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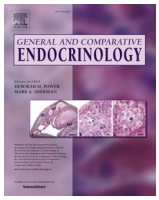

Research paper

Gonadotropin expression, pituitary and plasma levels in the reproductive cycle of wild and captive-reared greater amberjack (*Seriola dumerili*)

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ABSTRACT

We compared the endocrine status of the pituitary-gonad axis of wild and captive-reared greater amberjack (*Seriola dumerili*) during the reproductive cycle (April – July), reporting on the expression and release of the two gonadotropins for the first time in the Mediterranean Sea. Ovaries from wild females were characterized histologically as DEVELOPING in early May and SPAWNING capable in late May-July, the latter having a 3 to 4-fold higher gonadosomatic index (GSI). SPAWNING capable wild females exhibited an increase in pituitary follicle stimulating hormone (Fsh) content, plasma testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), while almost a 10-fold increase was observed in pituitary luteinizing hormone (Lh) content. An increasing trend of plasma 17β-estradiol (E_2) was also recorded between the two reproductive stages in wild females. Captive-reared females sampled during the reproductive cycle exhibited two additional reproductive categories, with REGRESSED females having extensive follicular atresia and fish in the REGENERATING stage having only primary oocytes in their ovaries. Pituitary content of Fsh and Lh, *fshb* and *lhb* expression and plasma levels of Fsh and Lh remained unchanged among the four reproductive stages in captive females, in contrast with plasma E_2 and T that decreased in the REGENERATING stage, and 17,20β-P which increased after the DEVELOPING stage. In general, no significant hormonal differences were recorded between captive-reared and wild DEVELOPING females, in contrast to SPAWNING capable females, where pituitary Lh content, plasma Fsh and T were found to be lower in females in captivity. Overall, the captive females lagged behind in reproductive development compared to the wild ones and this was perhaps related to the multiple handling of the sea cages where all the sampled fish were maintained. Between wild males in the DEVELOPING and SPAWNING capable stages, pituitary Lh content, plasma T and 17,20β-P, and GSI exhibited 3 to 4-fold increases, while an increasing trend of pituitary Fsh content, *lhb* expression levels and plasma 11-ketotestosterone (11-KT) was also observed, and an opposite trend was observed in plasma Lh. Captive males were allocated to one more category, with REGRESSED individuals having no spermatogenic capacity. During the SPAWNING capable phase, almost all measured parameters were lower in captive males compared to wild ones. More importantly, captive males showed significant differences from their wild counterparts throughout the reproductive season, starting already from the DEVELOPING stage. Therefore, it appears that captivity already exerted negative effects in males prior to the onset of the study and the multiple handling of the cage where sampled fish were reared. Overall, the present study demonstrated that female greater amberjack do undergo full vitellogenesis in captivity, albeit with some dysfunctions that may be related to the husbandry of the experiment, while males, on the other hand, may be more seriously affected by captivity even before the onset of the study.

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1. Introduction

Marine aquaculture is expected to provide an increasing contribution in reducing the gap between demand and supply of seafood (FAO, 2022), and aquaculture in general is a more environmentally friendly protein production system compared to livestock production (Nijdam et al., 2012). Almost 70 % of the 250 farmed finfish species in the world are dependent to a certain extend to wild resources for broodstock production and only a few species can be considered fully domesticated (Teletchea and Fontaine, 2014). In fact, reproduction control is one of the major bottlenecks in the domestication process of many fish species (Mylonas et al., 2010; Zohar and Mylonas, 2001).

To control reproductive maturation, the brain integrates (a) endogenous signals, such as developmental stage, body size, nutritional status and energy stores, and (b) environmental signals, such as photoperiod, temperature, landscape and social conditions, to regulate the synthesis and secretion of gonadotropin releasing hormone (GnRH), the main brain hormone controlling the reproductive axis in vertebrates (Karine et al., 2020; Munoz-Cueto et al., 2020; Parker and Cheung, 2020). Then, GnRH binds to the respective receptors expressed by the gonadotropic cells of the pituitary, and stimulates the synthesis and release of follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), the key factors regulating gonadal function (Levavi-Sivan et al., 2010; Zohar et al., 2010). The Fsh and Lh are heterodimers with a common α subunit and differing in their β subunits, and Lhβ is more phylogenetically conserved compared to Fshβ (Levavi-Sivan et al., 2010; Rosenfeld et al., 2007). In salmonids, which are synchronous spawning fishes (*i.e.* a single egg batch is produced during the annual reproductive cycle), Fsh has a predominant role in vitellogenesis/spermatogenesis, while Lh is mainly involved in the final stages of maturation (oocyte maturation and spermiation). However, the reproductive endocrinology of asynchronous or group-synchronous spawning fishes (*i.e.* fish that spawn many egg batches during the annual reproductive cycle) is more complex and the role of the two gonadotropins (GtHs) is ambiguous (Rosenfeld et al., 2007). Therefore, species-specific studies are needed to discriminate the specific function of Fsh and Lh in different fishes, especially the ones with interest in aquaculture production.

The greater amberjack (*Seriola dumerili*) is a very promising species for aquaculture diversification throughout the world (Crespo et al., 1994; Fakriadis et al., 2020b; Lazzari et al., 2000; Paxton et al., 1989), together with a number of congener species (Corriero et al., 2021a). The greater amberjack exhibits reproductive dysfunctions in captivity, since captive-reared breeders maintained either in tanks (Fakriadis et al., 2020b; Jerez et al., 2018; Jerez et al., 2006; Micale et al., 1999; Mylonas et al., 2004; Sarih et al., 2018) or in sea cages (Kozul et al., 2001; Zupa et al., 2017b) fail to reproduce, unless treated with spawning inducing hormones (Corriero et al., 2021a; Fakriadis et al., 2019; Fakriadis et al., 2020a; Fakriadis and Mylonas, 2021; Fakriadis et al., 2020b). Recently, a comparative study of reproductive development in wild and captive-reared greater amberjack showed a great susceptibility to husbandry-induced stress, associated with a significant long-term reduction in plasma testosterone (T), 17β-estradiol (E_2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in females (Zupa et al., 2017b). As a result, the gonadosomatic index (GSI) was significantly reduced at the peak of the reproductive season, and ovaries underwent extensive follicular atresia. Similarly, significant reductions in plasma T, 11-ketotestosterone (11-KT) and 17,20β-P were observed in males at the peak of spermatogenesis, again resulting in significant reductions in GSI (Zupa et al., 2017b), concomitant with elevations in plasma E_2, reduction in spermatogonial mitosis and high level of apoptosis even at the beginning of the reproductive season (Zupa et al., 2017a). Similar reproductive dysfunctions were observed in both male (Lavecchia et al., 2023) and female (unpublished data) hatchery-produced F1 generation greater amberjack, and these dysfunctions were associated to a wide dysregulation of gonad gene expression.

