Deliverable Report

Deliverable No:	D3.5		**Delivery Month:**	49
Deliverable Title	Description of the process of oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones and Vg, (e) nutritional status and (f) egg biochemical composition.			
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	P4. IOLR	P15. ULL	P23. ARGO
Status:	Delivered		**Expected month:**	46
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Objective

Description of the process of oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones and Vg, (e) nutritional status and (f) egg biochemical composition. The endocrine control of the reproductive cycle in female greater amberjack has not been described yet. This deliverable will provide an evaluation of the brain-pituitary-gonad axis during oogenesis in captive greater amberjack, as a way of assessing reproductive function and predicting spawning performance. The deliverable will (a) assess the size and the age at first sexual maturity of greater amberjack females; (b) evaluate captive fish body condition by using different parameters such as the condition index (CI) and the gonadosomatic index (GSI); (c) describe the reproductive cycle of captive fish in order to identify the optimal time for the administration of hormonal treatments for the induction of spawning; (d) identify the possible effects of captivity on the reproductive axis by measuring the pituitary and plasma levels of the two gonadotropins (FSH and LH), as well as the sex steroid (17β-estradiol, Testosterone, 17,20b-DHP) plasma concentration; (e) provide information on the nutritional status of captive females by measuring a key metabolic hormone (e.g. leptin) as well as Vg plasma level and oocyte yolk accumulation; and (f) assess egg composition by determining fatty acid, vitamin and carotenoid content.

Table of Contents

Description

The reproductive success of a fish species candidate to aquaculture 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 gonadotropins' (GtHs: follicle stimulating hormone, FSH; luteinizing hormone, LH) release, 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, the most common being the inhibition of final oocyte maturation and spawning for females and the production of a scarce quality sperm for males, respectively (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; Rodríguez-Barreto et al., 2014)).

In order to setup an adequate hormonal treatment and, more in general, proper rearing practises for a fish species candidate to aquaculture, 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, the effects of diet on broodstock body condition as well as on egg composition are key factors which contribute to reproductive success of a fish species in aquaculture.

The present deliverable describes female 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 by comparing ovarian development 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 wild and captive-reared specimens. The assessment of fish body condition index, the quantification of leptin (a key metabolic hormone) and vitellogenin plasma levels, the quantification of oocyte yolk accumulation and the evaluation of egg composition will help us to evaluate the effectiveness of the diet on

greater amberjack captive female body circumstance and nutritional status and, more in general, on their reproductive performance.

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

Pousis, C., Mylonas, C.C., De Virgilio, C., Gadaleta, G., Santamaria, N., Passantino, L., Zupa, R., Papadaki, M., Fakriadis, I., Ferreri, R., and Corriero, A. (**2017**). The observed oogenesis impairment in greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is not related to an insufficient liver transcription or oocyte uptake of vitellogenin. **Aquaculture Research** (published online on August 1st, 2017).

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., and Corriero, A. (**2017**). 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 size/age at first sexual maturity are essential components of models used in stock assessment of fish populations and provides 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 & Bard, 1983; Megalofonou & De Metrio, 2000).

There are several ways of modelling the growth of fish using age determination data (Ricker, 1975). The von Bertalanffy growth function has been by far the most studied and most used of all length-age models 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 interaction may also operate (Munro et al., 1990).

The endocrine control of the reproduction in fishes, similar to the other vertebrates, is regulated by brain–pituitary–gonadal (BPG) axis (Mittelholzer et al., 2009), which involves three component parts: (a) Gonadotropin-releasing hormone (GnRH) neurons projecting from the hypothalamus to the pituitary; (b) gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins (GtHs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH); (c) the somatic cells of the gonads (theca and granulosa cells in the ovary).

Fish female reproductive cycle, similar to other vertebrates, can be divided into two main phases. The proliferation, growth and differentiation of the gametes (i.e. vitellogenesis), constitute the first phase, while the maturation and preparation of the oocytes (i.e. oocyte maturation) for release and insemination constitute the second phase (Mylonas et al., 2010). Gonadotropic regulation of gametogenesis in female fish is

characterized by increasing FSH levels during oocyte growth and peak levels of LH at final maturation and ovulation (Kumar & Trant, 2004; Kim et al., 2005).

During vitellogenesis, the glycolipophosphoprotein vitellogenin (Vtg) is synthesized by the liver under the stimulation of 17ß-estradiol (E_2) produced by the developing ovary, and then is transported via bloodstream to the ovary to be incorporated as yolk granules in the oocyte. LH and FSH have similar potencies in stimulating E_2 (Planas et al., 2000). Vitellogenesis is a crucial step for fish reproduction, since embryo survival depends on the amount and the quality of yolk incorporated in eggs (Bobe & Labbé, 2010).

During vitellogenesis not only large quantities of proteins must be made available for transfer to the developing oocytes but also lipids and carotenoids. Lipid mobilization towards gonadal maturation also depends upon E_2 and testosterone (T), plasma levels. These mobilized lipids are particularly rich in both saturated and monoenoic fatty acids for energy provision and highly unsaturated fatty acids (HUFA or LC-PUFA) as essential fatty acids (EFA; mainly EPA (20:5n-3), ARA (20:4n-6) and DHA(22:6n-3). EPA and ARA are precursors of eicosanoids, a group of active compounds with very important physiological functions such as reproduction (Tocher 2003). ARA derived eicosanoids have also shown to be involved in pheromone attraction, steroidogenesis, and oocytes maturation (Henrotte et al., 2011; Sorbera et al., 2001). Thus, supplying proper levels and ratios of EFA on broodstock diet is vital not only to produce eggs with the suitable contents of these fatty acids to ensure embryo and larvae development (Sargent et al., 2002; Tocher 2010), but also in the regulation of reproductive physiology (Henrotte et al., 2011; Sorbera et al., 2001).

Effects of captivity on reproductive axis

Among the major bottlenecks for the incorporation of a new species in the aquaculture industry, reproductive dysfunctions affect frequently fish in captivity, hindering the production of viable eggs. Reproductive dysfunctions commonly involve an inadequate pituitary GtHs synthesis and/or release (Zohar & 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 females, captivity-induced reproductive dysfunctions may result in the lack of final oocyte maturation and spawning (Bobe & Labbé, 2010; Mylonas et al., 2010). Reproductive dysfunctions have been also documented in captive-reared greater amberjack confined in sea cages or tanks (Díaz et al. 1997; Micale et al., 1999; Mylonas et al., 2004).

