Deliverable Report

Deliverable No:	D3.6		**Delivery Month:**	49
Deliverable Title	Description of the process of spermatogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of testicular development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones, (e) proliferation and apoptosis of germ cells, (f) sperm quality and (g) fish nutritional status..			
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Objective

The objective of the present deliverable was the description of the process of spermatogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of testicular development, c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones, (e) proliferation and apoptosis of germ cells, (f) sperm quality and (g) fish nutritional status. The endocrine control of the reproductive cycle in male greater amberjack has not been described yet. This deliverable will provide an evaluation of the brain-pituitary-gonad axis during spermatogenesis in captive greater amberjack, as a way of assessing reproductive function and predicting spawning performance. The deliverable will (a) assess the size and age at first sexual maturity of greater amberjack males; (b) evaluate captive fish body condition by using parameters such as the condition index (CI); (c) describe the reproductive cycle of captive fish in order to get a synchronized effect of the hormonal treatment in both sexes; (d) identify the possible effects of captivity on reproductive axis by measuring the pituitary and plasma levels of the two gonadotropins (FSH and LH), as well as sex steroid plasma concentrations (testosterone, T, 11-ketotestosterone, 11-KT and 17α,20β-dihydroxy-4-pregnen-3-one, 17β-DHP); (e) identify the possible effects of captivity on spermatogenesis in terms of proliferation and apoptosis of germ cells as well as on sperm quality (motility, velocity, ATP content, etc); and (f) provide information on the nutritional status of captive males by measuring a key metabolic hormone (*e.g.* leptin).

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Description

The reproductive success of a fish species depends on the correct functioning of the brain-pituitary-gonad reproductive axis (Mylonas et al., 2010). Combination of captivity-induced stress and lack of appropriate 'natural' spawning conditions may produce an insufficient pituitary stimulation by hypothalamic gonadotropin hormone-releasing hormone (GnRH) causing an insufficient gonadotropin release (GTHs: follicle stimulating hormone, FSH; luteinizing hormone, LH), and a consequent negative effect on sex steroid production by gonads (Zohar & Mylonas, 2001; Mylonas et al., 2010). As a result, fish reared in captivity may exhibit reproductive dysfunctions, which in males eventually could result in the production of sperm of variable quality (Mylonas et al., 2010). These dysfunctions can be overcome by hormonal therapies and modulation of environmental parameters (Mylonas et al., 2010) as well as by a proper broodstock diet (Izquierdo et al., 2001).

In order to set up an adequate hormonal treatment and, proper rearing practices for a fish species candidate for aquaculture production, it is necessary to study its growth, puberty, as well as the effect of confinement on reproductive axis and, in particular, on pituitary GTHs release, gametogenesis and reproductive cycle. Besides, broodstock diet is a key factor that contributes to reproductive success of a fish species in aquaculture.

The present deliverable describes male greater amberjack growth and size at first maturity in order to get useful information for broodstock constitution. Moreover, the effects of confinement in captivity on the functioning of greater amberjack brain-pituitary-gonad axis will be assessed both at the macroscopic (gonadosomatic index) and microscopic (histological) levels, pituitary and plasma GTHs content, and sex steroid plasma concentrations during different phases of the reproductive cycle in captive-reared specimens. The assessment of fish body condition index and the quantification of leptin (a key metabolic hormone) will help us evaluate the effectiveness of the diet on greater amberjack captive male nutritional status and their reproductive performance.

The work described in this deliverable has resulted in two publications to international scientific journals:

Zupa, P., Fauvel, C., Mylonas, C.C., Pousis, C., Santamaría, C.A., Papadaki, M., Fakriadis, I., V., C., **2017a**. Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack, *Seriola dumerili* (Risso, 1810). **Journal of Animal Science** 95, 4085-4100.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., Corriero, A., **2017b**. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). **PLoS ONE** 12, e0169645.

Background

Age determination, growth and first sexual maturity

The main objective of age and growth studies in fish is to estimate their mean size at each age class and determine their growth parameters (Mather et al., 1995). Fish growth rate and sexual maturity are essential components of models used in stock assessment of fish populations (Megalofonou, 2000; Corriero et al., 2005) and provide useful basic information when a new species has to be introduced in aquaculture.

Basically, two methods can be used to estimate the age of fish: size analyses of caught individuals (cohort analysis) or interpretation of the discontinuities of different hard structures (or hard parts) of the fish. The reading of hard parts, such as otoliths, scales, spines and vertebrae is based on the number of marks, usually called annuli, which are interpreted as periodic events (Sella 1929; Compeàn-Jimenez and Bard, 1983; Megalofonou and De Metrio, 2000).

Age determination data are commonly used to model fish growth (Ricker, 1975). Among the proposed length-age models, the von Bertalanffy growth function has been by far the most applied in fish biology (King, 1995).

Reproductive cycle

Seasonality is a common feature of teleost reproduction and it is most marked at higher latitudes. Seasonal cycles are principally entrained by photoperiod and temperature but a range of other variables including lunar phase and rainfall as well as social interactions may also operate (Munro et al., 1990).

The endocrine control of reproduction in fishes, similar to the other vertebrates, is regulated by the brain through the hypothalamic–pituitary–gonadal (HPG) axis (Mittelholzer et al., 2009). This axis involves three component parts, GnRH neurons projecting from the hypothalamus to the pituitary, gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the somatic cells of the gonads (Leydig and Sertoli cells in the testis).

Spermatogenesis is a complex and long-term process in which spermatogonia proliferate and become mature spermatozoa through a series of events involving mitosis, meiosis and cellular differentiation (Schulz et al., 2010). The control of spermatogenesis by the HPG axis seems to be limited to a restricted number of processes (Schulz and Miura, 2002): 1) the balance between self-renewal and the production of differentiating germ cells, through which it is possible to determine whether and how many germ cells enter the spermatogenesis process; 2) the recruitment of early spermatogonia into the rapid proliferation phase and their entrance into meiosis; 3) the loss of germ cells via apoptosis; 4) Sertoli cell proliferation and differentiation which determine germ cell production capacity.

Effects of captivity on reproductive axis

Reproductive dysfunctions are often observed in fish reared in captivity and involve an inadequate pituitary gonadotropin (GtH) synthesis and/or release (Zohar and Mylonas, 2001; Mylonas et al., 2010; Berkovich et

al., 2013), which has been attributed to captivity-induced stress, lack of suitable environmental conditions (Mylonas et al., 2010) and/or nutritional deficiencies (Izquierdo et al., 2001). In males, captivity-induced reproductive dysfunctions may result in a qualitative and quantitative decrease of sperm output (Rurangwa et al., 2004; Cabrita et al., 2009; Bobe and Labbé, 2010) and, therefore, in unsuccessful spawning and production of fertilized eggs.

Previous studies revealed that Atlantic bluefin tuna reared in captivity had a low capacity to reach full testicular development and maturation, since their relative testicular mass was lower and their seminiferous lobules were smaller than in wild fish (Corriero et al., 2007b, 2009). Moreover, a delay in proliferating activity, as well as an increase of apoptosis, was demonstrated in male germ cells of captive-reared Atlantic bluefin tuna compared to wild fish. The observed spermatogenesis impairment that finally resulted in the production of low-quality sperm, was ascribed to an insufficient GTHs release from the pituitary and to the consequent low androgen synthesis and secretion (Corriero et al., 2009; Zupa et al., 2013).

Nutritional status

Reproduction is a complex process that can be affected/modulated by several factors such as genetic background, environmental conditions and nutrition. Therefore, characterizing broodstock nutritional status is crucial in order to identify possible reproductive/metabolic dysfunctions occurring in fish reared in captivity (Izquierdo et al., 2001).

The peptide hormone leptin, the product of the obese gene, is a 16 kDa, 167 amino acid (aa) hormone, consisting of a 21 aa signal peptide and a 146 aa soluble protein (Zhang et al., 1994; 1997). In mammals, leptin is secreted into the bloodstream both as a free protein and as a protein – bound entity, primarily from adipocytes, and acts on the brain to regulate food intake and metabolism (Chen et al., 1996; Zhang et al., 1994; Pelleymounter et al., 1995; Tartaglia et al., 1995; Lee et al., 1996; Huising et al., 2006). In addition to its role in conveying signals of the energy stores to the central nervous system, leptin was found to interact with the endocrine system to provide critical information about the nutritional status and therefore to act as a permissive factor allowing the onset of energy demanding situations such as reproduction (Rosenbaum & Leibel, 1998; Fernandez-Fernandez et al., 2006).

Materials and methods

Age determination, growth and first sexual maturity of male greater amberjack

Sampling

During the months of June 2015 and 2016, when adult greater amberjack were supposed to be reproductively active (Zupa et al., 2017a, b), twenty-three juvenile greater amberjack were sampled on board a professional purse seine fishing vessel operating around the Pelagie Islands (Sicily, Italy). Soon after capture, fish biometric data (fork length, FL in cm; body mass, BM in kg; gonad mass, GM in g) were recorded (**Table 1**), and gonads and scales were sampled. The gonado-somatic index was calculated as: $GSI = 100xGMxBM^{-1}$. The gonads of five of the fish sampled did not have a macroscopic appearance clearly ascribable to ovaries or testes and these fish were classified as "Sex Indeterminate" and were not further processed. The gonads of eight fish were macroscopically classified as ovaries and therefore they were used for the determination of the first sexual maturity of females (see D3.5). The gonads of ten fish were macroscopically classified as testes and were processed for the determination of the first sexual maturity of males.