The aim of the present study was to examine for the first time the expression of *fshb* and *lhb* in the pituitary, and the pituitary and plasma levels of Fsh and Lh during the reproductive cycle of greater amberjack in the Mediterranean Sea, in a comparison between breeders reared in captivity or captured in the wild during different times between April and July. The obtained data was examined together with GSI values, gonadal histology and plasma sex steroid levels reported previously (Zupa et al., 2017a; Zupa et al., 2017b), but now reorganized and analyzed according to gonadal stage of reproductive development -instead of simply time of sampling during the reproductive season. This was done in order to obtain further insights into the endocrine dysfunction and at what reproductive stage it occurs in wild-caught greater amberjack reared in captivity.

2. Materials and methods

2.1. Broodstock maintenance, sampling and welfare

The present study was based on the analysis of 33 breeders sampled in the wild (14 males and 19 females) and 24 breeders captured as juveniles from the wild and reared in captivity until reproductive maturity (12 males and 12 females). The fish were sampled at three different times of the reproductive cycle in 2014 and 2015 expecting to sample fish at different stages of reproductive maturation including spawning: in late April-early May (wild females n = 5, wild males n = 5, captive females n = 4, captive males n = 4), in late May-early June (wild females n = 2, wild males n = 4, captive females n = 4, captive males n = 4) and in late June-July (wild females n = 12, wild males n = 5, captive females n = 4, captive males n = 4).

Wild fish were caught by commercial purse-seiners around the Pelagie Islands (Sicily, Italy) and sampled on board immediately after death for biometric data (fork length (FL), body mass (BM) and gonad mass (GM)) and blood, gonads and pituitaries were collected and preserved. Captive-reared individuals were captured as juveniles from the wild in 2011 (0–1 + year of age) in the area of Astakos (Ionian Sea, Greece) and were reared in commercial sea cages. In September 2013, the fish were transferred to the sea-cage facilities of Argosaronikos Fishfarming S.A. (Salamina Island, Greece), where they were reared in a sea cage for two years according to standard farming practices (Zupa et al., 2017b) and were sampled during the reproductive season of 2015.

Before each of the three samplings, all captive-reared fish were confined in a plastic sack of 20 m^3 and then were tranquilized with 0.01 ml l^{-1} clove oil dissolved in ethanol at a 1:10 ratio. Then, with the objective of sampling four males and four females at each sampling, fish of unknown sex 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. Subsequently, fish were sexed using a gonadal biopsy and a blood sample was obtained from the caudal vasculature using a heparinized syringe. Then the fish were euthanized by decapitation, were placed in crushed ice and transferred to the farm facility for further collection of biometric data and tissue samples. The GSI of all sampled fish was calculated as GSI % = 100 GM BM^{-1}.

For the present study, ethical approval was not required according to Italian decree n. 26 of 04 March 2014 and Greek presidential decree 56/2013, since fish were either obtained from a registered aquaculture facility during regular fish rearing operations or from the commercial fishery. Captive-reared fish were reared at a registered aquaculture facility for 3 years, according to routine farming practices, before they were used for this study. Each fish was first anaesthetized with clove oil for 10 min and then painlessly sacrificed by decapitation. Wild greater amberjacks were captured by the commercial purse seine fishing vessel "Graziella" authorized to catch pelagic fish by the port authority of Porto Empedocle (Agrigento, Italy). No specific permission was required because these fish were commercially caught during routine fishing operations, placed on ice by the fishermen and left to die. 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).

2.2. Histological analysis

One-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 then stained with hematoxylin-eosin. The evaluation of the ovaries was based on the most advanced oocyte population, the presence of post-ovulatory follicles (POF) and the occurrence of follicular apoptosis/atresia. The evaluation of the testes was based on the presence of spermatocysts with different germ cell types, the presence of luminal spermatozoa and the abundance of somatic intertubular tissue. No quantification of tubule area or sperm amount is reported, and these parameters were subjectively assessed through the simple observation of the histological slides.

After histological examination of the gonads, fish were grouped according to their reproductive stage (Table 1) and were classified as DEVELOPING (gonads were undergoing gametogenesis, but were not yet ready to spawn), SPAWNING capable (well-developed gonads, able developmentally to complete maturation and spawn), REGRESSED (reproductive development ceased) or REGENERATING (reproductively inactive), following the criteria of Brown-Peterson et al. (2011).

2.3. Sex steroid plasma levels

Plasma levels of 17β-estradiol (E_2), 17,20β-dihydroxy-4-pregnen-3-one (17, 20β-P) testosterone (T), and 11-ketotestosterone (11-KT) were measured with the use of enzyme-linked immunosorbent assays (ELISAs) according to (Rodríguez et al., 2000), (Nash et al., 2000) and (Cuisset et al., 1994), with some modifications and using reagents from Cayman Chemical Company (USA). For the steroid extraction, 200 μl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing, samples were frozen for 10 min at −80 °C and the supernatant organic phase was collected in new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in 600 μl of Reaction Buffer (dilution 1:2).

Table 1
Biometric data (mean ± SD) of captive-reared and wild greater amberjack sampled during three periods of the reproductive season (late April-early May, late May-early June, late June-July) in the Mediterranean Sea (modified by (Zupa et al., 2017b)). Reproductive stage evaluation was done after histological examination of the ovaries following the criteria of Brown-Peterson et al. (2011) (F: female, M: male).

Fish origin	Sex	N	Reproductive stage	Fork length (cm)	Body mass (kg)	GSI (%)
captive	F	4	DEVELOPING	95 ± 6	12.9 ± 1.9	1.0 ± 0.2
wild	F	5	DEVELOPING	108 ± 6	16.3 ± 3.2	1.1 ± 0.3
captive	M	4	DEVELOPING	95 ± 4	13.1 ± 1.5	0.5 ± 0.1
wild	M	5	DEVELOPING	113 ± 2	17.3 ± 2.3	2.3 ± 0.4
captive	F	2	SPAWNING capable	99 ± 3	12.6 ± 0.4	6.2 ± 1.2
wild	F	14	SPAWNING capable	103 ± 7	14.2 ± 3.6	4.8 ± 1.5
captive	M	3	SPAWNING capable	97 ± 7	12.4 ± 2.6	4.4 ± 0.4
wild	M	9	SPAWNING capable	105 ± 9	14.7 ± 3.9	6.5 ± 2.4
captive	F	4	REGRESSED	99 ± 5	13.3 ± 2.3	1.7 ± 0.7
captive	M	5	REGRESSED	95 ± 2	11.8 ± 1.6	1.3 ± 0.5
captive	F	2	REGENERATING	94 ± 3	10.0 ± 2.5	1.1 ± 0.0

2.4. Pituitary and plasma concentrations of gonadotropins

Pituitaries were homogenized individually in 200 μl of cold ultrapure H_20 and used for RNA extraction (60 μl) and GtH measurements (140 μl). The latter fraction was diluted (v:v) with 140 μl of x2 PBS and separated in two aliquots and stored at −80 °C for further analyses. Plasma was separated from the blood by centrifugation (5000 rpm for 5 min) and stored at −80 °C for further analyses.

