Deliverable Report

Deliverable No:	D6.1		**Delivery Month:**	24
Deliverable Title	Computer Assisted Sperm Analysis (CASA) for wreckfish sperm			
WP No:	6		**WP Lead beneficiary:**	P8. IEO
WP Title:	Reproduction and Genetics – Wreckfish			
Task No:	6.4		**Task Lead beneficiary:**	P14. IFREMER
Task Title:	Evaluation of sperm characteristics and cryopreservation protocols			
Other beneficiaries:	P1. HCMR	P3. IRTA	P8. IEO	P14. IFREMER
P15. ULL	P19. CMRM	P32. MC2		
Status:	Delivered		**Expected month:**	24
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Lead Scientist preparing the Deliverable: Fauvel, C. (IFREMER),

Other Scientists participating: Peleteiro, B. (IEO), Vilar, A. (MC2), Mylonas, C.C. (HCMR)

Objective: The main goal of this deliverable was to provide a tool for an objective assessment of sperm quality of male wreckfish (*Polyprion americanus*), subject to captivity during the full reproductive season, by establishing a Computer Assisted Sperm Analysis (CASA) for the evaluation of wreckfish sperm. The best adapted CASA parameters for wreckfish sperm analyses were determined and reported to end users to optimize their abilities to check fertility potential of the semen in the course of their future spawning induction experiments. Moreover a movie describing the procedure of sperm activation and CASA analysis is proposed on the website of the project.

Background

In preliminary experiments, wild caught wreckfish exhibited a high growth rate and a good behavior in captivity (Papandroulakis et al., 2004; Peleteiro et al., 2010), which indicated that this species was an interesting candidate for aquaculture. However, these preliminary experiments were based on a few individuals and the catches have remained very low and scattered so that few organisms are available to research the potential of wreckfish culture. Therefore, the understanding of wreckfish reproduction in captivity is a prerequisite to both control reproduction in captivity and provide individuals for research of the species' biology and aquaculture potential.

There exists a scarcity of knowledge on the biology of the wreckfish and particularly for wreckfish reproduction. The species in the wild was reported to mature at around 10-13 years and spawn during the winter in both hemispheres (Sedberry et al., 1999; Peres & Klipell, 2003; Roberts, 1989) in deep waters from

300 to 850 m (Glukhov and Zaferman, 1982; Sedberry et al., 1999). Due to those extreme environmental conditions associated with successful reproduction (hydrostatic pressure, light, deep water location), the actual environmental requirements for reproduction are unknown and the environment provided for broodstock management in controlled conditions has been empirical. However, in captivity wreckfish have been observed to spawn spontaneously only in very large aquaria and both fertilized eggs and larvae were collected, but in an inconsistent and unreliable way (observed in DIVERSIFY). The first experience with wreckfish in aquaculture facilities, demonstrated that final maturation and ovulation required hormonal stimulation and some eggs could be released spontaneously, but only eggs obtained by hand-stripping resulted in fertilized eggs and embryos (Papandroulakis et al., 2004; Fauvel et al., 2008). These observations demonstrated that strip-spawning and artificial fertilization may be the preferred method for a reliable production of embryos for the wreckfish propagation in aquaculture.

To develop artificial fertilization the availability of live and efficient wreckfish sperm at the right time is mandatory. In order to anticipate this need, it was proposed to characterize wreckfish sperm in a simple but objective manner. The characterization would provide the base information needed in order to prepare for the use of fresh or cryopreserved sperm in artificial fertilization protocols. This information has applications both at experimental and production scale, as is the case in salmonid and most of flatfish production (*e.g.*, turbot, *Scophtalmus* maximus; Atlantic halibut, *Hippoglossus hippoglossus*), which are produced entirely using artificial fertilization in aquaculture. Moreover, this research work also helps transferring know-how about sperm assessment methods and understanding of gamete biology from laboratory to the fish farming industry. The present deliverable is a report of the applied methods and the results obtained. However, due to the restricted number of males available in captivity, the characteristics of wreckfish sperm described below are indicative, as data is required from more animals to confirm the results through powerful statistical analyses.