Table 1. Biometric data and estimated age of sex indeterminate and male wild greater amberjack sampled around the Pelagie Islands (Italy) during the reproductive seasons 2015 and 2016.

Fish origin	Sampling Date	Sex	FL (cm)	BM (kg)	GM (g)	GSI (%)	Estimated Age (years)
wild	29/06/2015	Indeterminate	35.0	1.0	nd	nd	1
		Indeterminate	35.0	1.0	nd	nd	1
		Indeterminate	36.0	1.0	nd	nd	1
		Indeterminate	38.0	1.1	nd	nd	1
		Indeterminate	39.0	1.2	nd	nd	1
wild	06/06/2016	m	58.0	2.99	3	0.10	2
		m	61.0	3.38	4	0.12	2
		m	65.0	3.89	2	0.05	2
		m	68.5	4.85	9	0.19	2
		m	71.5	5.10	17	0.33	2
wild	10/06/2016	m	80.0	6.69	67	1.00	3
		m	84.0	8.58	182	2.12	3
		m	84.0	7.65	73	0.95	3
		m	88.0	9.37	220	2.35	3
		m	89.0	10.40	194	1.87	3

BM = body mass; nd = not determined; FL = fork length; GM = gonad mass; GSI = gonado-somatic index.

Age and growth

The age estimate of wild male greater amberjack was carried out through the analysis of the scales, which proved to be the easiest structures to be processed and read (see D3.3). From each specimen, a variable number of scales were treated according to the protocol reported by McCurdy et al. (2002). Briefly, scales were removed from the skin taken from a body area between the pectoral and first dorsal fin, rinsed in tap water and in 70% ethanol and finally placed between two microscope slides. Subsequently, they were observed with a binocular lens microscope Wild M3C (Leitz, Heerbrugg, Switzerland) under transmitted light, connected through a digital camera DC 300 (Leica, Wetzlar, Germany) to the image analyser Quantiment 500 W (Leica, Wetzlar, Germany).

Scales show typical dense concentric growth rings (circuli) whose arrangement displays periodical (seasonal) variations: circuli density increases and circuli crowd during the slow growth season (winter) when they tend to form a solid line or annulus. The age of the fish was estimated based on the number of annuli counted on their scales (Meunier, 2002).

In order to calculate the growth curve of wild male greater amberjack, along with the juvenile specimens, all the wild male adults (n = 14) sampled in 2014 and 2015 for the study of the reproductive cycle of the wild population, whose age was previously determined (see D3.3), were used. Estimates of male greater amberjack theoretical growth in length were obtained by fitting the von Bertalanffy growth model (Bertalanffy von, 1938) to the mean lengths at estimated age:

$FL_t = FL_\infty [1 - e^{-k(t-t_0)}]$

where, FL_t = predicted fork length at age t; FL_∞ = mean asymptotic fork length; k = growth constant ($year^{-1}$); and t_0 = theoretical age at which the fish would have been 0 length.

The theoretical longevity of the species was calculated using Pauly and Munro's (1984) formula:

$AGE_{MAX} = 3/k$

First sexual maturity

Each specimen was classified as immature or mature on the basis of the histological appearance of the testes. For this purpose, testis samples were fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were stained with haematoxylin-eosin and observed under light microscope.

For each testis, the type of spermatogenic cysts was recorded and the amount of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively (Corriero et al., 2009; Zupa et al., 2017b). Fish were considered reproductively active (mature) if their testes had all stages of spermatogenesis in the germinal epithelium as well as large amount of luminal spermatozoa (testes in advanced spermatogenesis stage).

Reproductive cycle and reproductive dysfunctions in captive-reared greater amberjack males

Sampling

For the study of the reproductive cycle of captive-reared greater amberjack males, 12 adult specimens, belonging to a broodstock captured in 2011 in the area of Astakos (Ionian Sea, Greece) were used. In order to carry out the activities planned in WP3, these fish were transferred to Argosaronikos Fish Farm (Salamina Island, Greece) (P23. ARGO) in September 2013. The fish were initially fed raw fish and, once transferred to ARGO, they were reared in sea cages and fed on Vitalis Cal (Skretting). Captive-reared greater amberjack sampling program took place in 2015 and it was aimed at obtaining samples from four males during each of three periods of the reproductive cycle, which were identified on the basis of the available literature on the wild Mediterranean population (Mandich et al., 2004; Sley et al., 2014): early gametogenesis (EARLY, late April), advanced gametogenesis (ADVANCED, early June), spawning (SPAWNING, early July). All the fish destined to be sampled were confined in a small cage area using a PVC curtain and anesthetized lightly with about 0.01 ml l^{-1} clove oil. The fish were then gently guided into a PVC stretcher, brought on board of a service vessel and anesthetized deeply with about 0.03 ml l^{-1} clove oil. Fish were then placed on crushed ice and transferred to the farm facility where biometric data (fork length, FL in cm; body mass, BM in kg; gonad mass, GM in g) were recorded (**Table 2**). From each fish the following biological samples were taken: blood, pituitary and gonads.

Table 2. Biometric data of captive-reared male greater amberjack sampled at Argosaronikos Fish Farm (Salamina Island, Greece).

Fish origin	Sampling Date	SST (°C)	FL (cm)	BM (kg)	GM (g)
Early Gametogenesis (EARLY)					
captive	24/04/2015	17.5	92	12	65
			94	12	60
			94	13	60
			101	15	95
Advanced Gametogenesis (ADVANCED)					
captive	04/06/2015	20.0	90	9	370
			97	14	295
			98	13	600
			103	15	690
Spawning (SPAWNING)					
captive	02/07/2015	25.5	91	10	70
			95	11	155
			96	13	140
			96	12	130

BM = body mass; FL = fork length; GM = gonad mass; SST = sea surface temperature.

Reproductive cycle

The gonado-somatic index was calculated as GSI = 100xGMxBM^{-1}. The reproductive state was assessed by means of histological observations of testis sections. For this purpose, testis samples were fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were stained with haematoxylin-eosin and observed under light microscope.

For the classification of the reproductive state, the different germ cell types were identified on the basis of their morphological characteristics, the type of spermatogenic cysts was recorded and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively (Corriero et al. 2007b; Zupa et al., 2017b).

Germ cell proliferation and apoptosis

Proliferating germ cells were identified on testis sections by using the immunohistochemical localization of PCNA (Proliferating Cell Nuclear Antigen), a polymerase delta accessory protein that is synthesized in late G1 and S phases of the cell cycle and is, therefore, used as a nuclear marker of proliferation. For the PCNA detection, 5-µm thick testicular sections were deparaffinized in xylene, rehydrated through graded ethanol solutions and pretreated for 10 min with 3% H_2O_2 to inhibit endogenous peroxidase activity and then rinsed with distilled water and phosphate-buffered saline (PBS, 0.01 M, pH 7.4, containing 0.15 M NaCl). Non-specific binding sites for immunoglobulins were blocked by incubation for 30 min in normal horse serum (NHS) and then incubated overnight in moist chamber at 4°C with monoclonal antibodies to PCNA (ICN Pharmaceuticals, Milan, Italy) diluted 1:100 in PBS containing 0.1% bovine serum albumin. After rinsing for 10 min in PBS, immunohistochemical visualization was obtained using the Vectastain Universal Elite Kit (Vector). This method uses the avidin-biotin-peroxidase complex (ABC) procedure. Peroxidase activity was visualized by incubating for 10 min with a Vector DAB Peroxidase Substrate Kit (Vector), which produces a brown precipitate. To confirm the specificity of the immunoreaction, a control-staining procedure was carried out by replacement of the primary antibody with NHS and PBS.

Apoptotic germ cells were identified on testis sections by using the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method. Apoptotic cell labeling was obtained on 5-µm thick sections with an in situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany) used in accordance with the manufacturer's instructions. Prior to incubation with the reaction mixture, after their re-hydration through graded ethanol solutions, the sections were incubated in a permeabilization solution of 0.1% Triton X-100 in 0.1% sodium citrate for 8 min at 37°C. Terminal deoxynucleotidyl transferase was diluted 1:10 in TUNEL Dilution Buffer (Roche Diagnostics). A ready-to- use solution of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) (Roche Diagnostics) served as a substrate for the signal conversion.

The density of anti-PCNA positive spermatogonia (number of cells/ mm^2 testis tissue) and the density of anti-PCNA positive spermatocysts (i.e. number of cysts containing spermatogonia or primary spermatocytes/mm^2 testis tissue), as well as the surface occupied by TUNEL positive apoptotic cells (µm^2/mm^2 testis tissue), were measured on 5 randomly selected fields of each testicular section. All these measurements were performed on microphotographs taken with a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK).

