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Vitellogenin receptor and fatty acid profiles of individual lipid classes of oocytes from wild and captive-reared greater amberjack (*Seriola dumerili*) during the reproductive cycle

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ABSTRACT

The greater amberjack *Seriola dumerili* (Risso, 1810) is a large migratory pelagic fish occurring in tropical and temperate waters with a great potential for the world aquaculture industry. Previous studies showed that wild-caught female greater amberjack reared in sea cages and handled during the reproductive season, underwent extensive ovarian atresia. This atresia, however, was not related to an insufficient liver transcription or oocyte uptake of vitellogenin (Vtg). In the present study, the structure of two greater amberjack vitellogenin receptors, namely Vtgr (Lr8-) and Lrp13, was characterized. Moreover, *vtgr* and *lrp13* gene expression and the fatty acid profiles of specific phospholipids and neutral lipids were compared in the ovaries of wild and captive-reared greater amberjack during different phases of the reproductive cycle (*i.e.* early gametogenesis, advanced gametogenesis and spawning). Ovarian *vtgr* and *lrp13* transcription was more active during early gametogenesis, suggesting that vitellogenin receptor transcripts were synthesized by previtellogenic oocytes and remained in the cellular mRNA pool until oocytes resumed meiosis and entered into secondary growth (*i.e.* vitellogenesis). Rearing of wild-caught greater amberjack in captivity together with handling during the reproductive season was associated with a reduced *vtgr* and *lrp13* transcription and with a diminished capacity of oocytes in the early phase of gametogenesis (primary oocyte growth) to enter into vitellogenesis. During early gametogenesis, remarkable differences in the fatty acid composition were observed between wild and captive-reared individuals: all phospholipids of captive fish displayed dramatic increases of saturates (16:0 and 18:0) and decreases of arachidonic acid (ARA) and docosahexaenoic acid (DHA). The present study confirms the susceptibility of greater amberjack reproductive function to handling stress and suggests that the consequent extensive atresia of vitellogenic follicles originated during the primary oocytes growth when the capacity of oocytes to synthesize vitellogenin receptors was reduced. The study also suggests that this reduced capacity was associated with an altered oocyte phospholipid fatty acid composition during early gametogenesis.

1. Introduction

The greater amberjack *Seriola dumerili* (Risso, 1810) is a large migratory pelagic fish occurring in tropical and temperate waters [1], with a great potential for the world aquaculture industry [2]. One of the major bottlenecks for the incorporation of new species

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in the aquaculture industry is the control of reproductive function in captivity and the consistent production of high quality eggs [3]. Failure of greater amberjack females reared in the Mediterranean to complete oogenesis was documented long time ago [4–6], and more recent studies confirmed the existence of reproductive dysfunctions in captivity, when fish were not exposed to optimal conditions. In fact, captive-reared females that were exposed to handling due to the sampling operation carried out in the same cage during the previous phase of the reproductive cycle, showed a reduced ovarian relative mass (Gonadosomatic Index) and an extensive atresia of vitellogenic follicles during the natural spawning period of the wild population [7,8], and males showed an impairment of spermatogenesis with an increase of apoptosis and a reduced proliferation of germ cells, associated to abnormal sex steroid plasma concentrations [9]. As a result of the spermatogenesis impairment, sperm quality of captive-reared greater amberjack males appeared to be compromised [9].

However, successful spawning of greater amberjack reared in sea cages in the Mediterranean was induced through the administration of gonadotropin-releasing hormone agonist (GnRHa) when breeders were not handled during the early stages of the reproductive season prior to the administration of GnRHa [10], as well as in broodstocks of Atlantic Ocean origin reared in tanks in the Canary Islands [11,12]. Moreover, spontaneous spawning of a small number of breeders has been reported for captive-reared greater amberjack individuals reared in tanks in the Canary Islands under natural conditions of light and temperature [13].

In teleost fish, as in other oviparous animals, egg yolk is largely derived from vitellogenin (Vtg), a phospholipid-rich yolk protein precursor that is synthesized in the liver under 17β Estradiol (E_2) stimulation, taken up from the maternal circulation by the growing oocytes via receptors belonging to the low density lipoprotein receptor (LDLR) family [14,15]. Acanthomorph fishes produce three distinct Vtgs, referred to as vitellogenin A (VtgAa), vitellogenin B (VtgAb) and vitellogenin C (VtgC) [8,16,17].

Several biochemical studies have revealed multiple ovarian membrane proteins that specifically bind Vtg in salmonids [18] and in perciforms [19,20]. In the white perch (*Morone americana*) ovary, four Vtg receptor proteins were discovered: a receptor that binds only VtgA (VtgAar), two receptors that preferentially bind VtgAb (VtgAbr 116 kDa and VtgAbr 110.5 kDa), and a putative LDLR (pLDLR) that weakly and indiscriminately binds both VtgAa and VtgAb [19]. A white perch Vtg receptor orthologous to mammalian very low density lipoprotein receptor (VLDLR) has been named Vtgr or Lr8- due to the presence of eight ligand-binding repeats and because it is a spliced variant gene transcript of *vldlr* that does not encode the O-linked sugar domain [21–23]. Reading et al. [19] suggested that white perch Lr8- corresponds to one or both VtgAbr proteins based on the predicted molecular mass of the protein and on prior reports of fish and chicken Lr8-. Recently, Reading et al. [20] and Mushirobira et al. [24] described structure, expression, subcellular localization, and Vtg-binding properties of a receptor named Lrp13 that corresponds to VtgAar. Both VtgAbr (Lr8-) and Lrp13 have co-evolved in both oviparous and viviparous animals to sustain reproductive effort and to transport various ligands inside the cell [25,26]. In the present paper, the term “vitellogenin receptor” is used to generically refer to any receptor that binds vitellogenin, whereas specific vitellogenin receptors are mentioned using the appropriate acronyms.

The endocrine mechanism regulating vitellogenin receptor expression is not yet clarified, although a study using the medaka (*Oryzias latipes*) model revealed that E_2 exposure suppresses the expression of vitellogenin receptors in females [27], and in cultured ovarian follicles of largemouth bass (*Micropterus salmoides*), insulin, E_2 and 11-ketotestosterone (11-KT) have been also reported to be involved in the complex regulation of *vtgr* expression [28].

Long-chain polyunsaturated fatty acids (LC-PUFA) are relevant components of Vtg and play an important role during gametogenesis, oocyte maturation, embryo ontogeny and early larval development in marine fish [29–33]. During early gametogenesis, total lipids from ovaries of greater amberjack reared in sea cages contained 40% less arachidonic acid (ARA, 20:4n-6) than wild fish, causing strong imbalances of ARA/eicosapentaenoic acid (EPA, 20:5n-3) ratios [7].

