Deliverable Report

Deliverable No:	D3.4	**Delivery Month:**		32
Deliverable Title	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of greater amberjack sperm			
WP No:	3	**WP Lead beneficiary:**		P13. UNIBA
WP Title:	Reproduction and Genetics - greater amberjack			
Task No:	3.1	**Task Lead beneficiary:**		P13. UNIBA
Task Title:	Description of the reproductive cycle of greater amberjack			
Other beneficiaries:	P1. HCMR	P13. UNIBA	P14. IFREMER	P23. ARGO
Status:	Delivered	**Expected month:**	32	
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Objective:

The main goal of this deliverable was to establish a Computer Assisted Sperm Analysis (CASA) for the evaluation of greater amberjack (*Seriola dumerili*) sperm. The deliverable is a report containing the original characteristics of motility and concentration of amberjack sperm at different periods of the reproductive season. The analyses were based on the open source Image J software (NIH, USA) and are detailed in a user guide way so as to allow end users to develop by themselves future sperm quality assessment.

Background:

The annual aquaculture world production of Seriola sp is a significant input of fish culture production, which has been stabilized at around 140,000 t from the early eighties up to now. Notwithstanding, this production may be fragile since it has mainly relied on wild juvenile capture (http://www.fao.org/fishery/culturedspecies/Seriola/). The main reason of the limited development of self-sustained aquaculture of the species family is the difficulty to breed in captivity. In Europe, some of the reproductive dysfunctions of captive-reared greater amberjack have occasionally been overcome through the administration of human chorionic gonadotropins (hCG) (Pastor et al., 2000; Garcia et al., 2001; Kožul et al., 2001) or gonadotropin releasing hormone agonists (GnRHa) (Lazzari et al., 2000; Pastor et al., 2000; Garcia et al., 2001; Mylonas et al., 2004), and the rare occurrence of spontaneous spawning has been reported (Jerez et al., 2006). However, the absence of a significant aquaculture industry for greater amberjack in Europe is testament to the lack of a reliable technology for reproduction control in captivity for this species (Mylonas et al., 2004).

Since the beginning of the Diversify project, the consortium has obtained an increase of egg production, which, although sufficient for further developmental stage studies, has remained highly variable in terms of egg quantity and quality. The quality of eggs is generally characterized by their capacity to develop and particularly by the presence of an embryo so that egg quality integrates the quality of sperm as well. In order to better understand the main determinants of reproductive success in *Seriola* aquaculture, the present deliverable aimed at evaluating the actual state of sperm of captive males by analytical methods providing a more objective view of sperm quality and a more precise evaluation of paternal input in the determinism of reproductive success with particular reference to sperm quality variation along the reproductive season.

As a generality, except in Acipenseridae, fish spermatozoa lack an acrosome that allows fusion of sperm and egg membrane. Moreover, fish male gametes exhibit a reduced midpiece compared to that of species with internal fertilization. The reduced midpiece corresponds to a reduced number of mitochondria associated with cell respiration so that the respiration capacity hence the rate of energy production is limited. As a consequence, the motility of fish sperm generally presents a short duration. The gametes are immotile in the genital tract and they are activated and start swimming just at contact with external medium at the ejaculation. The process of activation is well known and corresponds to a reaction to abrupt variation of osmotic pressure between seminal fluid and external medium (seawater or freshwater according to species), which triggers flagellum movement through membrane ion exchange (Cosson et al., 2008). In the majority of fish, the fertilization occurs after a concomitant release of gametes by fish of both sexes associated to a mating behavior. Spermatozoa reach the oocyte membrane and penetrate it after crossing the chorion of the egg through one or several (until 7 in sturgeons) specific channels called micropyles (Zelazowska, 2010). Finally, in temperate fish, the spermatogenesis is generally discontinuous and spermiation can occur concomitantly or later on, according to species so that spermiation covers the entire reproductive period of

the females. However, the possible asynchrony between spermatogenesis and spermiation can induce variations of sperm quality along the reproductive season called ageing (Dreanno et al., 1999).