Nutritional state: plasma leptin

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 (Zhang et al., 1994; Pelleymounter et al., 1995; Tartaglia et al., 1995; Chen et al., 1996; 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).

Egg composition

Teleost gonads and consequently the eggs are particularly rich in both triacylglycerols and phospholipids which contain high quantities of polyunsaturated fatty acids (PUFA) of the n−3 series, mainly docosahexaenoic acid (DHA; 22:6n−3) and eicosapentaenoic acid (EPA, 20:5n−3) (Sargent et al., 2002).

Dietary uptake provides for the accumulation of lipid reserves in the liver and muscle and the consequent ovarian growth. Thus, the nutritional status of brood fish has great influence on the reproduction and egg quality of several fish species (Fernández Palacios et al., 1995; Almansa et al., 1999; Izquierdo et al., 2001), since n−3 PUFA derived directly from the dietary input of brood stock (Sargent, 1995) as well as from body reserves in the period of gonadogenesis are crucial to female fecundity and to embryo and early larval development, growth and survival (Sargent, 1995; Rodríguez-Barreto et al., 2014).

Material and methods

Age determination, growth and first sexual maturity

Sampling

During the months of June 2015 and 2016, when adult greater amberjack were supposed to be reproductively active (Pousis et al., 2017; Zupa et al., 2017b), 23 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 these 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. The gonads of ten fish were macroscopically classified as testes and therefore they were used for the determination of the first sexual maturity of males (see D3.6). The gonads of eight fish were macroscopically classified as ovaries and were processed for the determination of the first sexual maturity of females.

Table 1. Biometric data and estimated age of wild greater amberjack with indeterminate sex and juvenile females 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	f	59.0	3.09	6	0.19	2
		f	60.0	3.53	5	0.14	2
		f	66.0	3.89	10	0.26	2
		f	69.0	4.95	11	0.22	2
		f	69.0	4.20	15	0.36	2
wild	10/06/2016	f	86.0	8.69	43	0.49	3
		f	87.0	8.88	27	0.30	3
		f	87.0	9.17	288	3.14	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 juvenile 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 sampled individual, a variable number of scales were treated according to the following 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).

Estimate of female greater amberjack theoretical growth in length was 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

For the estimate of the first sexual maturity of female greater amberjack, both the juveniles (**Table 1**) and the wild adults sampled for the study of the reproductive cycle (D3.3), were used.

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

The classification of the reproductive state was based on the histological criteria reported by Schaefer (1998). Briefly, female greater amberjack were classified as mature if their ovaries contained: a) late vitellogenesis and/or more advanced oocyte stages; b) primary growth oocytes along with signs of previous reproductive activity (atretic vitellogenic follicles or post-ovulatory follicles).

The body length at median sexual maturity (L_{50}) was estimated by fitting a logistic function to the fraction of mature fish per 5 cm FL intervals by nonlinear regression. L_{50} was defined as the theoretical length in which 50% of the specimens were mature.

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

Sampling

For the study of the reproductive cycle of captive-reared greater amberjack females, 12 adult individuals 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, and will be referred to as captive-reared fish. 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 females 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): i.e. EARLY (late April), ADVANCED (early June), 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 female 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	87	10	85
			96	14	125
			97	14	155
			100	14	160
Advanced Gametogenesis (ADVANCED)					
captive	04/06/2015	20.0	97	13	335
			97	13	920
			101	12	660
			106	17	305
Spawning (SPAWNING)					
captive	02/07/2015	25.5	92	8	95
			95	11	135
			96	12	130
			97	12	140

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

Reproductive cycle

The gonado-somatic index was calculated as $GSI = 100xGMxBM^{-1}$.
For the evaluation of the reproductive state, one-cm thick ovary slices were cut from each individual and fixed in Bouin's solution. Samples were subsequently dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were than stained with haematoxylin-eosin, and observed under light microscope. The reproductive state was assessed by recording the most advanced oocyte stage for each specimen as well as the presence of postovulatory and atretic follicles.

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 & Schmittgen, 2001). The gene specific primers (GSPs) designed using the Primer Express 3.0 software (Applied Biosystems) are listed in D3.1.

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□. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl per well) for 0.5 h at 37□. 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□. 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□), 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□. 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 an homologous ELISA that was developed in the framework of this study (D3.2). Briefly, the recombinant greater amberjack FSH (*rec-ga*FSH) was produced using the *Pichia pastoris* yeast recombinant DNA expression system (Invitrogen, Carlsbad, CA). Using Ni-Affinity Chromatography (Amersham Biosciences), the rec-gaFSH was purified and 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 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 were 9.6% (n = 7).

Sex 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 testosterone (T), and 17,20β-dihydroxypren-4-en-3-one (17,20β-P) (a putative maturation-inducing steroid; MIS) in the plasma, already established and well-described enzyme-linked immunoassays (ELISA) were used (Nash et al., 2000; Rodríguez et al., 2000) with some modifications, and using reagents from Cayman Chemical Company (USA). For the quantification of 17β-estradiol (E_2), 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.

Vitellogenin plasma levels and oocyte yolk accumulation

For the quantification of vitellogenin (Vtg), 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. To this aim, a commercial Vitellogenin ELISA Kit (Biosense Laboratories), already successfully used for the determination of greater amberjack plasma vitellogenin concentrations (Mandich et al., 2004), was used according to the manufacturer's instructions with plasma diluted 1:250.

For the analysis of oocyte yolk accumulation, oocytes at early and late stage of vitellogenesis, having a large and centrally located nucleus were randomly selected. Oocyte diameter (µm) and surface occupied by yolk granules (μm^2) were measured from 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). In order to assess if a normal yolk accumulation occurred in oocytes of captive-reared specimens, yolk accumulation data were compared with those of the wild greater amberjack sampled for the study of the reproductive cycle (see D3.3).

Nutritional state

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 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 (D3.2). 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, yet failed to detect greater amberjack leptin extracted from liver samples (D3.2).

