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Running head: Spermatogenesis in captive greater amberjack

Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack *Seriola dumerili* (Risso, 1810)[1]

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ABSTRACT: The greater amberjack *Seriola dumerili* (Risso, 1810) is a promising candidate for the diversification European aquaculture production, but inconsistent reproduction in captivity prevents commercial production. Recent studies showed that greater amberjack confined in sea cages exhibited scarce gonad development and early interruption of gametogenic activity during the reproductive season. The aim of the present study was to improve our understanding on the observed impairment of spermatogenesis. Adult wild and captive-reared males were sampled during three different phases of the reproductive cycle: early gametogenesis (EARLY, late April-early May), advanced gametogenesis (ADVANCED; late May-early June) and spawning (SPAWNING, late June-July). Spermatogonial stem cells and proliferating germ cells were identified through the immunohistochemical localization of Pou5f1 and Proliferating Cell Nuclear Antigen, respectively. Apoptotic germ cells were identified throughout the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling method. Sperm quality of captive-reared fish was evaluated using computer-assisted sperm analysis. Captive-reared males exhibited seminiferous lobules of a smaller diameter, a precocious and progressive decrease of spermatogonial mitosis and high level of apoptosis at the beginning of the reproductive season. Except for E_2 that was many-fold higher in captive-reared fish during the EARLY phase, sex steroid plasma levels were lower in captive-reared males compared to wild individuals. Motile spermatozoa percentage of captive greater amberjack was lower than in other teleosts and a drastic decrease of spermatozoa motility duration, velocity and ATP content occurred along the reproductive season. An abnormal increase of sperm concentration as well as an increase of dead spermatozoa occurred during the SPAWNING phase, probably because of lack of sperm hydration and ejaculation, and consequent sperm ageing. The present study demonstrates the extreme susceptibility of greater amberjack to rearing stress and underscores the need for an improvement of the handling procedures to ameliorate gametogenesis dysfunctions in commercial aquaculture production.

Key words: germ cell apoptosis, germ cell proliferation, greater amberjack, rearing in captivity, *Seriola dumerili*, sperm quality.

INTRODUCTION

The greater amberjack *Seriola dumerili* (Risso 1810) is a highly valuable teleost considered as a promising aquaculture species. However, a proper commercial aquaculture production of the species has not developed so far, mainly due to its unpredictable reproduction in captivity (Micale et al., 1999; Garcia et al., 2001; Kožul et al., 2001; Mylonas et al., 2004), which prevented the development of hatchery production of juveniles and the conversion of the capture-based farming activity into a true aquaculture industry.

A renewed effort to develop a technology for the aquaculture production of greater amberjack is currently in progress within the EU Project DIVERSIFY (www.diversifyfish.eu). A comparative study on the reproductive development in captive-reared greater amberjack (Zupa et al., 2017), demonstrated a reduced testis development and an early cessation of spermatogenic activity; this gametogenesis impairment was associated with important changes in sex steroid plasma concentrations. Gametogenesis dysfunctions in fish reared in captivity involve an inadequate pituitary gonadotropin (GtH) synthesis and/or release (Zohar and Mylonas, 2001; Mylonas et al., 2010; Berkovich et al., 2013), which has been attributed to captivity-induced stress, lack of suitable environmental conditions (Mylonas et al., 2010) and/or nutritional deficiencies (Izquierdo et al., 2001). Spermatogenesis dysfunctions may result in a qualitative and quantitative decrease of sperm output (Rurangwa et al., 2004; Cabrita et al., 2009; Bobe and Labbé, 2010) and, therefore, in unsuccessful spawning and production of fertilized eggs.

The aim of the present study was a) to compare male germ cell proliferation and apoptosis in wild and captive-reared greater amberjack sampled in different phases of the reproductive cycle and b) to assess sperm quality of greater amberjack specimens reared in sea cages, in an effort to improve our understanding on the spermatogenesis impairment recently described in this species (Zupa et al., 2017).

MATERIALS AND METHODS

Sample collection

Ethical approval was not required because this study did not fall within the obligations contained in the Italian decree n. 26 of 04 March 2014 regarding the permission to carry out research studies on experimental animals, as the fish came from a registered aquaculture facility and from commercial catches. The research did not involve any experiments on live animals. Captive-reared fish originally came from the fishery at 0+ year of age, and were then reared at a registered aquaculture facility for 3 years, according to routine farming practices, before they were recruited for this study, sacrificed and sampled. No specific permission was required for wild greater amberjack sampling since these fish were commercially caught from an authorized purse-seine fishing vessel during routine fishing operations. Immediately after death, those fish considered suitable for the present study were purchased and sampled on board. The greater amberjack is classified as "Least Concern" in the IUCN Red List of Threatened Species (Smith-Vaniz et al., 2015).

In the present study, the same male wild and captive-reared greater amberjack used in Zupa et al. (2017) were analysed. A total of 14 wild and 12 captive-reared greater amberjack males were sampled in three different phases of the reproductive cycle that were determined according to the available literature (Mandich et al., 2004; Sley et al., 2014): early gametogenesis (EARLY, late April-early May), advanced gametogenesis (ADVANCED, late May-early June) and spawning (SPAWNING, late June-July). Wild fish were caught by a professional purse seine fishing vessel during two consecutive fishing seasons (2014-2015) around the Pelagic Islands (Sicily, Italy). Immediately after capture, fish were placed on ice and left to die before sampling. Captive-reared fish belonged to a broodstock captured in the

area of Astakos (Ionian Sea) in 2011, and transferred in a sea cage of Argosaronikos Fish Farm (Salamina Island, Greece) in September 2013. Fish were reared for two years according to standard farming practices and fed to apparent satiation with a commercial extruded broodstock diet (Vitalis Cal, Skretting SA, Norway) every other day. Before sampling, captive-reared fish were confined in a small cage area using a PVC curtain and then anesthetized lightly with 0.01 ml l^{-1} clove oil. Then, they were gently directed into a PVC stretcher, brought on board of a service vessel and anesthetized deeply with 0.03 ml l^{-1} clove oil for sex recognition by means of gonad cannulation. Subsequently, 10-20 ml blood samples were taken from the caudal vasculature using heparinized syringe and then transferred to 10 ml tubes containing 200 IU sodium heparin ml^{-1} of blood. Then, fish were euthanized by decapitation, placed in crushed ice and transferred to the farm facility for the subsequent sample collection.

Biometric data (fork length, FL, nearest cm; body mass, BM, nearest kg; testis mass, TM, nearest g), gonado-somatic index (GSI = 100*TM BM^{-1}) and reproductive state are reported in Table 1 (data already presented in Zupa et al., 2017). For the present study, one-cm thick cross sections were taken from one of the testis of each fish, fixed in 10% buffered formalin for further histological (germ cell type description), immunohistochemical (stem and proliferating germ cells) and apoptosis analysis. For the assessment of sperm quality, sperm samples were taken from all 12 captive-reared greater amberjack. After unsuccessful attempts to collect sperm by applying pressure on the fish's abdomen, samples of intratesticular semen were obtained by squeezing the dissected testes. Part of each sperm sample was left undiluted and part was diluted 1/3 (vol/vol) in modified Leibovitz medium according to Fauvel et al. (2012); all the samples were stored at 4°C until analysis, which took place within 30 min.