In addition to ARA, also docosahexaenoic acid (DHA, 22:6n-3) - considered as the most relevant essential fatty acid for fish egg quality [34] - was found to be much lower in total lipids of gonads from captive reared greater amberjack [7]. The importance of DHA- and ARA-rich phospholipids (*i.e.* phosphatidylcholine, PC; phosphatidylserine, PS; and phosphatidylethanolamine, PE) on gonad development and egg quality has been also highlighted by several authors [35–40]. In fact, two thirds of the lipid fraction of Vtg is made of PC [34] that is also the main phospholipid in mature ovaries and fertilized eggs [41].

The aim of the present study was to characterize the structures of Vtgr (Lr8-) and Lrp13 in the greater amberjack as well as to analyze the expression of the two genes and the fatty acid profiles of specific phospholipids and neutral lipids in the ovaries of wild and captive-reared specimens during different phases of the reproductive cycle, in order to improve our understanding of the regulation of these genes and to further investigate the mechanisms underlying the oogenesis impairment observed when these fish are exposed to adverse rearing conditions in captivity.

2. Material and method

2.1. Fish sampling

Twenty-one wild and twelve captive-reared greater amberjack females were sampled during 2014, 2015 and 2016 at three different phases of the reproductive cycle determined according to the available literature [42,43]: early gametogenesis, late April-early May (wild fish = 5; captive-reared fish = 4); advanced gametogenesis, late May-early June (wild fish = 4; captive-reared fish = 4)); spawning, late June-early July (wild fish = 12; captive-reared fish = 4). Wild fish were sampled on board a professional purse-seine fishing vessel operating around the Pelagie Islands (Sicily, Italy); captive-reared individuals belonged to a broodstock captured as juveniles (~1 kg body weight) in 2011 in the area of Astakos (Ionian Sea, Greece) and moved in September 2013 (5–7 kg in body weight) to a sea cage of Argosaronikos Fishfarming S.A. (Salamina Island, Greece), where they were reared for 2 years according to standard farming practices.

The fish were fed to apparent satiation every other day, using a commercial extruded broodstock diet (Vitalis Cal; Skretting, SA, Norway) (see Ref. [7] for proximate and fatty acid composition), until they were killed for research purposes during the three above mentioned phases of the reproductive season of 2015 (N = 4 per reproductive phase). For sampling, fish were herded into a PVC anesthetic bag (volume 10–15 m^3), where they were slightly anesthetized with 0.01 mL/L clove oil. Then, one-by-one they were gently directed into a PVC stretcher, brought on board of a service vessel and anesthetized deeply with 0.03 mL/L clove oil for sex recognition by means of gonad cannulation. Subsequently, four males and four females for each sampling time were euthanized by decapitation and were placed on crushed ice and transferred to the onshore farm facility for processing. The remaining fish in the population were then allowed to exit from the anesthetic bag and into their rearing cage, and the procedure was repeated again in the two subsequent samplings. For each fish, biometric data (fork

length, FL, nearest cm; body mass, BM, nearest kg; ovarian mass, OM, nearest g) were recorded and ovary samples were taken. Ovarian samples destined for molecular biology studies were stored in RNA later® at 4 °C and then transferred at −80 °C. Ovarian samples destined for basic histological and immunohistochemical analyses were fixed in Bouin's liquid for 4–6 h.

2.2. Histology and immunohistochemistry

Fixed ovary samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin wax. The assessment of the reproductive state was performed, according to Corriero et al. [44,45], on the basis of the most advanced oocyte stage, the occurrence of post-ovulatory (POFs) and atretic follicles observed in 5-μm thick, de-paraffinized sections stained with haematoxylin-eosin.

In order to compare the density of oocytes at late vitellogenesis stage (number of late vitellogenesis follicles/mm^2 ovary section) between wild and captive-reared greater amberjack, healthy oocytes at late vitellogenesis stage and atretic vitellogenic follicles were counted 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).

For the immunolocalization of Vtg and its derived yolk proteins, deparaffinized ovarian sections were hydrated and pre-treated for 30 min with 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase activity. The sections were treated for 30 min in normal serum (NS) to block non-specific binding sites for immunoglobulins and then incubated overnight in moist chamber at 4 °C with purified IgG fraction from rabbits immunised with the portion from amino acid 764 to amino acid 1025 of the Atlantic bluefin tuna (*Thunnus thynnus*) Vtg sequence [46]. Peptide synthesis, rabbit immunization, serum production and total IgG purification were performed by Agrisera AB (Vännäs, Sweden). The rabbit total IgG fraction was diluted 1:5000 in PBS containing 0.1% BSA. Normal serum, biotinylated secondary antibodies and avidin-biotin complex were contained in the Vectastain Universal Elite Kit (Vector, Burlingame, CA). Peroxidase activity was visualized by incubating for 10 min with Vector DAB Peroxidase Substrate Kit (Vector, Burlingame, Ca), which produces a brown precipitate. To confirm the specificity of the immunostaining, the following control staining procedures were carried out: (1) replacement of primary antibody with normal serum; (2) replacement of primary antibody with pre-immune rabbit serum.

2.3. RNA extraction and reverse transcription

RNA extractions from gonad samples were carried out with Qiagen RNaesy® Lipid Tissue Mini Kit (including the RNase Free DNase set) as described by the manufacturer. Frozen tissue (50 mg) was powdered under liquid nitrogen with a porcelain mortar and pestle. The RNA was resuspended in 50 μL of RNase free water and stored at −80 °C until used. In order to have an equal amount of total RNA to perform reverse transcription, quantification of the RNA was necessary. Quality and concentrations of the RNA preparation were determined in 1x agarose- Tris-acetate buffered 1% gels stained with ethidium bromide and by spectrophotometric measurements at 260, 280 and 230 nm using NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific Inc., MI., Italy), respectively. Reverse transcription of 1 μg of total RNA was performed using SuperScript® III Reverse Transcriptase as described by the manufacturer (Invitrogen). Random hexamer primers were used for the first-strand cDNA synthesis. cDNA was kept at −80 °C until used in the real-time PCR assay. Two microliters of cDNA were used for the PCR.

2.4. Cloning of greater amberjack vtgr and lrp13 cDNA

The complete nucleotide sequences of *vtgr* and *lrp13* were amplified from total cDNA by means of overlapping PCR reactions respectively. For cloning of *lrp13*, PCR amplification was conducted using primers that were designed according to the most conserved regions across Perciforms (Table 1). In order to clone *vtgr*, the same primer pairs used for Atlantic bluefin tuna were utilized [46]. All PCRs were performed on a PCR Sprint Thermal Cycler using ~50 ng cDNA, 10 pmoles of each oligonucleotide primer, 0.2 mM dNTP mix, 10× Taq polymerase buffer and 1.5 unit Taq Polymerase (Eppendorf). PCR-generated DNA fragments were resolved in 1x Tris-acetate buffered 1.2% agarose gels and visualized by ethidium bromide staining. RACE 5 ′and 3′ were necessary to complete the sequences of the mRNA (5'/3′ RACE Kit, 2nd Generation Roche Applied Science). Amplification product was excised from a 1.2% agarose gel and purified using Nucleo Spin extract II (Macherey-Nagel) and ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit; Invitrogen) and transformed into *Escherichia coli* competent cells (One Shot TOPO 10 chemically competent cells; Invitrogen). Approximately 20 μL of purified plasmid was sent to the eurofin Genomics Sequence Service (Ebersberg, Germany) for sequencing with M13 reverse and M13 forward primers.