Pituitary and plasma Lh levels were measured using the heterologous ELISA developed for striped bass (*Morone saxatilis*) Lh (Mañanos et al., 1997) that was modified for tuna species (Berkovich et al., 2013; Rosenfeld et al., 2012) and validated for the greater amberjack. For that purpose, pituitary extract derived from a captive amberjack female was assayed at four serial dilutions (1:50, 1:100, 1:200 and 1:400) and plasma samples of two wild caught fish were assayed at three serial dilutions (1:8, 1:16, 1:32). A clear linearity was obtained in the dilution of the pituitary of the captive greater amberjack female and the plasma samples derived from wild fish (see Supplementary Figure S1). Moreover, the dilution curves exhibited parallelism with the standard recombinant Lh (r-Lh) enabling the determination of Lh in this species. The sensitivity of the assay was 0.65 ng ml^{-1} and the respective inter- and intra- assay coefficients of variation were 8 % and 15 %, respectively. Ninety-six well polystyrene plates were coated with r-Lh (2.4 ng $well^{-1}$) and incubated overnight at 4 °C. The plates were then washed with PBST and blocked with BSA (2 % in PBST; 100 μl $well^{-1}$) for 0.5 h at 37 °C. The primary antibody (anti-striped bass Lh) was diluted 1:80,000 in PBST containing 2 % normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4 °C. Then the content in each tube was dispensed into the antigen-coated wells (100 μl $well^{-1}$ in duplicates). Following incubation (overnight at 4 °C) Affini-Pure Goat anti-Rabbit IgG (H + L) (Jackson Immunoresearch Laboratories Inc.) in 1 % NGS-PBS T was added (100 μl $well^{-1}$) for 0.5 h at 37 °C. Then, the wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 μl $well^{-1}$). The reaction was stopped after 20 to 40 min at room temperature by the addition of 100 μl of 1 N phosphoric acid and the absorbance was read at 450 nm.

The related pituitary and plasma Fsh levels were measured using a homologous ELISA that was developed specifically for greater amberjack. Briefly, recombinant greater amberjack Fsh (rec-gaFsh) standard antigen was produced using the *Pichia pastoris* yeast recombinant DNA expression system (Invitrogen, Carlsbad, CA). For that purpose, a translational fusion consisting of sequence encoding for gaFshβ mature peptide (Accession # LC019038.1) tandemly fused to the mice gonadotropin alpha subunit sequence (Accession #AF307151.1) and a chain of 6 histidine residues (6xHis-Tag), was synthetically synthesized (GenScript, Piscataway NJ. USA) and introduced into the *P. pastoris* expression vector, pPIC9K (Invitrogen). Following linearization with SalI, the constructed plasmid was used to transform the host strain GS115 his4 (auxotropihic for histidine) using the Micro-Pulser-Electroporator (Bio-Rad Laboratories) adjusted to yeast cells (Voltage- 2 kV; Time constant-3.7 ms). Transformant colonies selection, protein production and purification were as described in Berkovich at al. 2013. The purified rec-gaFsh was subjected to SDS-PAGE analysis that confirmed the presence of an intact protein at the expected size (see Supplementary Figure S2). The purified rec-gaFsh was used as antigen for both standardization and generation of the specific polyclonal antibodies. Antibodies against the purified rec-gaFsh were produced by a commercial company (Adar-Biotech, Ness Ziyona, Israel.). Two rabbits were immunized (4 subsequent immunizations) with 2 mg of rec-gaFsh. Then, rabbits were bled and the harvested sera was negatively purified on an affinity column against r-Lh antigen. The gaFsh ELISA was carried out as

described above for the Lh, with the following modifications: (1) Ninety-six well polystyrene plates were coated with rec-gaFsh (25 ng well^{-1}), (2) The primary antibody (anti-rec-gaFsh) was diluted 1:1,500 in PBST containing 2 % normal goat serum (NGS). Comparison of displacement curve, obtained with a serial dilution of pituitary extract (PE; x2, x4, x8, x16, x32, x64, x124) from wild-caught greater amberjack, and the rec-gaFsh standard curve, indicated linear responses (Supplementary Fig. S3A). Furthermore, no significant cross-reactivity with the r-Lh was detected (Supplementary Fig. S3B). The standard curve ranged from 100 ng ml^{-1} to 0.19 ng ml^{-1}. The intra-assay coefficient of variation for standard of 10 ng ml^{-1} in the same plate was 3.0 % (n = 10). The inter-assay coefficient of variation for the same plasma sample on different plates was 9.6 % (n = 7).

2.5. Pituitary gonadotropin gene expression levels

Total RNA was obtained from pituitary using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 μl of RNase free water and stored at –80 °C. Two μg of DNAse treated total RNA were reverse transcribed with random primers using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Branchburg, NJ) according to manufacturer's protocol. Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicate in 10 μl reaction volumes consisting of Fast SYBR Green Master Mix (Applied Biosystems). Amplification was carried out in a Fast Real Time PCR System (Applied Biosystems). Cycling parameters were as follows: 3 s at 95 °C, and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. The presence of a single amplicon was verified using a melting curve run following the PCR. To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR experiments included minus-reverse transcriptase controls (*i.e.*, PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = $2^{-\Delta\Delta Ct}$, Ct – threshold cycle (Livak and Schmittgen, 2001). The gene specific primers (GSPs) designed using the Primer Express 3.0 software (Applied Biosystems) are listed in Table 2.

2.6. Statistical analysis

Differences in the measured parameters among fish in different reproductive stage, or between fish from different origin (captive versus wild) within reproductive stage were tested using Welch's Analysis of Variance (ANOVA) followed by Tukey's HSD post hoc test. Data were transformed accordingly to meet the ANOVA assumptions, if not normally distributed. A level of $P \leq 0.05$ was set as minimum statistical significance for the ANOVA tests. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

3. Results

Both wild and captive females sampled in late April - early May, at sea surface temperatures between 17.5 and 18.1 °C, were found to be in the DEVELOPING reproductive stage. All females had mostly primary oocytes (po) and a few cortical alveoli (ca) oocytes, while some females showed also a few early vitellogenic oocytes (eVg) (Fig. 1A). One month later (late May – early June), when temperature reached 19.5–20.0 °C, 100 % of wild females were at the SPAWNING capable reproductive stage, since late Vg oocytes (Fig. 1C) and post-ovulatory follicles (not shown) were observed. At this time, 50 % of the captive females were at the SPAWNING capable stage, but with increased follicular atresia and absence of post-ovulatory follicles compared to wild fish. The remaining captive females were already REGRESSED. In the ovaries of these fish, almost all the advanced vitellogenic oocytes were atretic and only small vitellogenic oocytes (mid-vitellogenesis, midVg) were still intact (Fig. 1E). At temperatures of 23.4–23.8 °C in late June – early July, all wild females were at the SPAWNING capable stage having late vitellogenic oocytes and 17 % of the wild females had oocytes in maturation (not shown), expected to ovulate within 24 h. No follicular atresia was recorded, while post-ovulatory follicles were visible in almost all fish sampled. On the contrary, 50 % of the captive females at this time were categorized as REGRESSED with extensive follicular atresia concomitantly with a few eVg or midVg oocytes (Fig. 1E), while the ovaries of the other females were in the REGENERATION phase (Fig. 1G).

