Deliverable Report

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Table of Contents

Objective 2
Description 2
Background 2
Materials and Methods 5
Results 12
Discussion and Conclusions 31
References 34
Deviations: 39

Objective

Identification of possible reproductive dysfunction of gametogenesis of greater amberjack *Seriola dumerili* reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating and apoptotic germ cells, vitellogenin accumulation, yolk content in the oocytes and nutritional status. Reproductive maturation has been reported to be unreliable in greater amberjack reared in captivity. This deliverable characterized the reproductive dysfunction by comparing gametogenesis between wild and captive amberjack using methodologies to (a) assess the influence of captivity on spermatogenesis by looking at germ cell proliferation and apoptosis, and (b) evaluate the effects of captivity on vitellogenesis by monitoring liver vitellogenin (Vg) and vitellogenin receptor (VgR) genes expression, as well as oocyte yolk accumulation. Moreover the deliverable reports on the role of a key hormone related to nutritional status (e.g. leptin) in order to get information on captive fish nutritional status and, as a consequence, to get gametes, embryo and larvae of very high quality.

Description

It is well known that many fish species exhibit different reproductive dysfunctions when reared in captivity (Mylonas et al., 2010). These reproductive dysfunctions can be ascribed to an impairment of the reproductive axis (brain-pituitary-gonad axis, BPG), which probably results from the combination of captivity-induced stress (Sumpter et al., 1994), as well as the lack of appropriate 'natural' spawning conditions (Zohar, 1989).

The lack of a reliable reproduction has been acknowledged as one of the bottlenecks for the establishment of greater amberjack aquaculture industry in Europe. Although greater amberjack reproduction in captivity has been achieved after hormonal treatments (Mylonas et al., 2004) and the occurrence of spontaneous spawning has been reported (Jerez et al., 2006), a reliable technology for reproduction control in aquaculture has not been achieved for this species. The present deliverable describes the occurrence of reproductive dysfunctions in greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of male germ cell proliferation and apoptosis, liver Vg gene expression and oocyte yolk accumulation. Moreover, by comparing expression profiles of the gene encoding for the metabolic hormone leptin and gonad biochemical composition in captive-reared *vs*. wild individuals along the reproductive cycle, the current research efforts will be also directed to understand the fine-tuned interplay between nutritional status and reproductive function.

Background

Vitellogenesis

Vitellogenin is a high-density serum glycolipophosphoprotein synthesized by the liver under the stimulation of 17ß-estradiol (E2) produced by the developing ovary (Kwon et al., 1993; Ng &Idler, 1983; Sumpter, 1995; Susca et al., 2001). Vitellogenin is transported via the bloodstream from the liver into the follicular layer and endocytosed by the growing oocytes, in order to form the yolk proteins (Babin et al., 2007; Lubzens et al., 2010; Sargent, 1995; Tyler et al., 1988; Wallace, 1985). 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 (Bobe & Labbé, 2009; Brooks, 1997).

Evidence of the existence of multiple Vg genes in many different teleost species has been obtained through molecular cloning of cDNAs encoding two distinct Vgs, named VgA (or VgI) and VgB (or VgII) (Babin et al., 2007; LaFleur et al., 1995a, 1995b, 2005; Sawaguchi et al., 2005, 2006; Pousis et al., 2011). Both VgA and VgB genes encode proteins that undergo proteolytic cleavage during oocyte maturation, in

order to generate a pool of free amino acids and lipids for oocyte hydration (Cerdá et al., 2007) and for allocation of specific types of nutrients to the developing embryo (Sawaguchi et al., 2006). A third Vg gene (VgC) and protein has also been identified in fish (Finn et al., 2009; Sawaguchi et al., 2005, 2006), but this Vg does not undergo proteolytic cleavage during oocyte maturation and probably gives rise to nutrients at a later stage of embryonic development (Sawaguchi et al., 2006).

In UNIBA (P.13) laboratories, all the three Vg forms from the Atlantic bluefin tuna *Thunnus thynnus* were sequenced, and no significant differences in Vgs mRNA levels between wild and captive-reared individuals were found (Pousis et al., 2011).

Vitellogenin is specifically incorporated in the oocytes by receptor-mediated endocytosis through receptors belonging to the Low Density Lipoprotein Receptor (LDLR) family, which have been named Very Low Density Lipoprotein Receptors (VLDLR), VgR or LR8, due to the presence of eight Ligand-binding Repeats (Bujo et al., 1994; Hiramatsu et al., 2004). In the last years, the structural characteristics of this gene family have been described in several teleosts (Agulleiro et al., 2007; Davail et al., 1998; Hiramatsu et al., 2004; Li et al., 2003).

In UNIBA laboratories, the two isoforms of the Atlantic bluefin tuna VgR (VgR^+ and VgR^-) were sequenced (Pousis et al., 2012). The two isoforms were generated by differential splicing of an exon called the O-linked sugar domain corresponding to a 20-amino acid sequence in the mature protein. VgR^- relative expression levels were determined in the ovary of juvenile and adult Atlantic bluefin tuna during the reproductive cycle. Relative amounts of VgR^- were greater in juvenile females and in those adults having only previtellogenic oocytes. During the ripening period, greater VgR^- gene expression was observed in wild fish in comparison to fish reared in captivity, possibly because of differences in water temperature exposure, energy storage or inadequate diet in reared Atlantic bluefin tuna.

Spermatogenesis

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). Apoptosis (or programmed cell death) of germ cells is an integral component of normal testicular function in mammals as well as in fish. Even though in mammals apoptosis occurs mainly during testicular regression (Young & Nelson, 2001), in large pelagic teleosts such as the swordfish *Xiphias gladius* and the Atlantic bluefin tuna this process takes place throughout the reproductive season together with germ cell proliferation, and both are maximal at the onset of spermatogenesis, therefore suggesting a role of apoptosis in the quantitative control of the germ cell population as well as in preventing the development of aberrant germ cells (Corriero et al., 2007a; Zupa et al., 2013).

Previous studies on Atlantic bluefin tuna males reared in captivity revealed these individuals had a low capacity to reach full testicular development and maturation, since their relative testicular mass (gonadosomatic index, GSI) was lower than that of wild adult fish (Corriero et al., 2009), and the histological analysis of testes showed smaller seminiferous lobules in captive specimens, whose lumen was only partially filled with spermatozoa (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 (Zupa et al., 2013). In captive-reared Atlantic bluefin tuna, gonadotropin-releasing hormone agonist (GnRHa) administration through sustained-release delivery system was effective in stimulating spermatogonial proliferation and reducing apoptotic germ cells (Corriero et al., 2009).

Nutritional status

Reproduction is a very complex process that can be affected/modulated by several factors such as genetic background, environmental conditions or nutrition. In this sense, and in order to assess the reproductive

potential of wild vs. captive amberjack broodstocks and identify possible reproductive/metabolic dysfunctions during gametogenesis, characterization of broodstock nutritional status is crucial.

To further improve reproductive potential of captive greater amberjack broodstocks, and to gain insight of how physiological processes dictate reproduction in this species, the current research effort is directed to understand the fine-tuned interplay between energy stores and reproductive function through the analysis of the metabolic hormone leptin and gonad biochemical composition (proximate and fatty acid composition as well as carotenoid contents).

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; Huising et al., 2006; Lee et al., 1996; Pelleymounter et al., 1995; Tartaglia et al., 1995; Zhang et al., 1994;). 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 (Fernandez-Fernandez et al., 2006; Rosenbaum & Leibel, 1998). To date, all studies with teleosts have identified the liver as the major site for leptin expression, in contrast to mammalian species, where leptin was found to be predominantly expressed in the adipose tissue. In the current research we cloned a partial sequence encoding leptin in the greater amberjack (Deliverable D3.1), and examined its temporal expression patterns in captive-reared vs. wild fish during their natural reproductive season in the Mediterranean Sea.

Comparisons of proximate and fatty acid compositions from tissues of wild fish and their cultivated counterparts can provide a good estimation of the suitability of the diet offered to the broodstock (Cejas et al., 2004; Grigorakis et al., 2002; Pérez et al., 2007; Rodríguez-Barreto et al., 2012, 2014, 2015). The efficacy of dietary ingredients for broodstock has been ascribed to its superior protein quality, as well as its higher phospholipid and cholesterol content. In fact, two thirds of the lipid fraction in Vg is made of phospahatidylcholine (PC) (Sargent, 1995) that is also the main phospholipid in mature ovaries and fertilised eggs (Watanabe & Vasallo-Agius, 2003). Similarly, sperm is particularly rich in phosphatidylserine (PS) and phosphatidylethanolamine (PE). Regarding nutrition, dietary fatty acids have also proven to be particularly important in the reproduction of several species, since they determine gonad composition and function, affecting not only sperm and egg quality (Izquierdo et al., 2001; Tocher, 2010), but also being involved in the synthesis of eicosanoids, autocrine mediators in the reproductive process (Henrotte et al., 2011; Mercure & Van Der Kraak, 1996; Patiño et al., 2003; Sorbera et al., 2001; Stacey & Sorensen, 2005). Considering the importance of lipids on breeder diet, several studies have been performed in greater amberjack by the research group involved in DIVERSIFY (P15. ULL), leading to the formulation of a broodstock diet that approximates the ovary lipid composition of cultured fish to that from wild specimens, with some improvement of reproduction success under captive conditions (Rodríguez-Barreto et al., 2012, 2014, 2015). Carotenoids including astaxanthin, are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Guerin et al., 2003; Miki, 1991). Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. There is evidence to suggest that the presence of carotenoids mitigates deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. There is also evidence that carotenoids protection against oxidative damage is directly related to sperm functional fertility.

Together with nutrition, the effect of temperature on the reproductive performance of cultured finfish is well documented (Portz et al., 2006). This and other factors such as fish stocking density and water volume also influence fish physiology and welfare (Conte, 2004; Ellis et al., 2002; Mylonas et al., 2010), and may affect gonads proximate composition, and reproductive fitness (Rodrígue-Barreto et al., 2015).

Materials and Methods

Biometric data and biological samples collection

A total of 33 (14 males and 19 females) wild adult greater amberjack were sampled during the fishing seasons 2014 and 2015 onboard a professional purse seine fishing vessel operating around the Pelagic Islands (Sicily, Italy). Based on previous experience on other large pelagic fish and on the available literature regarding greater amberjack reproductive cycle (Mandich et al., 2004), the sampling campaigns were carried out during three supposed periods of the reproductive cycle: early gametogenesis (EG) (early May); advanced gametogenesis (AG) (late May); spawning (SP) (late June). Soon after capture, from each fish biometric data (fork length, FL in cm; body mass, BM in kg; gonad mass, GM in g) were recorded (**Table 1**) and the following biological samples were taken: brain, pituitary, liver, muscle, gonad and hard parts for age determination (spines, vertebrae, otoliths and scales). The gonado-somatic index was calculated as $GSI = 100xGMxBM^{-1}$.

Wild greater amberjack that were caught and reared in captivity were used in the present study and will be referred to as captive-reared. The captive-reared greater amberjack used in the present study belonged to a broodstock captured in 2011 in the area of Astakos (Ionian Sea, Greece) and transferred to Argosaronikos Fish Farm (Salamina Island, Greece) (P23. ARGO) in September 2014. 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 eight fish (four males and four females) during each of three periods of the reproductive cycle, i.e. EG (late April), AG (early June), SP (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. After sex determination by means of gonad cannulation, a blood sample was collected from each fish. Fish were then placed on crushed ice and transferred to the farm facility where biometric data were recorded (**Table 2**) and the same biological samples as for wild fish were taken.

Table 1. Biometric data and estimated age of wild greater amberjack sampled around the Pelagic Islands (Italy).

Sampling Date	Sex	Fork length (FL, cm)	Body Mass (BM, kg)	Gonad Mass (GM, g)	Age (years)
Early gametogenesis period					
01/05/2015 (SST = 18.1 °C)	m	111	14	300	6
	m	112	20	450	6
	m	112	15	300	6
	m	117	19	550	6
	m	113	19	400	6
	f	103	14	100	5
	f	112	19	200	6
	f	116	20	300	6
	f	103	15	200	5
	f	106	13	100	5
Advanced gametogenesis period					
31/05/2014 (SST = 19.3 °C)	m	124	22	1900	7
	m	102	13	650	5
	m	115	19	2200	6
	m	99	14	1150	5
	f	117	22	1650	6
	f	114	21	1600	6
Spawning period					
29/06/2015 (SST = 23.8 °C)	m	100	12	650	5
	m	102	14	700	5
	m	104	16	950	5
	f	101	14	500	4
	f	114	19	1000	6
	f	109	16	700	6
30/06/2014 (SST = 23.4 °C)	m	100	11	400	5
	m	99	11	577	4
	f	99	11	500	5
	f	100	12	490	5
	f	97	12	450	5
	f	100	12	400	5
	f	98	12	500	4
	f	96	12	390	4
	f	102	13	600	5
	f	104	14	950	5
	f	95	12	450	5

Table 2. Biometric data and estimated age of adult captivity-reared greater amberjack from Argosaronikos Fish Farm (Salamina Island, Greece).