Description

The analysis of wreckfish sperm took place in the Spring of 2014 in Galicia (NW Spain) using gametes collected from three different locations and in the Winter of 2015 from two locations of the same area. From 8 to 13 April 2014, sperm was collected from 6 males from P32.MC2 (Acuarium Finisterrae) facilities, from 2 males of Luso Hispana de Aquicultura (LHA) and finally from 2 males from the facilities of P8.IEO (Center of Vigo). The laboratory methods of analysis were shared between researchers of the different partners and the P14. IFREMER researcher in charge of this task at the IEO laboratories. In January 2015, some of the males of P32 and P8 were sampled again and a complementary transfer of know-how about sperm quality assessment was performed at the Acuarium Finisterrae facilities. Concomitantly, taking profit of regular maturation monitoring during 2014, P1. HCMR analyzed sperm quality of several wreckfish males according to its own usual routine field methods.

Routine field assessment of sperm quality (HCMR)

The semen was collected by hand-stripping. It was rapidly assessed for density and motility as described below. Moreover, aliquots of small volume (10 µl) of the semen were stored undiluted at 4°C and assessed for motility at different times post collection. The density of sperm was assessed after adequate dilution (from 1/21 to 1/4221 according to the initial concentration of the semen, by counting spermatozoa on a Neubauer haemocytometer under 200X magnification).

The initial motility of sperm was evaluated with the following steps: 1) a small volume of around 1µl of sperm was collected by plunging a syringe needle in the semen; 2) the 1 µl drop of sperm was deposited on a microscope slide; 3) a drop of seawater (50 µl) was deposited beside the sperm without making contact with the sperm; 4) contact between sperm and seawater was triggered by the depositing of a coverslip over both

drops; 5) after a rapid focus at 400X magnification, and the choice of an observation field showing adequate sperm number and motility, the initial motility was subjectively assessed at-a-sight, by attributing a mark on a motility scale of 10 steps, each of which represented a 10% motility class.

The duration of sperm motility was established as the time from when the sperm was activated to the time when only 5% of spermatozoa exhibited motility. For this measurement, the semen drop was quickly mixed with the water drop using a needle point, prior to placing the coverslip. Finally, the ability of sperm to survive was established by daily test of motility as above, the survival capacity was expressed as the number of days when more than 5% motility was observed.

Laboratory assessment of sperm quality (Ifremer, IEO, MC2)

Laboratory protocols to record pictures and movies of sperm motility were established in order to allow a delayed analysis of data, to provide more objective complementary data, to shift the time of analysis and finally to inter-calibrate the analyses between partners.

To estimate sperm concentration, the collected semen were diluted to 1/1000 in distilled water and 2 aliquots were deposited into a Thoma counting plate, for which the depth between slide and coverslip was 10 µm. The sedimentation of wreckfish spermatozoa was very quick and completed after only 3 min. After 5 min for complete sedimentation, pictures at X20 magnification were recorded using a digital camera. For this purpose, the microscope was focused to provide a homogenous background on which the grid of the haemocytometer was just detectable, whereas spermatozoa were highly contrasted without any care about the shape and the resolution of the cells, which decrease as field depth increases. For this, the condenser was set in a lower position with its diaphragm closed.

The analysis of concentration was very quick in case of homogeneous background (**Fig. 1,** upper left frame) using the free software ImageJ. After selection and crop of an area delimited by grid lines, the image was binarized by the application of a level of grey level threshold separating spermatozoa from background (automatic threshold proposes the red particles in the **Fig. 1** central frame). Finally, an analysis of particles was launched to provide a count of the number of particles (sperm) of interest (**Fig. 1,** upper right frame). Each particle can be recognized by an id number (**Fig. 1,** lower right frame). In case of several pictures recorded within the same experiment with the same microscope settings, a macro can be recorded to automatize the counting process.