Egg composition

Three pooled samples of eggs produced by the greater amberjack broodstock reared at Argosaronikos Fish farming S.A. (Salamina Island, Greece) for the execution of the Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean, were taken and

immediately frozen at -80ºC, until analysis. Moisture contents were determined in approximately 500 mg samples by thermal drying in an oven at 110°C, until constant weight. Total lipid (TL) was extracted by homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (Christie, 1982) and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (5: 5: 5: 2: 1.8, by volume) as developing solvent system for the polar lipid classes and isohexane/diethyl ether/acetic acid (22.5: 2.5: 0.25, by volume), for the neutral lipid separation. Lipid classes were visualized by charring at 160°C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, and quantified by scanning densitometry using a dual-wavelength flying spot scanner Shimadzu CS-9001PC (Shimadzu, Duisburg, Germany) (Olsen & Henderson, 1989). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic acid (90:10: 1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m 9 0.32 mm I.D. 9 0.25 lm; Sigma-Aldrich, Madrid, Spain). Helium was used as carrier gas and temperature programming was 50–50°C at 40°C min -1 slope, then from 150 to200°C at 2°C min $^{-1}$, to 214 °C at 1°C min -1 and, finally, to 230°C at 40°C min -1. Individual FAME and DMA were identified by reference to authentic standards, and further confirmation of FAMEs and DMAs identity was carried out by GC-MS (DSQ II; Thermo Electron Corp). Carotenoids were obtained according to the method of Barua et al. (1993), using ethyl acetate/ethanol (10mL, 1:1 v/v), ethyl acetate (5 ml) and hexane (10 ml) as extracting solvents. Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

Statistical analysis

Differences in GSI, GTHs and leptin gene expression, as well as GTHs and sex steroid plasma concentrations mean values between consecutive oocyte developmental stages were assessed by a two tailed Student's t-test. Prior to the Student's t-test, the raw data of GSI and surface occupied by yolk granule were arcsine-transformed, as appropriate with proportions (Sokal and Rohlf, 1981).

Differences in the mean values of vitellogenin plasma concentration were assessed between oocyte developmental stages by ANOVA followed by Bonferroni *post-hoc* test.

Diameter of oocytes at early and late vitellogenesis stage and Fulton's condition factor (K) were compared between wild and captive-reared specimens 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

Among the 13 juvenile greater amberjack sampled for the study of the first sexual maturity, five (all those classified as Sex Indeterminate) belonged to the age class 1, five to the age class 2 and 3 to the age class 3 (**Fig. 1; Table 1**)

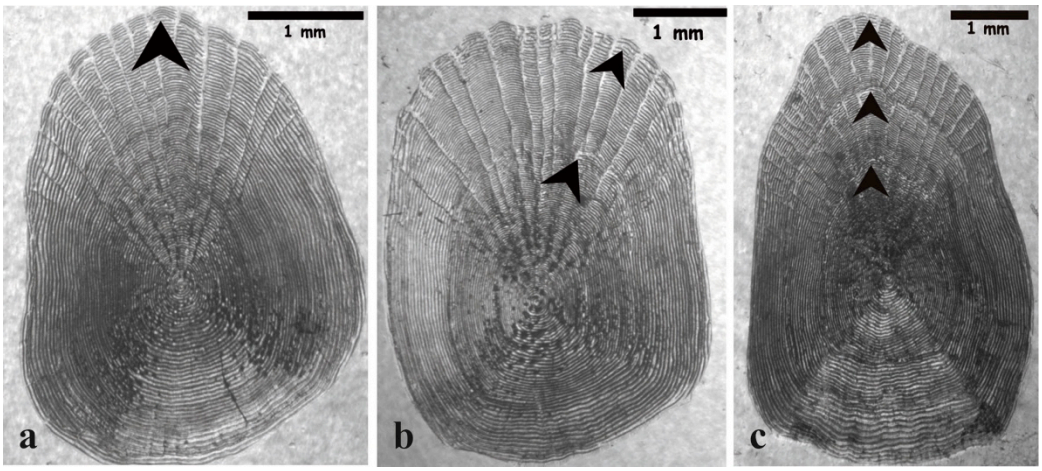

Figure 1. Images of scales from juvenile wild greater amberjack sampled in the Mediterranean Sea. (a) Sex indeterminate; age 1; 35 cm fork length. (b) Female; age 2; 59 cm fork length. (c) Female; age 3; 87 cm fork length. Magnification bar = 1 mm. Arrowheads: growth marks (annuli).

The parameters of the von Bertalanffy growth equation derived from the observed FLs-at-age were: FL_∞ = 121.5 cm; k = 0.40; t_0 = -0.09 (**Fig. 2**). The theoretical longevity was 8 years.

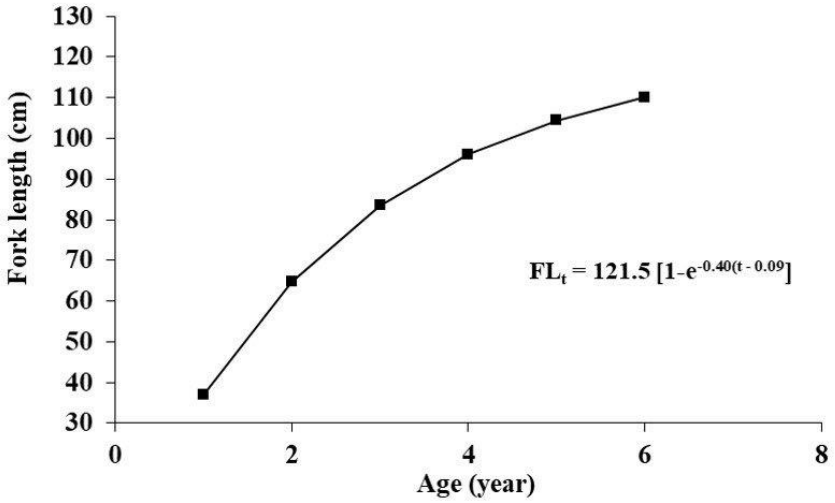

Figure 2. Von Bertalanffy growth curve. FL_t = predicted fork length at age t.

First sexual maturity

All the fish belonging to age class 2 showed only perinucleolar oocytes in their ovaries and were classified as immature. Among the fish belonging to age class 3, two had primary growth oocytes in their ovaries and were classified as reproductively inactive (immature) and one showed late vitellogenesis oocytes and it was classified as reproductively active (mature). Based on the above maturity results, the theoretical body length at median sexual maturity (L_{50}) calculated for female greater amberjack was 88.3 cm and 100% maturity was reached above 110 cm FL (**Fig. 3**).