Sampling Date	Sex	Fork length (FL, cm)	Body Mass (BM, kg)	Gonad Mass (GM, g)	Age (years)
Early gametogenesis period					
	m	101	15	95	4
	m	94	12	60	4
	m	92	12	65	4
24/04/2015	m	94	13	60	4
(SST = 17.5 °C)	f	87	10	85	4
	f	97	14	155	4
	f	96	14	125	4
	f	100	14	160	4
Advanced gametogenesis period					
	m	90	9	370	4
	m	97	14	295	4
	m	98	13	600	4
04/06/2015	m	103	15	690	4
(SST = 20.0 °C)	f	97	13	335	4
	f	97	13	920	4
	f	106	17	305	5
	f	101	12	660	4
Spawning period					
	m	96	13	140	4
	m	95	11	155	4
	m	91	10	70	4
02/07/2015	m	96	12	130	4
(SST = 25.5 °C)	f	92	8	95	4
	f	96	12	130	4
	f	95	11	135	4
	f	97	12	140	4

Age determination of wild and captive greater amberjack

For the age estimate of wild and captive-reared greater amberjack, different hard parts were tested: the first spiniform ray of the first dorsal fin (spine); caudal vertebrae; scales; otoliths (sagittae). A first screening was addressed to the identification of the most suitable structure for age determination purpose, i.e. the structure(s) showing clear alternation of bands of different optical density interpreted as periodical events (Meunier & Panfili, 2002). The spine proved to be unsuitable for age estimate because of the absence of any growth band, whereas clearly distinguishable growth bands were observed in all the other examined structures, as showed in **Figures 1, 2 and 3**.

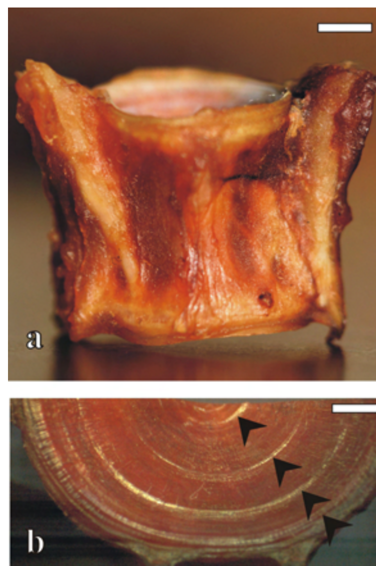

Figure 1. Images of a caudal vertebra from a 94-cm-FL greater amberjack sampled on 24 April 2015 in Argosaronikos Fish Farm (Salamina Island, Greece). (a) Dorsal view of the vertebra. (b) Cranial view of a longitudinal section of the vertebra showing growth marks (annuli) (arrowheads). Alizarin red S staining. Bar = 5 mm in (a) and 2.5 mm in (b).

Figure 2. Images of greater amberjack sagittae. (a) Lateral view of a sagitta from a 94-cm-FL specimen sampled on 24 April 2015 in Argosaronikos Fish Farm (Salamina Island, Greece). (b) Micrograph of a sectioned sagitta from a 124-cm-FL fish captured on 31 May 2015 around Pelagic Islands (Italy). (c) Particular of the rostrum of (b). Magnification bar = 3 mm in (a), 1 mm in (b) and 300 μm in (c). Arrowhead, annuli; a, antirostrum; p, postrostrum; r, rostum.

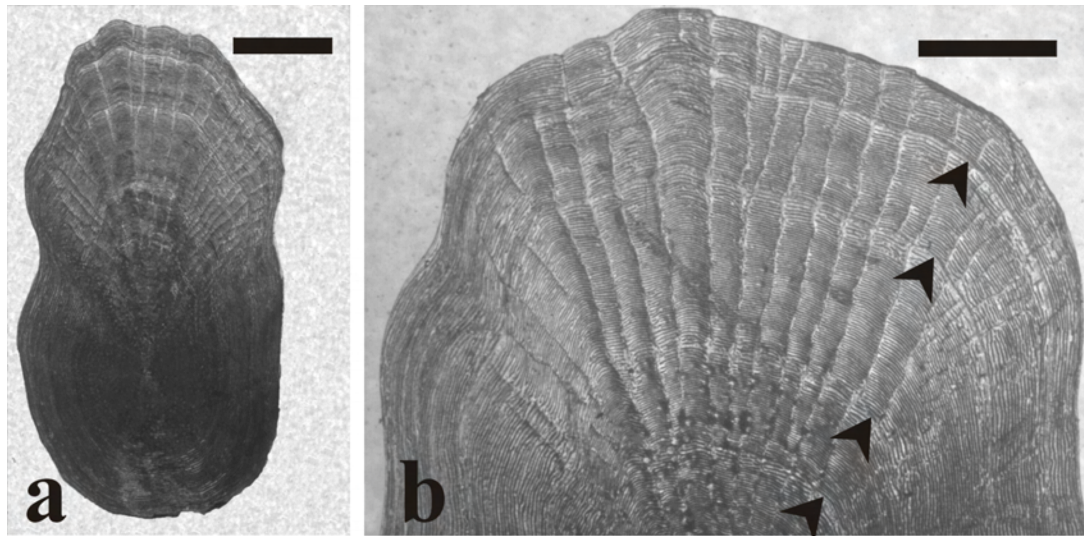

Figure 3. Images of greater amberjack scales. (a) Scale from a 95 cm specimen sampled on 24 April 2015 in Argosaronikos Fish Farm (Salamina Island, Greece). (b) Particular of (a) showing growth marks (annuli) (arrowheads). Magnification bar = 2 mm in (a) and 1 mm in (b).

Scales, cycloid and oblong in this species, proved to be the easiest hard parts to be processed and read and therefore greater amberjack age was estimated using only these structures, which were treated according to the following protocol: the 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 (McCurdy et al., 2002). The scales 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 its scales (Meunier, 2002).

Histological analysis of greater amberjack reproductive state and oocyte yolk accumulation

Greater amberjack reproductive state was assessed by means of light microscope observation of gonad histological sections. For this purpose, 1-cm thick gonad slices were cut and 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 Mallory’s trichrome.

The reproductive state of females was assessed by recording the most advanced oocyte stage for each specimen, according to the classification reported by Corriero et al. (2007b) for the Atlantic bluefin tuna. The presence of postovulatory and atretic follicles was also recorded.

For the classification of the reproductive state of males, the type of spermatogenic cysts was recorded, and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively, as reported by Corriero et al. (2007b).

In order to compare oocyte yolk accumulation in wild and captive-reared individuals, oocytes at early and late stage of vitellogenesis, having a large and centrally located nucleus were 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).

Proliferating and apoptotic germ cells detection

The identification of proliferating germ cells was performed on testicular samples from all the greater amberjack wild and captive-reared males sampled 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.

Detection of apoptotic germ cells was done on the same male specimens as above, 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, the sections, after their re-hydration through graded ethanol solutions, 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.

Seminiferous lobule diameter and quantification of germ cell proliferation and apoptosis

At least 50 seminiferous lobules were selected and measured randomly from the same testicular section used for germ cell proliferation and apoptosis analyses. 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 by using the same digital camera, light microscope as well as image analysis software used for oocyte yolk accumulation.

Liver vitellogenins, vitellogenin receptor and leptin real-time PCR

Once the greater amberjack homologous sequences were obtained (D3.1), Vgs (VgA, VgB and VgC), VgR, leptin (Lep) and β-actin specific primers were designed (**Table 3**) using the Primer3 software (Rozen & Skaletsky, 2000) and their specificity was checked with both in silico (the UCSC "In-Silico PCR" and the NCBI Primer-BLAST tools) and by means of agarose gel electrophoresis. It was confirmed that the VgA primer set did not produce any amplified fragments using as a template VgB or VgC cDNA, and vice versa. Total RNA was obtained from pituitary and liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Reverse transcription of 1000 ng of total RNA was performed using SuperScript III Reverse

Transcriptase (Invitrogen®) and diluted cDNA (1:10) was used in all following qPCR reactions. For gene expression analysis qRT-PCR experiments were carried out in triplicate using the QuantStudio™ 7 Flex System (Applied Biosystems®, Thermo Fisher SCIENTIFIC) using 1µl of diluted cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). No template controls were included as negative controls for each primer pair. The quantification of the β-actin gene was used as the endogenous control. Amplification parameters were as follows: hot start at 95°C for 15 min; 40 amplification cycles (95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec); dissociation curve step (95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec). Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied Biosystems®, Thermo Fisher SCIENTIFIC) and analyzed with the DART-PCR Excel workbook (Peirson et al., 2003). Actual amplification efficiency values (E) for each amplicon were used to correct Cq values before analyzing these data by the ΔCq method to compare relative expression results. Gene expression levels were calculated by: relative expression = $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001).

Table 3. Primers for greater amberjack real-time PCR

VGA FOR	GCAGCTTGAGACTGAGATCAG
VGA REV	GGTACAGAAACAGGCAGAGCT
VgB FOR	CAGCTGCTGGACCAGTCATC
VgB REV	CAGGAACCAAGATATTCTTGAGT
VgC FOR	GAGCCAGAATGTGCGCTGAG
VgC REV	GCGTGTGCTCATCGGATGTC
VgR FOR	GAGGACGAGGTCAACTGTGG
VgR REV	CCTGGCAGTCAACATCGTCG
Lep FOR	CCGTTAAGGGTGTCAGAGA
Lep REV	TTCCAGGTCCCTGTTGGTC
β-actin FOR	CCCTGTCCTGCTCACAGAGG
β-actin rev	CAAGTCCAGACGCAGGATGG

Gonads proximate and fatty acid composition, and carotenoid contents

To evaluate broodstock nutritional status, pieces of gonad from captive and wild greater amberjack, representative of all the reproductive stages, were immediately frozen and kept at -80ºC, until analysis. Dry matter, and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC, 2012). Moisture contents were determined in approximately 500 mg samples by thermal drying in an oven at 110°C, until constant weight. Protein was determined by sample digestion according to Kjeldahl method. Total lipid (TL) was extracted from the tissues and diet 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 to 200°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 only from gonads at advanced gametogenesis and spawning periods, 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

Mean values of (i) age; (ii) GSI, (iii) oocyte surface occupied by yolk granules, (iv) liver VgA, VgB and VgC as well as ovarian VgR gene expressions, (v) diameter of seminiferous lobules, (vi) density of anti-PCNA positive spermatogonia and anti-PCNA positive spermatocysts, (vii) surface occupied by apoptotic germ cells, were compared between wild and captive greater amberjack individuals by using one-way ANOVA ($P \leq 0.05$). Results are presented as means ± SE.

Leptin qRT-PCR data were analyzed using the JMP IN ® 8 statistical software (SAS Institute Inc., Cary, NC) for Windows 7.0 statistical package. One-Way ANOVA was employed to compare mean values ($P<0.05$). Unless otherwise indicated, all means are presented as mean values ± SE.

Gonad biochemical analyses are reported as means ± standard deviation (SD). All values presented as percentage were arcsine transformed. Normal distribution was checked for all data with the one-sample Kolmogorov–Smirnoff test and homogeneity of the variances with the Levene test. Differences between pairs of means were tested using Student's t-test. To compare more than two means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal-Wallis test was accomplished. The significant level for all the analysis was set at $P<0.05$. All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com).

Results

Age estimate

Individual estimated age is reported in **Table** 1 and **Table** 2. Mean age of wild individuals was higher than captive-reared fish (5.30 ± 0.12 *vs* 4.04 ± 0.04; $P < 0.05$).

Reproductive state assessment

a) females

Gonadosomatic index of both wild and captive-reared female grater amberjack showed significant seasonal changes with an increase from early gametogenesis (EG) to advanced gametogenesis (AG) and a decrease

during the spawning period (SP). Gonadosomatic index was higher in wild than in captive individuals in all the examined periods, although a statistical significant difference was detected only in SP (**Fig. 4**).