Both captive and wild males were in a DEVELOPING reproductive stage in late April – early May, having spermatocysts at all stages of development, with spermatocytes (sc), spermatids (st) and spermatozoa (sz) (Fig. 1B). One month later, all wild males were classified as SPAWNING capable, with large numbers of spermatozoa in their testes (Fig. 1D). On the contrary, only 75 % of the captive males were classified as SPAWNING capable, but appeared to have less spermatozoa in the lumen of the testicular lobules compared to their wild counterparts. Furthermore, 25 % of the captive males had already ceased their spermatogenic activity, showing thick somatic tissue around the seminiferous tubules, and were classified as REGRESSED (Fig. 1F). One month later in late June – early July, wild males were still categorized as SPAWNING capable, while all captive males were characterized as REGRESSED.

In wild and captive fish of both sexes, the GSI was significantly higher at the SPAWNING capable stage compared to the other stages (ANOVA, $P < 0.001$) (Fig. 2). Wild males at the DEVELOPING stage had higher GSI compared to captive fish at the same stage (ANOVA, $P < 0.05$).

Pituitary *fshb* (Fig. 3A) and *lhb* (Fig. 4A) expression levels and plasma Fsh (Fig. 3E) and Lh (Fig. 4E) remained unchanged both for captive and wild females among the different reproductive stages examined. The plasma Fsh levels were higher in wild females compared to the captive ones at the SPAWNING capable stage (ANOVA, $P < 0.05$). Additionally, a significant increase in pituitary Fsh (ANOVA, $P < 0.05$) (Fig. 3C) and Lh (ANOVA, $P < 0.001$) (Fig. 4C) content between the DEVELOPING and SPAWNING capable wild females was observed, as well as higher levels of pituitary Lh content in wild females compared to the captive ones at the SPAWNING capable stage (ANOVA, $P < 0.05$).

Regarding males, no significant differences were observed in *fshb* expression levels (Fig. 3B), pituitary Fsh content (Fig. 3D), plasma Fsh (Fig. 3F), *lhb* expression (Fig. 4B) and plasma Lh levels (Fig. 4F) among the reproductive stages of either captive or wild males (ANOVA, $P > 0.05$). However, significantly higher levels of plasma Fsh (ANOVA, $P < 0.05$) (Fig. 3F) and Lh (ANOVA, $P < 0.05$) (Fig. 4F) were recorded in wild compared to captive males in the DEVELOPING and SPAWNING

Table 2
Gene specific primers used to quantify the respective gene expression levels by real-time PCR.

		Primer sequence (5′ → 3′)	
Gene	Accession #	Forward	Reverse
lhβ	LC019039.1	CCCGGTGGCTTTGAGCTG	GTCGGGTTGCAGGCTCTC
fshβ	LC019038.1	GGGACTGGTCCTTTGAGGTGAA	GCGGCACACTTGCAGTTTC
18S	AY850370.1	GCGAAAGCATTTGCCAAGAATG	CGCTAGTTGGCATCGTTTATGG

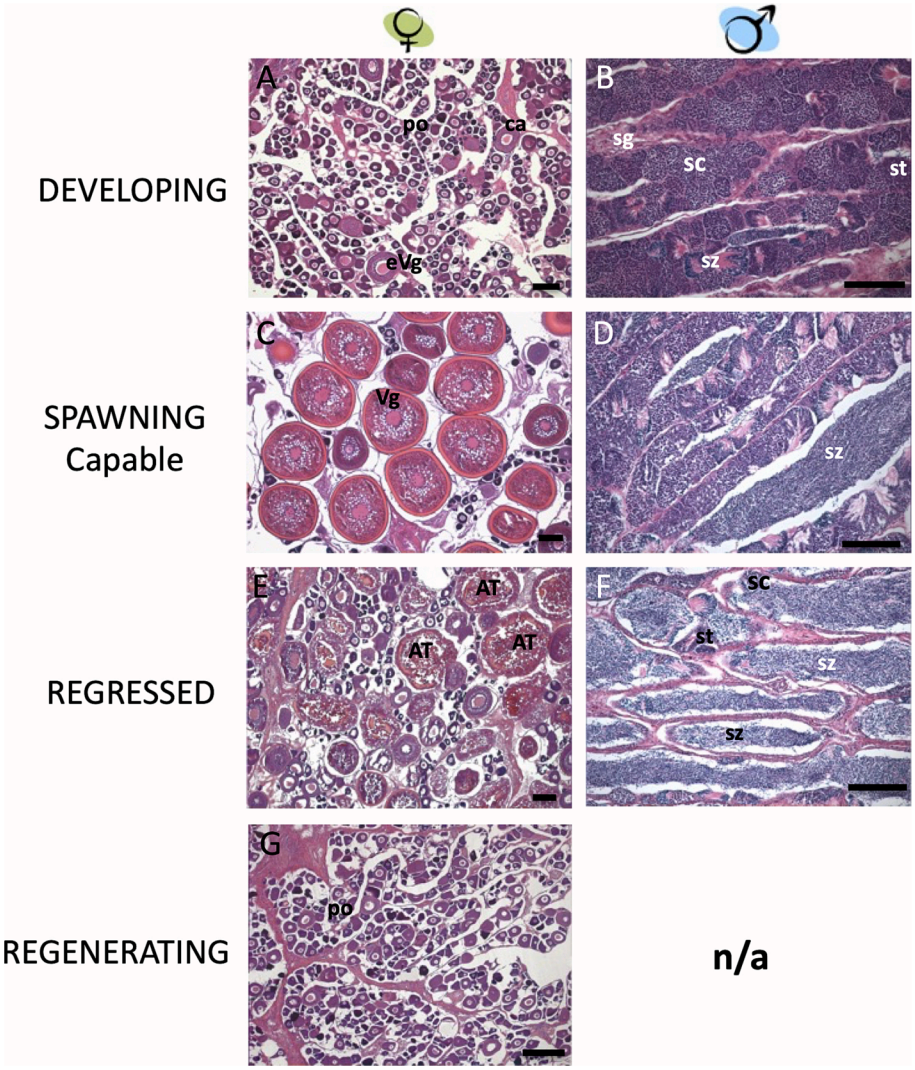

Fig. 1. Representative microphotographs of ovaries (A, C, E, G) and testes (B, D, F) at different reproductive stages (DEVELOPING, SPAWNING capable, REGRESSED and REGENERATING) from wild or captive-reared female and male greater amberjack (*Seriola dumerili*), sampled during three periods of the reproductive season in the Mediterranean Sea (late April-early May, late May-early June, late June-July). po: primary oocyte, ca: oocyte at cortical alveoli stage, Vg: late vitellogenesis oocyte, AT: atretic follicle, sg: spermatogonia, sc: spermatocytes, st: spermatids, sz: spermatozoa. Ovaries, bar = 150 μm; testes, bar = 100 μm.

capable stages, as well as pituitary Lh content (ANOVA, $P < 0.05$) in SPAWNING capable stage (Fig. 4D). Pituitary Lh content increased significantly for the wild males from the DEVELOPING to the SPAWNING capable stage (ANOVA, $P < 0.05$) (Fig. 4D), but not for the captive males, where an increase was observed only between the DEVELOPING and REGRESSED stages (ANOVA, $P < 0.05$). On the other hand, significantly lower *fshb* expression levels were recorded in the wild males at the DEVELOPING stage compared to their captive counterparts (ANOVA, $P < 0.05$) (Fig. 3B).