2.5. Real Time PCR

Once the greater amberjack homologous sequences were obtained, *vtgr* and *lrp13* specific primers were designed (Table 2) employing the Primer3 software and used to establish quantitative real-time PCR (qPCR) for gene expression analysis. For the relative quantification of *β-actin* where used the same primer pairs used by Pousis et al. [8].

Total RNA was obtained from ovary using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Reverse transcription of 1.500 μg of total RNA was performed using SuperScript III Reverse Transcriptase (Invitrogen®) and diluted cDNA (1:10) was used in all following qPCR reactions. The qRT-PCR experiments were carried out in triplicate using the QuantStudio™ 7 Flex System (Applied Biosystems®, Thermo Fisher SCIENTIFIC, Milan, Italy) using 1 μL of diluted (10^{-1}) cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). Thermal cycling conditions included an initial heat-denaturing step at 95 °C for 15 s, 40 cycles at 95 °C for 15 s, 60 °C for 30 s and at 95 °C for 15 s. Following the amplification, melting curves of the PCR products were determined from 60 to

Table 1
Primers for greater amberjack *lrp13* and *vtgr* cloning.

Target	Primer	Sequence reported in 5′–3′	Tm	Method
lrp13	FOR 1	CAGTTCCAGTGTGCCCATGG	62	Cloning
lrp13	FOR 2	ATGGCACAGATGAGAAGGATTG	62	Cloning
lrp13	FOR 3	GGCTGATGTTCTGGACAGAG	62	Cloning
lrp13	FOR 4	ACAACTTCACTCAGCTCCTTC	62	Cloning
lrp13	REV 1	CCTCACCAGTTCCAGTGTGG	62	Cloning
lrp13	REV 2	GTCTACTGGACAGATGAAAGG	62	Cloning
lrp13	REV 3	GTGGTGTGGAAGGATGCTGT	62	Cloning
lrp13	REV 4	GTGTGTGTTCACTGGGCTACA	62	Cloning
lrp13	Sp1	GGTGGTTTGGTGCAGCTC	58	3′ RACE
lrp13	Sp2	CGTCCCAACAGTCCATCTC	60	3′ RACE
lrp13	Sp3	GACACCATCGCACACCTGGC	68	3′ RACE
lrp13	Sp5	GTAGACTGCTCCGACCACTCG	70	5′ RACE
vtgr	Sp1	AGTTGACCTCGTCCTCTCC	60	3′ RACE
vtgr	Sp2	CGTCTGACCCGTCCTCAC	60	3′ RACE
vtgr	Sp3	GCCGTTCTGACACACAAAGTCC	68	3′ RACE
vtgr	Sp5	CCTGATGTGGAGAAACTGGCAAC	70	5′ RACE

Table 2
Primers for greater amberjack real-time PCR.

Target	Primer	Sequence reported in 5′–3′	Tm
vtgr	Forward	GAGGACGAGGTCAACTGTGG	64
vtgr	Reverse	CCTGGCAGTCAACATCGTCG	64
lrp13	Forward	GCCTGATGGCAACAATCTGAC	64
lrp13	Reverse	CAGGAACCAAGATATTCTTGAGT	64
β-actin	Forward	CCCTGTCCTGCTCACAGAGG	64
β-actin	Reverse	CAAGTCCAGACGCAGGATGG	64

95 °C to ascertain the specificity of the amplification. No template controls were included as negative controls for each primer pair. The quantification of the *β-actin* gene was used as the endogenous control to normalize initial RNA levels. Real-time PCR Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied Biosystems®, Thermo Fisher SCIENTIFIC) and analyzed through the spreadsheet working DART-PCR Excel version 1.0. This method of analyzing real-time PCR converts raw fluorescence data into R0 values, based upon the theory that fluorescence is proportional to DNA concentration. This allows an automatic calculation of amplification kinetics, as well as performing the subsequent calculations for the relative quantification and calculation of assay variability giving a final estimate of the efficiency of amplification of the primer pairs used in real-time reactions [47].

Amplification efficiency values (E) for each amplicon were used to correct Ct values before analysing these data by the ΔCt method to compare relative expression results. For all PCRs gene expression levels and the cycle threshold values were processed with the "Delta delta method" and were calculated by relative expression = 2-ΔΔCt [48].

2.6. Sequence analyses

The molecular weight (Mw) and the isoelectric point (pI) were predicted using the Compute pI/Mw tool [49–51]. The signal peptide sequence of the deduced protein was predicted using the SignalP 5.0 prediction tool (http://www.cbs.dtu.dk/services/SignalP) [52]. Comparisons of nucleotide and amino acid sequences with those of other fish, were performed using the CLUSTALW (https://www.ebi.ac.uk/Tools/msa/clustalo) program [53] and BLASTP 2.2.24+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [54,55].

2.7. Fatty acid analysis of lipid classes

Total lipid (TL) was extracted by sample homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch et al. [56]. The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT) [57].

Individual phospholipids and total neutral lipid (TNL) were separated by thin layer chromatography (TLC) in 20 × 20 cm silica plates (Merk, Darmstadt, Germany) using methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (25: 25: 25: 10: 9, by volume) as developing solvent system. After development, the plate was dried under vacuum and sprayed with 2′,7′-dichlorofluorescein. The bands corresponding to individual lipid classes and TNL were scraped off the plate and directly subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted with isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic acid (90:10:1, by volume) as developing system. Fatty acid methyl esters were separated and quantified through gas chromatography analysis as described by Zupa et al. [7].

2.8. Statistical analysis

Differences in oocyte density and fatty acid composition between wild and captive-reared specimens were assessed by Student's t-test, and results are presented as means ± SEM.

Ovarian *vtgr* and *lrp13* trascription levels were expressed as medians, interquartile range and range. Differences in *vtgr* and *lrp13* expression among the different groups were assessed through the Kruskall-Wallis test for non-parametric comparison; the multiple comparison Dunn's test was then used to assess differences in *vtgr* and *lrp13* between the following pairs of 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.

Statistical analyses were performed by STATA SE14 software and statistical significance was identified at $P \leq 0.05$.