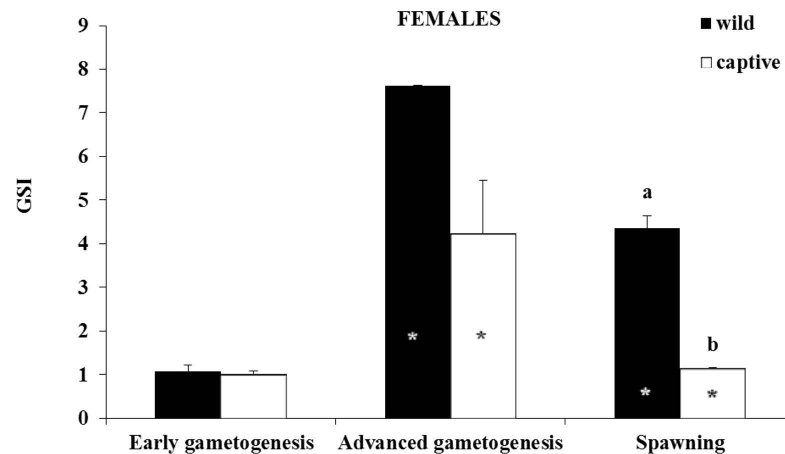

Figure 4. Mean (±SE) gonado-somatic index (GSI) of wild and captive-reared female greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period. Different letters indicate significant difference between wild and captive individuals in the same period of the reproductive cycle (ANOVA, $P < 0.05$).

Among the wild greater amberjack females captured during EG, one fish was at primary oocyte growth stage with perinucleolar oocytes as the most advanced stage in the ovary (**Fig. 5a**), two specimens showed oocytes at cortical alveoli stage (**Fig. 5b**), two fish had early vitellogenic oocytes as the most advanced stage (**Fig. 5c**). The two females sampled during AG showed late vitellogenic follicles along with post-ovulatory follicles (sign of recent spawning) in their ovaries (**Fig. 5d**). Among the 12 females sampled during the SP period, 10 had late vitellogenic follicles along with post-ovulatory follicles and two showed hydrated oocytes (**Fig. 5e**).

Among the female captive-reared greater amberjack sacrificed during EG, one showed ovaries with perinucleolar oocytes, and three had few early vitellogenic oocytes as the most advanced ovarian stage. All four individuals sampled during AG showed oocytes in late vitellogenesis stage, but three of them were affected by an extensive atresia (more than 50% of late vitellogenic follicles were atretic) (**Fig. 5f**). During the SP, three females had ovaries with late vitellogenic follicles undergoing extensive atresia and one showed only perinucleolar oocytes. All these fish were considered to be in a regressing condition.

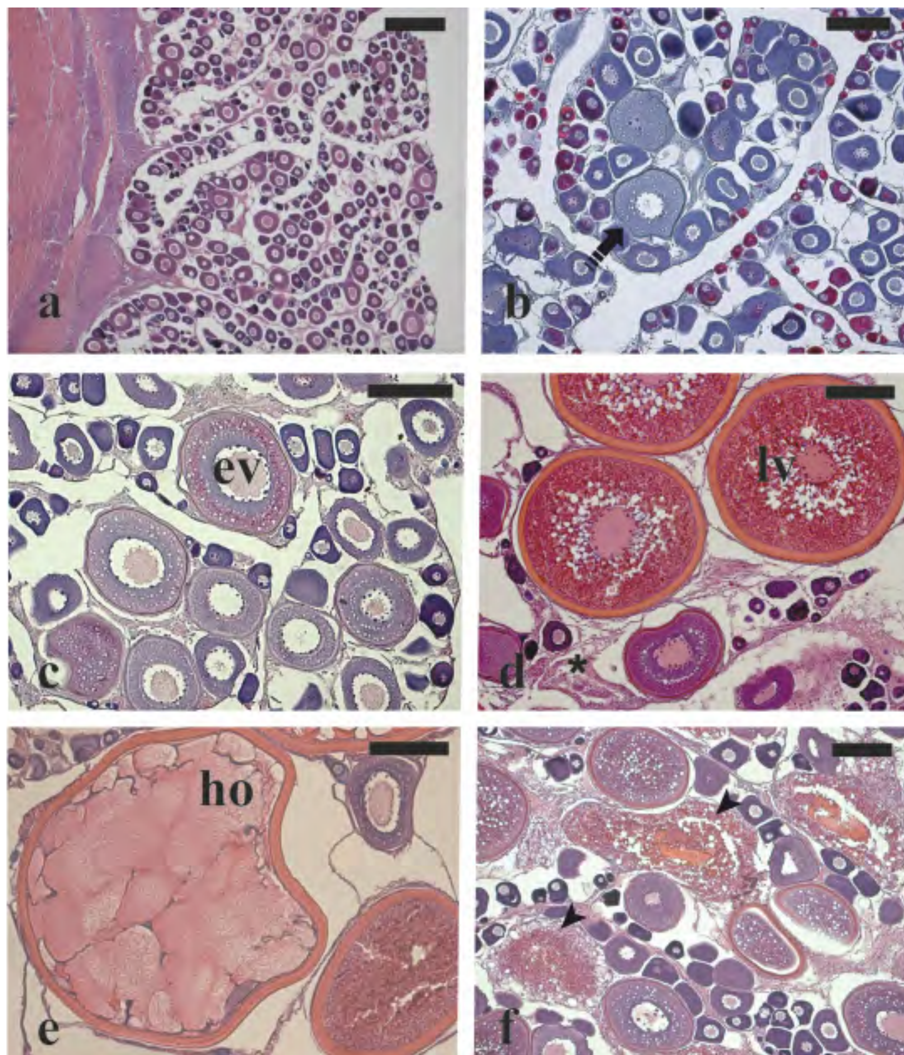

Figure 5. Micrographs of ovary sections from female greater amberjack sampled in three different periods of the reproductive season. (a) Wild individual sampled on 1 May 2015 showing perinucleolar oocytes as the most advanced stage in the ovary. (b) Cortical alveoli oocytes from the ovary of a wild specimen captured on 1 May 2015. (c) Early vitellogenic oocytes from the ovary of a wild individual sampled on 1 May 2015. (d) Late vitellogenic oocytes together with post-ovulatory follicles from a wild spawning fish caught on 31 May 2014. (e) Hydrated oocyte from a spawning wild fish sampled on 30 June 2014. (f) Extensive atresia of late vitellogenenic follicles in a captive-reared specimen sampled on 4 June 2015. Haematoxylin-eosin staining in (a), (c), (d), (e) and Mallory's trichrome staining in (b). Magnification bars = 300 µm in (a) and 150 µm in (b)-(f). Arrowhead: atretic late vitellogenic follicle; asterisk: post-ovulatory follicle; dashed arrow: cortical alveoli stage oocyte; ev: oocyte in early vitellogenesis stage; ho: hydrated oocyte; lv: oocyte in late vitellogenesis stage.

No statistical difference was found in the oocyte surface occupied by yolk granules between wild and captive-reared specimens (**Table 4**).

Table 4. Oocyte yolk accumulation in early and late vitellogenic follicles of wild and captive-reared greater amberjack.

Oocyte stage	Fish condition	Oocyte diameter (µm)	Yolk surface (µm^2)
Early vitellogenesis	Wild	362.5 ± 3.5	55584.9 ± 1513.4
	Captive-reared	356.5 ± 6.9	55760.8 ± 3238.2
Late vitellogenesis	Wild	453.7 ± 3.5	84660.1 ± 1368.3
	Captive-reared	453.0 ± 9.3	90790.6 ± 3650.1

b) males

Gonadosomatic index of both wild and captive-reared male greater amberjack increased significantly from EG to AG, and decreased during the SP. Gonadosomatic index was significantly higher in wild than in captive-reared males, in all the three considered periods (**Fig. 6**).

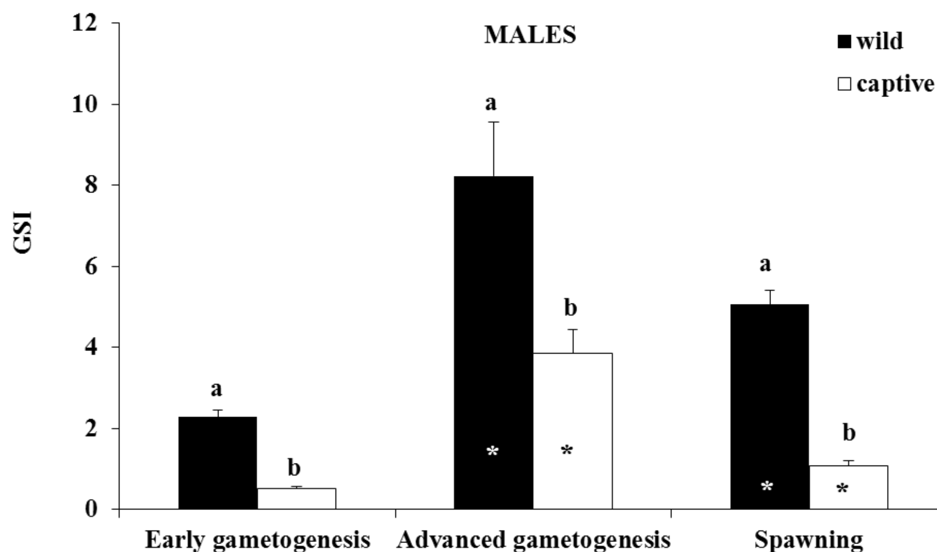

Figure 6. Mean (±SE) gonado-somatic index (GSI) of wild and captive adult male greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period. Different letters indicate significant difference between wild and captive individuals at the same period of the reproductive cycle (ANOVA, $P < 0.05$).

The testes of the five wild males caught during the EG period contained germ cells in all the spermatogenic stages and spermatozoa in the lumen of seminiferous lobules (**Fig. 7a**). All four wild males sampled during the AG period and four out of five fish sampled during the SP period showed all stages of spermatogenesis in the germinal epithelium as well as large amount of luminal spermatozoa (**Fig. 7b**). One of the fish sampled in the SP period was partially spent, showing seminiferous lobules with residual spermatozoa.

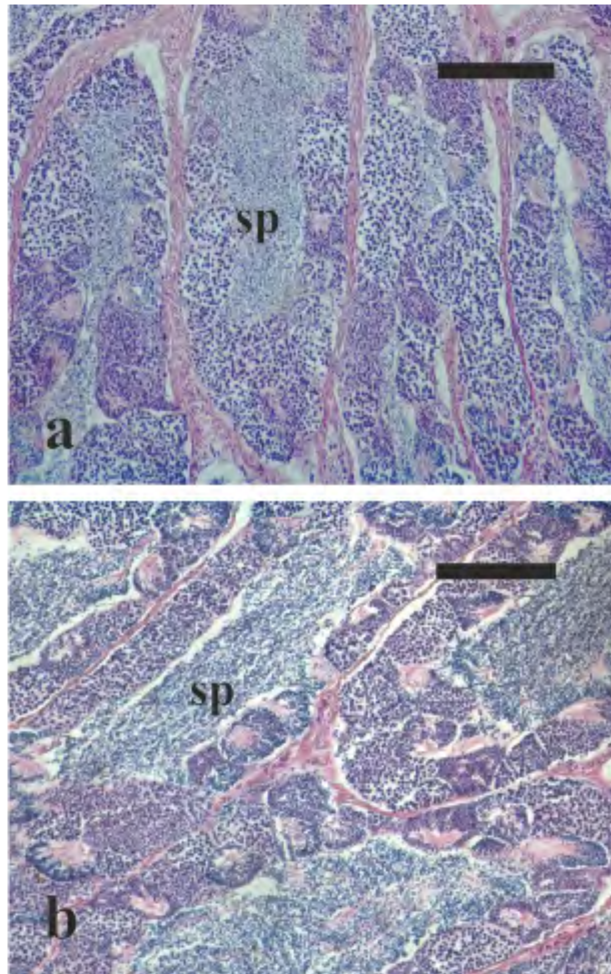

Figure 7. Micrographs of testis sections of wild greater amberjack sampled in two periods of the reproductive season. (a) Testis section from an individual sampled in early gametogenesis period showing the presence of all stages of spermatogenesis in the germinal epithelium and a limited amount of luminal spermatozoa. (b) Testis section from a fish caught during the advanced gametogenesis period, showing all stages of spermatogenesis as well as large amount of luminal spermatozoa. Haematoxylin-eosin staining. Magnification bars = 100 µm. sp: spermatozoa in the lumina of seminiferous lobules.