In order to objectively assess the motility through different parameters (percentage of mobile spermatozoa, swimming velocity and track linearity), samples of sperm were activated following a two-step procedure as recommended by Billard and Cosson (1992). The dilution protocol includes a first dilution that aims at 3 objectives: 1) adjusting the concentration of spermatozoa, 2) preventing gradual motility trigger when the activating medium has to penetrate sperm mass, and/or finally 3) improving the storage of semen. For this first dilution, a non-activating medium (NAM) is mandatory. In wreckfish, the NAM was either diluted seawater (1/3 v/v eg. approximatively 300 mOsm) complemented with 20 mg ml^{-1} Bovine Serum Albumin (BSA, Sigma Chimie, France), or a complex medium of Leibovitz L15 (Sigma Chimie, France) supplemented with sodium pyruvate, 6mg ml^{-1}, glutamine, 0.3mg ml^{-1} and adjusted to 250 mosm and pH 8.

The second dilution, called activation, aims at triggering the motility of spermatozoa. The use of seawater supplemented by 20 mg ml^{-1} BSA or by 0.2% Pluronic acid (P1300, Sigma-Aldrich) as an activating medium allows a natural motility, while it prevents spermatozoa sticking on glass. A final density after activation of 1 to $2*10^4$ spz μl^{-1} provides an adequate number of motile sperm for motility analysis.

In order to get this final concentration, 10 µl of fresh sperm were sampled with a positive displacement pipette (Socorex, Switzerland) and mixed with 240 µl of NAM. This pre-diluted sperm was then stored on ice until the analysis. For each motility assay, 10 µl of pre-diluted sperm was deposited on the wall of a 1.8 ml micro-centrifuge tube already filled with 990 µl activating medium (seawater +BSA). Care must be taken that the pre-diluted sperm placed on the tube wall does not make any contact with the activating medium.