3. Results

3.1. Histological and immunohistochemical analysis of the ovaries

Both wild (EW group) and captive-reared (EC group) greater amberjack sampled during the early phase of the reproductive cycle had ovaries containing perinucleolar and cortical alveoli stage oocytes. Two of the five specimens of the EW group and three of the four fish of the EC group showed also oocytes at the early vitellogenesis stage, having a minimum diameter of 200 μm, characterized by small eosinophilic (Fig. 1a) and anti-Vtg immunopositivity (Fig. 1b and c) in the peripheral ooplasm. Sparse anti-Vtg positive granulosa cells were also observed in ovarian follicles at this stage (Fig. 1b and c).

All the fish from the wild sampled in the advanced phase of the reproductive cycle (AW group) showed oocytes in advanced vitellogenesis (Fig. 2a) along with POFs. Oocytes in late vitellogenesis were present in all captive-reared females sampled in the same period (AC group); however, in three of them the majority of the oocytes were atretic (Fig. 2b). All the fish sampled in the wild during the spawning period (SW group) were in spawning condition showing either POFs or hydrated oocytes (Fig. 2c). Captive-reared specimens sampled in the same period were in regressed condition, showing primary growth oocytes and extensive atresia of vitellogenic oocytes. The occurrence of late vitellogenesis follicles in the ovary was significantly higher in wild than in captive-reared greater amberjack females (5.6 ± 0.3 *vs* 2.9 ± 0.4 oocytes/mm^2 ovary section; $P < 0.05$).

3.2. Molecular characterization of greater amberjack vtgr and lrp13

The complete greater amberjack *vtgr* cDNA was amplified from total cDNA by means of overlapping PCR reactions and deposited in Genbank with the accession number (MK111068). The nucleotide sequence of greater amberjack *vtgr* cDNA clone contained an open reading frame of 2532 bp encoding 844 amino acid residues. The amino acid sequence homology analysis showed high identities among Vtgr deduced amino acid sequences of greater amberjack and those of the European seabass (*Dicentrarchus labrax,* GenBank: AAO92396.1) (97%), largemouth bass (*Micropterus salmoides,* GenBank: HQ326241.1) (97%), Atlantic bluefin tuna (GenBank: HQ675023.1) (96%). Greater amberjack *vtgr* showed 100% identity to that of the greater amberjack *vldlr* derived from the genomic sequence (GenBank: XM022744486.1; [58]). Moreover, greater

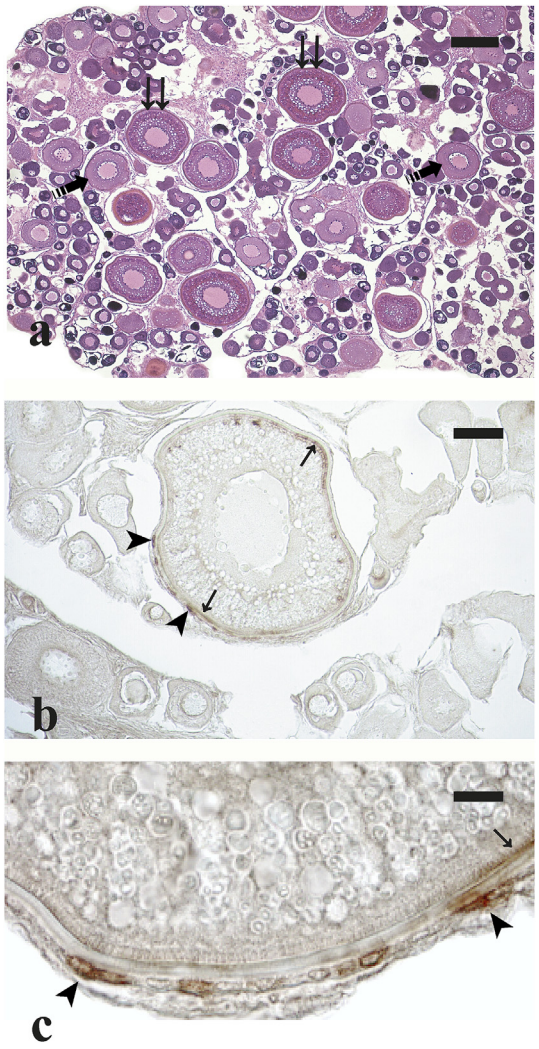

Fig. 1. Micrographs of ovary sections from greater amberjack individuals sampled during the early phase of the reproductive cycle. (a) Ovary section of a wild female showing cortical alveoli and early vitellogenic oocytes. Haematoxylin-eosin staining. (b) Ovary section from a captive-reared female with an early vitellogenic oocyte showing anti-vitellogenin immunostaining in the peripheral ooplasm and in some granulosa cells. Immunostaining with purified IgG fraction from rabbits immunised with the portion from amino acid 764 to amino acid 1025 of the Atlantic bluefin tuna vitellogenin sequence. (c) Higher magnification of part of the early vitellogenic oocyte shown in (b). Arrow, anti-vitellogenin positive ooplasm; arrowhead, anti-vitellogenin positive granulosa cell; dashed arrow, cortical alveoli oocyte; double arrow, early vitellogenic oocyte. Magnification bar = 200 μm in (a), 50 μm in (b) and 10 μm in (c).

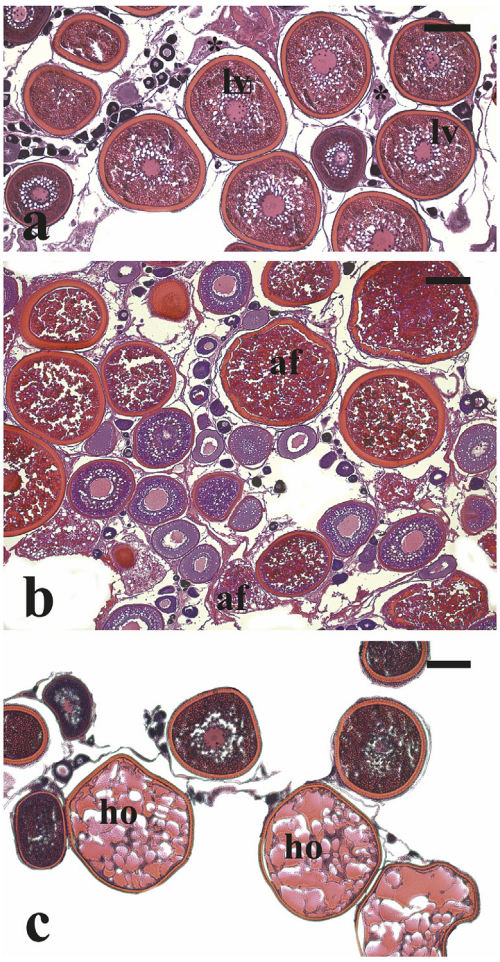

Fig. 2. Micrographs of ovary sections from greater amberjack individuals sampled during the advanced and spawning phases of the reproductive cycle. (a) Ovary section from a wild fish sampled during the advanced phase showing late vitellogenic oocytes as the most advanced stage. (b) Ovary section from a captive-reared fish sampled during the advanced phase showing atretic vitellogenic follicles. (c) Ovary section from a wild fish sampled during the spawning phase showing hydrated oocytes. Haematoxylin-eosin staining. Magnification bars = 200 μm af = atretic vitellogenic follicles; ho = hydrated oocytes; lv = late vitellogenic oocytes.

amberjack Vtgr protein has the same structural characteristics of the Atlantic bluefin tuna Vtgr-protein [46] and it is not further described in the present paper.