Those general features of sperm biology can be objectively assessed through different parameters illustrating the quality of the gametes which have been related to fertilization capacity in model or aquaculture species such as seabass (Fauvel et al, 1999; Fauvel et al 2010), turbot (Suquet et al., 1994), trout (Lahnsteiner et al., 1998) and sole (Cabrita et al., 2006). Among these parameters, the concentration of sperm indicates the availability of gametes for fertilization; the use of CASA (computer assisted sperm analysis) allows assessing the motility parameters such as the speed of spermatozoa, the variations of motility in relation to time after activation and the shape of the tracks. The evaluation of ATP illustrates the energetic content of the cells and their ability to swim before exhaustion (Christen et al., 1987) and finally, the integrity of the membranes can indicate the potential of activation at ejaculation hence the capacity of sperm to fertilize. These different parameters were applied to the case of the greater amberjack *Seriola dumerili* in order to better explain the conditions of reproduction in captivity and to suggest improvement of handling and spawning management for this species.

Description:

In order to describe the capacity of greater amberjack males to successfully breed, sperm quality was assessed through physical and biological features. Sperm was collected from the four captive fish sampled at each sampling date in P.23 (Argosaronikos) facilities in Salamina island (Greece) for gametogenesis evaluation in order to make a relation between physiological status of the gonad and gamete quality. Sampling for sperm assessment was performed by P1. HCMR and P14. IFREMER personnel who attended every sampling session (April 20-25th, June 3-8th and July 2-4th) after a short training and inter-calibration work held in P1 laboratory (HCMR Gournes, Crete). After unsuccessful attempts to collect sperm by stripping the fish, samples of sperm were directly extracted from the dissected gonads at fish slaughter. Sperm samples were stored dry or after dilution in modified Leibovitz from sperm collection until being processed for the following different purposes: concentration, motility, ATP content and viability assessments. The procedures for the different analyses are summarized below.

Concentration

On site

- Dilution 1/500 in tap water or physiological saline 9 g l^{-1}
- Deposit on counting cell (Thoma)
- Record several pictures after focusing so as to differentiate spermatozoa from any other particle or structure like the grid.

Laboratory

- Analyze with imageJ using particle analysis function after contrasting the image and selecting of spermatozoa only by binarization.

Motility **(according to Wilson Leedy and Ingermann, 2007)**

On site

- Establish "zero motility medium" by decreasing (physiological saline + Bovine Serum Albumin (BSA)) concentration if necessary, test the modified Leibovitz medium set for mullet, meagre and wreckfish (deliverable D 6.1)
- Dilute sperm in non-activating medium (1/3).
- Activate sperm in seawater (sw) containing 1.5-2% BSA. The activation consists of mixing 20 µl of diluted sperm into 1 ml sw-BSA for a final dilution of 1/500, v/v.
- Immediately deposit activated sperm under the video microscope into a pre-focused special cell (Leja cell, depth 10µm) and record motility until the cessation of any movement.

Laboratory

- CASA analysis, Image J settings were adjusted according to the quality of videos and camera specificities (after intercalibration between P1 and P14) following the didactic "VADEMECUM FOR THE ANALYSIS OF SPERM MOTILITY: GENERAL PROTOCOL AND PROPOSITIONS FOR GREY MULLET SPERM QUALITY ASSESSMENT " provided as the deliverable D7.1 of Diversify.

On site

- Prepare extraction medium (boiling Tris 100mM+EDTA 10 mM buffer pH7.75).

  Drop 1, 10 and 100 µl of sperm into tubes containing 1 ml extraction medium and let boil for 2 min, then store at -20°C.

Laboratory

- Analysis in Ifremer Brest using the luminescence assay kit ATP-lite (Perkin Elmer).