Histology, immunohistochemistry and identification of apoptotic germ cells

Testis slices were dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Four-μm thick sections were cut and stained with haematoxylin-eosin (H-E) or processed for immunohistochemistry and for detection of apoptotic cells. The identification of spermatogonial stem cells was carried out through the immunohistochemical identification of Pou5f1, a transcription factor involved in the maintenance and self-renewal of undifferentiated and pluripotent cells, which is considered a reliable molecular marker for spermatogonial stem cells in fish (Schulz et al., 2010; Lacerda et al., 2014). The identification of proliferating germ cells was performed through the immunohistochemical localization of the Proliferating Cell Nuclear Antigen (PCNA), 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.

The immunohistochemical detection of Pou5f1 and PCNA were performed using the same protocol, with the exception of an antigen retrieval procedure that was applied only to Pou5f1 immunostaining. This procedure was performed by boiling testis sections in citrate buffer (0.01 M, pH 6.0; 4x5 min cycles) in a microwave oven on high power (750 watts). Endogenous peroxidase was inhibited by treating sections for 10 min with 3% H_2O_2 and then rinsing them with distilled water and PBS (0.01 M, pH 7.4, containing 0.15 M NaCl). Subsequently, sections were incubated for 30 min in normal horse serum (NHS; Vector, Burlingame, Ca) to block non-specific binding sites for immunoglobulins and then incubated overnight in a moist chamber at 4°C with rabbit polyclonal antibodies raised against synthetic peptide of Pou5f1 (Abnova, Taipei, Taiwan) and monoclonal antibodies to PCNA (Santa Cruz Biotechnology Inc., Dallas, Texas). Anti Pou5f1 and anti PCNA antibodies were diluted 1:500 and 1:100, respectively, in PBS containing 0.1% BSA (Sigma-Aldrich, Milan, Italy). After rinsing for 10 min in PBS, immunohistochemical visualization was obtained using the Vectastain Universal Elite Kit (Vector, Burlingame, Ca). This method uses the avidin-biotin-peroxidase complex (ABC) procedure. Peroxidase activity was visualized by incubating for 10 min with a Vector

3,3'-diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector, Burlingame, Ca), 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.

The localization of apoptotic germ cells was performed using the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method with an in situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany) that was 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, Mannheim, Germany). A ready-to- use solution of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) (Roche Diagnostics, Mannheim, Germany) 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 sections used for germ cell proliferation and apoptosis analyses. The density of anti-PCNA positive single A spermatogonia (number of cells mm^{-2} germinal epithelium) and the density of anti-PCNA positive spermatocysts (i.e. number of cysts containing type A and type B spermatogonia or primary spermatocytes mm^{-2} germinal epithelium), as well as the surface occupied by TUNEL positive apoptotic cells (μm^2 mm^{-2} germinal epithelium), were measured on 5 randomly selected fields of each testicular section. All these parameters 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).

188

189 ***Sex steroid plasma level measurement***

190 Plasma was separated from the blood by centrifugation (2408 x *g* for 5 minutes at room
191 temperature) and then was kept at -80°C until assayed for sex steroid determination. The
192 quantification of testosterone (T), 11-Ketotestosterone (11-KT), and the putative maturation-
193 inducing steroid (MIS) 17,20β-dihydroxypren-4-en-3-one (17,20β-P) in the plasma was
194 already reported by Zupa et al. (2017). For the quantification of 17β-estradiol (E_2), the same
195 procedure was used, with reagents from Cayman Chemical Company (USA). For steroid
196 extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done
197 by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing,
198 samples were frozen for 10 min at -80°C and the supernatant organic phase was collected in
199 new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany).
200 Samples were reconstituted in reaction buffer for running in the ELISA.

201

202 ***Analysis of sperm quality***

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

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

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

Statistical analysis

Differences in GSI, mean diameter of seminiferous lobules, density of anti-PCNA positive single A spermatogonia, density of anti-PCNA positive spermatocysts (cysts containing type A and type B spermatogonia + cysts containing primary spermatocytes), surface occupied by apoptotic germ cells and sex steroid concentrations were evaluated by a two tailed Student's t-test in the following groups: wild specimens sampled in consecutive phases of the reproductive cycle; captive-reared specimens sampled in consecutive phases of the reproductive cycle; wild vs captive-reared specimens sampled in the same phase of the reproductive cycle. Prior to the Student's t-test, the raw data of apoptotic surfaces were square root-transformed, as appropriate with surface data (Sokal and Rohlf, 1981).

Differences in the sperm quality indexes between the three sampling phases were assessed either by ANOVA (after angular transformation in the case of % of motile spermatozoa), or by nested design ANOVA (in the case of sperm velocity where individual spermatozoa performances were taken into account for each male at the different sampling periods). Means were compared using Duncan's New Multiple Range (DNMR) post hoc test. All the results are presented as means ± SE, and the statistical probability significance was established at the $P < 0.05$ level.

RESULTS

Changes in gonado-somatic index and diameter of seminiferous lobules

The testicular development of wild and captive-reared greater amberjack during the sampling periods was evident by the observed significant changes in GSI and the diameter of seminiferous lobules (Table 2). Both in wild and captive-reared fish, GSI and seminiferous

lobule diameter increased significantly ($P < 0.05$) from EARLY to ADVANCED periods and decreased thereafter. Wild fish had higher GSI compared to captive-reared fish at all three phases ($P < 0.05$), and seminiferous lobules were significantly larger ($P < 0.05$) in wild than in captive-reared greater amberjack during the EARLY and SPAWNING phases.

Histological and immunohistochemical analysis of the testes

The immunohistochemical staining with antibodies against the stemness marker Pou5f1 labeled single intralobular cells (type A spermatogonia not making part of spermatocysts), 8.0 ± 0.1 µm in diameter, as well as sparse interlobular cells (Fig. 1a). Intralobular anti-Pou5f1 positive cells were more frequently observed beneath the tunica albuginea; in sections stained with H-E, they likely corresponded to cells having a spherical nucleus with heterochromatin dots and two nucleoli, surrounded by a thin acidophilic cytoplasm (Fig. 1b). A larger type of single A spermatogonia, 10.6 ± 0.2 µm in diameter, not immunoreactive to anti-Pou5f1, showed a roundish/ovoidal nucleus with a prevalent euchromatic appearance and sparse heterochromatic patches, with a preeminent nucleolus and an acidophilic cytoplasm (Fig. 1b). Spermatogonia contained in cysts appeared as two different cell types: larger cells (mean diameter 8.8 ± 2.0 µm) with one or more nucleoli and a moderately acidophilic cytoplasm making part of small cysts containing few cells (presumptively type A spermatogonia) (Fig. 1b); smaller cells (mean diameter: 4.8 ± 0.8 µm), showing a small heterochromatic nucleus and thin rim of a weakly acidophilic cytoplasm (Fig. 1b), making part of larger cysts (presumptively type B spermatogonia).

The appearance of primary (4.4 ± 0.9 µm) and secondary (3.3 ± 0.8 µm) spermatocytes differed according to the different phases of meiosis; metaphasic figures were often observed within spermatocyte I and spermatocyte II cysts (Fig. 1b). Spermatids had a mean diameter of 2.6 ± 0.7 µm and were characterized by a compacted and strongly basophilic nucleus (Fig. 1b).

Flagellated spermatozoa showed an oval head stained intensely with haematoxylin and were observed within cysts or in the lumina of seminiferous lobules after the cyst breakdown (Fig. 1b).