Plasma sex steroid levels in captive females exhibited significant differences among different reproductive stages (T, ANOVA, $P < 0.05$, Fig. 5A; E_2, ANOVA, $P < 0.05$, Fig. 5C; 17, 20β-P, ANOVA, $P < 0.001$, Fig. 5G), whereas in wild females, T (Fig. 5A) and 17, 20β-P (Fig. 5G) increased between the DEVELOPING and SPAWNING capable stages (ANOVA, $P < 0.001$). Specifically, plasma T (Fig. 5A) and E_2 (Fig. 5C) of captive females decreased at the REGENERATION stage, while 17, 20β-P (Fig. 5G) increased from the DEVELOPING to SPAWNING capable stages and remained unchanged at the other two later stages. In wild fish, plasma T increased from the DEVELOPING to the SPAWNING capable stage (Fig. 5A). Significantly higher values were observed in the wild females compare to the captive ones for plasma T (Fig. 5A) at the SPAWNING capable stage (ANOVA, $P < 0.01$) and for 17, 20β-P (Fig. 5G) (ANOVA, $P < 0.01$) at the DEVELOPING stage.

Some differences in plasma steroid levels of males were observed among different reproductive stages, for T (ANOVA, $P < 0.05$) (Fig. 5B), 11-KT (ANOVA, $P < 0.01$) (Fig. 5F) and 17, 20β-P (ANOVA, $P < 0.05$) (Fig. 5H) of captive males, and for T (ANOVA, $P < 0.05$) (Fig. 5B) and 17, 20β-P (ANOVA, $P < 0.01$) (Fig. 5H) of wild males. Specifically, plasma T and 11-KT of captive males decreased at the REGRESSED stage, while 17, 20β-P increased. In the wild males, plasma T (Fig. 5B) and 17, 20β-P (Fig. 5H) increased from the DEVELOPING to the SPAWNING capable stage. Significantly higher values in wild males compared to captive ones were observed for plasma T (one-way ANOVA, $P < 0.001$), 11-KT (ANOVA, $P < 0.05$) and 17, 20β-P (ANOVA, $P < 0.01$) in SPAWNING capable fish, and for 17, 20β-P (ANOVA, $P < 0.05$) in males at the DEVELOPING stage.

4. Discussion

Fish reproductive dysfunctions in captivity are more common in females and have been classified in three types (Mylonas et al., 2010; Zohar and Mylonas, 2001). These include (a) fish that fail completely to undergo vitellogenesis in captivity, (b) fish that undergo vitellogenesis, but do not enter oocyte maturation and (c) fish that mature and ovulate,

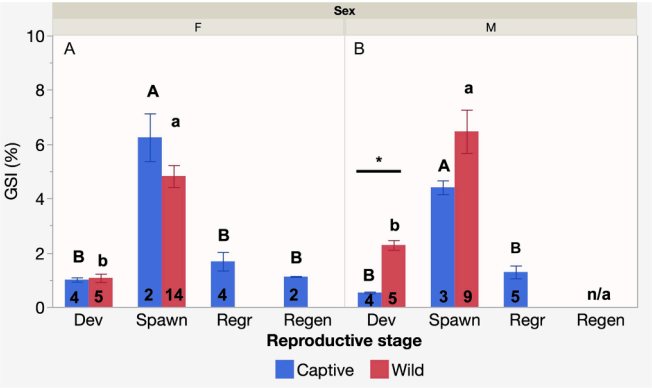

Fig. 2. Mean (±SEM) gonadosomatic index (GSI)(%) of captive (n = 12) and wild (n = 19) A. female (F), and captive (n = 12) and wild (n = 14) B. male (M) greater amberjack at different reproductive stages (DEVELOPING, SPAWNING capable, REGRESSED and REGENERATING). Different capital letters above the means indicate statistically significant differences among reproductive stages of captive fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Different lowercase letters above the means indicate statistically significant differences among reproductive stages of wild fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild fish of the same sex at the same reproductive stage (ANOVA, Tukey's HSD, $P \leq 0.05$). Numbers inside the bars are the number of samples for each mean. Reproductive stages: Dev – DEVELOPING, Spawn – SPAWNING capable, Regr – REGRESSED, Regen – REGENERATING.

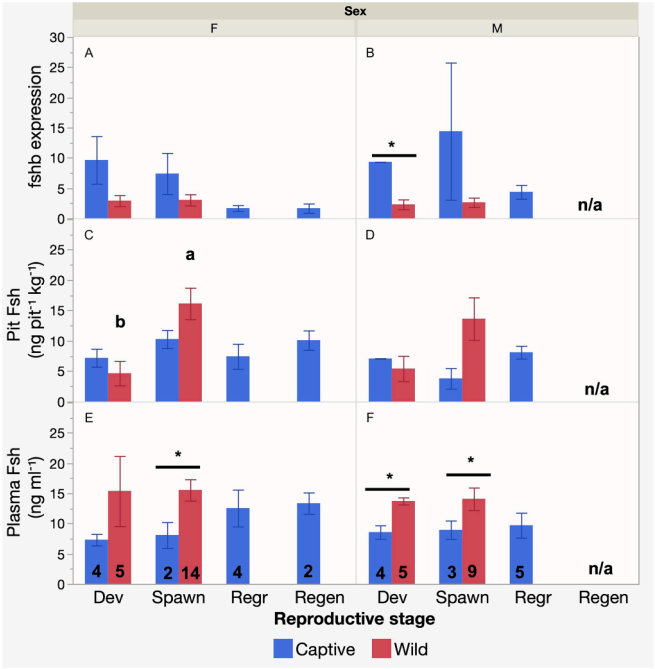

Fig. 3. Mean (±SEM) pituitary *fshb* expression (A, B), pituitary follicle stimulating hormone (Fsh) (ng pit^{-1} kg^{-1}) (C, D) and plasma Fsh (ng/ml) (E, F) of captive (n = 12) and wild (n = 19) female (F), and captive (n = 12) and wild (n = 14) male (M) greater amberjack at different reproductive stages (DEVELOPING, SPAWNING capable, REGRESSED and REGENERATING). Different capital letters above the means indicate statistically significant differences among reproductive stages of captive fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Different lowercase letters above the means indicate statistically significant differences among reproductive stages of wild fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild fish of the same sex at the same reproductive stage (ANOVA, Tukey's HSD, $P \leq 0.05$). Numbers inside the bars are the number of samples for each mean. Reproductive stages: Dev – DEVELOPING, Spawn – SPAWNING capable, Regr – REGRESSED, Regen – REGENERATING.