Membrane integrity **(according to Beirão et al., 2009)**

On site

- Dilute sperm to 1/25 in a solution of 9g l^{-1} NaCl (i.e 40 µl sperm in 1 ml solution), then add 2 µl of 25% glutaraldehyde, shake vigorously to prevent aggregation of spermatozoa.
- After 5 minutes, add 3ml of PS, store at 4°C until analysis and bring back to the laboratory.

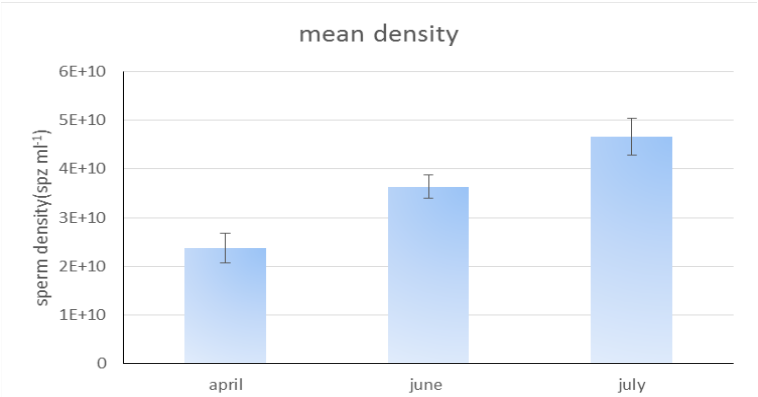
Laboratory

- Dilute original sybr14 to 1/5 in DMSO then dilute this mix to 1/10 in distilled water.
- Dilute 10 µl sperm to 1/100 and add 5 µl of diluted sybr14 and 1 µl stock solution of propidium iodide.
- Let in the dark for 10 min then observe with epifluorescence microscope and take pictures.
- Compare red and green particles.

The resulting dataset was complete and allowed studying the variations of the different quality indexes between the 3 sampling times by ANOVA (after angular transformation in the case of percentage analysis (motility)). Due to variations of motility recording quality on sampling site, the image treatment settings were adjusted to each sampling time and each sample (for example substraction of imperfect background using specific Image J functions) in a common work between P1and P14, while the parameters of CASA associated to motility evaluation were set common for all the analyses. The analytical work was co-performed by P1 and P14, as well as statistical analysis using Sigmastat as well as Statistica software.

Results

Sperm concentration



The concentration of sperm measure was from 2.3 to 4.6 10^{10} spz ml^{-1}, and is considered in the usual range for sperm of marine fish (Suquet et al., 1994). Sperm density showed a significant difference between April (presumed beginning) and July (presumed end of the reproductive period) (**Fig. 1** and **Table 1**).

Figure 1. Variations of greater amberjack mean sperm concentration with time based on the analysis of 4 males at each sampling.

	Apr	June	July
Apr		0,193934	0,017906
June	0,193934		0,311706
July	0,017906	0,311706	

Table 1. Crossed probabilities showing a significant difference in sperm concentration between April and July in captive-reared fish (n=4).

Sperm motility

The motion characteristics of sperm also changed along the reproductive period. The analysis of the main motility features by CASA also showed a classic behavior of greater amberjack sperm compared to other studied marine fish (**Fig. 2**). The maximal motility described by the percentage of swimming spermatozoa was reached within 20 seconds after activation by seawater whatever the rank in the season. However, the maximal motility showed significant variations between the 3 samplings. The duration of motility estimated

by the time when any movement ceases was between 1 and 11 minutes and also showed season-related significant differences. (**Fig. 3**) and correlatively the swimming speed of spermatozoa presented significant variations (**Fig. 4**).