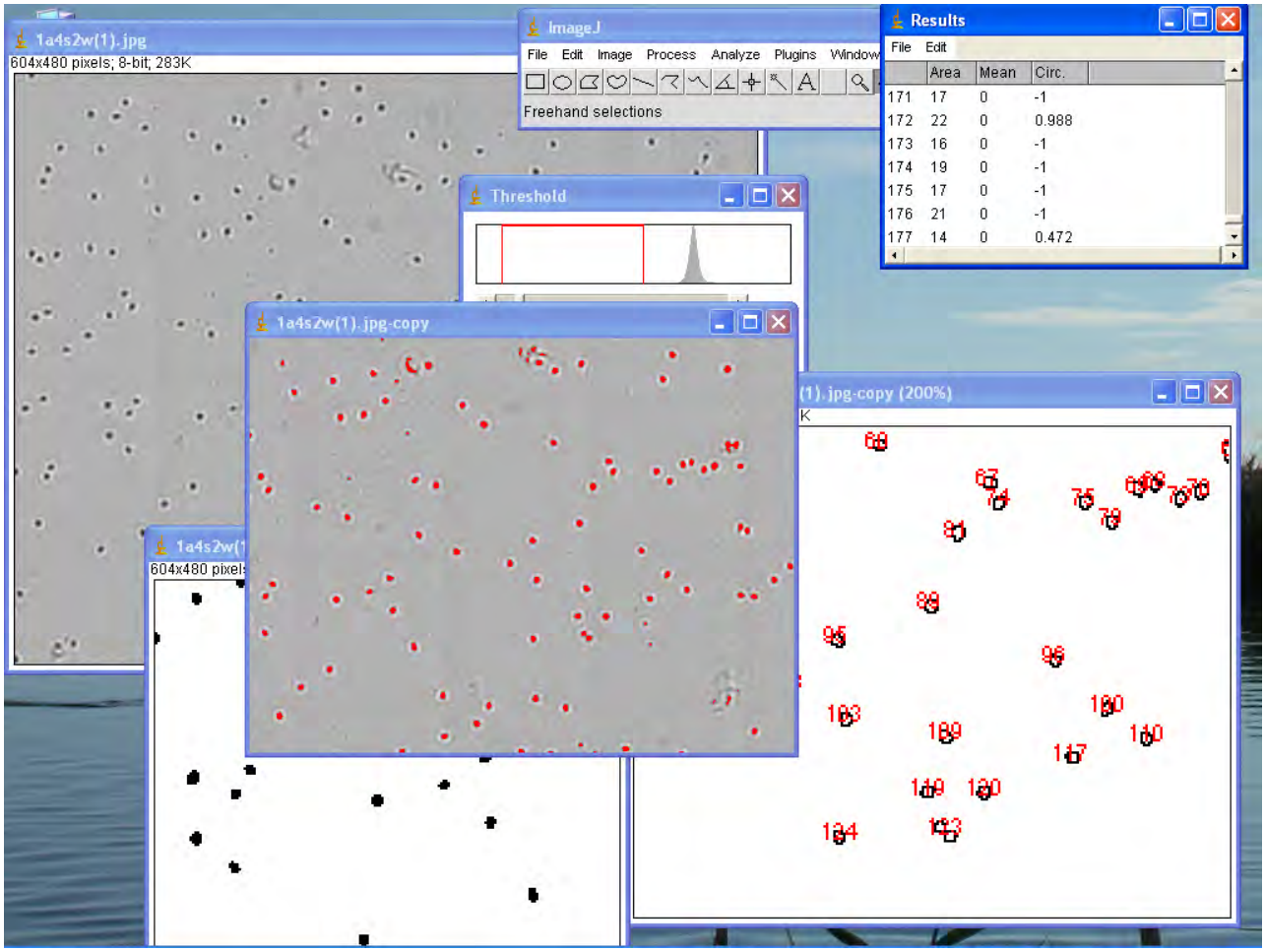

Figure 1. Snapshot of a screen representing the different steps of sperm count by image analysis using the free software Image J.

The motility was recorded using a simple microscope (Axiolab, Zeiss, Germany) set for dark field observation and equipped with a digital USB monochrom videocamera (DMK 22BUCO3, Imagingsource, Germany). The frame rate was set at 30 frames per second.

To activate the sperm, the tube was shaken strongly only once, in order to establish contact between spermatozoa and seawater. At the same time as the tube was shaken the video record was concomitantly launched in order to get the start point of sperm activation. At mixing, the drop was therefore diluted in seawater to 1/100 and the total dilution of sperm was 1/2500. A volume of 2 µl of activated sperm was immediately deposited into a pre-focused special cell (Leja cell, 10 µm depth). This method permits a first reliable assessment of the motility only 10 s after activation. The video recording was stopped when just 5% motile cells were observed at a glance within the population.

The analysis of the motility was run after fine-tuning the settings of image preparation and those of CASA plug-in according to the protocol exhaustively described in DIVERSIFY *Deliverable 7.1 Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of grey mullet sperm*, which includes the following items:

- Download of the software ImageJ and the installation of the CASA plug-in
- Movie process and upload for analysis
- Definition of sequences of interest
- Image treatment
- The use of the CASA plug-in
- The use of macros to repeat the whole analysis.

Wreckfish sperm characteristics

The mean concentration of wreckfish sperm was 2.41 $x10^{10}$ (sd: 0.4 $x10^{10}$, n=9) spermatozoa ml^{-1} in Galicia in January, while it remained around $1x10^{10}$ from April to September with no significant variation between sampling dates at the P1.HCMR facilities in Crete. In 2015, the concentration at these facilities reached higher values of up to $2x10^{10}$. In any case, the standard deviation was very high between and within males of the different locations, and it is not possible to conclude that there was a significant difference in sperm concentration between wreckfish in Crete and Galicia. These concentrations levels do not differ from earlier data (from 1.5 to 2.71 10^{10} spz ml^{-1}) from the P1. HCMR broodstock (2008 unpublished). Finally, the spermatozoa concentration in wreckfish stripped semen was of the same order of magnitude as that of pelagic fish such as European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) or meagre (*Argyrosomus regius*) and it was higher than that of sole (*Solea solea*) and turbot.