The four captive-reared males sampled during EG showed the presence of all stages of gametogenesis in the germinal epithelium and only rare luminal spermatozoa (**Fig. 8a**). Among the four captive-reared males sampled during AG, two had testes in active spermatogenesis and two had ceased their spermatogenic activity, showing only residual sperm cysts in the germinal epithelium and abundant spermatozoa in the lumen of seminiferous lobules (**Fig. 8b**). All four males sampled during SP period had ceased their spermatogenic activity and still showed a moderate amount of spermatozoa in the lumen of seminiferous lobules (**Fig. 8c**).

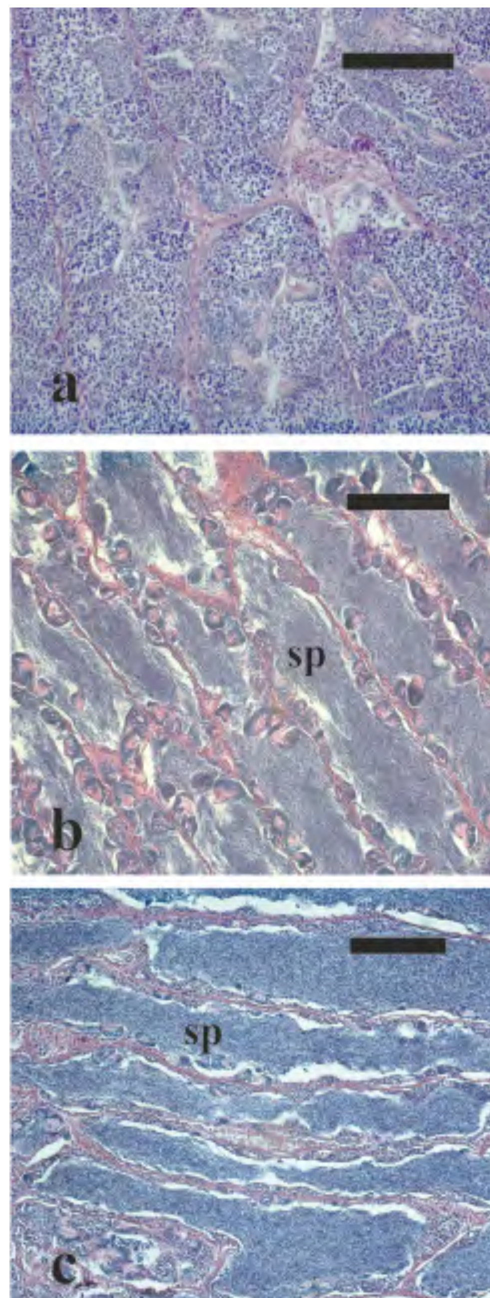

Figure 8. Micrographs of testicular sections of captive-reared greater amberjack sampled in three periods of the reproductive season. a) Testis section from an individual sampled in early gametogenesis period showing the presence of all stages of spermatogenesis. (b) Testis section from a fish sampled 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.

Comparative analysis of seminiferous lobule diameter, proliferating and apoptotic germ cells in wild and captive-reared greater amberjack

The diameter of seminiferous lobules of both wild and captive-reared greater amberjack showed significant monthly changes, with an increase from EG to AG, followed by a decrease during the SP period. The diameter of seminiferous lobules was significantly larger in wild than in captive-reared greater amberjack during both the EG and SP periods (**Fig. 9**).

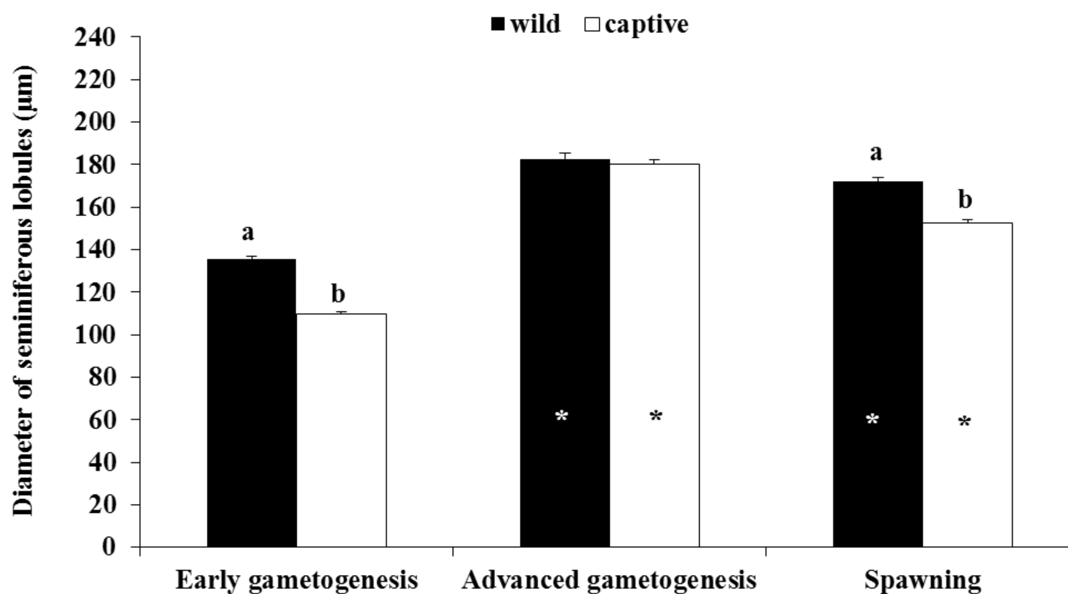

Figure 9. Mean (±SE) seminiferous lobule diameters of wild and captive-reared greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically different diameter versus the preceding period. Different letters indicate a statistically significant difference between wild and captive individuals within the same sampling period (ANOVA, $P < 0.05$).

Immunolocalization of PCNA in male germ cells was detected in single spermatogonia, as well as in cysts containing spermatogonia and primary spermatocytes (**Fig. 10**). A weak staining of the nuclei of secondary spermatocytes was also observed; however these cells were not taken into consideration in the comparative analysis of germ cell proliferation.

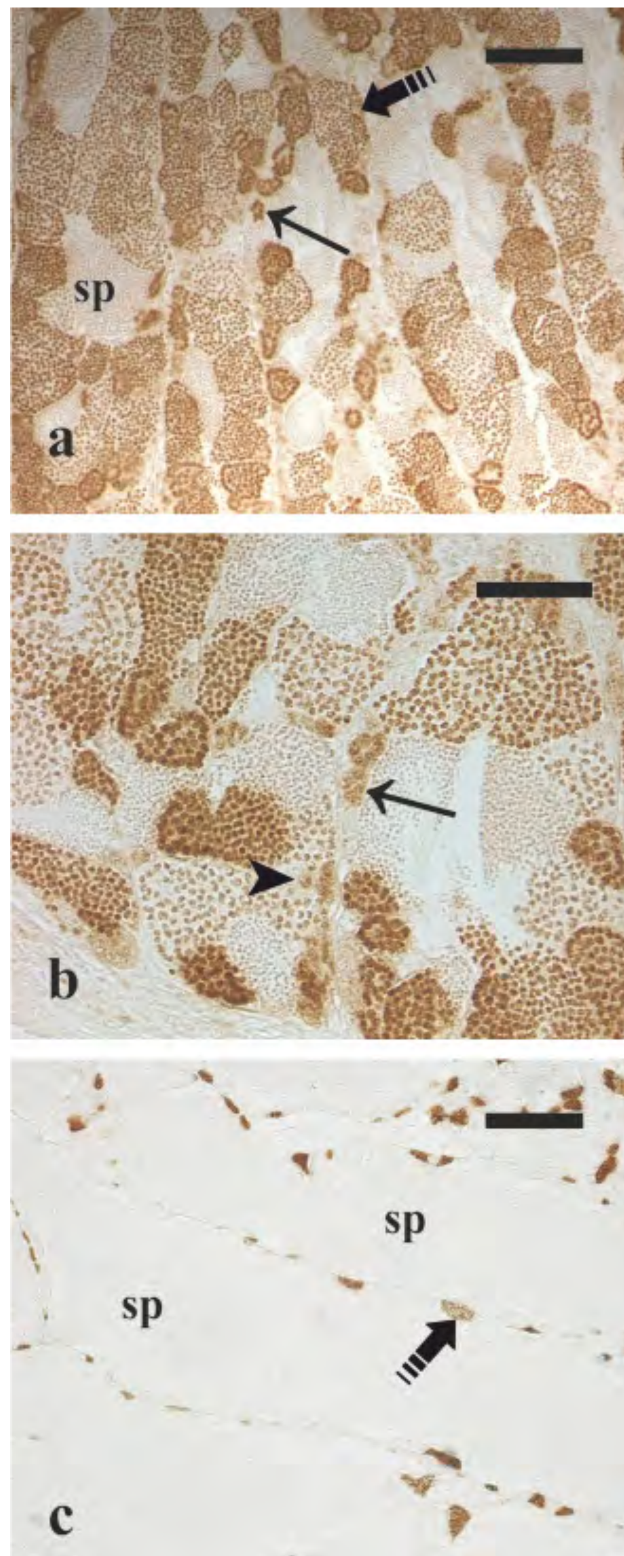

Figure 10. Micrographs from greater amberjack testis sections labelled with antibodies against PCNA. Nuclei of proliferating cells are stained in brown. (a-b) Wild greater amberjack sampled on 1 May 2015. (c) Captive-reared greater amberjack sampled on 4 June 2015. Magnification bars = 70 µm in (a), 40 µm in (b) and 150 µm in (c). Arrowhead: single spermatogonium; dashed arrow: spertmatocyte cysts; single arrow: spermatogonial cysts; sp: spermatozoa.

In captive-reared greater amberjack, the density of anti-PCNA spermatogonia was stable from EG to AG and decreased significantly during the SP period (**Fig. 11a**). A constant, statistically significant decrease of anti-PCNA positive spermatocysts density was observed in captive-reared specimens throughout the examined periods of the reproductive cycle (**Fig. 11b**). The density of anti-PCNA positive spermatocysts was higher in wild than in captive-reared specimens both during the EG and the SP periods (**Fig. 11b**).

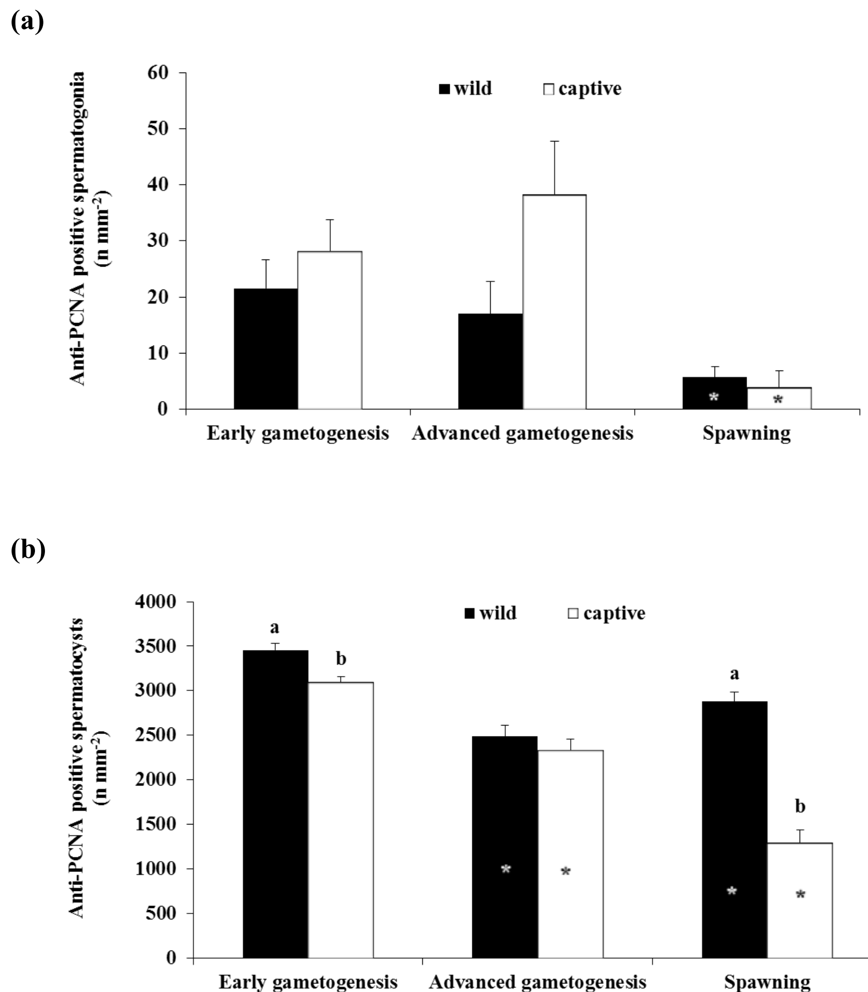

Figure 11. Changes in mean (±SE) anti-PCNA positive germ cell density during three periods of wild and captive-reared greater amberjack reproductive cycle. (a) Anti-PCNA positive single spermatogonia. (b) Anti-PCNA positive spermatocysts. Asterisks indicate statistically different mean density versus the preceding period. Different letters represent significant difference between wild and captive individuals within the same sampling period (ANOVA, $P < 0.05$).