Germ cell proliferation and apoptosis

Anti-PCNA immunostaining was observed in the nuclei of single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (Fig. 2a). A weak staining of the nuclei of secondary spermatocytes was also observed, but these cells were not included in the quantitative analysis. The relative quantification of anti-PCNA positive single A spermatogonia and spermatocysts throughout the sampling period is shown in Fig. 3. In wild greater amberjack, anti-PCNA positive single A spermatogonia gradually decreased throughout the three examined phases, although a statistically significant change ($P < 0.05$) was detected only at the SPAWNING stage (Fig. 3a); anti-PCNA positive spermatocysts decreased from the EARLY to the ADVANCED phase and then increased slightly in the SPAWNING phase ($P < 0.05$) (Fig. 3b). In captive-reared greater amberjack, the density of anti-PCNA single A spermatogonia was stable throughout the EARLY and the ADVANCED phase and decreased dramatically in the SPAWNING phase ($P < 0.05$) (Fig. 3a); a progressive decrease of anti-PCNA positive spermatocysts density was observed in captive-reared specimens throughout the examined phases of the reproductive cycle ($P < 0.05$) (Fig. 3b).

All the captive-reared and most of the wild greater amberjack showed TUNEL-positive germ cells. Apparently, the TUNEL reaction involved mainly single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (Fig. 2b). In wild males, the surface occupied by apoptotic germ cells increased significantly ($P < 0.05$) from the EARLY to the ADVANCED phase and remained stable thereafter, whereas in captive-reared individuals the surface occupied by apoptotic cells was already high at the EARLY

gametogenesis stage and remained unchanged during the three sampling phases, and was comparable to the highest levels of the wild specimens (Fig. 4).

Sex steroid plasma levels

The trend of T, 11-KT and 17,20β-P (reported by Zupa et al., 2017) and E_2 plasma levels of wild and captive-reared greater amberjack is shown in Fig. 5. Except for E_2, plasma levels of the analysed steroids were generally higher in wild than in captive-reared fish. Many-fold higher ($P < 0.05$) E_2 plasma levels were observed in captive-reared fish during the EARLY phase, but these levels decreased significantly in the following phases and were similar to wild fish.

Sperm quality in captive-reared greater amberjack

Spermatozoa concentration of captive-reared greater amberjack was stable throughout the EARLY ($2.3 \pm 0.5 \times 10^{10}$ spz ml^{-1}) and ADVANCED ($3.6 \pm 0.4 \times 10^{10}$ spz ml^{-1}) phases, and increased significantly during the SPAWNING period ($4.6 \pm 0.6 \times 10^{10}$ spz ml^{-1}; ANOVA, $P < 0.05$). For all the three sampling phases, the highest spermatozoa motility (%) was reached within the first 20 s after activation, and was followed by a progressive decrease until complete cessation of movement (Fig. 6a). However, sperm movement within the first 20 s presented variations linked to the sampling time, with the highest mean percentage of swimming spermatozoa recorded in the ADVANCED phase (59 ± 16.9 % of motile spz), and the lowest mean value registered in the SPAWNING phase (21 ± 9.7 % of motile spz). The mean VAP of the spermatozoa varied during the three different phases, with the highest mean value 10 s after activation recorded in the ADVANCED phase (102.7 ± 7.0 μm s^{-1}) and the lowest mean VAP during the SPAWNING phase (36.5 ± 3.3 μm s^{-1}); the highest maximum value of individual velocity was reached during the ADVANCED phase (164 μm s^{-1}) (Fig. 6b). Finally,

a progressive significant decrease ($P < 0.05$) of sperm motility duration was observed from the EARLY to the SPAWNING phase (Fig. 6c).

The ATP level of captive-reared greater amberjack sperm was generally very low, and close to the detection threshold for several samples (data not shown). A progressive, but not statistically significant, decrease of spermatozoa ATP concentration occurred from the EARLY phase (4.7 ± 1.7 n mole 10^{-9} spz) to the ADVANCED (1.9 ± 0.6 n mole 10^{-9} spz) and the SPAWNING phase (1.2 ± 0.4 n mole 10^{-9} spz). In terms of spermatozoa viability, there were significant variations among fish within each sampling time. Notwithstanding this individual variability, a significant increase ($P < 0.05$) of the proportion of dead and live spermatozoa was observed from the ADVANCED to the SPAWNING phase, while the proportion of dying spermatozoa did not vary significantly (Fig. 7).

DISCUSSION

The negative influence of captivity on reproductive function has been widely demonstrated in all vertebrate classes, including fishes (Zohar and Mylonas, 2001; Corriero et al., 2009; Mylonas et al., 2010; Schreck, 2010; Corriero et al., 2011; Rosenfeld et al., 2012; Zupa et al., 2013). As part of a larger effort to improve our understanding of the gametogenesis dysfunctions reported recently in greater amberjack maintained in captivity (Zupa et al., 2017), the present study adds further information on the effects of confinement in captivity on greater amberjack germ cell proliferation and apoptosis, as well as on sperm quality. Recently, Zupa et al. (2017) reported scarce gonad development, precocious cessation of the spermatogenic activity as well as abnormal T, 11-KT and 17,20β-P plasma levels. On the other hand, vitellogenesis proceeded without any noticeable impairment (C. Pousis, unpublished data). The present study suggests that the earlier reported dysfunctional spermatogenesis of captive-reared greater amberjack involves also smaller seminiferous lobules in the EARLY and

363 SPAWNING gametogenesis phases, an altered pattern of germ cell proliferation and an
364 increased amount of apoptotic germ cells, as well as abnormally high E_2 plasma concentrations
365 during the EARLY spermatogenesis phase.

366 The smaller seminiferous lobules of captive-reared greater amberjack reported here was
367 correlated with a lower GSI, and indicates a reduced capacity of the testes to develop and reach
368 full maturation. In turn, this reduced capacity to reach a full gonad development was likely
369 related to the lower sex steroid (T, 11-KT and 17,20β-P) plasma concentrations reported for
370 the same fish by Zupa et al. (2017). In terms of germ cell proliferation, the employed basic
371 histology and immunohistochemistry in the present study made it possible to distinguish and
372 describe three different type A spermatogonia, of which two types were single cells and a third
373 type was represented by cells being part of spermatocysts, type B spermatogonia, primary and
374 secondary spermatocytes, spermatids and spermatozoa. Only one of the two type A single
375 spermatogonia immunoreacted with anti Pou5f1 antibodies, revealing stemness properties.
376 This stem spermatogonium type, likely corresponding to the type A undifferentiated* (A_{und}*)
377 spermatogonium of the classification used by Schulz et al. (2010), is responsible for germ cell
378 self-renewal. The second type of A single spermatogonia found in greater amberjack are non-
379 stem spermatogonia whose activity is likely related to differentiation and rapid proliferation
380 towards meiosis. This cell type may correspond to the type A undifferentiated (A_{und})
381 spermatogonia referred by Schulz et al. (2010), although these authors did not exclude a
382 residual stem capacity for this cell type. In greater amberjack testis, anti-PCNA positive
383 undifferentiated single type A spermatogonia (both positive and negative cells to the stemness
384 marker Pou5f1), differentiated spermatogonia (type A and B spermatogonia being part of cysts)
385 and primary spermatocytes were detected during all the investigated reproductive phases.