Pituitary gonadotropin gene expression levels

Total RNA was obtained from pituitary using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 µl of RNase free water and stored at –80°C. The cDNA was prepared from 1.2 µg total RNA. Random hexamer primers were used for the cDNA synthesis using U SuperScript III Reverse Transcriptase (Invitrogen). Two µg of DNAse treated total RNA were reverse transcribed with random primers using the High Capacity cDNA Reverse Transcriptase kit (Applied

Biosystems, Branchburg, NJ) according to manufacturer's protocol. Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicate in 10 µl reaction volumes consisting of Fast SYBR Green Master Mix (Applied Biosystems). Amplification was carried out in a Fast RealTime PCR System (Applied Biosystems). Cycling parameters were as follows: 3 s at 95°C, and 40 cycles of 3 s at 95°C and 30 s at 60°C. The presence of a single amplicon was verified using a melting curve run following the PCR. To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR experiments included minus-reverse transcriptase controls (*i.e.* PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = 2DCt, Ct – threshold cycle (Livak and Schmittgen, 2001). The gene specific primers (GSPs) designed using the Primer Express 3.0 software (Applied Biosystems) are listed in ***D3.1. Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor)***.

Pituitary and plasma concentrations of gonadotropins

Pituitary and plasma LH levels were measured using the heterologous ELISA developed for striped bass LH (Mañanós et al., 1997) and validated for the greater amberjack (D3.2). Ninety-six well polystyrene plates were coated with recombinant LH (r-LH; 2.4 ng per well) and incubated overnight at 4°C. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl per well) for 0.5 h at 37°C. The primary antibody (anti-striped bass LH) was diluted 1:80,000 in PBST containing 2% normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4°C. Then the content in each tube was dispensed into the antigen-coated wells (100 µl per well in duplicate). Following an incubation (overnight at 4°C), AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch laboratories, inc.) in 1% NGS-PBS T was added (100µl per well) for 0.5 h at 37°C. The wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 µl per well). The reaction was stopped after 20 to 40 min at RT by the addition of 100µl of 1N phosphoric acid and the absorbance was read at 450 nm.

The related pituitary and plasma FSH levels were measured similarly using the homologous ELISA that was developed in the framework of this project. Briefly, the recombinant greater amberjack FSH (*rec-ga*FSH) was produced using the *Pichia pastoris* yeast recombinant DNA expression system (Invitrogen, Carlsbad, CA). Following purification on an affinity column (Amersham Biosciences), the rec-gaFSH was used as antigen for both standardization and generation of the specific polyclonal antibodies. The standard curve ranged from 100 ng/ml to 0.19 ng/ml. The assay detection limit was 0.78 ng/ml. The intra-assay coefficients of variation (CV) for standard of 10 ng/ml in the same plate were 3.0% (n = 10). The inter-assay CV for the same plasma sample on different plates was 9.6% (n = 7).

Steroid plasma levels

Plasma was separated from the blood by centrifugation (2408 x g for 5 minutes at room temperature) and then was kept at -80°C until assayed for sex steroid determination. For the quantification of T, 11-KT and 17,20β-P (a putative maturation-inducing steroid; MIS) in the plasma, already established and well-described enzyme-linked immunoassays (ELISA) were used (Cuisset et al., 1994; Nash et al., 2000; Rodríguez et al., 2000) with some modifications, and using reagents from Cayman Chemical Company (USA). For the quantification of E2, an ELISA kit was used (Cayman Chemical Company). For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing, samples were frozen for 10 min at -80°C and the supernatant organic phase was collected in new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in reaction buffer for running in the ELISA.

Sperm quality

Sperm quality analyses were carried out at the sampling site (Argosaronikos Fishfarms SA, Salamina Island, Greece), using a microscope (Nikon Eclipse 50i, Japan) equipped with a video camera (SONY SSC-DC58AP, Japan) recording 25 frames per second (FPS). In order to estimate spermatozoa concentration (spz ml^{-1}), sperm samples were diluted to 1/500 (vol/vol) in tap water and placed on a cell counting chamber (Thoma, 0.1 mm depth). Spermatozoa were allowed to settle on the counting chamber for 10 minutes, and then pictures were taken with the microscope at x20 magnification, focused so as to get highly contrasted spermatozoa and slightly apparent grid. The particles were then counted on a cropped part of each picture adjusted to a known number of squares using the free ImageJ software (NIH, USA).

In order to assess motility, a 20- µl sperm sample from each fish was diluted (1/10, vol/vol) initially in modified Leibovitz, and then activated by mixing with 1 ml seawater containing 2% BSA, for a final dilution of 1/500 (vol/vol); concomitantly, the 25 FPS video record (.avi format) was launched so as to record sperm activity from its beginning. Immediately after short mixing by a vigorous shake, 1 µl of activated sperm was placed in a pre-focused, 10 µm-deep, dedicated cell (Leja, Nieuw-Vennep, The Netherlands) on the microscope. The recording was interrupted at the cessation of any progressive spermatozoa movement. The time between activation and first possible motility analysis was around 10 seconds. For computer-assisted sperm analysis (CASA), the videos were subsequently transformed into sequences of frames using the free software 'Virtualdub' (www.virtualdub.org); then sequences of 2 seconds (*e.g.* 50 frames) were analyzed every 10 seconds using the plugin CASA developed by Wilson-Leedy and Ingermann (2007) for ImageJ (NIH, Bethesda, MD, USA). Due to variations of motility recording quality at the different times of sampling, the image treatment settings were adjusted to each sampling time and each sample, while the parameters of CASA associated to motility evaluation were common for all the analyses. The only motility features that showed variations among the three different phases of the reproductive cycle and were relevant were the motility (% of motile spz) and the velocity on smoothed trajectory called average path velocity (VAP; µm s^{-1}). Therefore, only these values are presented.

In order to determine spermatozoa ATP content, 1 and 10-µl aliquots of each sperm sample were prepared according to Boryshpolets et al. (2009) and assessed using ATPlite luminescence kit (Perkin-Helmer, Waltham, MA, USA). The integrity of spermatozoa plasma membrane was tested using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Eugene, OR, USA) after dilution to 1/100 (vol/vol) and pre-fixation for 4 minutes in 4% glutaraldehyde as described by Beirão et al. (2009). This procedure stains live, dying and dead spermatozoa in fluorescent green, green+red and red, respectively.

Nutritional Status

As indicator of fish body condition, Fulton's condition factor (K) was calculated according the following equation:

$K = BM\ 100/FL^3$

where, BM = body mass in g; FL = fork length in cm.

Fulton's condition factor of captive-reared greater amberjack was compared with that of wild individuals of the same age class sampled for the study of the reproductive cycle (D3.3).

Expression levels of liver leptin

Total RNA extraction from liver samples, cDNA synthesis and qPCRs to measure leptin transcript levels were carried out as described in the pituitary gonadotropin gene expression section (see above).

Leptin plasma concentration

Recombinant leptin was produced using the *P. pastoris* yeast recombinant DNA expression system. Following purification on an affinity column, the recombinant leptin was used to immunize rabbits and generate the specific polyclonal antibodies (Deliverable 3.2 in preparation). As attested by Western blot analyses the obtained antibodies exhibited high specificity to fish (produced herein) and human (ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel) recombinant leptin monomers, yet they failed to detect native fish leptin (*i.e.* greater amberjack and bluefin tuna) in liver samples (D3.2 in preparation).

Statistical analysis

Differences in GSI, GTHs and leptin gene expression, as well as GTHs and sex steroid plasma concentrations mean values between specimens sampled in consecutive phases of the reproductive cycle were assessed by a two tailed Student's t-test. Prior to the Student's t-test, the raw data of GSI and apoptotic surfaces were arcsine-transformed, as appropriate with proportions (Sokal and Rohlf, 1981). Differences in the sperm quality indexes among sampling phases were assessed either by ANOVA (after angular transformation in the case of % of motile spermatozoa), or by nested design ANOVA (in the case of sperm velocity where individual spermatozoa performances were taken into account for each male at the different sampling periods). Means were compared using Duncan's New Multiple Range (DNMR) post hoc test.

Fulton's condition factor (K) was compared with that of wild specimens sampled for the study of the reproductive cycle of the wild population (***Deliverable 3.3 Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating***) by two tailed Student's t-test. All the results are presented as means ± SE; the statistical probability significance was established at the $P \leq 0.05$ level.

Results

Age determination, growth and first sexual maturity

Age and growth

Among the 15 juvenile greater amberjack sampled for the study of the first sexual maturity, 5 belonged to the age class 1, 5 to the age class 2 and 5 to the age class 3 **(Fig. 1; Table 2**). The parameters of the von Bertalanffy growth equation, derived from the observed FLs-at-age of both juvenile and adult wild male greater amberjack, were: FL_∞ = 127.1 cm; k = 0.35; t_0 = -0.05 (**Fig. 2**). The theoretical longevity was 9 years.