The complete *lrp13* cDNA was amplified from total cDNA by means of overlapping PCR reactions. Full-length cDNA sequence encoding greater amberjack *lrp13* was deposited in GenBank with the accession number MH651044. The nucleotide sequence of greater amberjack *lrp13* cDNA clone contained an open reading frame of 4098 bp encoding 1365 amino acid residues (Fig. 3). The 5′ untranslated region (5′UTR) of the transcripts, extended from nucleotide 1 to 30 and the 3′ non-coding region (3′UTR) from nucleotide 4129 to 4177. The predicted mass of the theoretical mature protein was 148.03 kDa and the isoelectric point at 4.64. The *in silico* analysis of the Lrp13 deduced protein domains (Fig. 3) revealed typical characteristics of the LDLR gene superfamily members: (a) low-density lipoprotein receptor domain class A (LDLa); (b) calcium-binding epidermal growth factor-like domain (EGF_CA); (c) low-density lipoprotein-receptor YWTD domain (LY), (d) EGF-like domain (EGF); (e) LY domains; (f) a transmembrane domain; (g) a cytoplasmic domain.

The greater amberjack Lrp13 deduced amino acid sequence showed 100% identity with greater amberjack VLDLR amino acid sequence derived from the genomic sequence (GenBank: XM_022752886.1; [58]), 83% identity with withe perch (*Morone americana*, GenBank: KF387534.1) Lrp13 amino acid sequence and 63% identity with cutthroat trout (*Oncorhynchus clarkii*, GenBank: KR188876.1) amino acid sequence.

3.3. Relative quantification of lrp13 and vtgr

Gene expression values for *lrp13* and *vtgr* in wild and captive-reared fish are reported in Tables 3 and 4, respectively. The trend of *lrp13* expression levels in wild greater amberjack showed a slight (although not significant; P = 0.092) decrease from the early to the advanced gametogenesis phase and remained quite stable thereafter. In captive-reared fish, *lrp13* expression levels dropped significantly from the early to the advanced phase of gametogenesis

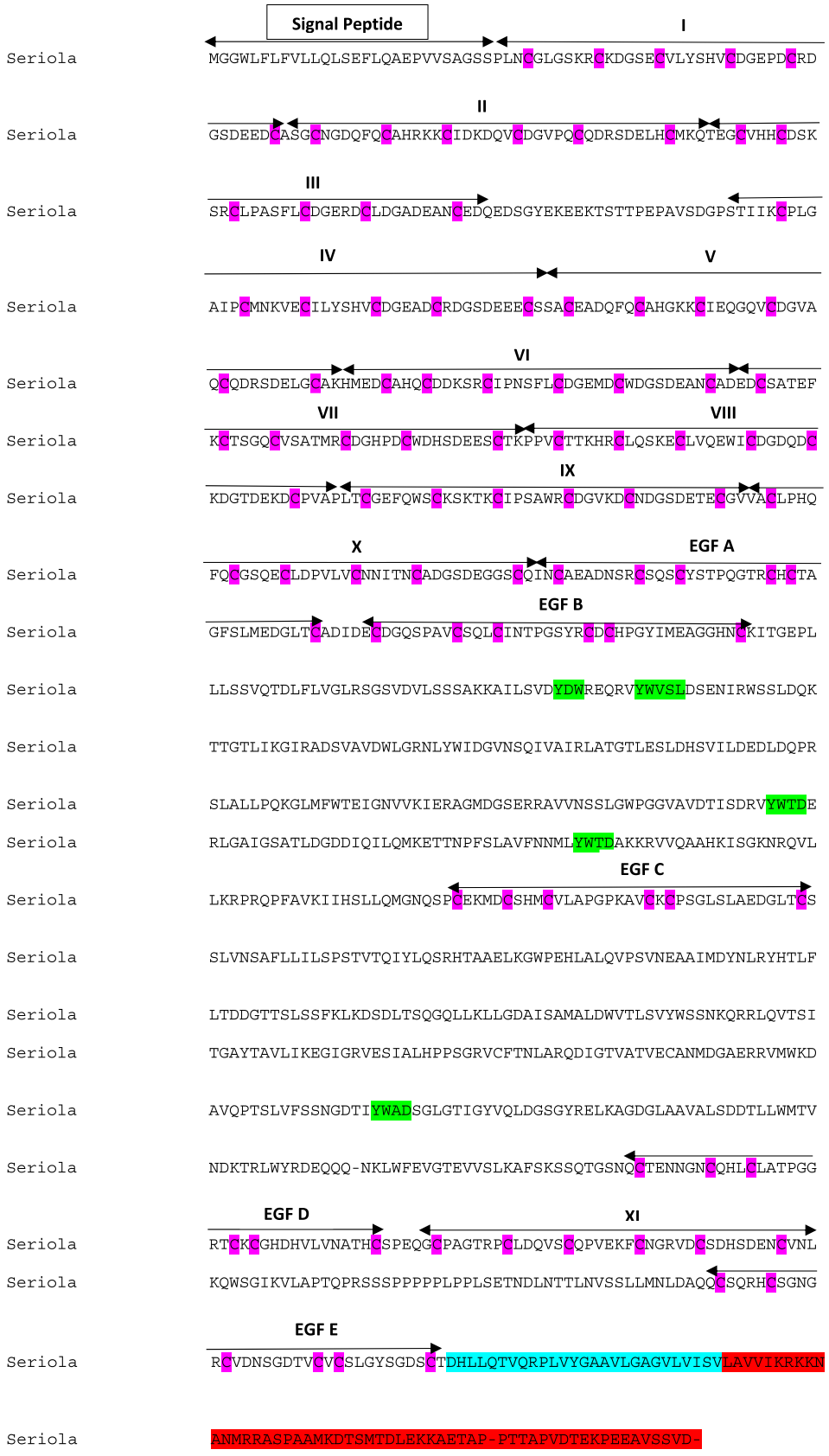

Fig. 3. Greater amberjack Lrp13 amino acid sequence numbered from the initiator, methionine. Arrows above the sequence indicate cysteine binding repeats, epidermal growth factor (EGF) homologue repeats sequences (A, B, C, D, E), transmembrane and cytosolic domains. Cysteine repeats, likely involved in protein folding, are numbered from I to XI and evidenced in violet. The (Y/F)WXD motif, found in multiple tandem repeats and implicated in the β-strand formation, is evidenced in green. The transmembrane and cytosolic domains in the C-terminal region are evidenced in light blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

($P < 0.05$) and remained in the same lower levels during the spawning phase. In all the examined phases and particularly during the spawning phase, *lrp13* expression level was lower in captive-reared compared with wild fish ($P < 0.01$). The trend of *vtgr* expression levels in wild greater amberjack was similar to that of *lrp13*, although no statistically significant differences were observed among the examined phases. The expression levels of *vtgr* of captive-reared greater amberjack were significantly lower than those of the wild counterpart during the early and advanced phases of gametogenesis.