386 In the present study, the density of proliferating single spermatogonia remained at the
387 highest levels throughout the EARLY and ADVANCED phases, and decreased dramatically

388 during the SPAWNING phase, with differences between wild and captive-reared individuals.
389 This trend of proliferating activity of single spermatogonia during the reproductive season is
390 coherent with the decreasing trend of T and 11-KT plasma levels during the SPAWNING phase
391 (Zupa et al., 2017). The absence of significant differences in the density of proliferating single
392 spermatogonia between wild and captive-reared greater amberjack is apparently in contrast
393 with the lower T and 11-KT plasma levels observed in fish kept in captivity (Zupa et al., 2017).
394 However, this apparent incongruence may be explained by i) an increased spermatogonial self-
395 renewal activity stimulated by abnormally high E_2 levels during the EARLY phase, and/or ii)
396 a diminished capacity of spermatogonia to proceed towards meiosis (lower density of PCNA
397 positive spermatocytes, see below), resulting in a comparatively higher amount of less
398 developed germ cells in captive-reared fish. The lower spermatogonial capacity of captive-
399 reared fish to proceed toward meiosis might have resulted from the combined effects of higher
400 E_2 and lower T/11-KT plasma concentrations. In fact, although E_2 in male fish stimulates
401 spermatogonial self-renewal (Miura et al., 1999; Schulz and Miura, 2002; Schulz et al., 2010),
402 supra-physiological concentrations of this hormone inhibit spermatogenesis via negative
403 feedback effects on the brain and the pituitary, involving down-regulation of the testicular
404 androgen production capacity (Schulz and Nóbrega, 2011).
405 In captive-reared individuals, the density of spermatocysts containing proliferating type
406 A and B spermatogonia plus primary spermatocytes was lower than in wild fish in the EARLY
407 and SPAWNING gametogenesis stages, and showed a progressive decrease from the EARLY
408 to the SPAWNING phase. This is in agreement with previous observations showing that
409 captive-reared greater amberjack were already in spent condition during the SPAWNING
410 phase of the wild population, and their T, 11-KT and 17,20β-P plasma levels were abnormally
411 low (Zupa et al., 2017). Besides promoting germ cell proliferation, spermiogenesis and
412 spermiation, androgens have been proposed to act as survival factors for germ cells, both in

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

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

438 a much reduced amount of arachidonic acid (Zupa et al., 2017) a molecule that stimulates
439 testosterone production by elevating cyclic adenosine monophosphate (cAMP) levels in a dose
440 dependent manner (Mercure and Van der Kraak, 1995; Mercure and Van der Kraak, 1996).
441 Moreover, from studies carried out in birds (Newman et al., 2008; Dickens et al., 2011) and in
442 mammals (Williams, 2012), it is known that the exposure to different types of stress can result
443 in an aromatase up-regulation with consequent increase of E_2 and decrease of androgens.
444 Another objective of this study was to assess if the above-described dysfunctions resulted
445 in a decreased sperm quality. Unfortunately, due to the difficulties to operate in the field, it
446 was not possible to collect and analyse sperm from the wild individuals collected on
447 commercial fishing vessels, which would have represented a valued reference for the
448 assessment of sperm taken from captive-reared specimens. Contrary to previous sperm
449 sampling attempts carried out in Croatia (Kožul et al., 2001), but consistent with attempts made
450 in Greece (Mylonas et al, 2004), in the present study it was not possible to take sperm by
451 stripping captive-reared greater amberjack. This failure was probably due to the strong
452 abdominal musculature of this species (Mylonas et al., 2004), but it also could be related to the
453 lack of significant testicular hydration. Indeed, during the dissection of the testes, it was
454 observed that the vasa deferens were not full of sperm and only a direct strong squeezing of
455 the testes allowed obtaining sperm, so that the following discussion actually refers to intra-
456 testicular sperm, which might lack complete maturation and hydration. The sperm
457 concentration of captive-reared greater amberjack measured in this study was in the upper
458 range of marine fish species (Suquet et al., 1994; Cosson et al., 2008a), which is consistent
459 with a lack of hydration that, if realized, would have resulted a physiological reduction of this
460 parameter towards the spawning season. To our knowledge, the observed increase of sperm
461 concentration during the SPAWNING phase has never been reported in any other fish species;
462 moreover, the histological analysis reported by Zupa et al. (2017) showed that while these

specimens had ceased their spermatogenic activity, they still retained a moderate amount of luminal spermatozoa in the testes. Altogether, these observations seem to support the hypothesis of the lack of proper sperm hydration in captive-reared greater amberjack, probably in response to low sex steroid levels. It is known that sperm hydration with seminal fluid and release via the sperm duct are under endocrine control, and a key-role in this process and in the intensification of sperm motility is played by 17,20β-P (Schulz and Miura, 2002; Scott et al., 2010; Milla et al, 2008), whose plasma levels were reported to be abnormally low in captive-reared greater amberjack during the ADVANCED and SPAWNING phases (Zupa et al., 2017).

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

488 percentage of dead spermatozoa increased significantly from the ADVANCED to the
489 SPAWNING phase, which is consistent with the lack of sperm hydration and, presumably
490 ejaculation, and consequent spermatozoa ageing.

491 In conclusion, the present study demonstrated that rearing in captivity affected
492 spermatogenesis in greater amberjack from its early phase, when a high level of germ cell
493 apoptosis was observed. A constant reduction of the rate of spermatogonia entering meiosis
494 resulted in a precocious cessation of the spermatogenic activity. As a consequence of this
495 spermatogenesis impairment, greater amberjack confined in captivity showed low sperm
496 quality, in terms of sperm density, motility and velocity, as well as of the ATP content and
497 membrane integrity. This study provides further information on the occurrence of severe
498 reproductive dysfunctions in captive-reared greater amberjack males reported by Zupa et al.
499 (2017), and further supports the need for an improvement of rearing technology. In particular,
500 handling procedures minimizing stress could be effective in alleviating reproductive
501 deficiencies.

502

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671

672 **Table 1.** Biometric data, gonado-somatic index (GSI) and maturity state of wild and captive-reared
673 greater amberjack males sampled during the reproductive season in the Mediterranean Sea.

Fish state	Sampling date	FL, cm	BM, kg	TM, g	GSI, %	Reproductive state
Early gametogenesis (EARLY)						
wild	01/05/2015	111	14	300	2.14	All spermatogenic stages; luminal spermatozoa
		112	20	450	2.25	
		112	15	300	2.00	
		113	19	400	2.10	
		117	19	550	2.89	
captive	24/04/2015	92	12	65	0.54	All spermatogenic stages; few luminal spermatozoa
		94	12	60	0.50	
		94	13	60	0.46	
		101	15	95	0.63	
Advanced gametogenesis (ADVANCED)						
wild	31/05/2014	99	14	1150	8.21	All spermatogenic stages; plenty of luminal spermatozoa
		102	13	650	5.00	
		115	19	2200	11.57	
		124	22	1900	8.63	
captive	04/06/2015	90	9	370	4.11	Ended spermatogenesis; plenty of luminal spermatozoa
		97	14	295	2.10	
		98	13	600	4.61	All spermatogenic stages; plenty of luminal spermatozoa
		103	15	690	4.60	
Spawning (SPAWNING)						
wild	29/06/2015	100	12	650	5.41	All spermatogenic stages; plenty of luminal spermatozoa
		102	14	700	5.00	
		104	16	950	5.93	
	30/06/2014	99	11	577	5.24	
		100	11	400	3.63	Partially spent; residual luminal spermatozoa
captive	02/07/2015	91	10	70	0.70	Ended spermatogenesis; small amount of luminal spermatozoa
		95	11	155	1.40	
		96	13	140	1.07	
		96	12	130	1.08	

674 Table modified from Zupa et al. (2017). BM: body mass; FL: fork length; TM: testis mass; GSI: gonado-somatic index

675

676 **Table 2.** Mean (±SE) gonado-somatic index (GSI) and seminiferous lobule diameters of wild
677 and captive-reared greater amberjack males sampled during the reproductive season in the
678 Mediterranean Sea.