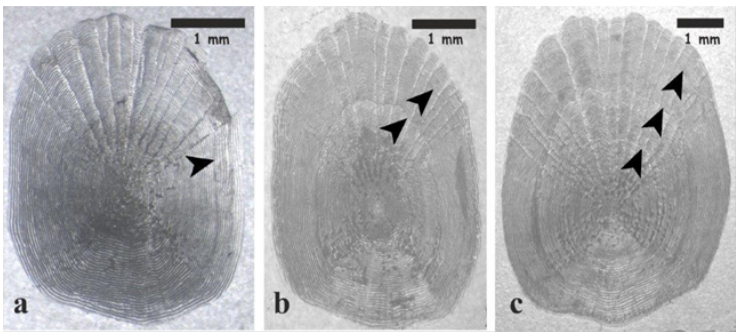

Figure 1. Images of scales from juvenile wild greater amberjack sampled in the Mediterranean Sea. (a) Age 1 specimen; 35 cm fork length; sex indeterminate. (b) Age 2 specimen; 61 cm fork length; male. (c) Age 3 specimen; 88 cm fork length; male. Magnification bar = 1 mm. Arrowheads: growth marks (annuli).

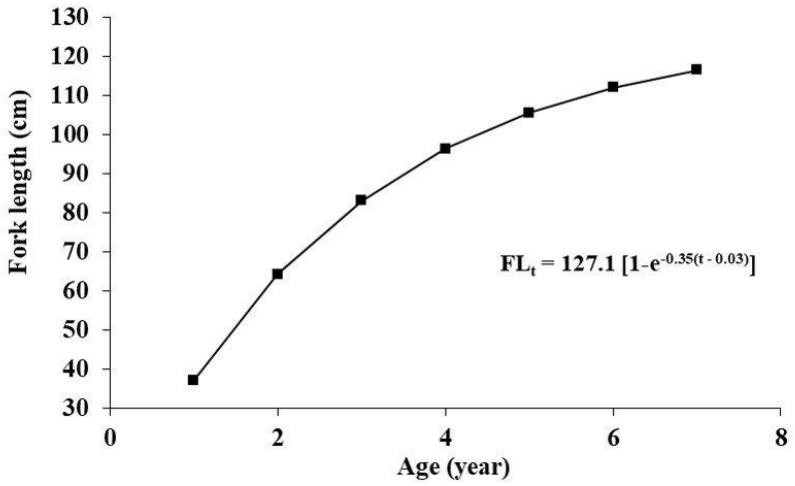

Figure 2. Von Bertalanffy growth curve. FL_t = predicted fork length at age t.

First sexual maturity

The gonads of age 1 fish did not have a macroscopic appearance clearly ascribable to ovaries or testes and then these fish were classified as "Sex Indeterminate" and were not further processed. All the fish belonging to age class 2 were immature since they showed testes in quiescent/early spermatogenesis stage (**Fig. 3**). All the fish belonging to the age class 3 were classified as mature as their testes were in a histological condition similar to those of adults in advanced spermatogenesis stage, showing all the stages of spermatogenesis and abundant luminal spermatozoa (see the following section for a description of the testes of adult specimens).

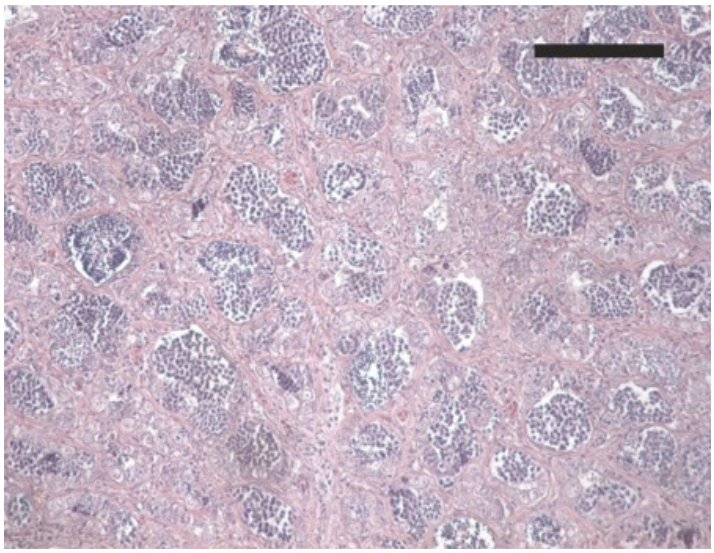

Figure 3. Micrographs of a testis sections from an immature, age 2, greater amberjack. Seminiferous epithelium is dominated by immature germ cells (mainly spermatogonia) and a small amount of luminal spermatozoa is visible. Haematoxylin-eosin (H-E) staining. Magnification bar = 100 µm.

Reproductive cycle and reproductive dysfunctions in captive-reared greater amberjack males

Morphological description of male germ cells

Two types of single A spermatogonia were identified in testis sections: a smaller cell type (diameter 8.0 ± 0.1 µm), having a spherical nucleus, with heterochromatin dots and two nucleoli, surrounded by a thin acidophilic cytoplasm (**Fig. 4**); a larger cell type, (diameter 10.6 ± 0.2 µm), showing a roundish/ovoidal nucleus with a prevalent euchromatic appearance and sparse heterochromatic patches, with a preeminent nucleolus and an acidophilic cytoplasm (**Fig. 4**).

Spermatogonia contained in cysts also appeared as two different cell types: larger cells (mean diameter 8.8 ± 2.0 µm) with one or more nucleoli and a moderately acidophilic cytoplasm making part of small cysts containing few cells (presumptively type A spermatogonia) (**Fig. 4**); smaller cells (mean diameter: 4.8 ± 0.8 µm), showing a small heterochromatic nucleus and thin rim of a weakly acidophilic cytoplasm (**Fig. 4**), making part of larger cysts (presumptively type B spermatogonia).

The appearance of primary (4.4 ± 0.9 µm) and secondary (3.3 ± 0.8 µm) spermatocytes differed according to the different phases of meiosis; metaphasic figures were often observed within spermatocyte I and spermatocyte II cysts (**Fig. 4**). Spermatids had a mean diameter of 2.6 ± 0.7 µm and were characterized by a compacted and strongly basophilic nucleus (**Fig. 4**). Flagellated spermatozoa showed an oval head stained intensely with haematoxylin and were observed within cysts or in the lumina of seminiferous lobules after the cyst breakdown (**Fig. 4**).

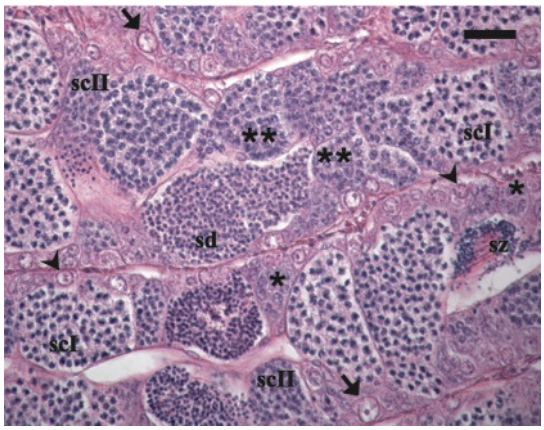

Figure 4. Micrograph of a testis section from a greater amberjack showing the different germ cell types. Haematoxylin-eosin (H-E) staining. Magnification bar = 25 µm. Single A spermatogonia are indicated by black arrows (presumptively differentiated and undifferentiated stem spermatogonia, respectively). Asterisk: type A spermatogonial cyst; double asterisk: type B spermatogonial cyst; sd: spermatid cyst; scI: primary spermatocyte cyst; scII: secondary spermatocyte cyst; sz: spermatozoa.

Reproductive cycle

The testes of the four captive-reared specimens sampled during the EARLY period contained germ cells in all spermatogenic stages, as well as spermatozoa in the seminiferous lobules (**Fig. 5a**). The testes of the fish sampled during the ADVANCED phase had seminiferous lobules in different conditions: in some cases, the germinal epithelium was in active spermatogenesis showing all the spermatogenic stages, in other cases the spermatogenic activity was ceased and only residual sperm cysts were visible. All the testes sampled in this period had a moderate/abundant amount of luminal spermatozoa (**Fig. 5b**). In the presumed SPAWNING phase, all the four captive-reared males had already ceased their spermatogenic activity, still showing a moderate amount of spermatozoa in the lumen of seminiferous lobules (**Fig. 5c**).