TUNEL-positive germ cells were observed in the germinal epithelium of the majority of the specimens analyzed. Apparently, the TUNEL reaction involved mainly spermatogonia and primary spermatocytes (**Fig. 12**).

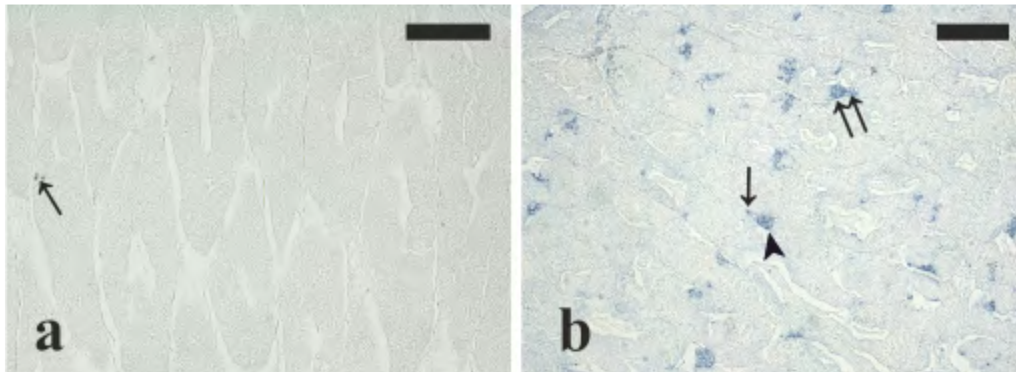

Figure 12. TUNEL-stained testis sections from wild (a) and captive-reared (b) greater amberjack sampled during the early gametogenesis phase. Apoptotic cells appear as dark blue dots. Magnification bar = 150 µm. Arrowhead: spermatogonial cyst; single arrow: single spermatogonia; double arrows: spermatocyte cysts.

In wild greater amberjack, the surface occupied by apoptotic germ cells increased significantly from EG to AG and remained stable thereafter (**Fig. 13**). In captive-reared individuals, the surface occupied by apoptotic cells was stable during the three sampling periods and comparable to the highest levels of the wild specimens (**Fig. 13**).

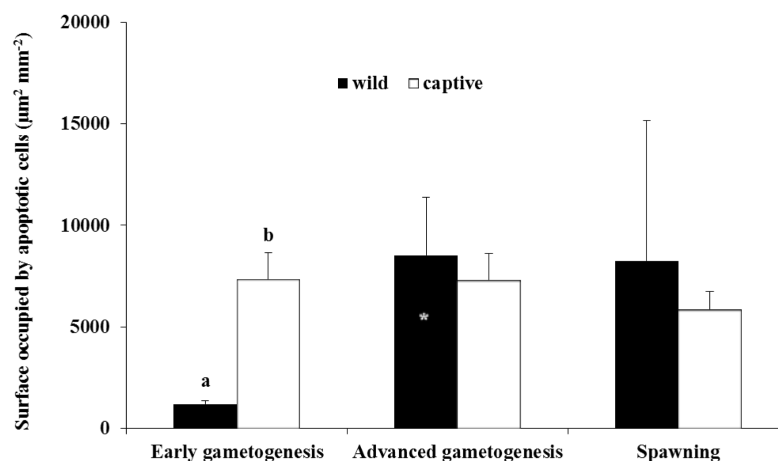

Figure 13. Changes in mean (±SE) surface occupied by apoptotic germ cells in wild and captive-reared male greater amberjack sampled in three periods of the reproductive cycle. Asterisk indicates statistically significant difference versus the previous period. Different letters indicate significant difference between wild and captive individuals sampled in the same period (ANOVA, $P < 0.05$).

Liver vitellogenins and ovarian vitellogenin receptor gene expression

In wild specimens, Vgs mRNA relative levels increased significantly from EG to AG period and then showed a slight, not significant, decrease in samples caught during the SP period.

In captive-reared greater amberjack Vg transcript levels showed the same temporal trend of wild specimens, although, due to a high individual variability, the changes were not statistically significant (**Fig. 14**).

During EG, Vg transcript levels were higher in captive-reared than in wild specimens, whereas in AG as well as during SP Vg transcript levels of captive-reared specimens were lower than those of wild individuals. In particular, VgA, VgB and VgC levels dropped dramatically in captive-reared individuals sampled in the SP period. The low Vgs levels in these specimens were congruent with the cessation of the reproductive state, which was shown by the histological analysis of the ovaries.

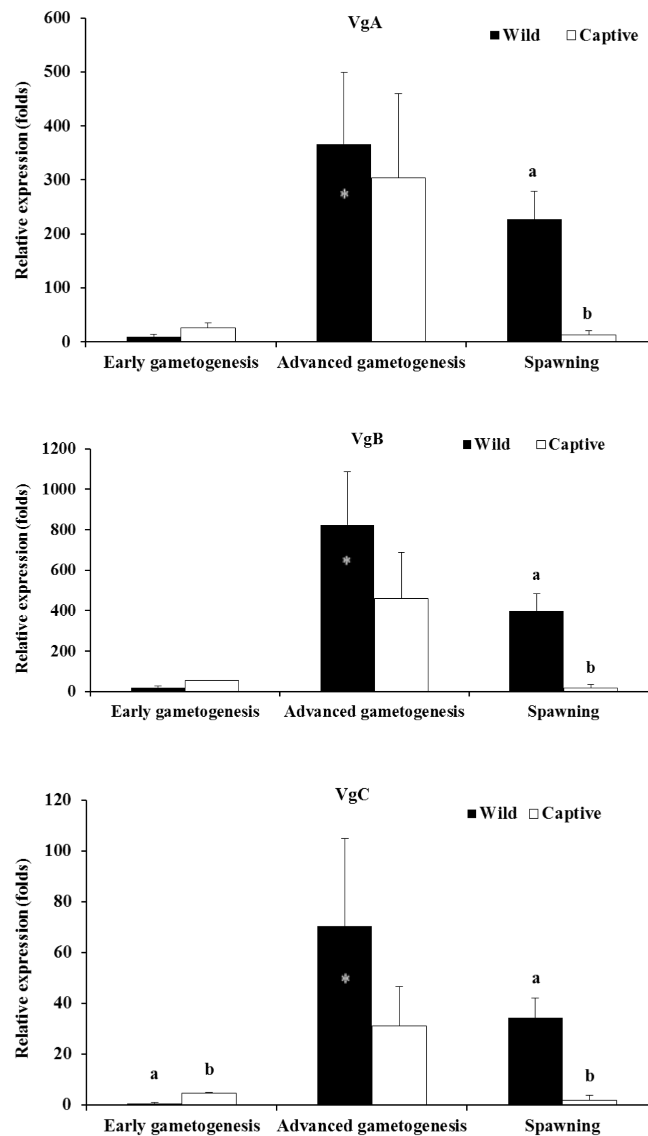

Figure 14. Mean (±SE) transcription levels of vitellogenin A (VgA), vitellogenin B (VgB) and vitellogenin C (VgC) during three periods of the reproductive cycle of wild and captive-reared greater amberjack. Asterisks indicate statistically different transcription levels compared with the previous sampling period (ANOVA, $P < 0.05$). Different letters above bars indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period (ANOVA, $P < 0.05$).

In captive-reared greater amberjack, the highest VgR mRNA relative levels were recorded at the time of the first sampling period (EG), when oocytes at cortical alveoli stage together with rare early vitellogenic oocytes were observed in the ovaries (**Fig. 15**). The VgR mRNA relative levels decreased significantly from EG to AG (**Fig. 15**), when the ovaries had oocytes in advanced vitellogenesis and most of them (three out of four) showed extensive atresia of vitellogenic follicles. Spent individuals sampled during SP showed a new, significant, increase of VgR mRNA relative levels.

The trend of VgR mRNA relative levels in wild greater amberjack was similar to that of captive-reared specimens, although with less pronounced, statistically not significant, changes.

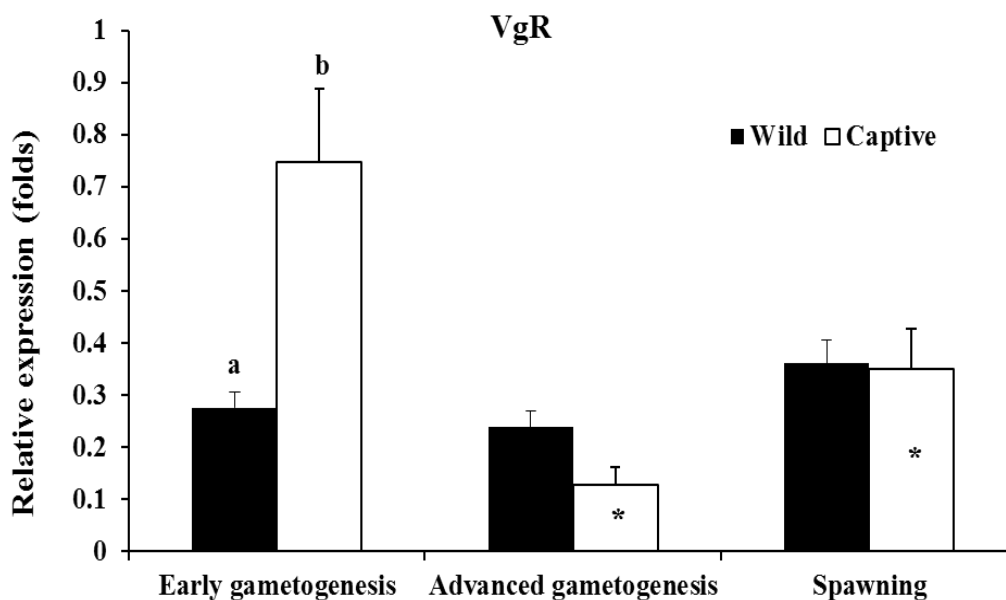

Figure 15. Mean (±SE) transcription levels of vitellogenin receptor (VgR) during three periods of the reproductive cycle of wild and captive-reared greater amberjack. Asterisks indicate statistically different transcription levels compared with the previous sampling period (ANOVA, $P < 0.05$). Different letters above bars indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period (ANOVA, $P < 0.05$).

Liver leptin gene expression

Quantitative real-time PCR (qRT-PCR) analyses of liver leptin mRNA demonstrated that transcript levels in both wild and captive-reared fish were minimal during the advanced stages of gametogenesis and maximal at spawning (**Fig. 16**). Interestingly, during early stages of gametogenesis, liver leptin mRNA levels were significantly ($P < 0.05$) higher in captive females than in cognate wild females (**Fig. 16a**). Nonetheless, the equivalent gene expression levels in captive and wild males did not significantly differ from each other (**Fig. 16b**).