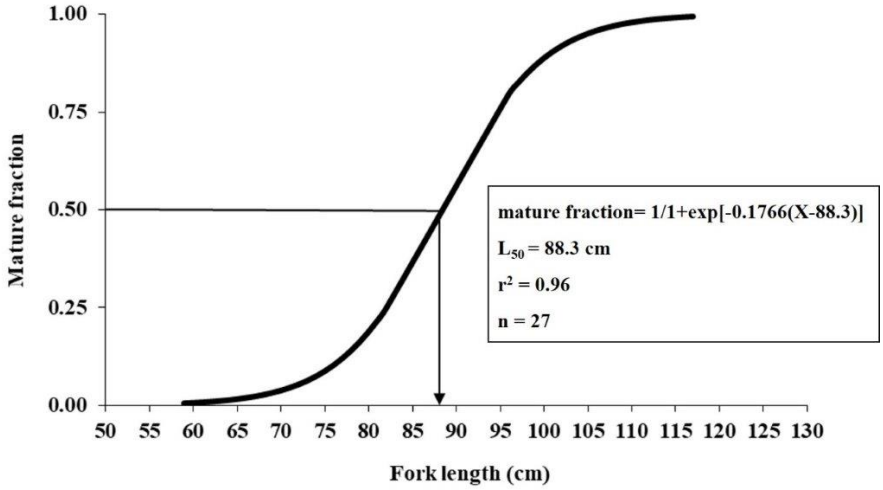

Figure 3. Fraction of mature female greater amberjack by fork length. Arrow indicates body length at median sexual maturity (L_{50}); n = sample size.

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

Morphological description of female germ cells

In the examined samples, oogonia along with the following oocyte developmental stages were observed: chromatin-nucleolus, perinucleolar, lipid, cortical alveoli, early vitellogenesis, late vitellogenesis (**Fig. 4**). Neither hydrated oocytes nor postovulatory follicles were found in any specimen. Different oocyte development stages were identified in the ovaries of captive-reared female greater amberjack.

Oogonia (**Fig. 4a**) (diameter 8-13 µm), often found in small clusters, were rounded cells with a large central euchromatic nucleus containing sparse eterochromatic patches.

Primary growth oocytes. Chromatin-nucleolus stage oocytes (**Fig. 4a**) (diameter 15-30 µm), had a slightly basophilic ooplasm and a large eccentric nucleus showing chromatin strands and sparse eterochromatic patches. Squamous follicular cells were associated to oocytes at this stage. Perinucleolar stage oocytes (**Fig. 4b**) (diameter 30-120 µm) were characterized by the presence of several nucleoli adjoining the nuclear envelope. Ooplasm basophily was strong in smaller perinucleolar oocytes and decreased with oocyte development. Flat follicular cells surrounded the oocytes at this stage. Oocytes at lipid/cortical alveoli stage (**Fig. 4c**) (diameter 120-200 µm) showed a further reduction of ooplasm basophily, small lipid droplets and the appearance of a thin zona radiata. Oocyte growth was associated to the increase of lipid globules, the appearance of cortical alveoli in the peripheral ooplasm and zona radiata thickening.

Secondary growth oocytes. Early vitellogenic oocytes (**Fig. 4d**) (diameter 200-400 µm) were characterized by the appearance of eosinophilic yolk globules in the peripheral ooplasm and a further increase of the zona radiata thickness. Follicular cells surrounding oocytes at this stage became cubic. In late vitellogenic oocytes (**Fig. 4e**) (diameter 400-550 µm) yolk granules progressively filled the entire ooplasm and the zona radiata further increased in thickness.

Alpha atretic vitellogenic follicles (**Fig. 4f**) displayed zona radiata fragmentation, coalescence of yolk globule and nucleus disintegration; in beta atretic follicles zona radiata and yolk globules were completely reabsorbed.

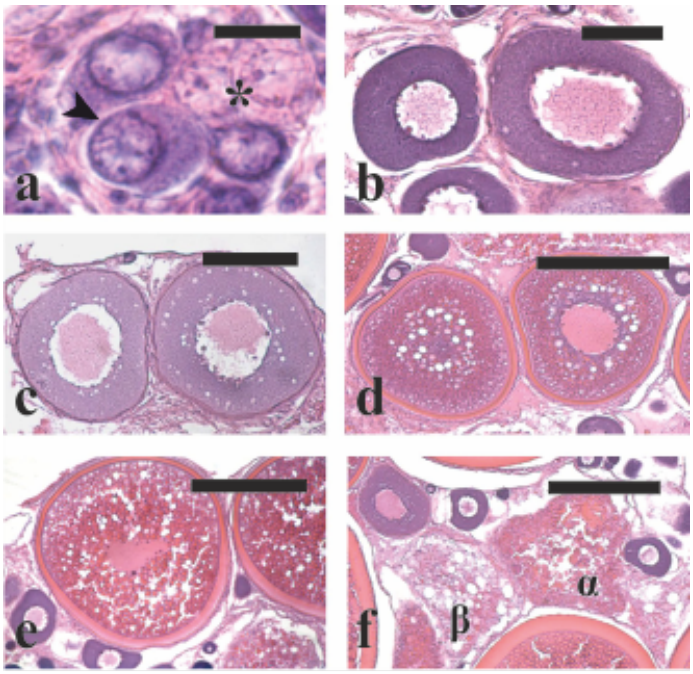

Figure 4. Micrographs of ovary sections from different captive-reared greater amberjack females sampled at Argosaronikos Fish Farm (Salamina Island, Greece) showing oogonia and oocytes in different developmental stages. a) Oogonia (asterisk) and chromatin-nucleolus stage oocytes (arrowhead). b) Perinucleolar stage oocytes. c) Cortical alveoli stage oocytes. d) Early vitellogenic oocytes. e) Late vitellogenic oocyte. f) α and β atretic vitellogenic follicles. Haematoxylin-eosin staining. Magnification bars = 10 µm in (a), 50 µm in (b), 100 µm in (c), and 200 µm in (d), (e), (f).

Reproductive cycle

Gonado-somatic index changed according to the oocyte maturation stage (**Fig. 5**). Increasing GSI values were found in individuals showing primary growth, early vitellogenesis and late vitellogenesis stage oocytes, respectively. The lowest GSI values were observed in spent fish sampled in July.