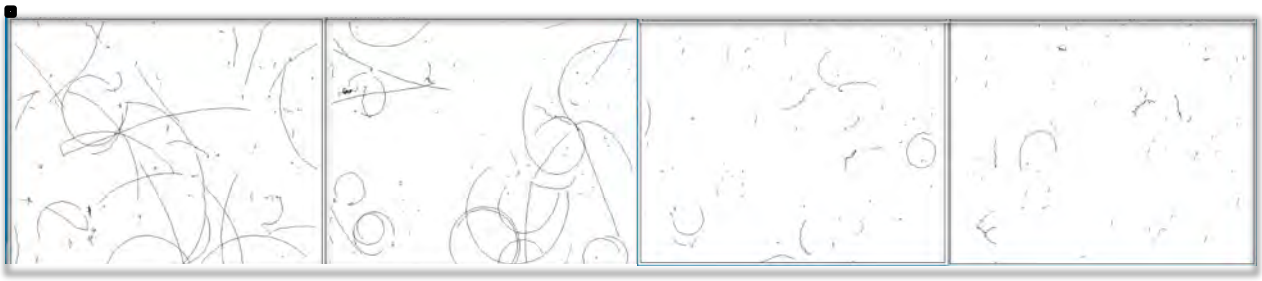


Figure 2. Illustration of spermatozoa tracks generated by CASA showing the decrease of motility and speed with time after activation as well as the disappearance of linear tracks.

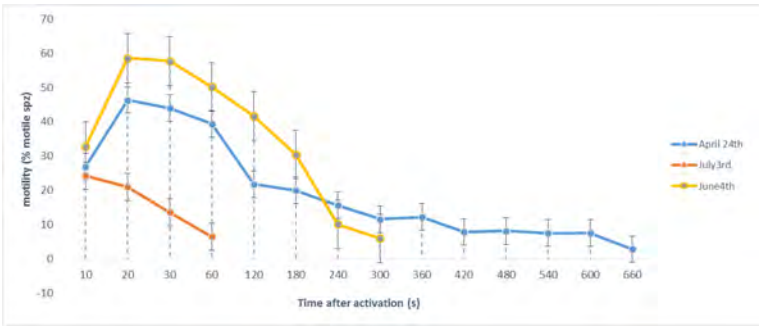


Figure 3. Mean motility of sperm of greater amberjack males (n=4) at different periods of reproductive season showing a usual pattern of spermatozoa behavior featured by general activation driving to a maximum of motile sperm and then a decrease of the percentage of motile sperm until any movement ceases (error bars: error type (n=4).

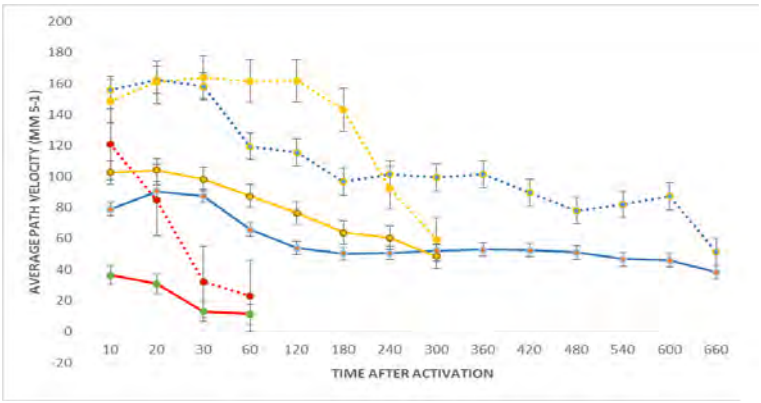


Figure 4. Mean (full line) and maximum (dotted line) speed of spermatozoa assessed by the Velocity Average Path (VAP) in µm sec^{-1} at different sampling times during reproductive period. Blue corresponds to April, Yellow to June and Red to July. Error bars represent SE (n=4).