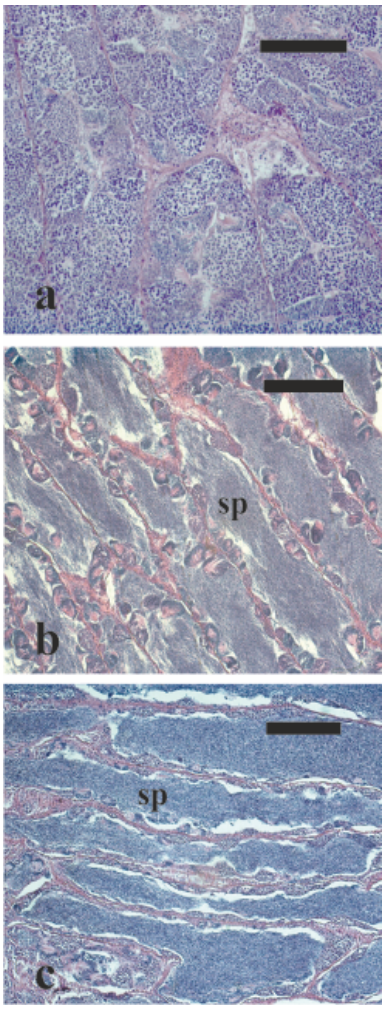

Figure 5. Micrographs of testis sections from captive-reared greater amberjack sampled in three periods of the reproductive season. a) Testis section from an individual sampled in early gametogenesis period showing the presence of all stages of spermatogenesis. (b) Testis section from a fish sampled in the advanced gametogenesis period showing an arrested spermatogenesis state, with residual sperm cysts in the germinal epithelium and abundant spermatozoa in the lumen of seminiferous lobules. (c) Testicular sections from a specimen caught during the spawning period showing a moderate amount of spermatozoa in the lumen of seminiferous lobules. Haematoxylin-eosin staining. Magnification bars = 100 µm in (a), 200 µm in (b) and (c). sp: spermatozoa in the lumina of seminiferous lobules.

Gonado-somatic index of all the analysed captive-reared greater amberjack is reported in **Table 1**. Mean GSI increased significantly ($P<0.05$) from the EARLY to the ADVANCED spermatogenesis phase and decreased significantly ($P<0.05$) during the SPAWNING phase **(Fig. 6)**.

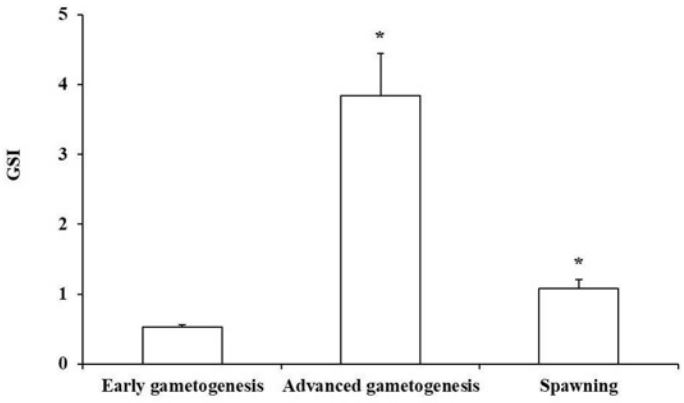

Figure 6. Mean (± SE) gonado-somatic index (GSI) of captive-reared greater amberjack males during three phases of the reproductive season. Asterisks indicate statistically significant differences versus the preceding phase ($P < 0.05$).

Germ cell proliferation and apoptosis

Anti-PCNA immunostaining was observed in the nuclei of single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (**Fig. 7a**). A weak staining of the nuclei of secondary spermatocytes was also observed, but these cells were not included in the quantitative analysis. The relative quantification of anti-PCNA positive single A spermatogonia and spermatocysts throughout the sampling period is shown in **Figure 8**. The density of anti-PCNA single A spermatogonia was stable throughout the EARLY and the ADVANCED phase and decreased dramatically in the SPAWNING phase ($P < 0.05$) (**Fig. 8a**); a progressive decrease of anti-PCNA positive spermatocysts density was observed throughout the examined phases of the reproductive cycle ($P < 0.05$) (**Fig. 8b**).

All the captive-reared greater amberjack showed TUNEL-positive germ cells. Apparently, the TUNEL reaction involved mainly single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (**Fig. 7b**). No significant difference in the surface occupied by apoptotic cells was observed during the three sampling phases (**Fig. 9**).

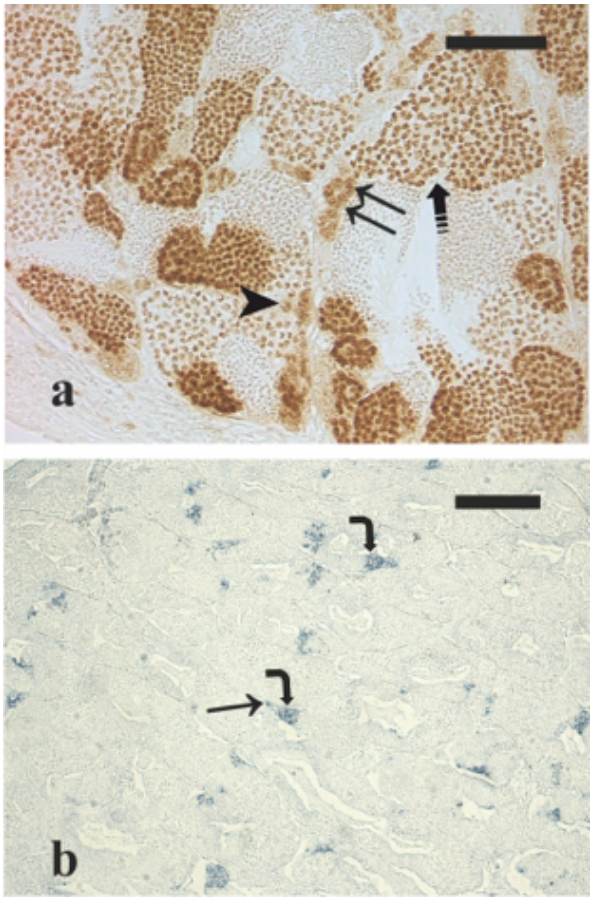

Figure 7. Micrographs of greater amberjack testis sections. (a) Testis section immunostained with antibodies against the Proliferating Cell Nuclear Antigen (PCNA), which stains brown the nuclei of proliferating cells. Magnification bar = 40 µm. Arrowhead: anti-PCNA positive single spermatogonium; double arrow: anti-PCNA positive spermatogonial cyst; dashed arrow: primary spermatocyte cyst. (b) Testis section stained with the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method, with apoptotic cells appearing as dark blue dots. Magnification bar = 150 µm. Arrow: TUNEL positive single spermatogonium; curved arrow: TUNEL positive spermatocysts.

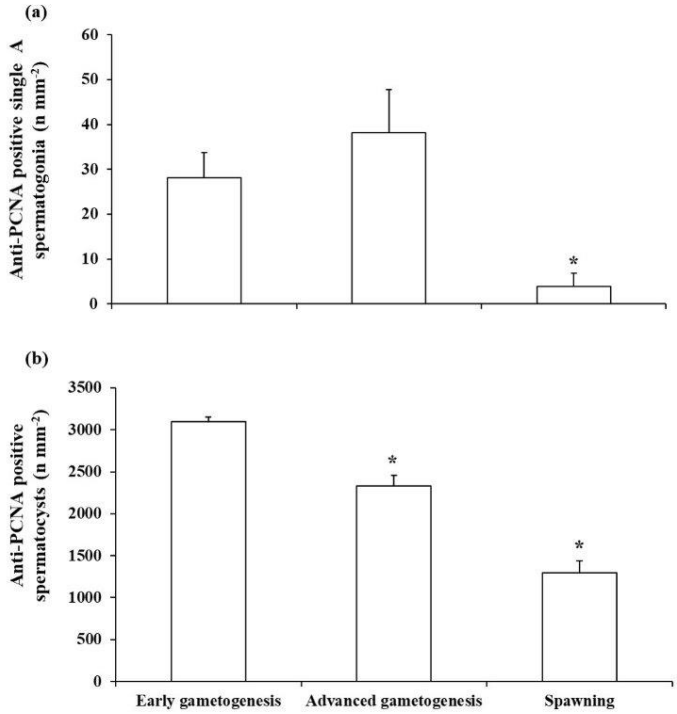

Figure 8. Changes in mean (±SE) anti-PCNA positive germ cell density in captive-reared male greater amberjack during three phases of the reproductive season. (a) Anti-PCNA positive single A spermatogonia. (b) Anti-PCNA positive spermatocysts. Asterisks indicate statistically significant differences versus the preceding phase (Student's t-test, $P < 0.05$).

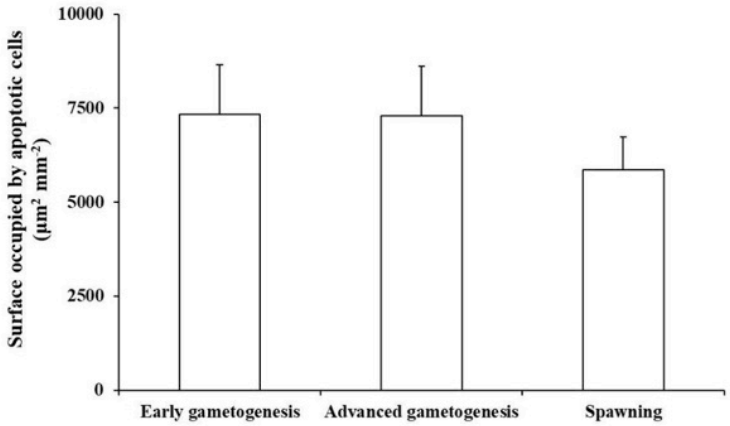

Figure 9. Mean (±SE) surface occupied by apoptotic germ cells in captive-reared male greater amberjack during three phases of the reproductive season. No statistical difference was observed between consecutive phases of the reproductive cycle (Student's t-test, $P < 0.05$).