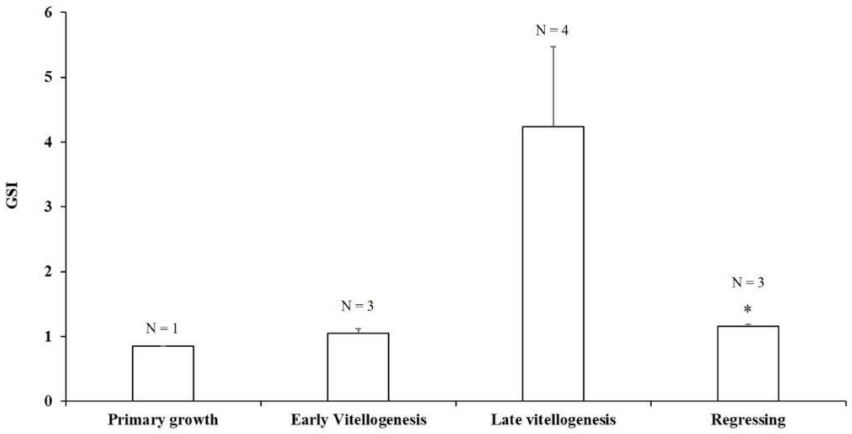

Figure 5. Mean (±SE) gonado-somatic index (GSI) of captive-reared female greater amberjack sampled at Argosaronikos Fish Farm (Salamina Island, Greece). Asterisks indicate statistically significant differences versus the preceding stage ($P < 0.05$).

Among the four greater amberjack specimens sampled during the EARLY phase (late April), one had ovaries with primary growth oocytes and three showed few early vitellogenic oocytes. In the ADVANCED phase, the four sampled specimens had oocytes at late vitellogenesis and three of them displayed major α atresia (> 50% of vitellogenic oocytes were in α atresia). In the SPAWNING phase, among the four sampled fish, three showed ovaries with late vitellogenic oocytes undergoing extensive atresia and one showed only perinucleolar oocytes, indicating that all these animals were in a regressed condition.

Pituitary and plasma concentrations of gonadotropins

Pituitary FSH content did not vary significantly ($P > 0.05$) in captive reared greater amberjack females during all the examined reproductive phases (**Table 3**). Likewise, circulating FSH levels, were steady and relatively low in females at early and late vitellogenesis, however, significantly ($P < 0.05$) increased in the regressing females. Although not statistically significant, the pituitary FSHβ transcript levels exhibited an opposed trend; maximal and minimal levels coinciding with early vitellogenic and regressing females, respectively.

The pituitary LH content gradually increased reaching its maximum (53.91 ± 9.36 ng/pituitary/kg BW) in the regressing females (**Table 3**). The circulating LH levels were steady and relatively low during early and late vitellogenesis. Maximal circulating LH levels were detected in regressing females (63.7 ± 44.8 ng/ml). Nonetheless, the latter result lacks statistical significance due to markedly high variability within the specific sampling group. Thus, the plasma LH levels in the regressing females ranged between 10 to 200 ng/ml. The pituitary LHβ gene expression did not vary significantly during the examined reproductive phases.

Sex steroid plasma levels

Sex steroids plasma concentrations of captive-reared greater amberjack females are reported in **Table 3**.

Testosterone and E_2 plasma concentrations increased constantly from primary oocyte growth to late vitellogenesis stage and then significantly decreased in specimens having ovaries in regressed conditions. A constant increase was shown by 17,20β-P from primary oocyte growth to the regression phase.

Vitellogenin plasma concentration and oocyte yolk accumulation

A statistically significant increase ($P < 0.05$) of vitellogenin plasma concentration was observed from early to late vitellogenesis stage, followed by a significant decrease ($P < 0.05$) in females with regressed ovaries (**Table 3**).

The surface occupied by yolk granules did not differ between captive-reared and wild specimens, neither in early vitellogenic nor in late vitellogenic follicles (**Table 4**).

Table 3. Intra-pituitary gonadotropin concentrations and plasma concentrations of sex steroids, vitellogenin and leptin in female greater amberjack reared in captivity at Argosaronikos Fish Farm (Salamina Island, Greece).

	Ovarian development stage			
	Primary growth (n = 1)	**Early Vitellogenesis (n = 3)**	**Late vitellogenesis (n = 4)**	**Regressing (n = 4)**
Pituitary FSH (ng/pituitary/kg BW)	8.8	6.4 ± 2.1	6.8 ± 2.5	9.1 ± 1.7
Pituitary LH (ng/pituitary/kg BW)	24.8	19.8 ± 13.8	36.8 ± 12.1	53.9 ± 9.4*
Plasma FSH (ng ml^{-1})	7.38	7.3 ± 1.3	8.3 ± 0.9	14.9 ± 2.3*
Plasma LH (ng ml^{-1})	49.1	36.1 ± 23.4	12.5 ± 3.3	63.7 ± 44.8
Pituitary FSHβ mRNA	7.8	10.6 ± 6.6	5.3 ± 2.9	1.8 ± 0.4
Pituitary LHβ mRNA	5.9	5.13 ± 2.7	3.6 ± 1.5	3.1 ± 1.1
Plasma T (ng ml^{-1})	0.2	0.3 ± 0.1	0.7 ± 0.2	0.2 ± 0.1*
Plasma E_2 (ng ml^{-1})	0.5	0.7 ± 0.1	1.9 ± 0.5	0.4 ± 0.2*
17,20β-P (ng ml^{-1})	0.1	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1*
Plama vitellogenin (mg ml^{-1})	2.5	2.4 ± 0.1	4.3 ± 0.1*	2.7 ± 0.2*
Plasma Leptin	ND	ND	ND	ND
Liver leptin mRNA	21.71	27.82 ± 17.90	2.34 ± 0.86	75.79 ± 32.80*

FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone; E_2, 17β-estradiol; 17,20β-P, 17,20β-dihydroxypren-4-en-3-one. ND, not determined. Asterisks = statistically significant difference versus the previous ovarian development stage ($P < 0.05$).

Table 4 Diameter of early and late vitellogenic oocytes, and surface occupied by yolk globules in wild and captive-reared greater amberjack.