Fish State	Early spermatogenesis (EARLY)	Advanced spermatogenesis (ADVANCED)	Spawning (SPAWNING)
GSI (%)			
Wild	2.3 ± 0.2^{a}	$8.3 \pm 1.3^{*, a}$	$5.1 \pm 0.4^{*, a}$
Captive	0.5 ± 0.1^{b}	$3.8 \pm 0.6^{*, b}$	$1.1 \pm 0.1^{*, b}$
Seminiferous lobule diameter (μm)			
Wild	135.6 ± 1.3^{a}	$182.6 \pm 2.6^{*}$	$171.9 \pm 1.8^{*, a}$
Captive	109.7 ± 1.1^{b}	$180.0 \pm 2.2^{*}$	$152.5 \pm 1.8^{*, b}$

679 Asterisks indicate statistically significant differences versus the preceding phase within the same group (wild or
680 captive-reared). Different letters indicate significant differences between wild and captive-reared individuals at
681 the same phase of the reproductive cycle (Student's t-test, $P < 0.05$).
682

Figure 1

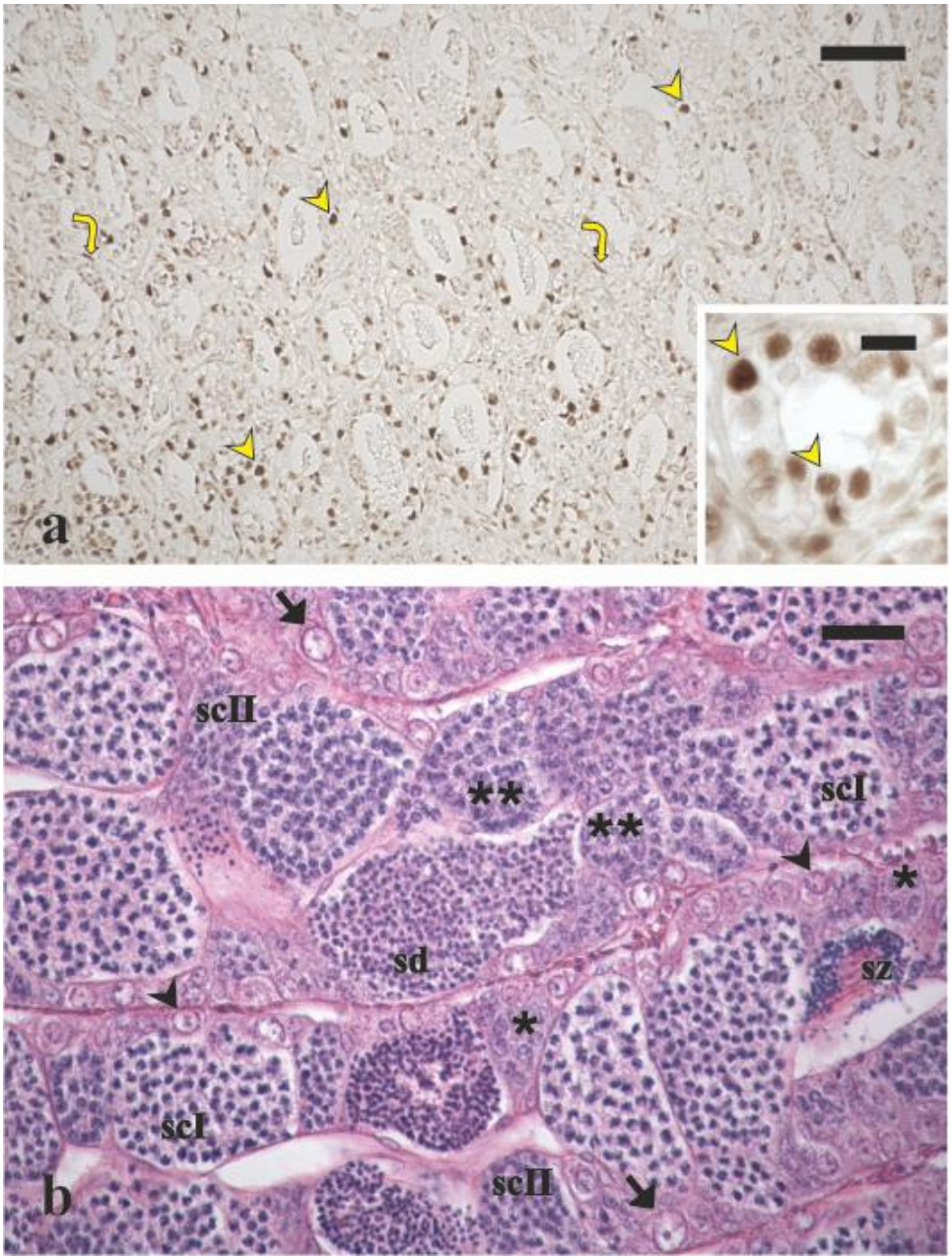

Figure 2

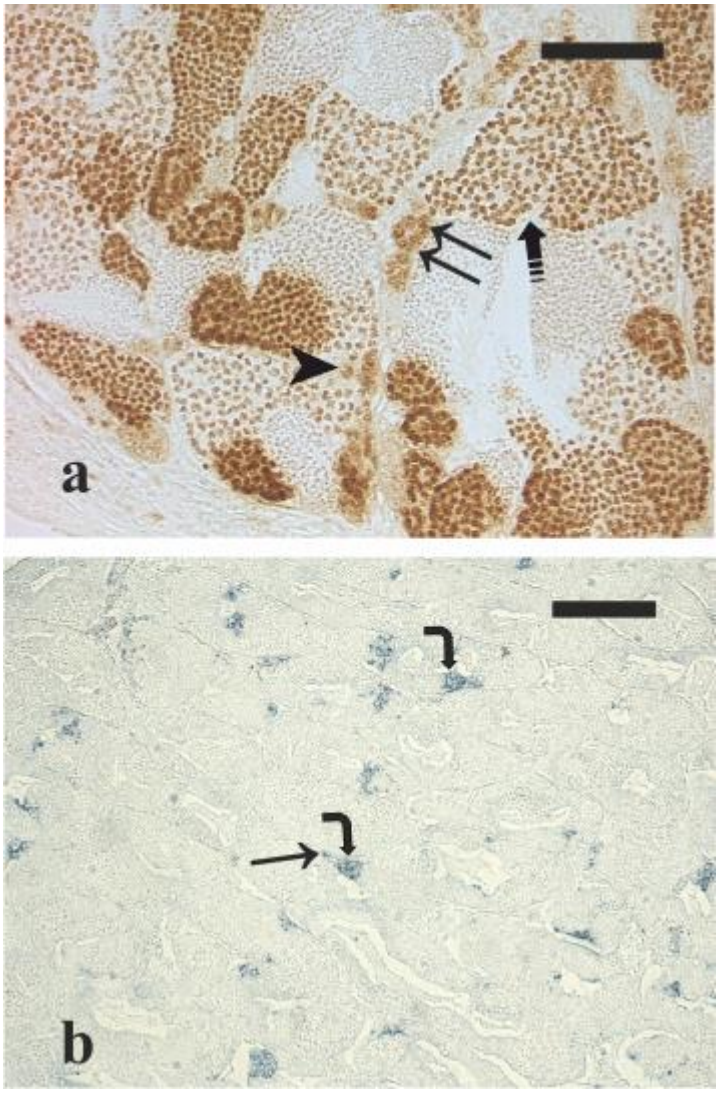

Figure 3

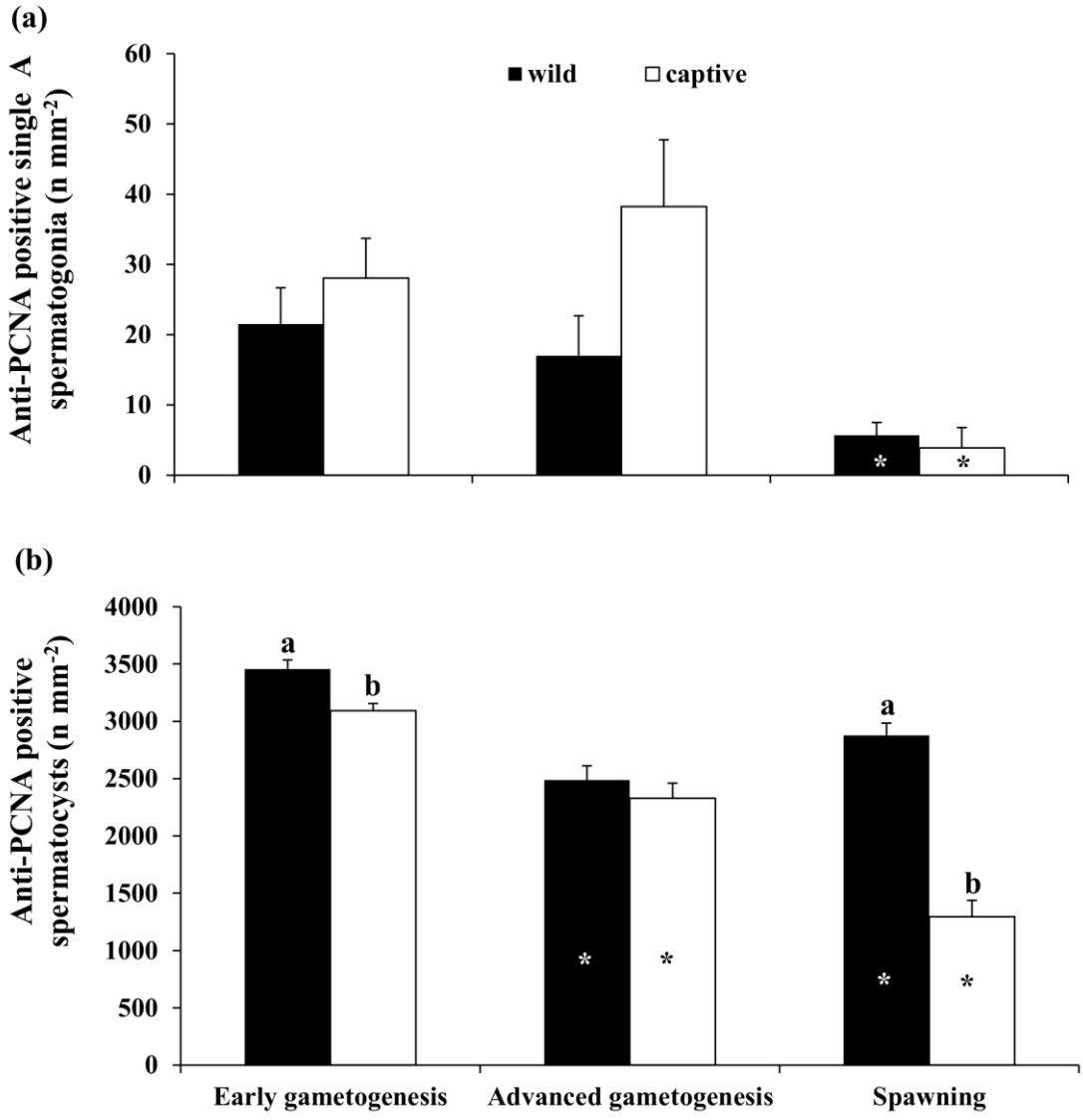
(a)
Anti-PCNA positive single A spermatogonia (n mm-2)
wild
captive
60
50
40
30
20
10
0
*
*
(b)
Anti-PCNA positive spermatocysts (n mm-2)
4000
3500
3000
2500
2000
1500
1000
500
0
a
b
a
b
*
*
*
*
Early gametogenesis
Advanced gametogenesis
Spawning

Figure 4

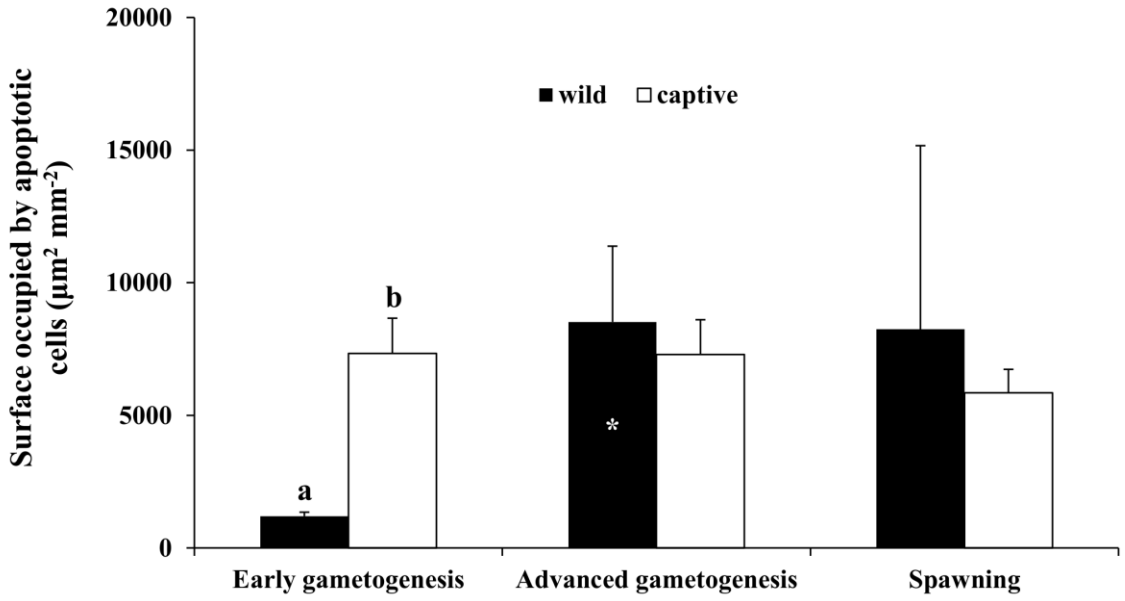
Surface occupied by apoptotic cells (µm² mm⁻²)
20000
15000
10000
5000
0
wild
captive
a
b
*
Early gametogenesis
Advanced gametogenesis
Spawning