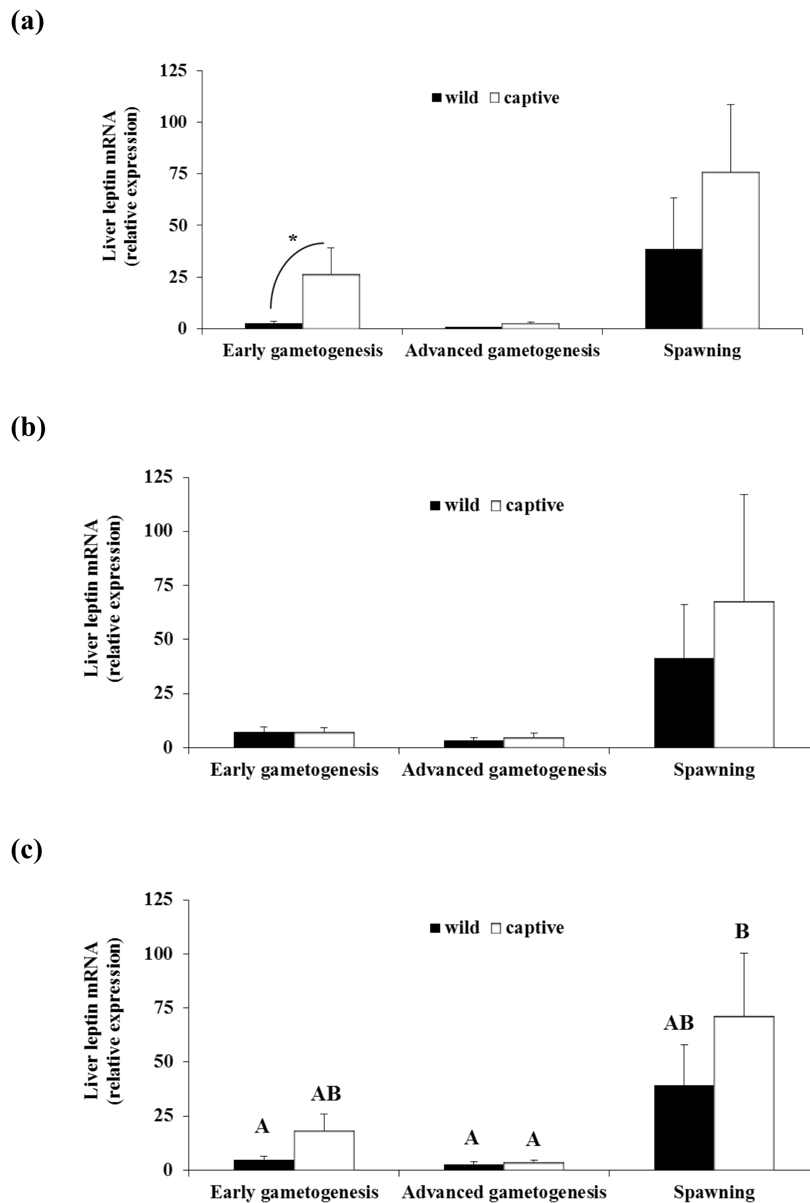

Figure 16. Mean (±SE) transcription levels of liver leptin during three periods of the reproductive cycle of wild and captive-reared greater amberjack (a) females, (b) males, (c) females and males. Asterisks indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period (ANOVA, $P < 0.05$). Different letters above bars indicate statistically different transcription levels (ANOVA, $P < 0.05$).

Gonad biochemical composition (proximate and fatty acid composition and carotenoids contents)

Proximate composition

Proximate composition of ovaries and testes from wild and captive-reared adult greater amberjack were similar (**Tables 5 and 6**).

Table 5. Proximate composition of wild greater amberjack gonads sampled around the Pelagic Islands (Italy).

Sampling Date	Sex	Fork length (FL, cm)	Moisture (M, %)	Lipid DW (TL, %)	Protein DW (TP, %)
Early gametogenesis period					
01/05/2015 (SST = 18.1 °C)	M	111	83.66	10.37	78.10
	M	112	83.97	11.23	76.34
	M	112	83.67	12.65	78.77
	M	117	83.87	17.48	80.86
	M	113	84.36	13.19	78.81
	F	103	81.93	7.99	80.54
	F	112	81.53	9.74	81.84
	F	116	80.39	8.71	81.90
	F	103	81.57	7.31	82.72
	F	106	81.36	7.38	84.69
Advanced gametogenesis period					
31/05/2014 (SST = 19.3 °C)	M	124	84.64	23.01	79.66
	M	102	84.49	14.93	81.77
	M	115	85.09	11.56	79.74
	M	99	84.52	12.47	82.39
	F	117	73.28	17.08	67.87
	F	114	72.52	17.80	66,81
Spawning period					
29/06/2015 (SST = 23.8 °C)	M	100	84.38	11.94	81.21
	M	102	84.77	11.66	81.52
	M	104	84.09	12.08	83.72
	F	101	73.10	15.51	70.45
	F	114	71.43	17.50	69.51
	F	109	73.10	15.18	69.65
30/06/2014 (SST = 23.4 °C)	M	100	86.01	11.82	80,66
	M	99	85.09	13.03	84.50
	F	99	73.89	17.59	66.35
	F	100	74.51	16.15	67.74
	F	97	72.52	18.11	65.49
	F	100	70.88	17.34	71.39
	F	102	71.94	20.11	65.30
	F	104	79.55	17.57	67.07

Table 6. Proximate composition of adult greater amberjack gonads reared in captivity in Argosaronikos Fish Farm (Salamina Island, Greece).

Sampling Date	Sex	Fork length (FL, cm)	Moisture (M, %)	Lipid DW (TL, %)	Protein DW (TP, %)
Early gametogenesis period					
	M	101	83.55	17.47	77.38
	m	94	83.71	13.67	76.62
	m	92	83.74	11.05	75.83
24/04/2015	m	94	83.61	14.71	69.75
(SST = 17.5 °C)	f	87	80.81	5.61	84.34
	f	97	81.11	6.73	81.40
	f	96	81.06	9.54	81.69
	f	100	79.76	9.57	78.36
Advanced gametogenesis period					
	M	90	83.02	17.75	79.80
	m	97	83.55	15.22	86.43
	m	98	82.79	14.51	87.67
04/06/2015	m	103	83.85	13.27	85.98
(SST = 20.0 °C)	f	97	72.53	18.65	62.06
	f	97	68.26	20.59	67.23
	f	106	75.64	18.01	71.21
	f	101	69.47	19.45	68.32
Spawning period					
	M	96	80.35	7.54	85.99
	M	95	83.33	13.54	83.85
	M	91	81.23	14.03	83.66
02/07/2015	M	96	82.25	11.79	80.65
(SST = 25.5 °C)	F	92	81.43	12.03	79.61
	F	96	78.97	16.57	66.74
	F	95	74.61	20.40	67.06
	F	97	77.73	15,16	71.69

a) females

Amongst the wild female gonads sampled during EG, one fish with oocytes at primary growth stage also presented a comparatively high ovary content of proteins, whereas ovaries from fish with early vitellogenic oocytes contained a relatively higher amount of lipids. The gonads from two wild females sampled at AG showed late vitellogenic follicles along with post ovulatory follicles (sign of recent spawning), coinciding

with much lower contents of moisture and proteins and an important increment in ovarian fat levels. Finally, among the 9 analyzed wild females sampled during the SP period, 8 had late vitellogenic follicles along with post ovulatory follicles and one showed hydrated oocytes, the latter containing the highest amount of moisture (**Table 5**).

Among the female captive-reared greater amberjack sacrificed during EG, the one showing ovaries with perinucleolar oocytes, also displayed a lower content of lipids *vs* a higher one of proteins. Ovaries from captive females sampled during AG did not show a clear relationship between oocyte stage and proximal composition. During the SP period, the ovary that showed only perinucleolar oocytes displayed the highest contents of moisture and proteins and the lowest one of lipids (**Table 6**).

The rearing conditions were not associated to any significant change in the general proximate composition of ovaries, with the exception of moisture content of captive fish, which was slightly higher than that of wild gonads during SP period (**Fig. 17 a**). The evolution pattern of ovaries proximate composition during the reproductive cycle was quite similar between wild and captive counterparts, with a clear decrement of moisture and proteins, associated to a significant increment of lipids at AG compared to EG and SP (**Fig. 17 a, b and c**).

b) males

There were not clear differences among the proximate composition of testes of the five wild males caught during the EG period, with the exception of the largest AG wild male, which displayed a clearly higher fat content in testes. Proximate compositions of wild males sampled during the AG did not differ from the previous sampling period (**Table 5**).

Captive-reared males sampled during EG and AG did not show clear differences in terms of testes proximal composition. Composition of SP testes was also relatively homogeneous with higher contents of proteins than those from the previous sampling periods (**Table 6**).

The rearing conditions did not seem to be associated to any change in the general proximate composition of testes, with the exception of moisture content of captive fish that was slightly lower than that of wild fish during AG and SP (**Fig. 18 a**). The evolution pattern of testes proximate composition of wild and captive individuals during the reproductive cycle was quite similar, with no significant differences in terms of lipid contents and only a slightly significant increment of proteins observed in wild fish sampled during AG compared to EG (**Fig. 18 b and c**).

Proximate composition was more variable in ovaries than in testes, with lipid contents ranging from 6-8% to 20%.

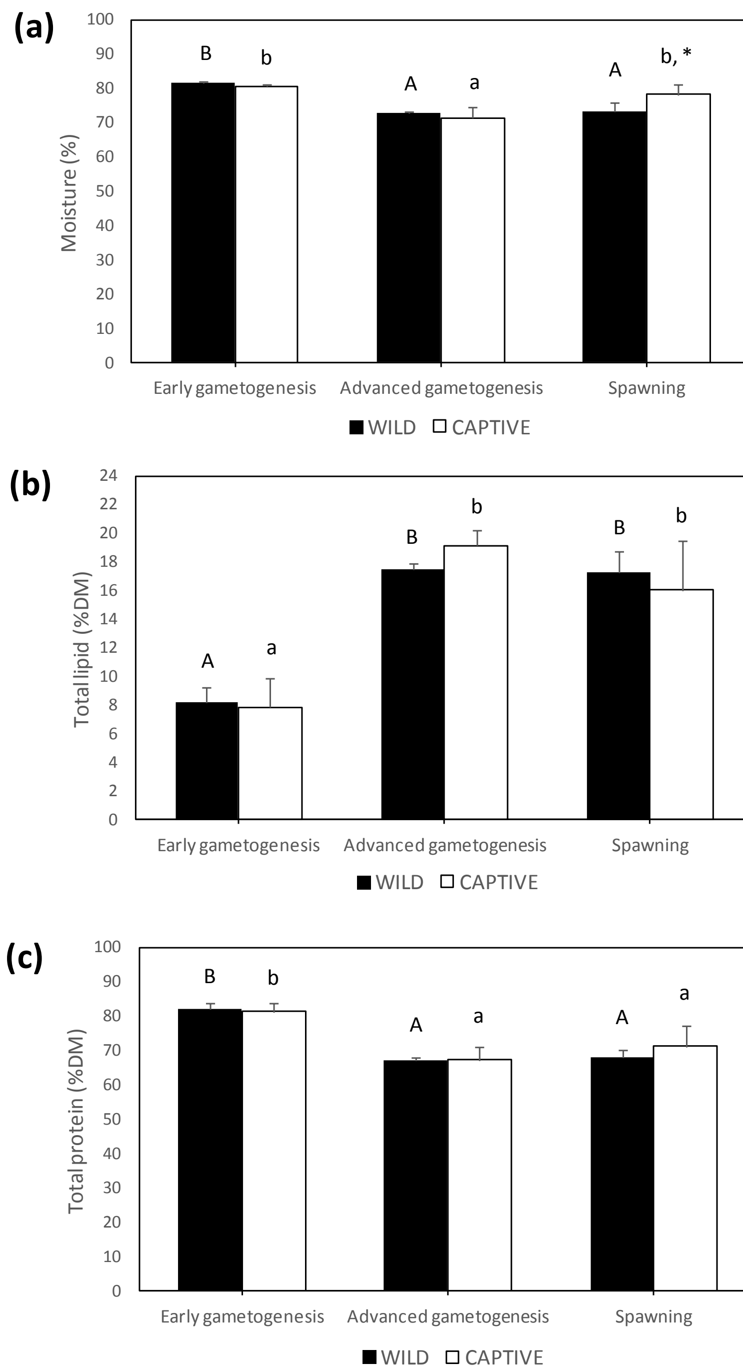

Figure 17. Mean (±SD) levels of ovaries (a) Moisture, (b) Total Lipids and (c) Total Proteins of wild and captive-reared greater amberjack broodstock at three periods of the reproductive cycle. Asterisks indicate statistical differences between wild and captive-reared specimens within a specific sampling period (Student`s t-Test, $P < 0.05$). Different uppercase and lowercase letters indicate statistical differences among the three periods for wild or captive fish, respectively (ANOVA, $P < 0.05$).