Pituitary and plasma concentrations of gonadotropins

Pituitary and circulating levels of FSH (**Table 3**) as well as pituitary expression levels of the FSHβ (**Table 4**) did not vary significantly ($P > 0.05$) during the three examined reproductive phases of greater amberjack reared in captivity. Conversely, the pituitary LH content gradually increased reaching its maximum (23.42 ± 3.68) at the spent stage (**Table 3**). However, cognate pituitary LHβ gene expression (**Table 4**) and plasma LH levels (**Table 3**), exhibited no significant variation during the inspected reproductive stages.

Sex steroid plasma concentrations

The trend of sex steroid plasma levels during the three examined phases of captive-reared greater amberjack reproductive cycle is reported in **Table 3**. Testosterone, 11-KT and 17,20β-P plasma levels showed a progressive decrease from the EARLY to the SPAWNING phase. Unexpectedly high E_2 plasma levels were observed during the EARLY phase, but these levels decreased significantly in the following phases.

Table 3. Pituitary gonadotropin concentrations and plasma gonadotropin, steroid and leptin concentrations in captive-reared male greater amberjack during three phases of the reproductive cycle.

	Testicular maturity stage		
	Early spermatogenesis (n = 4)	Advanced spermatogenesis (n = 4)	Spent (n = 4)
Pituitary FSH (ng/pit/kg BW)	7.09 ± 0.04	4.5 ± 1.38	8.51 ± 1.26
Pituitary LH (ng/pit/kg BW)	8.45 ± 1.04	22.3 ± 5.11	23.42 ± 3.68*
Plasma FSH (ng ml^{-1})	8.57 ± 1.11	7.52 ± 1.79	11.34 ± 1.65
Plasma LH (ng ml^{-1})	15.71 ± 5.75	8.56 ± 2.94	16.96 ± 5.94
Testosterone (T, ng ml^{-1})	0.7 ± 0.16	0.4 ± 0.10	0.2 ± 0.04
11-Ketotestoterone (11-KT, ng ml^{-1})	2.3 ± 0.63	0.8 ± 0.15	0.2 ± 0.04*
17β-estradiol (E_2, ng ml^{-1})	5.4 ± 1.95	0.7 ± 0.26*	1.1 ± 0.65
17,20β-dihydroxypren-4-en-3-one (17,20β-P, ng ml^{-1})	0.4 ± 0.36	0.1 ± 0.01	0.5 ± 0.13*
Plasma Leptin	ND	ND	ND

Asterisks = statistically significant difference versus the previous testicular maturity stage (Student' s t-test, $P < 0.05$). ND, not determined.

Leptin plasma concentration

The circulating levels of leptin could not be detected due to technical problems, which hampered the establishment of the related ELISA. Although not statistically significant, liver leptin mRNA levels appear to be relatively low at early and during spermatogenesis and elevated at the spent stage (**Table 4**).

Table 4. Pituitary gonadotropin and liver leptin gene expression in captive-reared male greater amberjack during three phases of the reproductive cycle.

	Testicular maturity stage		
	Early spermatogenesis (n = 4)	Advanced spermatogenesis (n = 4)	Spent (n = 4)
Pituitary FSHβ (relative units)	6.24 ± 3.1	6.91 ± 0.56	3.6 ± 1.1
Pituitary LHβ (relative units)	3.02 ± 1.51	4.11 ± 1.46	3.98 ± 1.37
Leptin (relative units)	7.22 ± 2.04	4.56 ± 2.2	67.7 ± 49.3

Sperm quality

Spermatozoa concentration of captive-reared greater amberjack was stable throughout the EARLY (2.3 ± 0.5 x 10^{10} spz ml^{-1}) and ADVANCED (3.6 ± 0.4 x 10^{10} spz ml^{-1}) phases, and increased significantly during the

SPAWNING period (4.6 ± 0.6 x 10^{10} spz ml^{-1}; ANOVA, $P < 0.05$). For all the three sampling phases, the highest spermatozoa motility (%) was reached within the first 20 s after activation, and was followed by a progressive decrease until complete cessation of movement (**Fig. 10a**). However, sperm movement within the first 20 s presented variations linked to the sampling time, with the highest mean percentage of swimming spermatozoa recorded in the ADVANCED phase (59 ± 16.9 % of motile spz), and the lowest mean value registered in the SPAWNING phase (21 ± 9.7 % of motile spz). The mean VAP of the spermatozoa varied during the three different phases, with the highest mean value 10 s after activation recorded in the ADVANCED phase (102.7 ± 7.0 µm s-1) and the lowest mean VAP during the SPAWNING phase (36.5 ± 3.3 µm s-1); the highest maximum value of individual velocity was reached during the ADVANCED phase (164 µm s-1) (**Fig. 10b**). Finally, a progressive significant decrease ($P < 0.05$) of sperm motility duration was observed from the EARLY to the SPAWNING phase (**Fig. 10c**).

The ATP level of captive-reared greater amberjack sperm was generally very low, and close to the detection threshold for several samples (data not shown). A progressive, but not statistically significant, decrease of spermatozoa ATP concentration occurred from the EARLY phase (4.7 ± 1.7 n mole 10^{-9} spz) to the ADVANCED (1.9 ± 0.6 n mole 10^{-9} spz) and the SPAWNING phase (1.2 ± 0.4 n mole 10^{-9} spz). In terms of spermatozoa viability, there were significant variations among fish within each sampling time. Notwithstanding this individual variability, a significant increase ($P < 0.05$) of the proportion of dead and live spermatozoa was observed from the ADVANCED to the SPAWNING phase, while the proportion of dying spermatozoa did not vary significantly (**Fig. 11**).

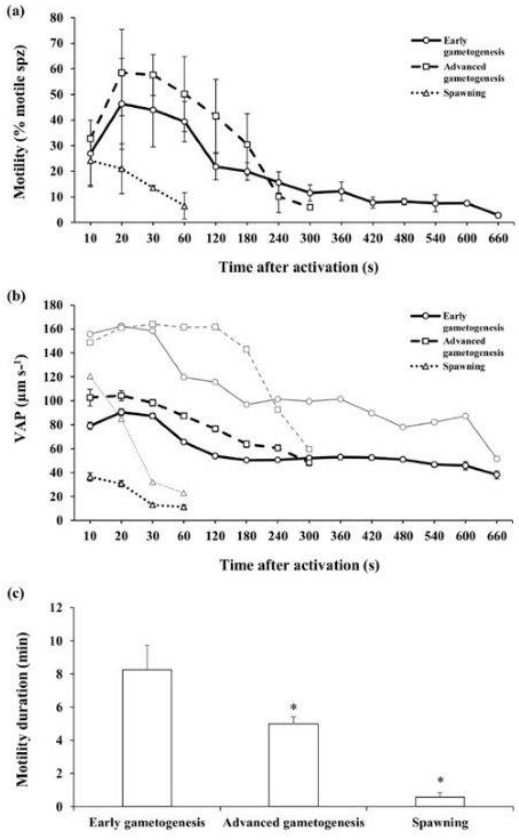

Figure 10. (a) Sperm motility percentage, (b) average path velocity (VAP), and (c) motility duration in captive-reared greater amberjack during three phases of the reproductive season. In (b), black lines illustrate the mean VAP of sperm population for each phase, while grey lines show the maximum value of individual sperm velocity recorded. In (c), black asterisks indicate significant differences versus the preceding phase (ANOVA, $P < 0.05$).

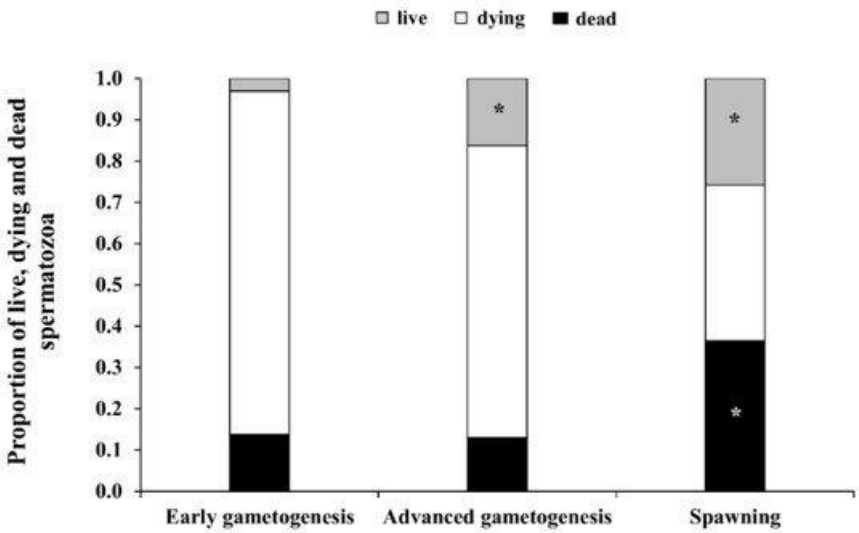

Figure 11. Proportion of live/dying/dead spermatozoa in captive-reared greater amberjack during three phases of the reproductive season. Black and white asterisks indicate significant differences versus the preceding phase within the same spermatozoa condition (ANOVA, $P < 0.05$).