Fresh sperm from Vigo was used to set the parameters of the ImageJ CASA plug-in. The movies were recorded as .avi files. They were transformed into image sequences using the free software Virtualdub, then, short sequences of 60 frames every minute (*e.g.*, 2 sec/min) were analyzed using the plug-in developed by Wilson-Leedy and Ingermann (2007). When the procedure was established and parameters adjusted for the first sequence at 10 s,, the method was recorded as a macro including 2 parts:

1) General frame treatment in order to binarize the images (what is spermatozoa is black, what is not is white) of sequence 1 at 10 s post activation (**Fig. 2**, green part) and

2) Actual CASA analysis (**Fig. 2**, pink part).

The macro was then applied to the following sequences until motility completion.

```
//run("Brightness/Contrast...");
 setMinAndMax(160, 162);
 run("Apply LUT", "stack");
 setAutoThreshold("Default dark");
 //run("Threshold...");
 setThreshold(129, 255);
 setOption("BlackBackground", false);
 run("Convert to Mask", "method=Default background=Dark calculate");
run("CASA ", "a,=0 b,=1000 c,=5 d,=30 e,=20 f,=20.000000000 g,=20 h,=5.00000 i,=1 j,=25.000000000 k,=25.000000000 l,=35.000000000 m,=80.000000000 n,=80.000000000 o,=50.000000000 p,=60.000000000 q,=30 r,=460 s,=0 t,=1");
String.copyResults();
```

Figure 2. Script of the macro established for automated CASA analysis of wreckfish sperm using Image J software and the CASA plug-in.

This analysis provides a large number of individual data about spermatozoa among which only some have been chosen for the current study, the other being irrelevant, due to the performances of the camera such as the curvilinear velocity or instant speed and wooble or lateral head displacement, which require a video camera recording at least 100 frame s^{-1} (FPS). Therefore in the current study, sperm motility was qualified by the percentage of motile spermatozoa, the average path velocity or VAP estimated as the mean instant speed recorded for a determined number of successive frames (*e.g.*, the speed on a smoothed track), the straight line velocity or VSL expressing the progressive straight-line movement within the time interval of the analysis and the linearity, which illustrates the curvature of the trajectories and is obtained from the ratio VSL/VAP. Finally the duration of motility was also considered for sperm quality assessment. The different parameters are explained in **Fig. 3A**.

The fresh sperm showed a high initial percentage of mobile spermatozoa at activation, which had regularly decreased with time for 5 minutes. The mean initial VAP or mean velocity along smoothed trajectory was around 230µm per second, which progressively decreased to 0 after 5 minutes (**Fig. 3C**). The velocity of spermatozoa was one of the highest reported for marine fish and the trajectories vary from straight forward at activation to progressive circling as the speed decreased as illustrated in **Fig. 4**. This illustration is corroborated by the decrease of linearity of the trajectories calculated by the ratio between the average path velocity and straight-line velocity (**Fig. 3D**).