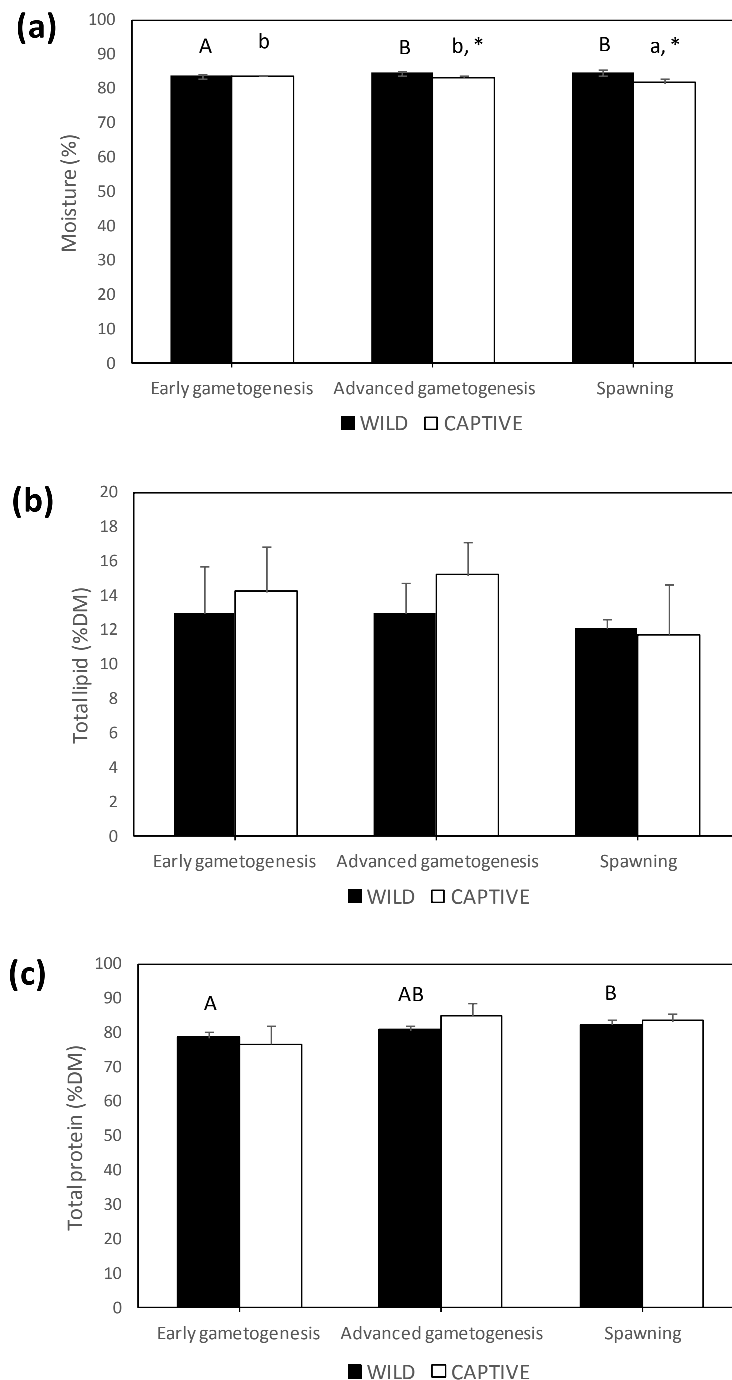

Figure 18. Mean (±SD) levels of testes (a) Moisture, (b) Total Lipids and (c) Total Proteins of wild and captive-reared greater amberjack broodstock at three periods of the reproductive cycle. Asterisks indicate statistical differences between wild and captive-reared specimens within a specific sampling period (Student`s t-Test, $P < 0.05$). Different uppercase and lowercase letters indicate statistical differences among the three periods for wild or captive fish, respectively (ANOVA, $P < 0.05$).

Carotenoid contents in ovaries and testes sampled at advanced gametogenesis period

The dietary regime of captive-reared fish, which consisted of a commercial extruded broodstock diet (Vitalis-Cal, Skretting SA, Norway) provided a significantly higher mean level of carotenoids than in wild specimens (**Fig. 19**). Surprisingly, carotenoid content in testes from captive-reared fish was the reverse of the wild fish where ovaries were significantly richer than testes in these antioxidant pigments.

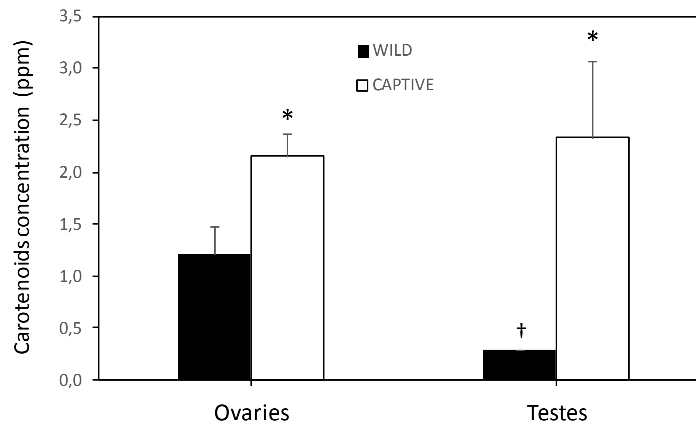

Figure 19. Mean (±SD) levels of carotenoids in ovaries and testes of wild and captive-reared greater amberjack sampled during the spawning period. Asterisks indicate statistically different levels between wild and captive-reared specimens; (†) denotes differences between sexes (Student`s t-Test, P < 0.05).

Gonad lipid classes and fatty acid composition

The main lipid classes and fatty acid compositions varied amongst the gonads from wild and captive adult greater amberjack males and females and across the three different periods of the reproductive cycle (**Tables 7 and 8**).

Table 7. Mean values of main lipid classes of gonads from wild and captive adult greater amberjack males and females sampled at three different periods of the reproductive cycle.

	Early gametogenesis				Advanced gametogenesis				Spawning period			
	Male		Female		Male		Female		Male		Female	
	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive
PC	27.6±2.9	20.5±1.8 *	21.6±1.5 b	18.4±2.2	25.8±1.1	22.9±3.0	18.2±1.0 a	17.7±1.8	27.8±0.8	23.1±0.9 *	17.2±1.1 a	17.3±0.6
PS	7.8±1.2 a	4.6±0.9 *,a	4.5±1.2 b	4.5±1.7 b	12.3±0.9 b	8.8±1.3 *,b	1.8±0.3 a	1.5±0.3 a	9.7±1.7 a	8.8±0.8 b	1.4±0.4 a	3.0±0.9 *,b
PI	5.9±0.4 b	5.0±1.1 a	4.9±0.8 b	3.8±0.7	1.7±0.6 a	6.8±0.7 *,b	2.4±0.4 a	2.9±0.4	5.6±3.1 b	7.1±0.3 b	2.4±0.3 a	3.4±0.7 *
PE	20.0±0.9 a	14.1±2.0 *,a	12.2±1.0 c	11.8±2.3 c	22.1±0.5 b	21.2±2.6 b	7.0±0.1 b	5.9±0.5 *,a	21.4±0.7 a	21.9±1.4 b	6.1±0.8 a	7.9±1.5 b
TPL	64.6±4.3 ab	47.4±5.3 *,a	47.8±2.8 b	43.8±7.1 c	63.5±0.5 a	63.8±7.4 b	32.3±1.2 a	30.8±0.6 *,a	67.6±3.4 b	65.7±1.6 b	28.6±3.1 a	35.6±3.8 *,b
Chol	18.7±1.0 a	16.5±2.3 a	16.2±1.3 b	15.1±2.1 ab	22.5±1.4 b	22.5±2.3 b	13.0±0.9 a	14.6±0.9 *,a	18.4±8.5 a	22.9±1.4 b	12.8±0.7 a	19.2±4.2 *,b
TG	8.7±1.9 c	28.9±8.6 *,b	21.0±1.5 a	26.3±8.6 b	3.0±0.9 b	2.8±0.6 a	24.7±0.5 b	26.4±1.8 b	1.8±0.7 a	4.4±1.6 *,a	22.2±1.8 a	21.3±2.6 b
TNL	35.4±4.3 ab	52.6±5.3 *,b	52.2±2.8 a	56.2±7.1 a	36.5±0.5 b	36.2±7.4 a	67.7±1.2 b	69.2±0.6 *,b	32.4±3.4 a	34.3±1.6 a	71.4±3.1 b	64.4±3.8 *,a

Values are expressed as mean ± SD (for a variable number of data). Asterisk indicates significant differences for a particular lipid class and period between wild and captive-reared broodstock (t-Student, P < 0.05). Letters within a row denote significant differences along the reproductive cycle for each sex (P < 0.05). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; Chol, Cholesterol; TG, triacylglicerides; TNL, total neutral lipids.

The rearing conditions failed to provide the nutritional requirements to the testes of captive-reared fish to resemble wild testes content of total polar lipids and specific lipid class proportions. This was particularly evident for males sampled at EG as the testes displayed more than three times the amount of triacylglycerols (TG) present in the wild counterparts, with the consequent decrement in proportions of PE and PS. Total Polar Lipids and PE were especially high in fish testes and tended to be preserved at AG and SP in wild and captive fish. A marked decrease in PI was evident from EG to AG in wild testes and ovaries. On the contrary, PI levels exhibited a significant increase from EG to SP in captive-reared testes.

Table 8. Mean values of main fatty acids of gonads from wild and captive adult greater amberjack males and females sampled at three different periods of the reproductive cycle.

	Early gametogenesis				Advanced gametogenesis				Spawning period			
	Male		Female		Male		Female		Male		Female	
	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive
16:0	22.9±1.0	19.6±1.0 *,a	20.3±0.9 b	17.9±0.5 *,b	23.1±0.4	22.1±0.3 b	16.3±1.8 a	15.2±0.9 a	22.6±1.3	22.2±2.3 b	17.4±0.7 a	17.5±3.4 b
18:1[1]	19.6±2.9	23.7±3.1 b	17.9±2.1 a	22.1±3.0 a	16.4±2.3	18.3±2.3 a	24.8±1.6 c	26.7±1.3 b	17.2±0.6	18.8±1.9 a	22.1±1.1 b	23.4±4.2 ab
18:2n-6	1.1±0.2 b	7.1±0.9 *,b	1.0±0.2 a	6.5±0.6 *,a	0.8±0.1 a	5.0±1.2 *,a	1.0±0.3 a	10.1±0.5 *	1.0±0.1 b	5.8±2.0 *,a	1.8±0.2 b	8.1±2.1 *,a
20:4n-6	4.1±0.8	2.3±0.4 *,a	5.9±1.0 b	3.4±1.2 *,b	4.3±0.4	2.7±0.5 *,a	3.7±0.5 a	2.1±0.4 *,a	5.4±1.0	5.0±1.4 *,b	4.9±0.6 b	4.0±0.6 b
20:5n-3	3.7±0.4 b	4.6±0.8	3.9±0.4 ab	5.3±0.5 *	2.9±0.4 a	4.8±1.0 *	3.3±0.6 a	5.1±1.3 *	2.8±0.5 ab	3.8±0.8	4.3±0.5 b	4.3±0.6
22:6n-3	26.2±3.6 a	18.1±3.0 *,a	27.3±1.8 b	19.4±3.9 *	32.9±1.9 b	26.9±3.5 *,b	31.2±3.5 b	21.1±1.6 *	26.9±2.2 a	24.6±3.6 b	22.9±1.9 a	23.3±2.9
DHA/EPA	7.1±0.6 a	3.9±0.3 *,a	7.1±1.2 b	3.6±0.5 *,a	11.3±1.0 b	5.7±0.7 *,b	9.4±0.9 c	4.4±1.3 *,ab	10.0±1.9 b	6.7±0.7 *,b	5.4±0.8 a	5.8±0.6 b
ARA/EPA	1.1±0.2 a	0.5±0.0 *,a	1.6±0.4	0.6±0.2 *,a	1.5±0.3 ab	0.6±0.1 *,b	1.1±0.2	0.4±0.1 *,a	2.0±0.7 b	1.5±0.8 c	1.2±0.2	1.0±0.2 b

Values are expressed as mean ± SD (for a variable number of data). Asterisk indicates significant differences in fatty acids between wild and captive-reared broodstock (t-Student, P<0.05). Letters within a row denote significant differences along the reproductive cycle for each sex (P<0.05). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.ù

Differences in terms of fatty acid composition, among total lipids from wild and captive gonads were particularly evident at EG and AG periods, with testes and ovaries of captive fish displaying around 30-40% less docosahexaenoic acid (DHA) and arachidonic acid (ARA), and clearly higher contents of 18:2n-6 (**Table 8**). As a consequence, DHA/eicosapentaeonic acid (EPA) and ARA/EPA ratios, also suffer marked decrements in the gonads of the captive fish. Among the three sampled periods, the most evident differences were a significant increase in DHA from EG to AG displayed by the testes of wild and captive-reared specimens. However, at SP the DHA content did not return to values observed at EG in the captive males, as it did in the wild fish. Similarly, the wild ovaries displayed an increase in DHA between EG and AG, returning at SP to EG levels. This DHA increment between EG and AG was not observed in the captive-reared females.