Oocyte stage	Fish condition	Oocyte diameter (µm)	Yolk surface (µm²)
Early vitellogenesis	Wild	362.5 ± 3.5	55,580 ± 1,513
	Captive-reared	356.5 ± 6.9	55,761 ± 3,238
Late vitellogenesis	Wild	453.7 ± 3.5	84,660 ± 1,368
	Captive-reared	453.0 ± 9.3	90,791 ± 3,650

No statistical significance existed in oocyte diameter and yolk surface between captive-reared and wild specimens (P>0.05).

Nutritional state

The body condition of captive-reared greater amberjack, expressed as Fulton's condition factor, was not significantly different from that of wild females of the same age class (**Fig. 6**).

Leptin plasma concentration and liver gene expression

The circulating levels of leptin could not be detected due to technical problems, which hampered the establishment of the related ELISA. At the gene expression levels, liver leptin transcripts reach their minimum in females undergoing late vitellogenesis and climax in the regressing females (Table 3).

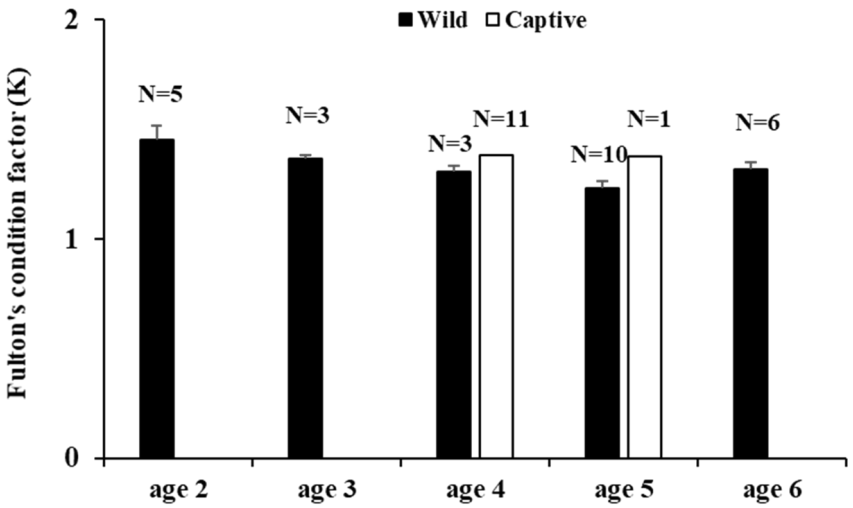

Figure 6. Body condition index calculated by using the Fulton's condition factor (K) in wild and captive-reared female greater amberjack. No statistical difference was found between captive-reared and wild individuals of the same age (P > 0.05).

Egg composition

Total contents of carotenoids, moisture and lipids from eggs of captive-reared females are given in **Table 5**. It also contains percentages of lipid classes with total neutral lipid classes (TNL) being particularly abundant, mainly sterol esters (SE) and triacylglycerides (TAG). Among total polar lipids (TPL) phosphatidylcholine (PC) followed by phosphatidylethanolamine are the most relevant lipid classes. In terms of the fatty acid profile of total lipids (TL) (**Table 6**), monounsaturates (MUFA; mainly 18:1n-9) followed by n-3 HUFA fatty acids, with a triplicate DHA (22:6n-3) content compared to EPA (20:5n-3), stand out for their abundance. Finally saturated fatty acids, whose main representative is 16:0 are also prominent in eggs. At the individual level, 18: 2n-6 is also particularly high.

When this profile is compared with that of polar lipids (TPL), it is observed that the presence of saturates is enhanced, and that n-3 HUFA, which becomes the most abundant group, and particularly the DHA content in this lipid fraction is also enhanced in a particular way. Arachidonic acid (20: 4n-6) also evidences this increase in the eggs polar lipids.

Table 5. Total carotenoid, moisture, total lipid content and main lipid classes of eggs from captive-reared greater amberjack sampled at Argosaronikos Fish farming S.A. (Salamina Island, Greece).

Carotenoid content (ppm)	2.03 ± 1.01
Moisture (%)	90.25 ± 0.74
Lipid content (% DM)	19.37 ± 2.66
Lipid clases (% total lipid)	
PC	13.98 ± 0.22
PS	0.41 ± 0.26
PI	1.31 ± 0.03
PE	4.29 ± 0.53
TPL	21.48 ± 0.70
Chol	8.97 ± 0.83
TAG	26.26 ± 1.40
SE	41. 21 ± 0.35
TNL	78.52 ± 0.70

Results are expressed as mean ± SD (n=3). DM, dry matter; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TPL, total polar lipids; Chol, cholesterol; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

Table 6. Main fatty acid composition of total lipids (TL) and total polar lipids (TPL) of eggs from captive-reared greater amberjack sampled at Argosaronikos Fishfarming S.A. (Salamina Island, Greece).

	TL	TPL
Total FA (μg mg egg^{-1} DM)	130.10±12.86	
14:0	1.49 ± 0.16	0.65 ± 0.06
16:0	15.69 ± 0.20	22.00 ± 0.84
18:0	4.26 ± 0.17	8.46 ± 0.24
Total SFA	22.08 ± 0.01	31.80 ± 1.02
16:1[1]	5.45 ± 0.34	2.56 ± 0.37
18:1[2]	29.47 ± 1.10	13.82 ± 0.25
20:1[2]	0.75 ± 0.06	0.57 ± 0.03
Total MUFA	35.67 ± 0.74	16.94 ± 0.22
18:2n-6	10.18 ± 0.52	4.44 ± 0.19
20:4n-6	1.38 ± 0.09	2.81 ± 0.09
Total n-6 PUFA	12.57 ± 0.33	8.22 ± 0.05
18:3n-3	1.35 ± 0.06	0.50 ± 0.02
18:4n-3	0.50 ± 0.04	0.19 ± 0.03
20:4n-3	0.47 ± 0.03	0.28 ± 0.01
20:5n-3	5.16 ± 0.62	7.59 ± 0.55
22:5n-3	1.74 ± 0.32	2.36 ± 0.13
22:6n-3	17.93 ± 0.28	29.44 ± 0.67
Total n-3 PUFA	27.34 ± 1.04	40.36 ± 1.08
Total n-3 HUFA	25.50 ± 1.02	39.68 ± 1.05
n-3/n-6	2.18 ± 0.14	4.91 ± 0.15
DHA/EPA	3.50 ± 0.37	3.89 ± 0.27
ARA/EPA	0.27 ± 0.02	0.37 ± 0.02