3.4. Fatty acid profiles of lipid classes

There were significant differences between wild and captive-reared greater amberjack in the fatty acid profiles of five different lipid fractions (PC, PS, PI, PE and TNL) in the ovaries of fish during the three analyzed periods of the reproductive cycle (Table 5). Other relevant fatty acids were oleic acid (18:1n-9), which varied in a similar way along the three periods in all lipid classes from both fish groups, being particularly abundant in the TNL fraction (20–30%), and linoleic acid (18:2n-6), also highly abundant in (Supplementary file 2) TNL from captive fish, ranging from 9.6 to 12.4% compared to values of 1.4–2.7% in wild specimens.

Remarkable differences in the fatty acid composition between ovaries of wild and captive individuals were found during early gametogenesis for each single lipid fraction analyzed (Table 5). At this period, all phospholipids of captive fish displayed dramatic increases of saturates (16:0 and 18:0) and decreases of ARA and DHA compared to their wild counterpart. Specifically, notable reductions of DHA in PC and that of ARA in PI were observed (23.4 ± 2.1 *vs* 2.5 ± 0.0 and 27.7 ± 1.1 *vs* 8.8 ± 0.9, respectively). TNL also displayed this reduction in ARA and DHA levels, although their content of saturates remained stable. It is also worth highlighting the constant proportion of EPA at this stage, decreasing exclusively in the PC of reared specimens (5.6 ± 0.7 *vs* 1.2 ± 0.0).

The fatty acid profile of ovaries from captive fish resembled more closely that of their wild counterparts at advanced gametogenesis. The only differences found were the lower ARA in PC and TNL fractions, and a 2 to 4-fold increase of EPA in all lipid fractions of the captive-reared group (Table 5).

At spawning, the proportion of DHA in ovaries of captive fish was lower in PC (22.7 ± 0.9 *vs* 28.2 ± 0.5), PS (18.2 ± 1.6 *vs* 23.0 ± 0.2) and PE (32.7 ± 0.6 *vs* 35.5 ± 0.3) but higher in their TNL fraction (19.3 ± 0.4 *vs* 16.1 ± 1.0) whereas EPA levels increased in PS and PI.

Table 3
Relative expression of *lrp13* gene normalized to that of *β-actin* in the ovaries of wild and captive-reared greater amberjack sampled during different phases of the reproductive cycle.

Fish group	Median	Interquartile range	Range
EW (N = 5)	0.28	0.21–0.31	0.12–0.43
EC (N = 4)	0.20	0.12–0.28	0.09–0.30
AW (N = 4)	0.13	0.09–0.17	0.09–0.17
AC (N = 4)	0.05†	0.04–0.07	0.04–0.08
SW (N = 12)	0.19	0.15–0.23	0.12–0.34
SC (N = 4)	0.02*	0.01–0.03	0.00–0.04

The asterisk indicates a significant difference (Dunn's test, $P < 0.05$) between wild and captive fish sampled in the same period of the reproductive cycle. The dagger indicates a statistically different gene expression in captive-reared fish compared with the previous period of the reproductive cycle. EW, wild fish sampled in early gametogenesis; EC, captive-reared fish sampled in early gametogenesis; AW, wild fish sampled in advanced gametogenesis; AC, captive-reared fish sampled in advanced gametogenesis; SW, wild fish sampled during the spawning period; SC, captive-reared fish sampled during the spawning period.

Table 4
Relative expression of *vtgr* gene normalized to that of *β-actin* in the ovaries of wild and captive-reared greater amberjack sampled during different phases of the reproductive cycle.

Fish group	Median	Interquartile range	range
EW (N = 5)	0.21	0.15–0.25	0.14–0.38
EC (N = 4)	0.10*	0.09–0.11	0.09–0.12
AW (N = 4)	0.14	0.11–0.17	0.11–0.17
AC (N = 4)	0.05*	0.04–0.07	0.04–0.09
SW (N = 12)	0.11	0.07–0.12	0.05–0.13
SC (N = 4)	0.10	0.07–0.18	0.06–0.25

Asterisks indicate significant difference (Dunn's test, $P < 0.05$) between wild and captive fish sampled in the same period of the reproductive cycle. EW, wild fish sampled in early gametogenesis; EC, captive-reared fish sampled in early gametogenesis; AW, wild fish sampled in advanced gametogenesis; AC, captive-reared fish sampled in advanced gametogenesis; SW, wild fish sampled during the spawning period; SC, captive-reared fish sampled during the spawning period.

4. Discussion

In the present study, the complete greater amberjack *vtgr* and *lrp13* cDNA was amplified from total cDNA by means of overlapping PCR reactions. Ovarian expression of *vtgr* and *lrp13* genes, as well as fatty acid profiles of oocyte structural phospholipids, exhibited significant differences between wild and captive-reared specimens during the reproductive period, underlining the potential reproductive dysfunction that may take place when greater amberjack are not exposed to optimal rearing conditions and/or handling in captivity.

In teleost fish, as in other oviparous vertebrates, Vtg is incorporated in the oocytes by receptor-mediated endocytosis through receptors belonging to the Low Density Lipoprotein Receptor (LDLR) family. These receptors have been given different names: VLDLR, Vtg Receptors (Vtgr) or Lr8- [21,59]. The structural characteristics of these receptors have been described in several teleosts [21,46,60–62], showing that they are produced from a highly conserved gene among teleost. Recently, Lrp13, a novel vertebrate lipoprotein receptor that binds Vtgs was sequenced from striped bass, white perch, zebrafish (*Danio rerio*) and cutthroat trout ovaries [20]. In the present study, *lrp13* was sequenced from greater amberjack ovaries and the deduced amino acid sequence showed high similarity and homology with that reported by Reading et al. [20] in the striped bass and white perch, thus supporting their findings that the two main egg yolk precursors, VtgAa and VtgAb are selectively bound by different receptors. The greater amberjack Lrp13 described in the present study showed all the expected structural characteristics of this receptor as demonstrated by the *in silico analysis*: low-density lipoprotein receptor domains class A (LDLa); calcium-binding epidermal growth factor-like domains; low-density lipoprotein-receptor YWTD domains; a transmembrane domain; a cytoplasmic domain.