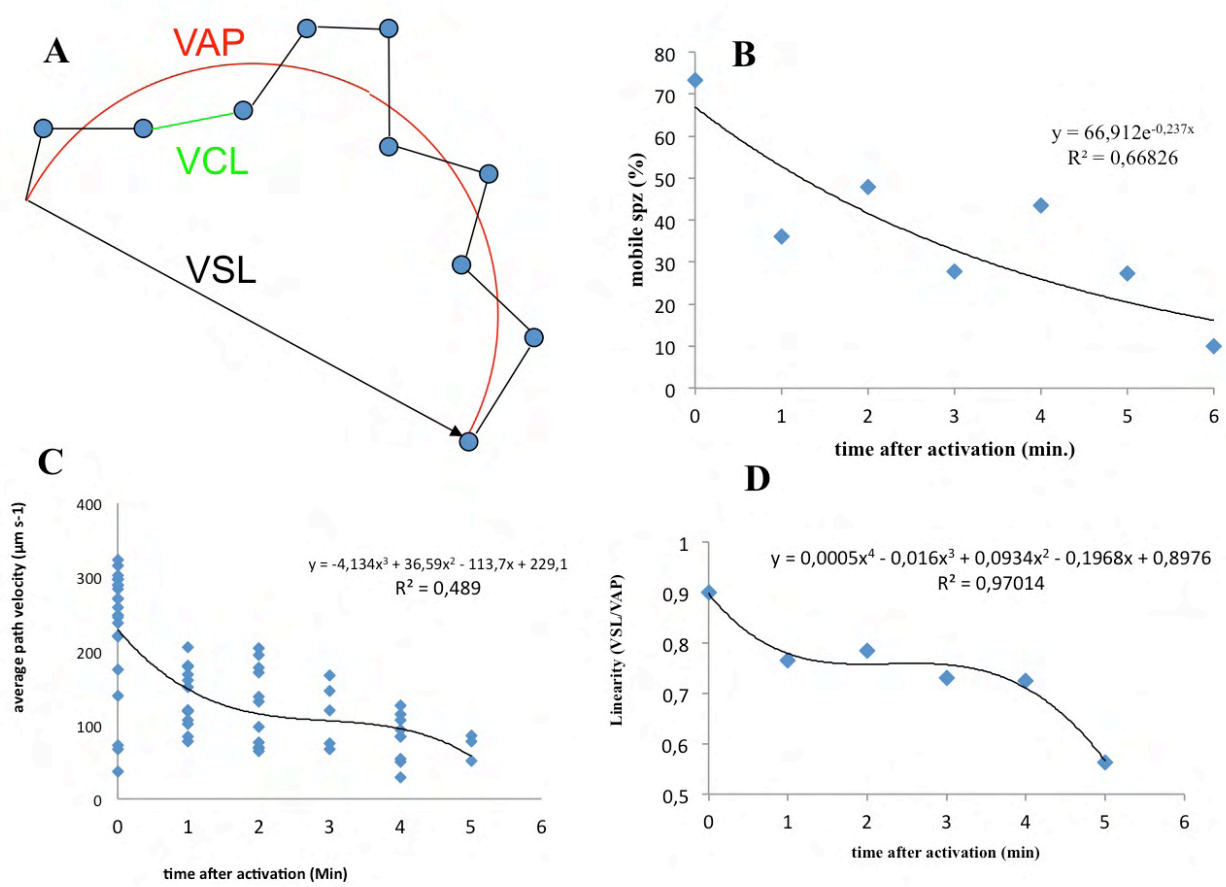

Figure 3. Motility parameters of wreckfish sperm: A) schematic representation of spermatozoa movement illustrating the three parameters of velocity generated by CASA; B) Variations of the percentage of swimming spermatozoa (spz) with time; C) average path velocity decrease with time after activation; D) decrease of linearity of spermatozoa trajectories after activation.