Results are expressed as mean ± SD (n=3). [1], mainly n-7 isomer; [2], mainly n-9 isomer. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Discussion and Conclusions

The age analysis, carried out through the examination of the scales, showed that the 13 juvenile greater amberjack specimens sampled for the study of age, growth and sexual maturity were 1 to 3 years old. The age of 19 wild adult females had been previously estimated (D3.3) and ranged between 4 and 6 years. The parameters of the von Bertalanffy equation, calculated using all the 13 juveniles and 19 adult females, indicate a theoretical maximum length of 121.5 cm FL and a theoretical longevity of 8 years. The theoretical length-at-age data, calculated applying the von Bertalanffy equation, confirm a very rapid growth of female greater amberjack during the first years of age: 37 cm FL at age 1; 65 cm FL at age 2; 84 cm FL at age 3,

corresponding to a theoretical body weight of 0.7 kg, 3.2 kg and 6.3 kg, respectively [(weight-at-age calculations made using the total length(TL)-weight relationship provided by Kožul et al. (2001b) 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 somehow contradictory (Marino et al., 1995a, b; Micale et al., 1999; Kožul et al., 2001a; Sley et al., 2014). In the present study, although based on a limited number of specimens, we found that the median at first sexual maturity (L_{50}) of female greater amberjack is 88 con FL and all individuals larger than 110 cm FL are reproductively active. In term of age, our data indicate that female greater amberjack start to reproduce at the age of 3 years and the whole population is sexually mature by the age of 5 years. These data are in overall agreement with Marino et al. (1995a) and Kožul et al. 2001a) that reported that 12.5-40%, 80-85% and 100% female greater amberjack are reproductively active at the age of 3, 4 and 5 years, respectively.

Reproductive dysfunctions have been documented in a number of captive-reared fish species, in both females and males. The most common dysfunctions in females are: absence of gonadal development (Tesch, 2003); failure of oocytes to undergo oocyte maturation (also referred to as final oocyte maturation) once vitellogenesis is completed (Tucker, 1994; Mylonas et al., 1997; Corriero et al., 2007); or absence of spawning (Bromage et al., 1992). Atresia of vitellogenic oocyte and failure to undergo oocyte maturation have been attributed to an insufficient pituitary luteinizing hormone (LH) release, and to the consequent steroid withdrawal, occurring in captive conditions (Mylonas & Zohar, 2001; Rosenfeld et al., 2012). These dysfunctions have been associated to a combination of factors, such as captivity-induced stress, lack of appropriate spawning environment and nutritional deficiencies [an exhaustive review on reproductive dysfunctions, their causes and therapeutic treatments is provided by Zohar and Mylonas (2001) and Mylonas et al. (2010)].

One of the main aims of the present work was to describe the reproductive cycle and to identify possible reproductive dysfunctions in greater amberjack reared in captivity. The study of the reproductive cycle of captive-reared greater amberjack females showed that in late April ovaries were in an overall maturity stage comparable to that of wild individuals sampled in the same period (cfr. D3.3 and Zupa et al., 20017b), having oocytes in primary growth or in early stage of vitellogenesis. However, the subsequent gonad maturation phase appeared to be seriously impaired in captivity, since, at the time of the second sampling campaign (early June), an extensive atresia of late vitellogenic oocytes affected the ovaries. In early July, when wild greater amberjack population was still in spawning condition (see D3.3 and Zupa et al., 2017b), all the four reared fish had ceased their reproductive activity. The histological changes observed in the ovaries of captive-reared greater amberjack during the three sampling periods were well described by GSI that increased from late April to early June and decreased in early July.

The histological and biometric (GSI) data on gonad maturation of captive reared greater amberjack were in close agreement with the trend of sex steroid plasma concentrations. In the present study, all the three analyzed steroids showed an increase from early to late vitellogenesis, which is typical of an asynchronous spawner such as the greater amberjack (Rinchard et al., 1993; García-López et al., 2006; Vazirzadeh et al., 2014). In greater amberjack reared in captivity, both T and E_2 showed a similar trend to that of the wild population; however, the two steroids were lower in captive-reared fish in all the examined phases (cfr. D3.3 and Zupa et al., 2017b). The gonadotropin analyses further attest this notion. Relatively low pituitary FSH and LH content and consequently reduced levels of these hormones in the circulation of captive-reared greater amberjack during vitellogenesis and final oocyte maturation, compared to equivalent levels measured in wild fish, appears to distract the formers from exploiting their maximal reproductive potential.

Altogether, the comparative analysis of GSI, histological observations, pituitary and circulating gonadotropins and sex steroid plasma levels in the present study indicate a severe adverse effect of confinement in captivity on greater amberjack reproductive axis, with consequent gametogenesis impairment. The negative effects of confinement were glaring in fish with oocytes in late vitellogenesis stage, perhaps because the fish sampled at this stage had already been manipulated once, as they were kept

together in the same sea cage, and resulted in an extensive oocyte atresia that prevented any further oocyte development.

In teleost fish, as well as in other oviparous animals, the process of vitellogenesis consists of an ordered sequence of events. These include the liver synthesis of different vitellogenins (egg yolk precursor proteins, Vtgs) under estrogen stimulation (Ng & Idler 1983; Susca et al., 2001; Sawaguchi et al., 2006), their release in the bloodstream as homodimers lipoprotein complex (Finn, 2007), their uptake by developing oocytes through endocytosis mediated by receptors belonging to the low density lipoprotein receptor (LDLR) family (Stifani et al. 1990), and their final cleavage into egg yolk proteins that are accumulated in yolk globules or platelets (Wallace & Selman, 1990). Yolk content is an important determinant of egg and larval quality in fish, as it represents the major nutrient for the developing embryo/larva during the first days of endogenous feeding (Brooks et al., 1997; Bobe & Labbé, 2010). Multiple vitellogenins have been described in a number of fish species including the white perch *Morone americana* (Gmelin, 1789) (Schilling et al., 2014), the Atlantic bluefin tuna *Thunnus thynnus* (Pousis et al., 2011), the grey mullet *Mugil cephalus* (Amano et al., 2008), the zebrafish *Danio rerio* (Wangn et al., 2005) and the red seabream *Pagrus major* (Sawaguchi et al., 2006).