Sperm viability

The use of a dedicated molecular probes kit (live/dead sperm kit) showed that there was also a variation with time of the integrity of spermatozoa membranes. The application of fluorescent markers of DNA with differential penetration of the cell in relation to its life/dead status drive to three different categories: live (green fluorescence), dying (green and orange) and dead (orange) (**Fig. 5**). A Nested Design ANOVA showed significant differences between randomly chosen males at each sampling time (**Fig. 6**). Notwithstanding this individual variability, a significant increase of the percentages of dead and live spermatozoa was observed from May to July while dying spermatozoa did not show significant difference between sampling periods (P<0.05) (**Fig. 7**).

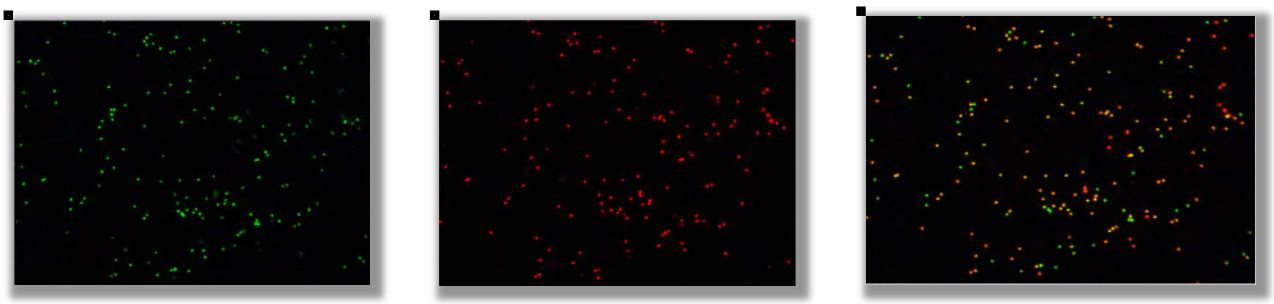


Figure 5. Estimation of sperm viability of greater amberjack by differential fluorescence of Sybr 14 (green) and propidium iodide (red). The superposition of the 2 images with a slight shift (right) allows distinguishing live (green), dead (red) and dying (both colors).