Discussion and Conclusions

The aim of the present work was to identify possible reproductive dysfunctions in captive-reared greater amberjack that were caught from the wild and reared in captivity. This study was based on: a) gonad maturation assessment through histological analysis; b) trend of vitellogenic process, from liver vitellogenin synthesis to oocyte yolk accumulation; c) evaluation of germ cells proliferation and apoptosis. Moreover, the possible effects of captivity on fish nutritional state was evaluated since this parameter is

considered a key requisite to get high quality gamete production in aquaculture (Izquierdo et al., 2001; Rodríguez-Barreto et al., 2012; Tocher et al., 2008).

On the basis of the available literature on greater amberjack reproduction in the Mediterranean Sea (Grau et al., 1996; Mandich et al., 2004; Mylonas et al., 2004), wild and captive-reared specimens were sampled during three supposed periods of reproductive cycle: early gametogenesis, late gametogenesis and spawning. The histological analysis of wild greater amberjack gonads showed that gonad development starts earlier than mid May, the gonad recrudescence period reported for the same area by Mandich et al. (2004). In fact, on 01 May, wild greater amberjack females exhibited early vitellogenic oocytes and males were in a condition that can be classified as mid spermatogenesis, showing all the spermatogenetic stages and spermatozoa in seminiferous lobule lumina.

Gonado-somatic index of captive-reared greater amberjack was significantly lower for both sexes in all the three considered periods compared to wild fish and this might be indicative *per se* that the process of gonad development was somehow impaired in captivity. Another possibility may be that due to the young age of the fish (almost all fish were 4 years old), their reproductive capacity had not reached its optimum. This stock of fish was also examined for their reproductive maturation status the year before (2014, when they were mainly 3 years old) and at the time the males were in spermiation and the females in vitellogenesis. So this was not the pubertal year of this broodstock, but it was their first post-pubertal reproductive season.

The histological analysis of the ovaries showed that captive-reared females sampled during the supposed period of early gametogenesis, were slightly late in the oocyte development process, since their ovaries had very few early vitellogenic oocytes compared to wild female in the same period. An extensive atresia of late vitellogenics follicles affected the ovaries of captive females sampled during the supposed advanced gametogenesis and spawning periods, thus preventing any further oocyte development.

Liver Vgs mRNA relative levels increased during advanced gametogenesis period in both wild and captive-reared females, in concomitance with the process of Vg oocyte uptake; however Vgs expression levels were slightly lower in captive-reared compared to wild fish. In captive-reared greater amberjack, ovary VgR mRNA relative levels were higher at the beginning of vitellogenesis, decreased significantly during advanced gametogenesis period and then rose up during the spawning period. Captive-reared greater amberjack sampled during early gametogenesis showed much higher VgR relative levels compared to wild specimens. This finding can be related to the slightly different oocyte development stage observed in the two groups of fish. In fact, one of the four captive-reared fish sampled in this period had perinucleolar oocytes and the other three fish had cortical alveoli oocyte stage and only few early vitellogenic oocytes, whereas all the wild females sampled in the same period had a higher amount of early vitellogenic oocytes and, consequently, a higher GSI. These findings confirm our previous observation that VgR mRNA is mainly synthesised in previtellogenic oocytes and it is stored in the ooplasm until the functional protein is required for Vg uptake (Pousis et al., 2012). Therefore the higher levels of VgR mRNA observed in captive greater amberjack can be related to an even slight difference in oocyte maturity stage rather than to any effect induced by rearing in captivity. This is further corroborated by the evidence that a normal yolk accumulation process occurred in the oocytes of captive-reared specimens.

The histological analysis of greater amberjack testes did not show any striking difference between wild and captive reared individuals during early and advanced gametogenesis periods; on the contrary all the sampled captive-reared males were spent during the spawning season of the wild population. In wild male greater amberjack, germ cell proliferating activity was rather constant from the early gametogenesis to the spawning period. On the contrary, in captive-reared individuals, germ cell proliferating activity showed a progressive decrease during the three examined periods. Changes in the pattern of germ cell proliferation, already reported for captive-reared Atlantic bluefin tuna, might be related to endocrine dysfunctions, in turn depending on suboptimal environmental conditions at the rearing sites, as well as on captivity-induced stress (Zupa et al., 2013, 2014).

In agreement with our previous findings on Atlantic bluefin tuna (Corriero et al., 2009; Zupa et al., 2013, 2014) and swordfish (Corriero et al., 2007a), apoptotic spermatogonia and primary spermatocytes were observed in most of the examined testis sections. In greater amberjack wild populations, as well as in wild

Atlantic bluefin tuna and swordfish, apoptotic germ cell density was higher during the peak of spermatogenic activity than both in the recrudescence and spawning periods, indicating a role of apoptosis in the quantitative control of germ cell populations as well as in the prevention of the maturation of aberrant germ cells (Corriero et al., 2007a; Zupa et al., 2013). Differently from their wild counterpart, captive-reared greater amberjack showed the highest density of apoptotic germ cells during the early gametogenesis period. The influence of captivity-induced stress on reproductive axis functioning has been widely demonstrated in all vertebrate classes, including fishes (Schreck, 2010), and the negative effect of confinement on gonadotropin-induced release of 11-ketotestosterone (11-KT), the predominant androgen in fish which acts as a germ cell survival factor, has been already hypothesized in Atlantic bluefin tuna (Corriero et al., 2009).

Transcript profiles of liver leptin showed relatively low and unchangeable levels throughout gametogenesis (April – May), and a dramatic elevation during spawning (June-July). Similar patterns, at the protein level, were detected in the cod-like freshwater turbot *Lota lota* (Mustonen et al., 2002). In the cited study, the circulating leptin-immunoreactive peptide levels were relatively low prior to and during reproduction and increased after spawning. It appears that the increase in the levels of leptin towards the end of the spawning cycle is a seasonal event helping the fish to recover from the exertion of reproduction, while re-absorbing the gonads and reorganizing the body energy storages.

Not only nutrition, but other rearing conditions may affect the pattern of energy usage and reserves mobilization towards reproduction as well. Whereas artificial diets and confinement conditions have been reported to increase tissue fat composition (Rodríguez-Barreto et al., 2012, 2014, 2015), stocking density is widely recognized as a critical husbandry factor in intensive aquaculture because it represents a potential source of chronic stress that can impact metabolism and growth (Ellis et al., 2002; Portz et al., 2006). In spite of the above mentioned gametogenesis impairment of the captive fish, the mobilization of energy reserves /nutrients, as lipids and proteins, seemed not to be committed in captive-reared fish along the reproductive cycle, being quite similar to that of wild counterparts. A clear reduction of moisture and proteins, accompanied by a significant increase of lipids at AG compared to EG and SP was observed for wild and captive-reared females, whereas for males no variations were recorded in terms of lipid contents and only a slight, yet significant, increase of proteins was achieved in AG wild testes, with respect to values observed at EG. In this sense, the sea cage-rearing conditions and the Vitalis Cal (Skretting) dietary regime seem to preserve energy, and total protein and lipid mobilization towards the gonads during the reproductive cycle of greater amberjack. Carotenoids were also actively mobilized into the gonads during sexual reproductive activity of wild and captive specimens, their presence probably mitigating deleterious oxidative damage to the developing embryo and larval visual function as well as adequate chromatophore responses. According to the bibliography, carotenoids may also protect the rich LC-PUFA sperm from oxidative damage, preserving sperm functional motility and fertility (Mansour et al., 2006).

In spite of these considerations of total protein and lipid mobilization, the rearing conditions compared to the wild, captive-reared male gonads had different total polar lipid contents, and specific lipid class and fatty acid profiles. This was particularly evident for males sampled at EG, whose testes displayed more than three times the amount of TG present in the wild counterparts with the consequent decrement in proportions of PE and PS. DHA and ARA were also 30-40% lower in these samples. Sperm is particularly rich in PS and PE, which influence membrane fluidity and male proper reproductive function. These phospholipids contain high levels of di-DHA (22:6n-3) a molecular species that is known to positively improve sperm motility and the efficiency of membrane fusion events, such as those taking place between spermatozoa and oocytes, or during synapses and visual accommodation. Since sperm fatty acid composition depends upon the essential fatty acid content of broodstock diet (Asturiano et al., 1999; Labbé et al., 1993), fertilization could be affected by lipid profiles. Studies by Asturiano et al. (2001) showed that male European sea bass *Dicentrarchus labrax* fed commercial HUFA-enriched diets exhibited more successful reproductive performance in terms of length of spermiation, total milt production, milt spermatozoa density, and fertilization than fish fed with a non-enriched diet. 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 (Almansa et al., 1999; Bruce et al.,

1999; Fernández-Palacios et al., 1995; Li & Olsen, 2015; Navas et al., 1997; Olsen et al., 2014; Rodríguez et al., 1998) mostly in relation to female fish. In addition, there is strong evidence for HUFAs, particularly 20:5n-3 (EPA) and 20:4n-6 (ARA), via metabolites formed from the cyclooxygenase and lipoxygenase pathways, being involved in steroidogenesis and oocyte maturation in vertebrates (Sorbera et al., 1998). In vitro, ARA stimulates testicular testosterone production in goldfish testes and ovaries by conversion to prostaglandin (Henrotte et al., 2011; Mercure & Van Der Kraak, 1996; Patiño et al., 2003; Stacey & Sorensen, 2005; Wade et al., 1994; Wade & Van Der Kraak, 1993). Therefore, since EPA and ARA compete for the same enzymatic complex to generate different series of prostanoids within different biological activities, the relative proportions of these two fatty acids are even more important than the level of each fatty acid in broodstock diet, as imbalances in the EPA/ARA ratio could lead to deregulated production of different mediators involved in reproduction. The PI is known as the main depot of ARA in fish (Sargent et al., 2002) and in the present study there was a marked decrement of PI in wild testes from EG to AG, which did not occur in the captive counterparts. The DHA is also known as the most relevant essential fatty acid (EFA) in sperm and egg quality (Sargent et al., 2002), but the important increase of DHA observed in wild testes and ovaries from EG to AG and its further decrease at SP was not observed in the captive fish. All these factors point out to a potential impairment of reproductive success.

In conclusion, captive-reared greater amberjack have been shown to suffer significant reproductive dysfunctions with impaired oogenesis and spermatogenesis. Females were affected by extensive atresia of vitellogenic oocytes and failed to undergo oocyte maturation; males displayed a precocious decrease of germ cell proliferation, an abnormal increase of apoptosis during the recrudescence period (early gametogenesis) and the complete cessation of spermatogenic activity much earlier compared with the wild population**.** Additionally the proportions of total polar lipids, and specific lipid classes and EFA proportions particularly differed among EG wild and captive-reared fish gonads, with the latter displaying clearly lower contents of specific phospholipids, EFA and DHA/EPA and ARA/EPA ratios, all crucial factors for reproductive success and sperm and egg and larval quality. The later nutritional differences were presumably the result exclusively of differences in the diet between wild and captive fish. It is necessary to contemplate as to the reason(s) for the impaired reproductive function in the captive fish –which could be caused by non-dietary reasons, and whether this was due to differences in age between the wild and captive fish, or differences in the "rearing conditions". As mentioned in the introduction, the captive environment is often associated with reproductive dysfunctions, but it was our initial expectation (or objective) that maintaining greater amberjack under low stocking densities in a sea cage, would expose to the fish to as "natural" conditions a possible, thus not affecting negatively their reproductive function. However, what could not be avoided was the sampling-associated stress, since all fish sampled at the three different times from the captive broodstock were maintained in the same cage. It is conceivable that at least part of the observed reproductive impairment was due to this multiple handling of the stock and not necessarily due to the overall rearing conditions and husbandry. If this is true, then it is perhaps still conceivable to maintain greater amberjack in sea cages in captivity, and expect them to undergo gametogenesis in a similar way as in the wild. Another captive-reared stock –albeit probably 1 year older than the one used in the present study- was maintained in almost identical conditions during the year, but without any handling until the spawning season, and reached final stages of gametogenesis and produced fertilized eggs upon stimulation with spawning inducing agents.

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