but do not spawn properly. Of these dysfunctions, the most common is the lack or unreliable oocyte maturation, ovulation and spawning caused by inadequate pituitary Lh synthesis and/or release at the completion of vitellogenesis. In males, reproductive dysfunctions are less severe, and in most species spermatogenesis is completed with spermiation, but fish may fail to produce adequate amounts of sperm and sometimes produce sperm of reduced quality (Mylonas et al., 2017). Here, we present the most complete comparative study to date, on the function of the endocrine reproductive axis of greater amberjack in the wild and in farming conditions, including gene expression, pituitary and plasma levels of Fsh and Lh, with the objective of gaining more insight on the endocrine involvement in the observed dysfunctions of captive breeders, which has been reported extensively in recent studies (Fakriadis et al., 2020b; Pousis et al., 2018; Pousis et al., 2019; Zupa et al., 2017a). To achieve this, we report new data for the first time on the function of the pituitary, together with previously reported data on gonadal histology and plasma sex steroid levels, all analyzed with a new approach of using the histological classification of each sample obtained over the whole reproductive season, as opposed to classifying samples with the sampling time (*e.g.* early gametogenesis, advanced gametogenesis and spawning) that was done originally (Zupa et al., 2017b). We believe this approach allows for a better identification of the stage and possible endocrine cause of the reproductive dysfunction.

Between DEVELOPING and SPAWNING capable wild females, a 3 to 4-fold increase of the GSI was recorded. At the same time a corresponding increase was recorded for pituitary Fsh content and plasma T, while almost a 10-fold increase was observed in pituitary Lh content. In general, in both captive and wild fish Lh levels were higher compared to Fsh for all the examined parameters (pituitary RNA expression, pituitary and plasma protein levels), which is in agreement with observations in zebrafish (*Danio rerio*) and carp (*Cyrpinus carpio*) (Hollander-Cohen et al., 2018). However, some of the analyzed parameters (*fshb* and *lhb* expression levels, plasma Fsh and Lh) did not change significantly among different reproductive stages. Taking all the above into account we can assume that Fsh and Lh plasma levels, as well as *fshb* and *lhb* expression levels, do not reflect the levels of the available GtHs in the pituitary. This was also observed in European seabass (*Dicentrarchus labrax*), where pituitary content of GtHs increased towards the spawning season and showed overlapping profiles for Fsh and Lh, but their secretion was quite different (Mazón et al., 2015). The same was observed also in the short finned eel (*Anguila australis*) where *fshb* and *lhb* expression levels variations were not reflected in the plasma protein levels (Nguyen et al., 2019). The absence of any covariation trend of GtH mRNA levels and GtH plasma levels was reported also in previtellogenic greater amberjack females (Nyuji et al., 2018). In the latter study, *fshb* and *lhβ* gene expression levels and plasma Fsh and Lh varied significantly during the annual reproductive cycle of the greater amberjack, contrary to what was observed here. However, in April and June they did not change significantly, neither a correlation was established between the plasma levels and the gene expression levels (Nyuji et al., 2016). The absolute values recorded for the Fsh and Lh plasma levels in the latter study were similar to the wilds of the present one. In contrast, lower levels of E_2 (4-fold) and GSI (2-fold) were recorded, possibly related with the questionable age of maturity of those fish or the captivity induced stress (Nyuji et al., 2016), as it was observed also here.

The captive females of the present study fell into four categories of reproductive development according to their ovarian histological analysis, compared to only two of the wild females. Extensive follicular atresia was observed from late May onwards, with the majority of the fish that had already ceased their reproductive development, showing signs of REGRESSED or REGENERATING ovaries. Such condition was not observed in fish sampled from the wild in the same period. According to an earlier study, this dysfunction of captive females was not related to an insufficient liver synthesis or reduced oocyte uptake of vitellogenin (Pousis et al., 2018). Pituitary content of Fsh and Lh, *fshb*

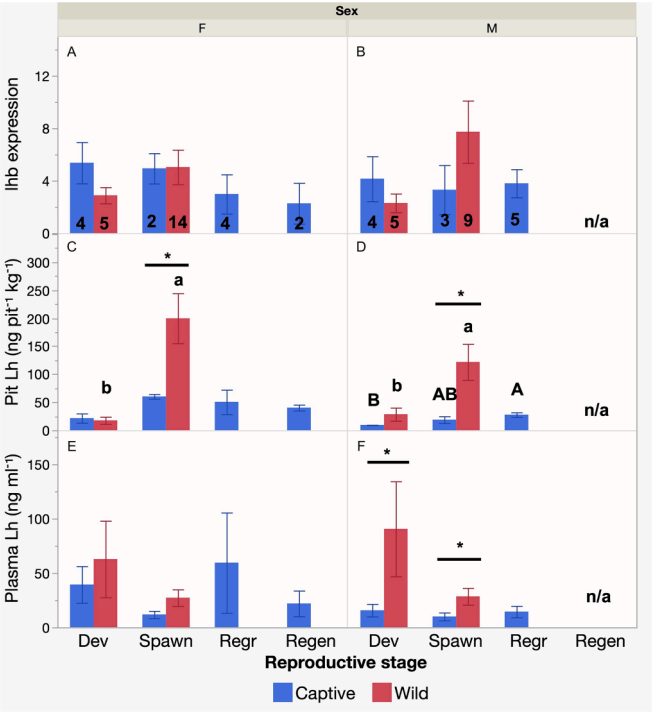

Fig. 4. Mean (±SEM) pituitary *lhb* expression (A, B), pituitary luteinizing hormone (Lh) (ng pit^{-1} kg^{-1})(C, D) and plasma Lh (ng/ml)(E, F) of captive (n = 12) and wild (n = 19) female (F), and captive (n = 12) and wild (n = 14) male (M) greater amberjack at different reproductive stages (DEVELOPING, SPAWNING capable, REGRESSED and REGENERATING). Different capital letters above the means indicate statistically significant differences among reproductive stages of captive fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Different lowercase letters above the means indicate statistically significant differences among reproductive stages of wild fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild fish of the same sex at the same reproductive stage (ANOVA, Tukey's HSD, $P \leq 0.05$). Numbers inside the bars are the number of samples for each mean. Reproductive stages: Dev – DEVELOPING, Spawn – SPAWNING capable, Regr – REGRESSED, Regen – REGENERATING.