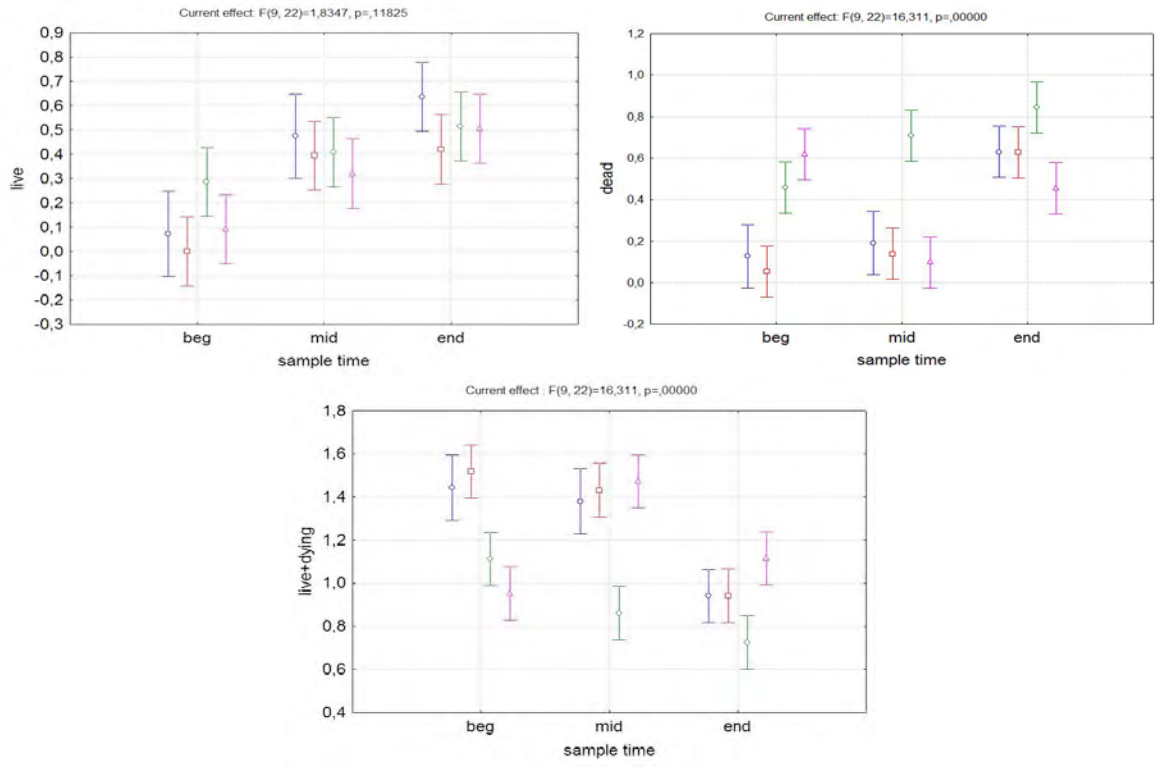


Figure 6. Male individual effect in the analysis of variance of sperm survival among reproductive season. Error bars indicate 95% confidence intervals.

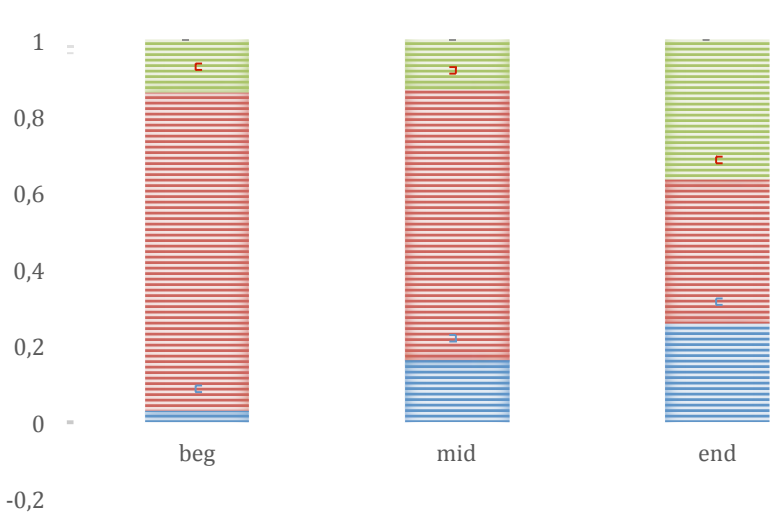


Figure 7. Repartition of sperm survival status expressed as % of the population at the different sampling period of reproductive season (blue= live, red= dying and green= dead). There is no significant decrease of sperm survival (wounded+live) along the reproductive season. Error bars show SE (n=4).

Sperm ATP content

The ATP content of spermatozoa was analyzed in P14 laboratory in Brest. The ATP level in sperm at the different points of the season remained very low and very variable in the whole batch and at the limit of detection and was not possible to distinguish significant differences between samplings (**Fig. 8**). This result corroborates the quite low performance of sperm whatever the period along the reproductive season and may be explained by the fact that we only observed intratesticular sperm, which may lack terminal maturation. However, a problem in sample conditioning or transportation cannot be excluded.

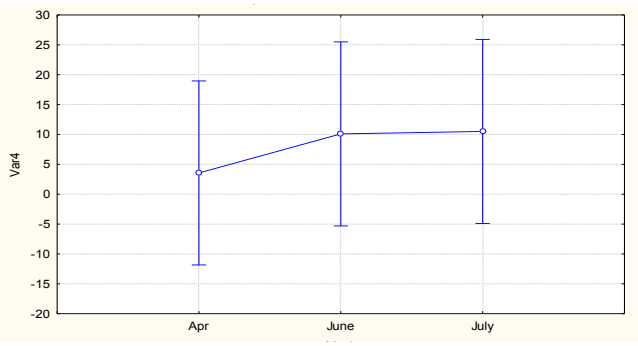


Figure 8. Mean ATP level (nmole 10^{-9}spz) with error bars showing 95% confidence interval (n=4) of sperm collected at three different samplings along reproductive season.