The pattern of seasonal changes in *vtgr* and *lrp13* expression in wild greater amberjack showed that the gene transcription is more active in fish sampled during early gametogenesis (early May) when the dominant oocyte population was represented by primary growth oocytes and Vtg starts to be internalized in some oocytes, as shown by ovary immunostaining with anti-Vtg antibodies. This is in agreement with previous observations in the Atlantic bluefin tuna [46], rainbow trout (*Oncorhynchus mykiss*) [63], white perch [21] and eel (*Anguilla anguilla*) [64], as well as with findings by Reading et al. [20], who found by *in situ* hybridization *lrp13* transcripts in striped bass previtellogenic oocytes and suggested that vitellogenin receptor transcripts were synthesized in the early phase of gametogenesis by previtellogenic oocytes and remained in the cell mRNA pool during all the oocyte previtellogenic arrest, being finally

Table 5
Mean values ± (SEM) of most variable fatty acids (% total FA) of individual lipid classes of ovaries from wild and captive-reared greater amberjack sampled at three periods of the reproductive cycle.

	EG		AG		SP	
	EW	EC	EW	EC	EW	EC
PC						
16:0	32.1 ± 1.0	59.9 ± 7.1*	29.2 ± 0.4	25.9 ± 0.9	27.3 ± 0.4	28.2 ± 0.3
18:0	3.4 ± 0.3	7.5 ± 0.5*	7.7 ± 0.0	6.7 ± 0.8	6.3 ± 0.2	4.6 ± 0.5
20:4n-6	5.4 ± 0.2	1.2 ± 0.2*	4.3 ± 0.2	2.5 ± 0.3*	5.4 ± 0.3	4.1 ± 0.5
20:5n-3	5.6 ± 0.7	1.2 ± 0.0*	4.5 ± 0.0	8.3 ± 1.1*	6.2 ± 0.3	5.9 ± 1.2
22:6n-3	23.4 ± 2.1	2.5 ± 0.0*	31.4 ± 1.8	27.5 ± 0.5	28.2 ± 0.5	22.7 ± 0.9*
PS						
16:0	8.8 ± 0.2	11.3 ± 0.7*	15.7 ± 0.3	9.4 ± 1.4	8.8 ± 0.7	8.8 ± 0.6
18:0	30.9 ± 1.6	46.8 ± 4.4*	23.8 ± 1.9	25.8 ± 1.6	26.3 ± 0.7	27.4 ± 1.4
20:4n-6	5.7 ± 0.7	2.9 ± 0.3*	3.6 ± 0.4	5.0 ± 0.7	5.0 ± 0.6	10.7 ± 0.9*
20:5n-3	2.1 ± 0.1	1.9 ± 0.4	0.9 ± 0.0	3.6 ± 0.2*	1.4 ± 0.2	3.3 ± 0.4*
22:6n-3	25.4 ± 1.1	11.7 ± 3.0*	16.0 ± 0.1	24.3 ± 0.7	23.0 ± 0.2	18.2 ± 1.6*
PI						
16:0	4.9 ± 0.2	7.3 ± 0.4*	7.5 ± 1.0	5.7 ± 0.1	6.1 ± 0.2	5.7 ± 0.1
18:0	29.4 ± 0.6	49.0 ± 3.5*	26.5 ± 0.2	25.5 ± 1.1	25.4 ± 0.4	26.1 ± 0.4
20:4n-6	27.7 ± 1.1	8.8 ± 0.9*	22.0 ± 1.7	22.8 ± 0.4	23.6 ± 0.5	23.8 ± 1.1
20:5n-3	1.5 ± 0.1	1.2 ± 0.3	1.5 ± 0.1	4.8 ± 0.6*	1.6 ± 0.1	3.2 ± 0.4*
22:6n-3	14.3 ± 1.3	9.5 ± 1.4*	9.1 ± 1.2	13.8 ± 0.5*	14.2 ± 0.5	12.8 ± 1.2
PE						
16:0	10.3 ± 0.1	21.3 ± 0.5*	15.0 ± 0.1	10.3 ± 0.5	12.2 ± 0.5	7.8 ± 0.8*
18:0	7.3 ± 0.4	17.2 ± 1.3*	12.1 ± 0.2	9.3 ± 0.5	10.0 ± 0.3	6.5 ± 0.4*
20:4n-6	7.1 ± 0.5	3.8 ± 0.4*	5.8 ± 0.7	3.9 ± 0.7	7.4 ± 0.3	8.5 ± 0.5
20:5n-3	3.5 ± 0.1	3.4 ± 0.4	2.7 ± 0.0	5.5 ± 0.4*	2.9 ± 0.2	4.8 ± 0.7
22:6n-3	32.4 ± 1.5	14.5 ± 2.1*	34.3 ± 1.4	35.4 ± 1.0	35.5 ± 0.3	32.7 ± 0.6*
TNL						
16:0	15.6 ± 1.2	15.2 ± 1.3	17.2 ± 0.6	11.8 ± 0.4*	13.9 ± 0.5	13.2 ± 0.8
18:0	3.0 ± 0.2	4.0 ± 0.4	4.1 ± 0.2	2.6 ± 0.1	4.7 ± 0.1	3.0 ± 0.2*
20:4n-6	2.6 ± 0.2	1.3 ± 0.3*	2.0 ± 0.0	1.2 ± 0.1*	2.6 ± 0.1	1.7 ± 0.1*
20:5n-3	2.8 ± 0.5	3.2 ± 0.3	2.4 ± 0.1	4.4 ± 0.5*	3.9 ± 0.3	3.6 ± 0.8
22:6n-3	28.8 ± 1.4	13.4 ± 1.9*	15.0 ± 1.2	16.1 ± 1.5	16.1 ± 1.0	19.3 ± 0.4*

EG = early gametogenesis; AG, advanced gametogenesis; SP, spawning period. EW, wild fish; EC, captive-reared fish. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TNL, total neutral lipids. * Indicate significant differences (*t*-Student, $P < 0.05$) among wild and captive-reared specimens for each fatty acid within a particular phase of the reproductive cycle.

translated into functionally active proteins only when oocytes resumed meiosis and entered on their secondary growth.

Compared to the wild population, captive-reared greater amberjack showed significantly lower *vtgr* transcript levels during the early and advanced gametogenesis phases, and lower *lrp13* gene expression during the spawning phase. This finding adds another reproductive dysfunction to the gametogenesis impairment already reported in greater amberjack under rearing conditions: low relative gonad weight and sex steroid plasma levels; extensive oocyte atresia during the advanced gametogenesis phase; precocious spermatogenesis arrest during the spawning phase [7,9]. Rearing in captivity has been reported in many fishes to result in major atresia of vitellogenic follicles during late gametogenesis and consequent incapacity of oocytes to proceed towards maturation and ovulation. We have recently reported that in captive-reared greater amberjack, also spermatogenesis is impaired at an early stage [9], when concomitantly with a many-fold higher plasma E_2 concentration, spermatogonial mitosis decreased and germ cell apoptosis increased.