In our previous study (D3.3 and Pousis et al., 2017), the expression of three genes encoding for vitellogenins (VtgA, VtgB and VtgC) was compared between wild and captive-reared greater amberjack and no effect on Vtgs gene expression could be ascribed to the rearing conditions. In the present study, Vtg plasma concentrations were found to be in the normal range of the wild population during the reproductive season (Mandich et al., 2004) and no difference was found in the amount of yolk accumulated in oocytes of wild and captive-reared greater amberjack. Altogether, the biomolecular, biochemical and histological data on vitellogenin gene expression, vitellogenin synthesis and vitellogenin uptake (yolk accumulation) by developing oocytes exclude any effect of the rearing condition on the vitellogenic process.

In captive-reared greater amberjack, plasma concentration of 17, 20β-P, which is known to be essential for oocyte maturation in different fish species, was about half than that of wild specimens during the late vitellogenesis stage (cfr. D3.3 and Zupa et al., 2017b). Therefore, it is possible that the observed extensive atresia affecting oocytes in late vitellogenesis in greater amberjack reared in captivity, was related to the low 17, 20β-P plasma concentration.

The efficacy of dietary ingredients for broodstock has been ascribed to its superior protein quality, as well as its higher phospholipid and cholesterol content. The importance of PC, PE and n-3 HUFA, and DHA/EPA/ARA ratios on gonad development, and egg quality (fecundity, hatching and larvae survival rates), has also been highlighted by many authors (Fernández-Palacios et al., 1995; Navas et al., 1997; Rodríguez et al., 1998; Almansa et al., 1999; Bruce et al., 1999; Olsen et al., 2014; Li & Olsen, 2015). In fact, two thirds of the lipid fraction in Vtg is made of phospahatidylcholine (PC) (Sargent, 1995) that is also the main phospholipid in mature ovaries and fertilised eggs (Watanabe & Vasallo-Agius, 2003). Dietary fatty acids have also proven to be particularly important in the reproduction of several species, since they determine gonad composition and function, affecting sperm and egg quality (Izquierdo et al., 2001; Tocher, 2010, Zupa et al., 2017a).

The importance of a high dietary input of DHA and DHA/EPA ratios of 2-3/1 have been also pointed out in previous studies (Rodríguez et al., 1997; Sorgeloos et al., 2001) as a limiting factor for marine larval performance. In fact, the increase in dietary DHA and phospholipids effectively improves stress tolerance of red sea bream (*Pagrus major*) and marbled sole (*Limanda yokohamae*) (Kanazawa, 1997) or red porgy (*Pagrus pagrus*) (Roo et al., 2009). This fact has been correlated with a higher efficiency in oxygen transport and better gill membrane fluidity properties, to recover normal oxygen levels after the stress test in those larvae fed with high levels of DHA which in turn showed a higher DHA content on these tissues. Thus, Sargent et al. (1999) reported the existence of tissue specific fatty acid compositions in its polar lipid fraction, probably related to its biological function, e.g., higher concentration of 22:6n-3 in neural and visual tissues. On the contrary, total fatty acid compositions including neutral and free fatty acid contents are mostly determined by the levels of fatty acids available from the diet. Therefore, not only total DHA content but also its lipid form should be considered to identify HUFA requirements. In this sense, Olsen et al. (2014)

found that cod larvae fed with rotifers with a high dietary n-3 HUFA content, particularly DHA, provided within the phospholipids fraction were more efficiently incorporated to larval tissues, than those supplied as triacylglicerides (TAG) resulting in a higher larval growth and survival.

Carotenoids including astaxanthin, are also widely present in fish gonads and eggs. They are precursors of vitamin A and are involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Miki, 1991; Guerin et al., 2003). Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. There is evidence to suggest that the presence of carotenoids mitigate deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. Specifically, carotenoids are found to be a determining factor for good egg quality in seriola (Watanabe et al., 2003). Carotenoids may perform a biological role similar to that of α-tocopherol (as a naturally occuring antioxidant), protecting tissues and reactive compounds from oxidative damage hence its importance in preventing LC-HUFA peroxidation (Guerin et al., 2003).

In the present study, egg biochemical analysis demonstrated that the dietary regime of captive fish (Vitali-Cal, Skretting), covers even in excess the amount of carotenoids found in wild specimens. In addition taking into account that viable greater amberjack wild eggs have around 17% of total lipid (TL) in DM: 30% TG and 20% PL, with 26% of DHA and 5% of EPA in both TL and PL, and 3 and 4% of AA in TL and PL, respectively (Rodríguez-Barreto et al., 2014), it can be concluded that except for the high contents of 18:2n-6 and the lower levels of 20:4n-6, the dietary regime and the resultant eggs were quite similar in terms of total polar lipids, EPA and DHA levels and ratios, to those present in the wild counterparts (Rodríguez-Barreto et al., 2014).

In conclusion, the present study demonstrated that rearing in captivity can affect oogenesis in greater amberjack, resulting in extensive oocyte atresia of vitellogenic process. The observed oogenesis impairment was related to a malfunctioning of the reproductive axis, which involved low levels of both, FSH and to a greater extent, LH and as a result low plasma steroid concentrations, particularly 17, 20β-P, the hormone responsible for oocyte maturation and spawning. However, another broodstock of the same source and age was maintained under identical conditions in the same facility for the execution of task 3.2 and, during June, it reached advanced stages of gametogenesis to be able to be induced to spawn and produce fertilized eggs whose quality, in terms of total polar lipids, EPA and DHA levels and ratios, were equivalent to those found in wild specimens. We suppose that the repeated sampling operations in the rearing cage might have played a major role in the observed reproductive dysfunction, thus underlying the extreme susceptibility of this species to the handling stress and the need for a careful management of greater amberjack broodstocks.

References

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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 they 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).

Also, during the gonadal development there is an important mobilization of nutrients, mainly protein, lipids, with specific lipid classes and fatty acids, and carotenoids, from the liver and the muscle to the gonads and towards the eggs. Therefore, the analysis of the proximal composition (protein, fat content, moisture and ash), and also the complete profile of lipid classes and fatty acids and total contents of carotenoids of the available samples of gonads and eggs was prioritized. There was not enough sample left for the foreseen determinations of vitamins C and E.

Co-funded by the Seventh Framework Programme of the European Union