Discussion

The current results were generated from a tight collaboration between four partners of the DIVERSIFY project of which three research teams (HCMR, UNIBA and IFREMER) and a private company (ARGO).

The joint study provided a complete set of data allowing a reliable analysis of the consequences of captivity on greater amberjack gamete quality.

Availability of sperm

Unlike in most cultured fish species, it was not possible to obtain any semen by pressure of male abdomen in captive greater amberjack. A similar situation was observed in captive Atlantic bluefin tuna while, for both species, spontaneous release of sperm has been usually observed during wild fish capture campaign. Since these large open sea pelagic fish species present a strong muscular abdominal wall contrary to other marine fish like seabasses, seabreams, sciaenids, it may be considered that greater amberjack and tuna have large but inexpressible milt volumes. However, in both cases, when dissecting testes of captive fish, some sperm was available but a strong squeezing of the testes, not only of the collector channel allowed collecting semen. Since movies of spontaneous release of sperm in tuna (Tokyo aquarium for example) showed a huge volume of sperm at ejaculation, a process of rapid mobilization of sperm and hydration of semen may be suspected just at the moment of mating.

The concentration of spermatozoa in greater amberjack semen was of the same order as that of most of the species. The concentration was low at the beginning of the reproductive season and was increasing then, stabilized by the middle of the season. Such a result confirms that a precocious use of greater amberjack males in the season may be unproductive for aquaculture. On the contrary, in seabass, the concentration of sperm was higher at the beginning of the reproductive season than at the end, but it was observed that if the males are stressed by successive stripping they may lose their ability to release sperm, while the concentration increases (Fauvel et al., 1999).

Motility

The greater amberjack spermatozoa present a classic behavior of fish sperm. Immotile in the genital tract, they are activated by a variation of osmotic pressure. In fish for which sperm is simply stripped, an immediate maximum motility is observed while in the present case, a period of increase of about 20 seconds was necessary to reach the climax of motility. Moreover, the maximum number of motile sperm hardly reaches 60% when it is generally 80 to 90% in most of the sperm samples of other species. This may due to the intra-testicular status of sperm, which did not seem sufficiently hydrated.

The decrease of the velocity of spermatozoa varies in relation to the season both in shape of the slope and in duration. For these two parameters, the pattern is very similar to what is generally observed in other fish but it is worth noting that the motility parameters reveal a poor swimming capacity at the beginning of the season, then the number of motile sperm and the speed are higher during full season with a compromise between motility duration and velocity which may be due to the very low energy content recorded along the experiment.

The motility duration was similar to that of trout or seabass but it was shorter than that of tuna or flat fish the motility of which can reach 20 minutes (Suquet et al., 1994). This short duration may be due to the very low ATP content.

Finally, the use of kit to assay the viability based on the differential penetration of fluorescent markers of DNA revealed that in any case the number of intact spermatozoa significantly increased along the season but remained very low. A similar significant increase of dead spermatozoa was noticed while the number of

dying spermatozoa did not vary significantly. This last observation may explain the low quality parameters exposed above.

In conclusion, the current observations are in agreement with the descriptions of different gonadal dysfunctions observed in the same animals in the frame of the Diversify project showing problems of endocrine disruptions (androgens) and gametogenesis sustainability (apoptotic figures). The present information shows that the successful reproduction of the greater amberjack *Seriola dumerili* will require improvements in broodstock husbandry and probably heterologous stimulations to provide regular and sufficient fry for the aquaculture development of the species.

References

Beirão, J., Soares, F., Herraez, P., Dinis, M.T., and Cabrita, E. (2009). Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology *72*, 1251-1261.