The reduced capacity of captive-reared greater amberjack to transcribe vitellogenin receptor genes during previtellogenic growth was associated with a reduced number of vitellogenic oocytes during the following phase of the reproductive cycle. We reported previously that the vitellogenic process does not seem to be altered in greater amberjack under rearing conditions, since both *vtg* expression in the liver and oocyte yolk accumulation were similar to those of wild specimens [8]. However, in the present study we found that rearing in captivity together with handling during the early reproductive season was associated with a reduced transcription of vitellogenin receptor genes and with a diminished capacity of oocytes at the primary growth stage to enter vitellogenesis. In other words, the rearing conditions resulted in a reduced reproductive potential (fecundity), as well as lower sex steroid levels and the onset of follicular atresia [7]. Under these conditions, a hormonal treatment with GnRHa applied at the expected spawning period would not be expected to induce maturation, ovulation and spawning. In fact, when greater amberjack females at such a stage of ovarian development were induced to spawn during the spawning period (June) using a hormonal therapy with sustained-release GnRHa implants, the fish either failed to spawn or produced small numbers of eggs of very low fertilization success [65].

In teleost fishes, the early phase of gametogenesis seems to be independent of gonadotropin and sex steroids [[66] and references cited therein] and, in fact, greater amberjack showed low steroid plasma levels during this phase of the reproductive cycle [7]. This confirms further previous observations from other fishes indicating that *vtgr* transcription is not up regulated by estrogens [21,46,67,68]. The complex regulation of this phase of oogenesis, which involves endocrine and paracrine growth factors, is still far from been elucidated, therefore it is not possible to suggest any plausible hypothesis about the mechanism linking the stress caused by the handling in captivity to the reported reduction of *vtgr* and *lrp13* expression during primary oocyte growth. However, in the Senegalese sole (*Solea senegalensis*) liver, *vldlr* overexpression was associated to a high fat diet [69], which suggests a possible role of the diet in the regulation of Vtg receptor transcription. In captive-reared Atlantic bluefin tuna, the administration of an improved diet based on squid (*Loligo* and *Illex* spp.) to increase the content of high quality fatty acids and protein in the diet, was

associated with higher ovarian *vtgr* expression levels [46]. Considering that the ovaries of captive-reared greater amberjack showed a different phospholipid profile and a lower content of essential fatty acids in total lipids compared with the wild population [7], we cannot exclude the possibility that a sub-optimal diet may have played a role in the observed reduction of vitellogenin receptor transcription.

The entire period encompassed by pre-vitellogenic and vitellogenic growth seems to be a critical timeframe for captive broodstock management, since appropriate conditioning, handling and diet are required for oocyte growth and maturation and for the production of good quality eggs [17]. In the regulation of vitellogenesis, cues signaling inadequate nutritional status or specific fat reserves may constrain vitellogenesis processes including signaling for vitellogenin receptors building.

Dietary ARA (20:4n-6) is preferentially accumulated in the gonad of fish species [29,70] and may provide the material base for regulating maturation of the gonads, being more important for immature than for mature females in tongue sole (*Cynoglossus semilaevis*) [29]. Miura et al. [71] proposed that ARA-derived progestins have a role together with estrogens in the regulation of early stages of oogenesis in fish. Although the role for progestins in early ovarian development is not clear, it has been also suggested that they regulate gene transcription in early ovarian follicles [72].

A reduced transcription of greater amberjack vitellogenin receptor genes and a diminished capacity of oocytes to enter into secondary growth coincided in time with the strong imbalances of fatty acids (higher saturates and lower ARA and DHA contents) found in the structural lipids of the ovaries from captive-reared fish. The relative proportions of fatty acids of each molecular species of phospholipids is of great importance, because cell membrane competency is highly dependent on fluidity, ion transport, enzyme function and protein-bound membrane interactions regulated by fatty acids [73].

Omega-3 fatty acids, and also ARA have specific functions based on their structural characteristics. These functions include serving as ligands for several receptors, and components of membrane glycerophospholipids (GPLs). Since ω-3 FAs (especially DHA) are highly flexible, the levels of DHA of GPLs may affect membrane biophysical properties such as fluidity, flexibility, and thickness [74]. Arachidonic acid bounds specifically PI and once it becomes free through the action of a phospholipase A, it also modulates the function of ion channels, and several receptors and enzymes, via activation as well as inhibition of complex pathways [75]. All phospholipids analyzed at early gametogenesis of captive greater amberjack in the present study were deficient in both DHA and ARA.

Functional vitellogenin receptors localized in coated pits on the surface of oocytes are able to internalize in vitellogenic oocytes high amounts of Vtg and other ligands they recognize. Therefore, the mechanisms controlling the expression and modulation of vitellogenin receptors, will be key determinants in effecting oocyte growth. Developmental changes in the maximum number of binding sites is crucial [76] and the composition of the lipid matrix with high content of phospholipids, particularly PE, with DHA chains is critical for membrane receptors. Polyunsaturated DHA displays rapid structural conversions and there is growing evidence for a role of specific DHA-receptors interactions [77]. Docosahexaenoic acid has been pointed out as particularly important in immature ovaries of tongue sole, although the mechanisms in which it is involved have not yet been elucidated [29]. Therefore, from all these data it is evident that compared to greater amberjack breeders sampled in the wild, captive-reared females had a deficiency of DHA and ARA in any of the structural GLPs analyzed and that the important increases of SFA also observed, changed completely the scenario for building up sufficient and functional membrane structures, including receptor domains. It is, therefore, tempting to speculate that these fatty acid imbalances contributed partially to the poor reproductive condition of the captive individuals.

In conclusion, the present study confirmed the presence also in the greater amberjack of the recently discovered receptor Lrp13. The reproductive dysfunctions observed in wild-caught individuals reared in captivity arose during the early phase of oogenesis, when transcription of vitellogenin receptor genes appeared to be reduced. At the same time, dietary deficiencies of the essential fatty acids DHA and ARA had altered widely the fatty acid profiles of the structural phospholipids of the developing oocytes. This seems to suggest that a nutritional deficiency might be co-responsible for the observed oogenesis impairment, caused by a handling-induced stress of captive-reared fish.

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Authors' contributions

CP contributed to sampling, molecular and statistical analyses, manuscript writing. CR contributed to lipid biochemical and statistical analyses, manuscript writing. PDR contributed to molecular analyses and manuscript writing. CDV contributed to molecular analyses and manuscript writing. JAP contributed to lipid biochemical and statistical analyses, manuscript writing. CCM contributed to experimental design, sampling and manuscript writing. RZ contributed to sampling, immunohistochemical analysis, manuscript writing and submission. LP contributed to histological analysis and manuscript writing. NS contributed to histological analysis and manuscript writing. LV contributed to manuscript writing. AC contributed to experimental design, sampling, statistical analysis, manuscript writing and submission.

Declaration of interest

The authors declare no competing or financial interests.

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