality are the concentration and motility of sperm, since they are related directly with the fertilization rate (Rurangwa *et al*., 2004). The selection of a methodology will depend on the available equipment, the precision, the efficiency and the time spent on the obtainment of results. For example, the sperm concentration can be evaluated quickly in a precise and low cost way through simple devices like Neubauer chamber, although the main disadvantage in the use of this method is that it requires more time compared to spectrometry, fluorescent microscopy and cytometry to get an estimation, the last one being the most expensive and not commonly available in laboratories (Fauvel *et al*., 2010).

In the case of motility, this can be evaluated through both the categorical subjective method or the quantitative method Computer Assisted Sperm Analysis (CASA). However, in the practice, the application of categorical method may be affected by the lack of experience of the observer, the evaluation criteria and the mode of interpreting. In contrast, CASA software provides further analysis with minor error, but implies a greater investment.

On the other hand, though viability and morphology of sperm are also basic and essential parameters, these are not normally used due to the stain-dependent variability of the results, since the dyes present differences in the permeability of live, dead or malformed cells, and therefore, in their detection. For this reason, the specific fluorescent DNA markers become useful tools in order to carry out a more precise evaluation. However, a greater economic investment must be purchased because of the sophisticated and expensive equipment that is required.

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