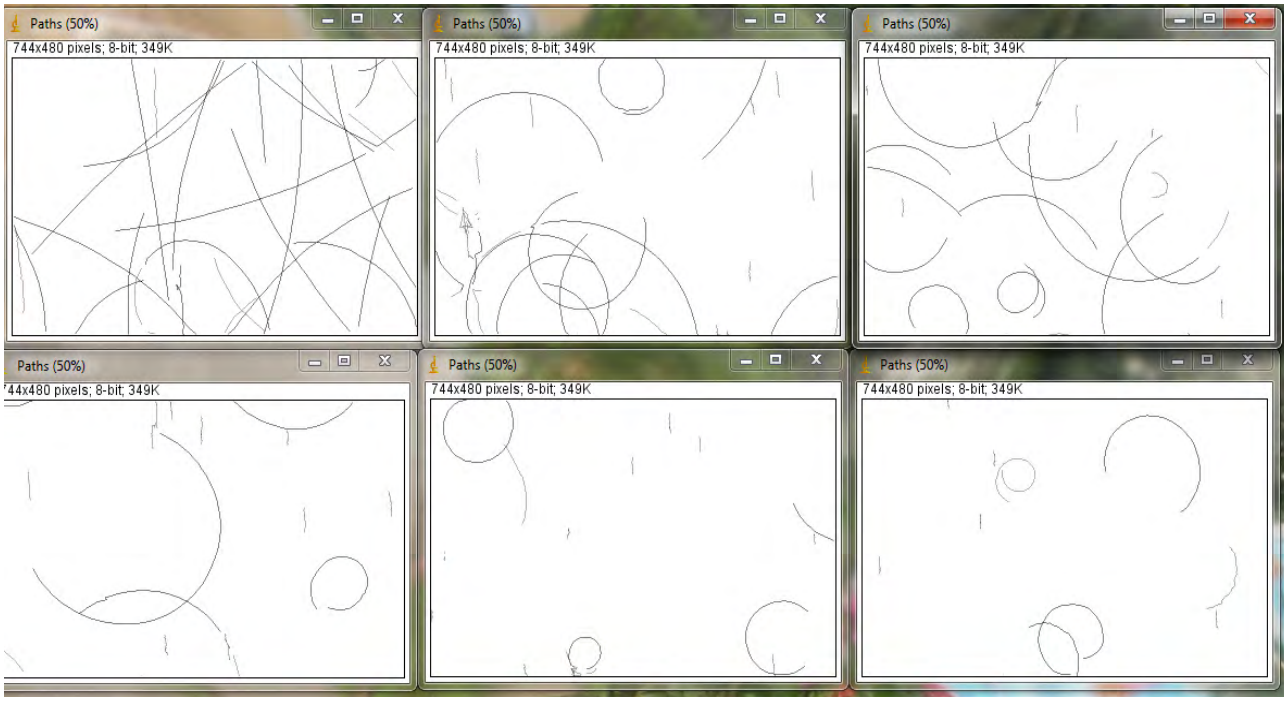

Figure 4. Spermatozoa path tracks of 2 s, generated by Computer Assisted Aperm Analysis (CASA plug-in of ImageJ) at 10 sec after activation, and at every minute for 5 min (starting from the upper left to the right) after sperm activation in wreckfish using the setting described above.

Conclusion and perspectives

As programmed in the DOW, a method for the objective assessment of sperm quality through the analysis of motility was implemented for wreckfish that complemented and confirmed field assessment usually implemented in broodstock rearing facilities. The preliminary analyses demonstrated that sperm of captive wreckfish shares a common pattern of motility with both marine and freshwater fish, based on a general activation of all the sperm at the same time of ejaculation in activating environment, then a decrease with time down to zero in a rapid lapse of time from 30 sec to more than 20 min due to exhaustion of energetic stores badly compensated by respiration.

Wreckfish males produced a high volume of easily expressible milt with a concentration considered as medium range for marine fish and of course much higher than that of flatfish. On the top of those general features, the setup of a CASA protocol adapted to wreckfish sperm demonstrated that wreckfish sperm exhibits a high percentage of motile cells at activation and one of the highest initial speeds recorded for fish sperm. This high speed was associated with a long swimming duration compared to other marine fish. The long duration exhibited a double trajectory shape. The first trajectory was straight (associated with the search of target eggs) and then the trajectory began bending, which was interpreted as a phase of searching for the micropyle on the egg surface. Moreover, the results obtained by CASA are in agreement with field observations obtained by human inspection under the microscope, and complement them by objective data that can be more easily statistically analyzed.

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Wilson-Leedy, J.G.and Ingermann, R.L. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology. 67(3):661-72.

Deviations: No deviations

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