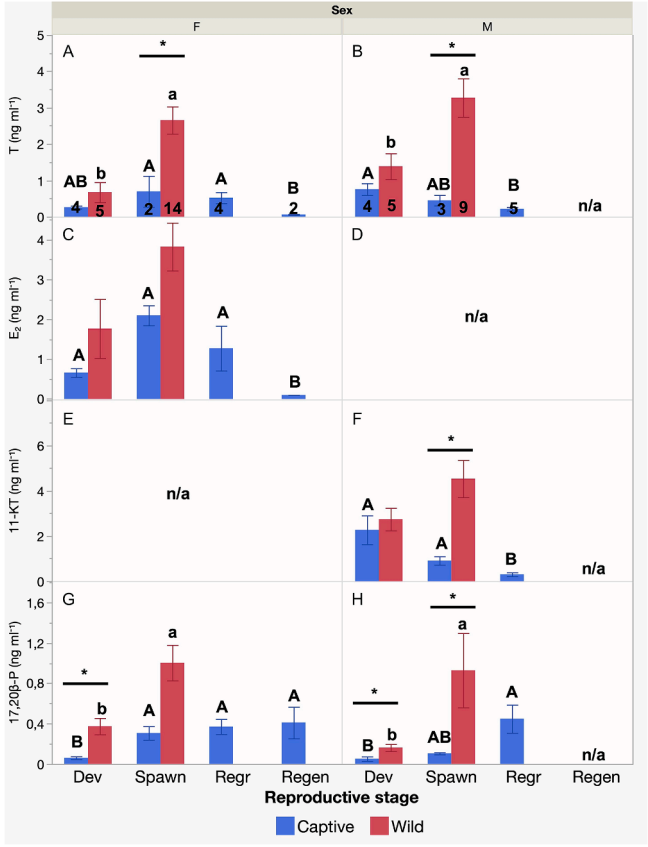

Fig. 5. Mean (±SEM) plasma testosterone (T) (ng/ml) (A, B), 17-β Estradiol (E_2) (ng/ml) (C, D), 11-ketotestosterone (11-KT)(ng/ml) (E, F) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) (ng/ml) (G, H) of captive (n = 12) and wild (n = 19) female (F), and captive (n = 12) and wild (n = 14) male (M) greater amberjack at different reproductive stages (DEVELOPING, SPAWNING capable, REGRESSED and REGENERATING). Different capital letters above the means indicate statistically significant differences among reproductive stages of captive fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Different lowercase letters above the means indicate statistically significant differences among reproductive stages of wild fish (ANOVA, Tukey's HSD, $P \leq 0.05$). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild fish of the same sex at the same reproductive stage (ANOVA, Tukey's HSD, $P \leq 0.05$). Numbers inside the bars are the number of samples for each mean. Reproductive stages: Dev – DEVELOPING, Spawn – SPAWNING capable, Regr – REGRESSED, Regen – REGENERATING.

and *lhb* gene expression levels and plasma levels of Fsh and Lh remained unchanged, in contrast with plasma E_2 and T that decreased during the REGENARATION stage, and 17,20β-P which showed the opposite pattern. No significant differences, except in 17,20β-P, were recorded in captive compared to the wild females in DEVELOPING stage, in contrast to SPAWNING capable stage, when pituitary Lh content, plasma Fsh and T were significantly lower. Considering all the above, a clear pituitary Lh deficiency in captive females, concomitantly with significantly reduced plasma Fsh and T levels may have resulted in the incapacity of vitellogenic oocytes to undergo maturation.

It is well known that Fsh acts as an anti-apoptotic factor for ovarian follicle cells in females (Luckenbach et al., 2011) and germ cells in males (Corriero et al., 2009; Zupa et al., 2023) so the low Fsh plasma levels observed in captive fish at SPAWNING capable compared to the wild ones of the present study might have been responsible for the extensive atresia of advanced vitellogenic follicles. In fact, in teleost fish, apoptosis of follicular cells is the first step of the complex process through which vitellogenic follicles undergo atresia (Corriero et al., 2021b). Decreased levels of pituitary and plasma Fsh in fish with follicular atresia have been observed also in European seabass compared to fish at late vitellogenesis stage (Molés et al., 2011). Interestingly, in 25 % of the sampled captive females at late May – July, similar GSI values were recorded with the wild fish, albeit with extensive follicular atresia present. Almost the same reproductive dysfunction was found in a similar study with captive and wild female jack mackerel (*Trachurus japonicus*) -which belongs to the same family of Carangidae (Imanaga et al., 2014). This dysfunction was attributed to captivity induced stress, when some of the captive fish concluded vitellogenesis and others not, showing high percentages of follicular atresia. Taking this into account, we can assume that, as proposed for jack mackerel, there are individuals that are better adapted to captive conditions and others that are more affected by the confinement-induced stress. This may occur when the process of domestication of a newly cultured species starts, using wild-caught or first generation individuals (Imanaga et al., 2014). We consider this phenomenon exemplified by the gilthead seabream (*Sparus aurata*), which is fully domesticated today and presents no reproductive related problems whatsoever (Pavlidis and Mylonas, 2011), but 50 years ago showed similar reproductive dysfunctions (Zohar and Mylonas, 2001).

The gene expression levels of both GtHs and their plasma levels did not change during reproductive development in either captive or wild breeders, showing that these concentrations may not reflect the maturation status of the ovaries or testes. Differences were observed in pituitary GtH content, although this is clearly independent from the circulating levels of the two hormones. Pituitary GtH mRNA levels were

considered also as a poor indicator of the reproductive stage of the fish, compared to plasma levels of GtHs, in the Senegalese sole (*Solea senegalensis*) (Chauvigne et al., 2016), gilthead seabream (Meiri et al., 2004) and European seabass (Mazón et al., 2015), contrary to what has been observed in salmonids (Swanson et al., 2003). In a congener of greater amberjack, the Japanese yellowtail (*Seriola quinqueradiata*), *fshb* was strongly expressed in early vitellogenesis and spermatogenesis, while *lhb* was expressed significantly at late vitellogenesis and spermatogenesis, and during spermiation (Rahman et al., 2003). In the present study, however, the absence of significant changes may be related with the limited number of samplings and a short sampling period.

High levels of Lh have been reported in many species during oocyte maturation (Levavi-Sivan et al., 2010; Swanson et al., 2003), however the high levels of Fsh at the same period may reflect the next generation of oocytes entering vitellogenesis (Aizen et al., 2007). In tilapia (*Oreochromis niloticus*) females, a mouth-brooding group-synchronous species with a spawning frequency of almost 12 days, when spawned eggs were removed from the mouth of the fish, both plasma Fsh and Lh demonstrated two peaks, the first one on day 2 and the second, and highest, on day 12 (Aizen et al., 2007). The greater amberjack has been shown to spawn almost every 5 days in the Southeastern U.S.A. Atlantic coast (Harris et al., 2007). Presumably, fluctuations of gene expression and plasma GtH levels would have been observed if we were able to follow a captive population of fish at the frequency of oocyte batch development. However, this is still not possible with the current state of domestication of greater amberjack compared to gilthead seabream or tilapia. Interestingly, plasma Fsh was shown to increase significantly in the greater amberjack at the end of the spawning season (August) and was related with the start of a new gametogenic cycle (Nyuji et al., 2016).