Nutritional status

Body condition

Body condition of captive-reared greater amberjack, expressed as Fulton's condition factor (K), was not significantly different from that of wild males of all the available age classes analysed for the study of the reproductive cycle of the wild population (D3.3) (**Fig. 12**).

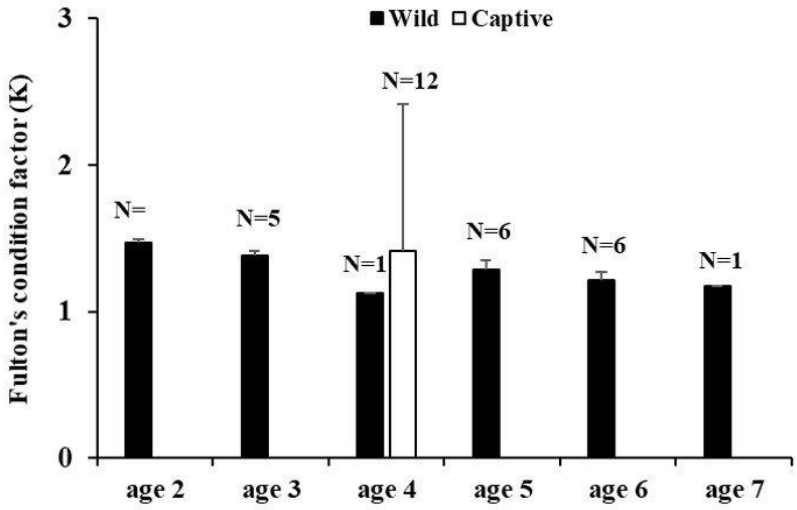

Figure 12. Fulton's condition factor (K) in wild and captive-reared greater amberjack grouped by age class.

Discussion and Conclusions

The age analysis, carried out through the examination of the scales, showed that the 15 sampled juvenile greater amberjack (sex indeterminate and males) were 1 to 3 years old. The age of 14 wild adult males had been previously estimated (Deliverable 3.3) and ranged between 4 and 7 years. The parameters of the von Bertalanffy equation, calculated using all the 23 juveniles and 14 adult males, indicate a theoretical maximum length of 127 cm FL and a theoretical longevity of 9 years. The theoretical length-at-age data, calculated applying the von Bertalanffy equation, confirm a very rapid growth of male greater amberjack during the first years of life: 37 cm FL at age 1; 64 cm FL at age 2; 83 cm FL at age 3, corresponding to a theoretical body weight of 0.7 kg at age 1; 3.2 kg at age 2, 6.4 kg at age 3 [(the calculation was made using the total length (TL)-weight relationship provided by Kozul et al. (2001) for greater amberjack specimens sampled in the Adriatic Sea, after conversion of FL to TL according to the correlation provided by Sley et al. (2014)].

The available literature data on greater amberjack first sexual maturity in the Mediterranean Sea are scarce and somehow contradictory, and refer mainly to females (Marino et al., 1995; Micale et al., 1999; Sley et al., 2014). In the Adriatic Sea, all males with TL > 107 cm (corresponding to FL > 88 cm) were reported to be sexually mature (Kozul et al., 2001). In the present study, all age 3 and older male greater amberjack appeared to be reproductively active. The size of the smallest mature fish was 80 cm FL, 6.7 kg total body weight.

The study of the reproductive cycle of captive-reared greater amberjack showed that during late April testes were in early spermatogenesis stage, showing an overall maturity stage comparable to that of wild individuals sampled in the same period (cfr. Deliverable 3.3 and Zupa et al., 2017b). However, the subsequent maturation phase appeared to be somehow impaired in captivity, since part of the fish (50%) sampled in early June had precociously ceased their spermatogenic activity. In early July, when the wild population was still in spawning condition (cfr. Deliverable 3.3 and Zupa et al., 2017a, b), greater amberjack reared in captivity had totally ceased their spermatogenic activity. Gonad development of captive-reared greater amberjack was well described by the GSI, which increased from late April to early June and decreased thereafter. However, GSI values of captive-reared greater amberjack were lower than in the wild population during the second and third sampling periods (cfr. Deliverable 3.3 and Zupa et al., 2017b). The GSI data, corroborated by the observed lower seminiferous lobule diameter (D3.3 and Zupa et al., 2017a), testify that greater amberjack reared in captivity did not reach a full testicular development.

Gonadosomatic index and histological data on gonad maturation of captive reared greater amberjack were in close agreement with the observed changes of sex steroid plasma concentrations during the three investigated phases of the reproductive cycle. All the androgens examined in the present study were constantly lower in captive than in wild fish (cfr. Deliverable 3.3 and Zupa et al., 2017a, b). As for many male teleost fish (Kime et al., 1982; Weltzien et al., 2002; Corriero et al., 2007b), 11-KT proved to be the prominent androgen in greater amberjack, always having higher plasma levels than T. Regarding 17,20β-P, plasma levels showed a surprising increase between the ADVANCED and SPAWNING phases, in concomitance with a GSI decrease and testis regression. The same was found to happen in tench *Tinca tinca*, where an inexplicable peak of 17,20β-P found in males with regressed gonads (Pinollos et al., 2003). The existing literature on 17,20β-P in greater amberjack and other related species has been limited mostly to females (Ouchi et al., 1985, 1989; Mandich et al., 2004); the only study including males, carried out on the congener yellowtail kingfish *Seriola lalandi*, failed to find any difference in the 17,20β-P values between the different reproductive stages (Poortenaar et al., 2001). The generally low 17,20β-P values observed both in the present study and in other studies on greater amberjack and related species (Ouchi et al., 1985; Poortenaar et al., 2001; Mandich et al., 2004) may suggest that this hormone is rapidly catabolized in the fish gonad and may still exist in the fish blood in different forms (glucuronated, sulfonated or reduced) (Scott et al., 1994; Vermeirssen et al., 1996, 1998) and it is not detected by the techniques used for the free steroids. Recent studies suggest that this hormone may play a role in stimulating meiosis or may be released into the water to act as a pheromone (Poortenaar et al., 2001; Antonopoulou et al., 2011; Scott et al., 2013). Therefore, in contrast to T and 11-KT, it cannot be considered as a trustworthy indicator of reproductive stage of development in male greater amberjack. Moreover, circulating 17,20β-P levels significantly increased in

response to handling stress in the black bream *Acanthopagrus butcheri* (Haddy et al., 1999) and in the greenback flounder *Rhombosolea tapirina* (Pankhurst et al., 2000) and a positive correlation between cortisol and 17,20β-P was found in sexually mature silver seabream *Pagrus auratus* (Carragher et al., 1991). The increase of 17,20β-P plasma levels observed in captive-reared greater amberjack with regressed testes in the present study, could then be associated to the handling stress due to sampling operations.

In greater amberjack testes, anti-PCNA positive undifferentiated single type A spermatogonia, differentiated spermatogonia (type A and B spermatogonia being part of cysts) and primary spermatocytes were detected during all the investigated reproductive phases.

In the present study, the density of proliferating single spermatogonia remained at the highest levels throughout the EARLY and ADVANCED phases, and decreased dramatically during the SPAWNING phase. This trend of proliferating activity of single spermatogonia during the reproductive season is coherent with the decreasing trend of T and 11-KT plasma levels.

The density of spermatocysts containing proliferating type A and B spermatogonia plus primary spermatocytes showed a progressive decrease from the EARLY to the SPAWNING phase. This is in agreement with the observation that captive-reared greater amberjack were already in spent condition during the SPAWNING phase of the wild population, and their T, 11-KT and 17,20β-P plasma levels were abnormally low. The low spermatogonial capacity of captive-reared fish to proceed toward meiosis might have resulted from the combined effects of higher E_2 and lower T/11-KT plasma concentrations. In fact, although E_2 in male fish stimulates spermatogonial self-renewal (Miura et al., 1999; Schulz and Miura, 2002; Schulz et al., 2010), supra-physiological concentrations of this hormone inhibit spermatogenesis via negative feedback effects on the brain and the pituitary, involving down-regulation of the testicular androgen production capacity (Schulz and Nóbrega, 2011).

Besides promoting germ cell proliferation, spermiogenesis and spermiation, androgens have been proposed to act as survival factors for germ cells, both in mammals (Young and Nelson, 2001) and in fish (Corriero et al., 2009; Zupa et al., 2013, 2014). Withdrawal of androgens induces apoptosis in the testis (Nandi et al., 1999; Woolveridge et al., 1999) and reintroduction of steroid hormones can reduce apoptotic cell death (Nandi et al., 1999). In the present study, apoptotic germ cells, spermatogonia and primary spermatocytes, were observed in all the specimens analysed. In captive-reared greater amberjack, a high density of germ cell apoptosis was observed at the beginning of the reproductive season (EARLY phase). The high incidence of apoptosis at the onset of spermatogenesis, far from playing a physiological role in the quantitative control of germ cell populations and in the prevention of aberrant germ cell development, as proposed for other large pelagic fish such as the Atlantic bluefin tuna *Thunnus thynnus* (Corriero et al., 2009; Zupa et al., 2013, 2014) and the swordfish *Xiphias gladius* (Corriero et al., 2007a), was likely correlated to the observed low androgen and high E_2 plasma levels and might be co-responsible for the reduced sperm concentration (see below). Incidentally, the administration of high doses of E_2 in male gilthead seabream *Sparus aurata* induced apoptosis of spermatogonia (Chaves-Pozo et al., 2007).