Boryshpolets, S., Dzyuba, B., Stejskal, V., Linhart, O. (2009). Dynamics of ATP and movement in Eurasian perch (*Perca fluviatilis* L.) sperm in conditions of decreasing osmolality. Theriogenology *72*,851-859.

Cabrita, E., Soares, F. and Dinis, E.M.T. (2006). Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture *261*, 967-975.

Christen, R, Gatti, J.L., and Billard, R. (1987). Trout sperm motility: the transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur. J. Biochem. *166*, 667-671.

Cosson, J. (2008). The motility apparatus of fish spermatozoa. Fish spermatology- Alpha Science International Ltd Oxford (Alavi, S.M.H., Cosson ,J., Coward, K., and Rafiee G. eds), 281-316 pp.

Dreanno, C., Suquet, M., Fauvel, C., Le Coz, J.R., Dorange, G., Quéméner, L., and Billard, R. (1999). Effect of aging process on the quality of seabass (*Dicentrarchus labrax*) semen. J. Appl. Ichthyol. *15*, 176-180.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., and Suquet, M. (1999). Characteristics of sperm of captive seabass in relation to its fertilization potential. J. Fish Biol. *54*, 356-369.

Fauvel, C., Suquet, M., Cosson, J. (2010). Evaluation of fish sperm quality. J. Appl. Ichthyol. *26*, 636-643.

Garcia, A., Díaz, M.V., and Agulleiro, B. (2001). Induccion hormonal de la puesta y desarrollo embrionario de la seriola Mediterranea (*Seriola dumerilii*, Risso). Monogr. Inst. Canar. Cienc. Mar. *4*, 561-566.

Jerez, S., Samper, M., Santamaría, F.J., Villamandos, J.E., Cejas, J.R., and Felipe, B.C. (2006). Natural spawning of greater amberjack (*Seriola dumerili*) kept in captivity in the Canary Islands. Aquaculture *252*, 199-207.

Kožul, V., Skaramuka, B., Glamuzina, B., Glavic, N., and Tutman, P. (2001). Comparative gonadogenesis and hormonal induction of spawning of cultured and wild Mediterranean amberjack (*Seriola dumerilii*, Risso 1810). Sci. Mar. *65,* 215-220.

Lahnsteiner, F., Berger, B., Weismann, T., and Patzner, R.A. (1998). Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture *163*, 163-181.

Lazzari, A., Fusari, A., Boglione, A., Marino, G., and Di Francesco, M. (2000). Recent advances in reproductional and rearing aspects of *Seriola dumerilii*. In: Basurco B, editor. Mediterranean Marine

Aquaculture Finfish Species Diversification, Cahiers Options Méditerranéennes, vol. 47. Zaragoza, Spain: CIHEAM. 241-247 pp.

Mylonas, C.C., Papandroulakis, N., Smboukis, A., Papadaki ,M., and Divanach, P. (2004). Induction of spawning of cultured greater amberjack (*Seriola dumerili*) using GnRHa implants. Aquaculture *237*, 141-154.

Pastor, E., Grau, A., Riera, F., Pou, S., Massuti, E., Grau, A.M. (2000). Experiences in the culture of new species in the 'Estacion de Acuicultura' of the Balearic Government (1980–1998). In: Basurco B, editor. Cahiers Options Méditerranéennes. Mediterranean Marine Aquaculture Finfish Species Diversification, vol. 47. Zaragoza, Spain: CIHEAM. 371-379 pp.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., and Fauvel C. (1994). Sperm features in turbot (*Scohthalmus maximus*): a comparison with other freshwater and marine fish species. Aquat. Living Resour. *7*, 283-294.

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*, 661-72.

Zelazowska, M. (2010). Formation and structure of egg envelopes in Russian sturgeon *Acipenser gueldenstaedtii* (Acipenseriformes: Acipenseridae). J Fish Biol.*76*, 694-706.

Deviations:

There were no deviations in this Deliverables.

Dr. Christian Fauvel sampling male greater amberjack at the facilities of P1. HCMR.

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