In teleost fishes, E_2 is considered responsible for oocyte growth while the progestins 17, 20β-P and/or 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P or 20β-S) induce oocyte maturation (Levavi-Sivan et al., 2010). In males, androgens (T, 11-KT) increase gradually as spermatogenesis proceeds and decrease at spermiation (Schulz et al., 2010). The progestins (17, 20β-P or 20βS) induce sperm maturation and release (Vizziano et al., 2008). Plasma sex steroids in greater amberjack were shown to peak at the end of May-early June in the Mediterranean, with E_2 levels being higher when females were at a maturing phase, mature or partially ovulated (Mandich et al., 2004), as observed in fish at late vitellogenesis in Japan (Nyuji et al., 2016). Plasma T levels peaked at the maturing phase and decreased significantly by 50 % at the mature and partially ovulated stages (Mandich et al., 2004). In Senegalese sole females, a group synchronous spawner also, plasma Fsh and Lh have been shown to peak in the middle of the spawning season, after the peak of the E_2 and T (Chauvigne et al., 2016). Considering the above information and the general pattern for GtHs and sex steroid profiles proposed by Habibi et al. (2007), we expected to be able to record clear peaks of the different sex steroid hormones. However, both the short half-life of these hormones in circulation (Gothilf et al., 1997) and the small number of samples that we were able to collect both in the wild and captive conditions may have affected our ability to detect statistically significant changes. The latter authors reported that in the absence of GtHs, females enter into follicular atresia, and this may be enhanced by the gonadal paracrine secretion of GnRH. Interestingly, the same follicular atresia at the late vitellogenesis stage that was present in tank-reared female greater amberjack (Fakriadis et al., 2020b) was possibly enhanced by the GnRHa treatment, since unfertilized eggs were spawned and follicular atresia was found to be increased 3 weeks after the GnRHa administration. This may be explained by the lack of the appropriate pituitary Lh content in these females, evidenced by their ovarian biopsies which were similar to the present study, and possibly their reproductive regression was at an irreversible phase, so any GnRHa administration at this point could not stimulate a proper oocyte maturation, ovulation and spawning.

Wild males were found to be at the same reproductive stages of females, as expected. Pituitary Lh content, plasma T, 17,20β-P and GSI of wild males increased by a 3 to 4-fold. A slight increasing trend of pituitary Fsh content, *lhb* expression levels and plasma 11-KT existed between the DEVELOPING and SPAWNING capable stages, while an opposite trend was observed in plasma Lh. No change between the two reproductive stages was observed in *fshb* expression levels and plasma Fsh. On the other hand, captive males fell into an additional category of REGRESSED testes, and showed significant differences in hormone levels from their wild counterparts even from the DEVELOPING phase in four out of 10 measured parameters. During the SPAWNING capable phase, the situation was even worst, since reduced levels of almost all measured parameters were observed between wild and captive males, being statistically significant or showing a strong downward trend. Taking all the above into account, the reproductive dysfunction in captive males seems to be more pronounced than in captive females and possibly not related to the handling stress of the experimental protocol, since prior to the first sampling, when all captive fish were at the DEVELOPING stage, no handling had been made. In the same captive fish, seminiferous lobules of a smaller diameter, a precocious and progressive decrease of spermatogonial mitosis and a high level of germ cell apoptosis, concomitant with a many-fold higher E_2 plasma levels was reported (Zupa et al., 2017a). In humans, apoptosis in male germ cells is suppressed by T, which is considered a survival factor (Erkkila et al., 1997), and the same is generally observed in non-human mammals (Schulz et al., 2001). In fish, it was suggested that E_2 regulates spermatogonial renewal, while T and 11-KT are involved in later stages of spermatogenesis (Miura and Miura, 2003). Although the role of estrogens in male fish fertility has been suggested of being neutral (Tang et al., 2017), the administration of high doses of E_2 in male gilthead seabream induced apoptosis of spermatogonia (Chaves-Pozo et al., 2007). Interestingly, higher sperm volumes and frequency of spermatozoa in cysts were found in mutant zebrafish, lacking *cyp19a1a* or both *cyp19a1a* and *cyp19a1b* - the genes that encode aromatase in fish which is the enzyme that catalyzes the final step of estradiol biosynthesis - compared to the wilds or *cyp19a1b* mutant (Tang et al., 2017). In men, estrogens were shown to have negative and positive feedback roles (Guercio et al., 2020). In fish, it is generally considered that high gonadal steroid plasma levels reduce *fshb* expression levels (Yaron et al., 2003), and this is opposite to what has been observed in the present study. In coho salmon, Fsh plasma levels and *fshb* expression levels decreased after E_2 administration (Dickey and Swanson, 1998). In the yellowtail kingfish (*Seriola lalandi*), females had increased Fsh plasma levels concomitantly with significantly higher E_2 levels (Nocillado et al., 2019) and this might be the cause of the *fshb* expression increase that was observed in the present study in captive males at the DEVELOPING stage, when the peak of E_2 was recorded. The same trend of *fshb* expression levels and E_2 towards ovarian development was observed also in greater amberjack females (Nyuji et al., 2016), but relevant studies in males are still lacking.

In general, it is assumed that in male fish Fsh regulates the early stages of spermatogenesis and Lh peaks at the onset of the spawning season (Schulz et al., 2010). In the Japanese yellowtail, plasma 11-KT and *fshb* and *lhb* expression levels peaked at late spermatogenesis, when their testes were at the same developmental stages with the DEVELOPING phase of the present study, and remained at the same high levels after entering the spawning season, when the GSI values peaked (Higuchi et al., 2016). In wild greater amberjack, T plasma levels peaked in mid-May and remained at the same levels until the end of June, while 11-KT levels increased in mid-May, but peaked in mid-June (Mandich et al., 2004). In the multiple spawner chub mackerel, it was proposed that Fsh may play a role in the early and late phases of spermatogenesis, while Lh is involved during late spermatogenesis and spermiation (Nyuji et al., 2012). In male salmonids, both *fshb* expression levels and Fsh plasma increase during early stages of maturation, while *lhb* expression levels and Lh plasma levels are very low at the early stages and increase sharply in the spawning season (Ciani et al., 2020).

The present study demonstrated that female greater amberjack may

undergo full vitellogenesis in captivity, although dysfunctions may occur due to multiple husbandry manipulations. On the other hand, males may be more seriously affected by captivity rearing even without any manipulations during the year. Although almost no significant differences were observed in captive-reared females compared to their wild counterparts in the measured endocrine parameters during the DEVELOPING phase -*i.e.* prior to any handling, during the spawning period most females had already aborted reproductive development and maturation. Males seemed to face reproductive dysfunctions even without handling, since significant differences were already present when compared to wild ones at the first sampling. We consider greater amberjack to be a "poorly spermiating" fish species (Mylonas et al., 2017), a category in which fish complete spermatogenesis in captivity, but produce small volumes of milt. This phenomenon is problematic in multiple spawning fish species or in species with a rather long female spawning period (Mylonas et al., 2017). Although spawning induction protocols have been proposed and implemented in greater amberjack, a spermiation enhancement method that will contribute to increasing the production of good quality sperm is still lacking and should be addressed in future research.

CRediT authorship contribution statement

Ioannis Fakriadis: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing, Conceptualization. **Iris Meiri-Ashkenazi:** Formal analysis, Methodology, Writing – review & editing, Data curation. **Chen Bracha:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Hanna Rosenfeld:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Writing – review & editing. **Aldo Corriero:** Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Rosa Zupa:** Data curation, Methodology, Investigation. **Chrysovalentinos Pousis:** Data curation, Investigation, Methodology. **Maria Papadaki:** Data curation, Investigation, Methodology. **Constantinos C. Mylonas:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114465.

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