Figure 5

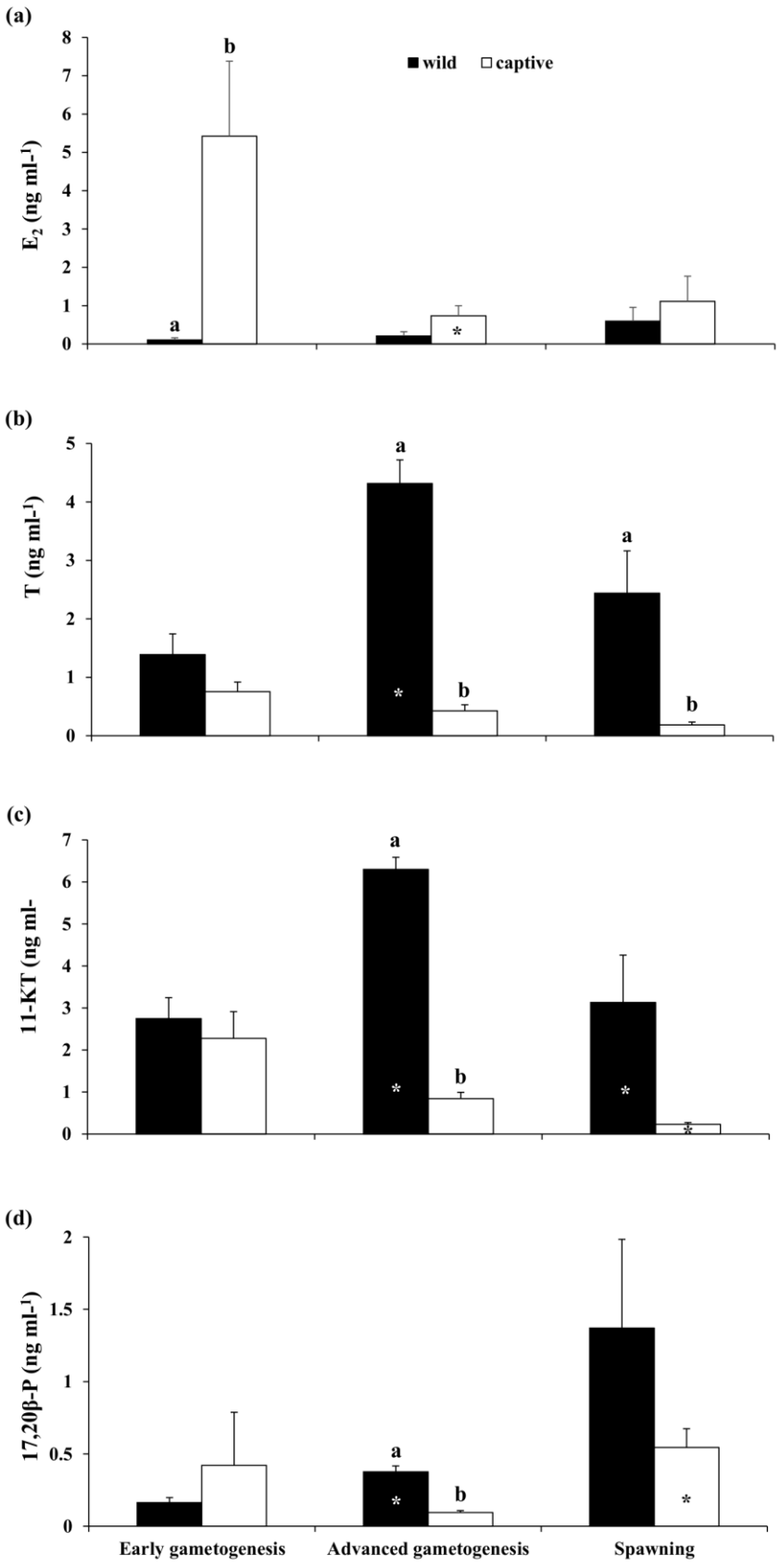
(a)
wild
captive
E_2 (ng ml^{-1})
a
b
*
(b)
T (ng ml^{-1})
a
b
a
b
*
(c)
11-KT (ng ml-
a
b
*
*
*
(d)
17,20β-P (ng ml^{-1})
a
b
*
*
Early gametogenesis
Advanced gametogenesis
Spawning

Figure 6

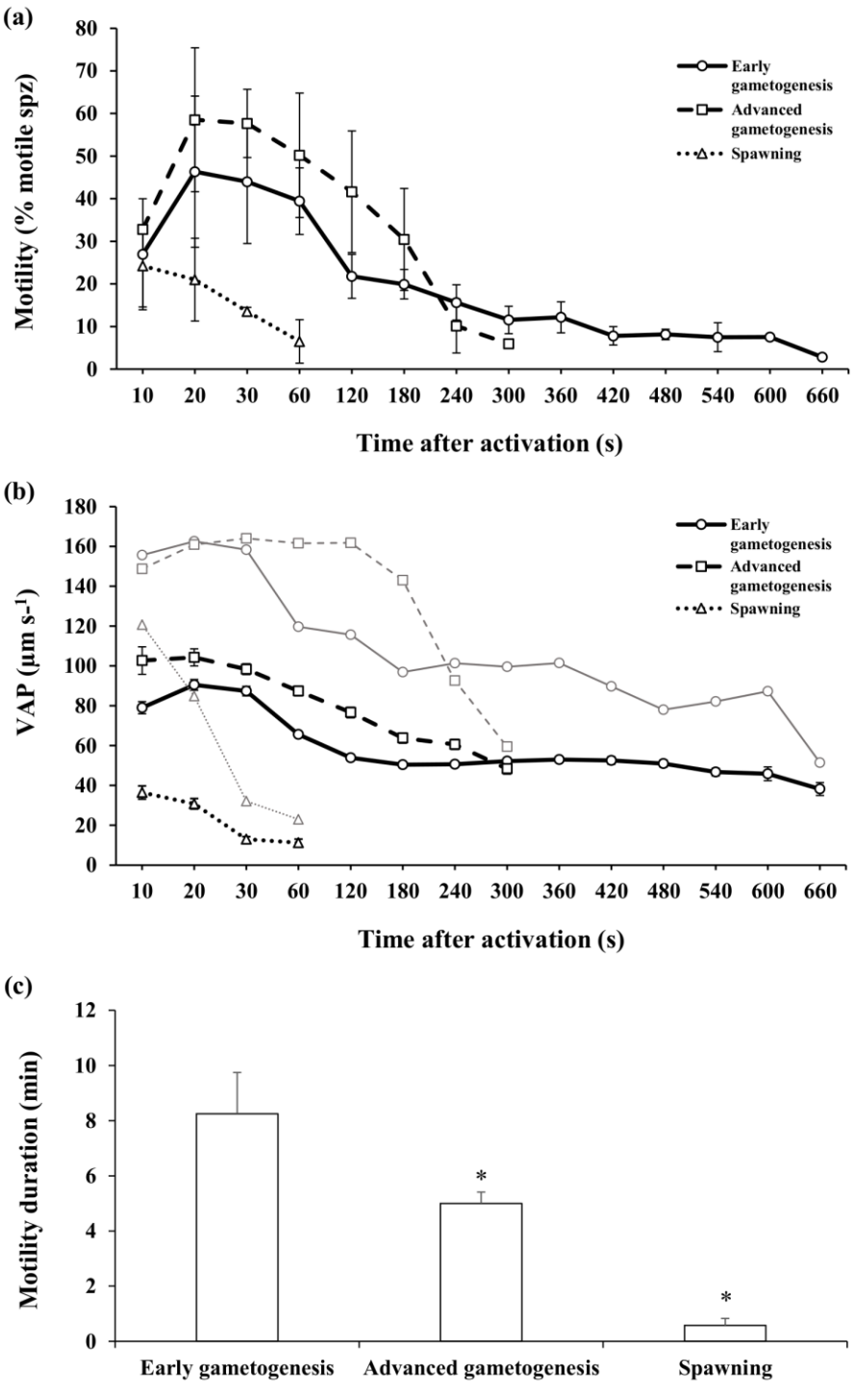
(a)
Motility (% motile spz)
Early gametogenesis
Advanced gametogenesis
Spawning
Time after activation (s)
(b)
VAP (µm s-1)
Early gametogenesis
Advanced gametogenesis
Spawning
Time after activation (s)
(c)
Motility duration (min)
Early gametogenesis
Advanced gametogenesis
Spawning