The gonadotropin analyses indicate relatively low pituitary FSH and LH content and consequently reduced levels of these hormones in the circulation of captive-reared greater amberjack undergoing spermatogenesis compared to levels measured in wild fish during the equivalent reproductive periods. It appears that the impaired gonadotropin synthesis and release distract captive-reared greater amberjack from exploiting their maximal reproductive potential.

In a mammalian model (rat), gonadotrophin withdrawal following hypophysectomy and the consequent decline of sex steroid circulating levels were found to induce testicular atrophy, reduction of germ cell proliferation and increase of apoptosis (Tapanainen et al., 1993). In wild-caught captive-reared Atlantic bluefin tuna, an increase of 11-KT plasma levels produced by gonadotropin-releasing hormone agonist (GnRHa) administration, stimulated spermatogonial proliferation and reduced the rate of apoptotic germ cells (Corriero et al., 2009). The observed low androgen levels found in captive-reared greater amberjack may have been caused by a reduced release of gonadotropins from the pituitary and/or an altered steroid metabolism. In fact, testes of greater amberjack reared in captivity were found to have a much reduced amount of arachidonic acid (Zupa et al., 2017b) a molecule that stimulates testosterone production by

elevating cyclic adenosine monophosphate (cAMP) levels in a dose dependent manner (Mercure and Van der Kraak, 1995, 1996). Moreover, in birds (Newman et al., 2008; Dickens et al., 2011) and mammals (Williams, 2012) the exposure to different types of stressing factors can result in aromatase up-regulation with the consequent increase of E_2 and decrease of androgens.

One objective of this study was to assess if the above-described dysfunctions resulted in a decreased sperm quality. In the present study it was not possible to take sperm by stripping captive-reared greater amberjack. This failure is believed to be due to the strong abdominal musculature of this species (Mylonas et al., 2004), and could be exacerbated by the lack of significant testicular hydration. It has been observed in various captive-reared greater amberjack broodstocks, that it is not possible to collect sperm by abdominal stripping even from males belonging to actively spawning broodstocks that produce large numbers of fertilized eggs daily (Fakriadis et al., 2017). In the case of the present study, however, our failure to collect sperm by stripping was probably more related to a lack of significant testicular hydration. Indeed, during the dissection of the testes, it was observed that the vasa deferentia were not full of sperm and only a direct strong squeezing of the testes allowed obtaining sperm, so that the following discussion actually refers to intra-testicular sperm, which might lack complete maturation and hydration. The sperm concentration of captive-reared greater amberjack measured in this study was in the upper range of marine fish species (Suquet et al., 1994; Cosson et al., 2008a), which is consistent with a lack of hydration that, if realized, would have resulted a physiological reduction of this parameter towards the spawning season. To our knowledge, the observed increase of sperm concentration during the SPAWNING phase has never been reported in any other fish species; moreover, the histological and immunohistochemical analyses showed that while these specimens had ceased their spermatogenic activity, they still retained a moderate amount of luminal spermatozoa in the testes. Altogether, these observations seem to support the hypothesis of the lack of proper sperm hydration in captive-reared greater amberjack, probably in response to low sex steroid levels. It is known that sperm hydration with seminal fluid and release via the sperm duct are under endocrine control, and a key-role in this process and in the intensification of sperm motility is played by 17,20β-P (Schulz and Miura, 2002; Scott et al., 2010; Milla et al, 2008), whose plasma levels were abnormally low in captive-reared greater amberjack during the ADVANCED and SPAWNING phases.

The sperm of captive-reared greater amberjack analysed in the present study showed a general motility pattern similar to other fishes, with high initial spermatozoa motility percentage and velocity at activation, followed by a decrease of both parameters until all movement ceased (Cosson et al., 2008a, b). However, despite the fact that the velocity of the faster spermatozoa in captive-reared greater amberjack sperm was similar to that of other species, such as the European sea bass *Dicentrarchus labrax* (Fauvel et al., 2012) and the Atlantic bluefin tuna (Zupa et al., 2013), the maximum sperm motility recorded (about 60% of motile spermatozoa during the ADVANCED phase) was lower compared to most other studied species (Cosson et al., 2008a), and the percentage of motile spermatozoa, motility duration and velocity declined drastically during the SPAWNING phase. Moreover, the sperm ATP content decreased in captive-reared greater amberjack from the EARLY to the SPAWNING phase. The ATP content is widely used as a sperm quality marker (Cosson et al., 2008a; Fauvel et al., 2010), since it is a key-limiting factor for maintaining motility (Christen et al., 1987; Cosson, 2010; Ulloa-Rodríguez et al., 2017). Therefore, the decrease of energy content observed in captive-reared greater amberjack in the present study might explain, at least partially, the lower percentage of motile spermatozoa. Finally, the assessment of sperm membrane integrity from captive-reared fish demonstrated that the percentage of dead spermatozoa increased significantly from the ADVANCED to the SPAWNING phase, which is consistent with the lack of sperm hydration and, presumably ejaculation, and consequent sperm ageing.

As to the underlying cause of the above-mentioned dysfunctions in sex hormone levels, germ cell proliferation and apoptosis, we can only speculate at this stage. The fish were acclimated for 2 years in the facilities and were maintained in a large-volume sea cage (640 m^3 rectangular shape) at low stocking densities (<1 kg m^{-3}). The site has excellent water circulation and low aquaculture production capacity (<500 mt $year^{-1}$) and the fish showed a good feeding behaviour and growth rate during the 2-year acclimation period, increasing in weight from 5-7 kg to 9-15 kg. Water temperatures during the year were typical of the Mediterranean Sea, ranging between 14.3 and 30.1°C. Apart from being maintained in a captive

environment preventing any migration, and being fed a commercial extruded broodstock diet as opposed to forage fish, the breeders were exposed to very low stress conditions during the whole year.

Although the overall greater amberjack nutrition status was not apparently affected by the rearing conditions, as evidenced by the normal body condition, it is conceivable that the offered food was not optimal for reproductive maturation and required some formulation adjustment, as suggested by Zupa et al. (2017b). In fact, another broodstock of the same source and age was maintained under identical conditions in the same facility, and during June it reached advanced stages of gametogenesis to be able to be induced to spawn and produce fertilized eggs using a gonadotropin releasing hormone agonist (GnRHa) therapy (Mylonas et al., 2017). This suggests that it is possible for captive-reared greater amberjack to undergo gametogenesis to an extent that viable gametes (eggs and sperm) can be spawned, producing viable progeny. Although similar evaluations of spermatogenesis were not undertaken on the males of this latter spawning stock, the fact that fertilized eggs were produced indicates that adequate amounts of viable sperm were produced by the males, who were also capable of engaging in normal breeding activities to fertilize the eggs produced by the females, albeit after an exogenous GnRHa therapy.

One aspect of the rearing conditions of the fish in the present study that might have exacerbated the progress of an already dysfunctional spermatogenesis was the management/handling stress to which the fish were exposed during the sampling process. Due to facility limitations, all fish used for the three samplings were maintained in the same sea cage. As a result of the sampling procedure (see Materials and Methods), except for the fish sacrificed during the first sampling, all other fish were exposed to a certain amount of handling or management stress a few weeks prior to their sampling for the study. We believe that this handling or management stress during the reproductive period was responsible for the deterioration of the spermatogenesis, which was already impaired at the beginning of the reproductive cycle (high plasma E_2 concentration and germ cell apoptosis during the EARLY phase).

In conclusion, the present study demonstrated that rearing in captivity affected spermatogenesis in greater amberjack from its early phase, when a high level of germ cell apoptosis was observed. Handling or mild management probably had an additional negative effect on spermatogenesis, resulting in a constant reduction of the rate of spermatogonia entering meiosis and in a precocious cessation of the spermatogenic activity. As a consequence of this spermatogenesis impairment, greater amberjack confined in captivity showed low sperm quality, in terms of sperm density, motility and velocity, as well as ATP content and membrane integrity. This study supports the need for an improvement of rearing technology. In particular, handling procedures minimizing stress could be effective in alleviating reproductive deficiencies.

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Deviations: According to the original plan, relatively high quantities of recombinant leptin were produced and purified, which enabled the generation of specific polyclonal antibodies. These antibodies were found to be highly specific to the recombinant antigen yet failed to detect native greater amberjack leptin. While it could be interesting to compare circulating leptin profiles during the reproductive cycle in wild vs. captive reared greater amberjack, the impact on the deliverable is negligible, particularly as we were able to follow the leptin gene expression levels and highlight a time window during which it appears to play a significant role (Zupa et al., 2017b).

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