Figure 7

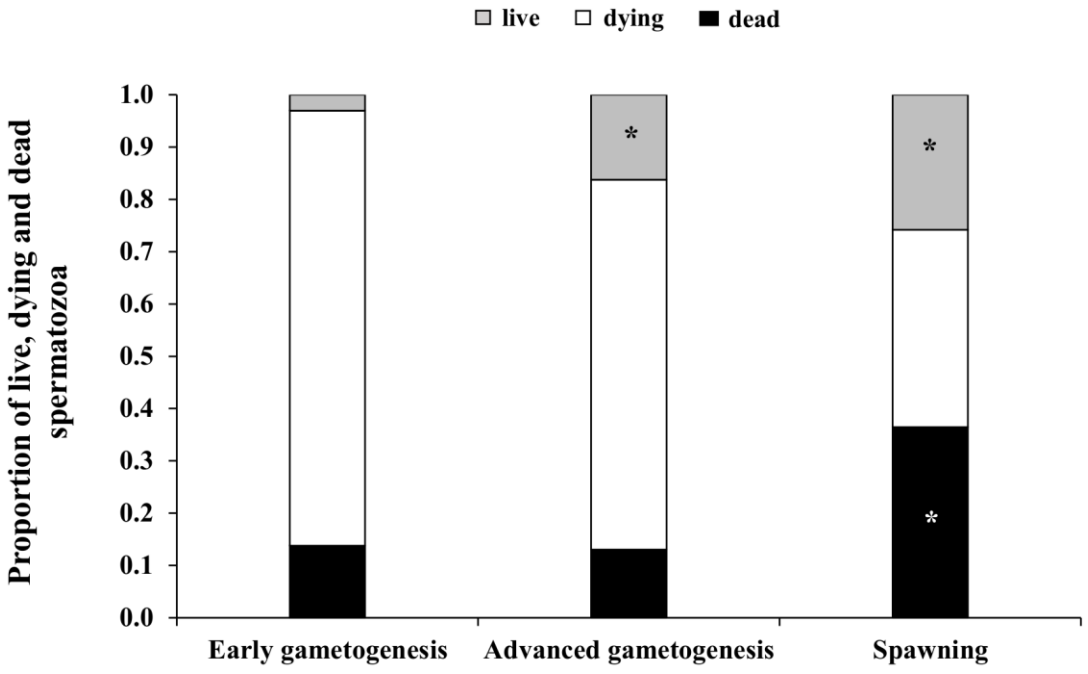
live
dying
dead
Proportion of live, dying and dead spermatozoa
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Early gametogenesis
Advanced gametogenesis
Spawning

Figure Captions

Figure 1. Micrographs of testis sections of greater amberjack sampled in different periods of the reproductive cycle. (a) Peripheral region of the testis and detail of a seminiferous lobule (inset) of an individual sampled in early May, immunostained with anti-Pou5f1 antibodies. Interlobular (yellow curved arrows) and intralobular (yellow arrowheads) positive cells are stained in brown. Magnification bar = 50 µm; in inset = 10 µm. (b) Testis section of an individual sampled in late May showing the different germ cell types. Haematoxylin-eosin (H-E) staining. Magnification bar = 25 µm. Single A spermatogonia are indicated by black arrows and arrowheads; the latter point to cells likely corresponding to the intralobular anti-Pou5f1 positive spermatogonia (undifferentiated stem spermatogonia) shown in (a). Asterisk: type A spermatogonial cyst; double asterisk: type B spermatogonial cyst; sd: spermatid cyst; scI: primary spermatocyte cyst; scII: secondary spermatocyte cyst; sz: spermatozoa.

Figure 2. Micrographs of greater amberjack testis sections sampled in different periods of the reproductive cycle. (a) Testis section of a wild individual sampled in early May, immunostained with antibodies against the Proliferating Cell Nuclear Antigen (PCNA), which stains brown the nuclei of proliferating cells. Magnification bar = 40 µm. Arrowhead: anti-PCNA positive single spermatogonium; double arrow: anti-PCNA positive spermatogonial cyst; dashed arrow: primary spermatocyte cyst. (b) Testis section of a captive-reared individual sampled during late April stained with the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method, with apoptotic cells appearing as dark blue dots. Magnification bar = 150 µm. Arrow: TUNEL positive single spermatogonium; curved arrow: TUNEL positive spermatocysts.

Figure 3. Changes in mean (±SE) anti-PCNA positive germ cell density in wild and captive-reared greater amberjack males during the reproductive season. (a) Anti-PCNA positive single A spermatogonia. (b) Anti-PCNA positive spermatocysts. White asterisks indicate statistically significant differences versus the preceding phase in wild fish. Black asterisks indicate statistically significant differences versus the preceding phase in captive fish. Different letters represent significant differences between wild and captive individuals within the same sampling phase. (Student's t-test, $P < 0.05$).

Figure 4. Changes in mean (±SE) surface occupied by apoptotic germ cells in wild and captive-reared male greater amberjack sampled during the reproductive season in the Mediterranean Sea. White asterisk indicates statistically significant difference versus the preceding phase in wild fish. Different letters indicate significant difference between wild and captive individuals sampled in the same phase. (Student's t-test, $P < 0.05$).

Figure 5. Mean (± SE) plasma (a) 17-β Estradiol (E_2), (b) Testosterone (T), (c) 11-Ketotestosterone (11-KT), and (d) 17,20β-dihydroxypren-4-en-3-one (17,20β-P) in wild and captive-reared greater amberjack males sampled during the reproductive season in the Mediterranean Sea. White asterisks indicate statistically significant differences versus the preceding phase in wild fish. Black asterisks indicate statistically significant differences versus the preceding phase in captive fish. Different letters indicate significant differences between wild and captive individuals sampled in the same phase. (Student's t-test, $P < 0.05$). Graphs (b), (c) and (d) have been taken from Zupa et al. (2017).

Figure 6. (a) Sperm motility percentage, (b) average path velocity (VAP), and (c) motility duration in captive-reared greater amberjack during three phases of the reproductive season in the Mediterranean Sea**.** In (b), black lines illustrate the mean VAP of sperm population for each phase, while grey lines show the maximum value of individual sperm velocity recorded. In (c), black asterisks indicate significant differences versus the preceding phase (ANOVA, $P < 0.05$).

Figure 7. Proportion of live/dying/dead spermatozoa in captive-reared greater amberjack during three phases of the reproductive season in the Mediterranean Sea. Black and white asterisks indicate significant differences versus the preceding phase within the same spermatozoa condition (ANOVA, $P < 0.05$).