https://doi.org/10.1016/j.theriogenology.2019.08.014

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.

Chrysovalentinos Pousis[a], Covadonga Rodríguez[b], Pasquale De Ruvo[c], Caterina De Virgilio[c], José A. Pérez[b], Constantinos C. Mylonas[d], Rosa Zupa[a], Letizia Passantino[a], Nicoletta Santamaria[a], Luisa Valentini[a], Aldo Corriero[a*]

[a]Department of Emergency and Organ Transplantation, Section of Veterinary Clinics and Animal Production, University of Bari Aldo Moro, S.P. per Casamassima km. 3, I-70010 Valenzano (Bari), Italy

[b]Departamento de Biología Animal, Edafología y Geología, Facultad de Ciencias, Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez s/n, La Laguna - Tenerife 38071, Spain

[c]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari Aldo Moro, Via E. Orabona, 4 - 70124 Bari, Italy

[d]Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Center for Marine Research P.O. Box 2214, Heraklion 71003, Crete, Greece

***Corresponding author:** Aldo Corriero, Department of Emergency and Organ Transplantation, Section of Veterinary Clinics and Animal Production, University of Bari Aldo Moro.

Email address: aldo.corriero@uniba.it.

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.

Key-words: Osteichthyes; Mediterranean Sea; oogenesis; vitellogenesis; reproduction; reproductive dysfunctions; phospholipids.

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 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

91 distinct Vtgs, referred to as vitellogenin A (VtgAa), vitellogenin B (VtgAb) and vitellogenin C
92 (VtgC) [8, 16-17].
93 Several biochemical studies have revealed multiple ovarian membrane proteins that specifically
94 bind Vtg in salmonids [18] and in perciforms [19, 20]. In the white perch (*Morone americana*)
95 ovary, four Vtg receptor proteins were discovered: a receptor that binds only VtgA (VtgAar), two
96 receptors that preferentially bind VtgAb (VtgAbr 116 kDa and VtgAbr 110.5 kDa), and a putative
97 LDLR (pLDLR) that weakly and indiscriminately binds both VtgAa and VtgAb [19]. A white perch
98 Vtg receptor orthologous to mammalian very low density lipoprotein receptor (VLDLR) has been
99 named Vtgr or Lr8- due to the presence of eight ligand-binding repeats and because it is a spliced
100 variant gene transcript of *vldlr* that does not encode the O-linked sugar domain [21-23]. Reading et
101 al. [19] suggested that white perch Lr8- corresponds to one or both VtgAbr proteins based on the
102 predicted molecular mass of the protein and on prior reports of fish and chicken Lr8-. Recently,
103 Reading et al. [20] and Mushirobira et al. [24] described structure, expression, subcellular
104 localization, and Vtg-binding properties of a receptor named Lrp13 that corresponds to VtgAar.
105 Both VtgAbr (Lr8-) and Lrp13 have co-evolved in both oviparous and viviparous animals to sustain
106 reproductive effort and to transport various ligands inside the cell [25, 26]. In the present paper, the
107 term “vitellogenin receptor” is used to generically refer to any receptor that binds vitellogenin,
108 whereas specific vitellogenin receptors are mentioned using the appropriate acronyms.
109 The endocrine mechanism regulating vitellogenin receptor expression is not yet clarified,
110 although a study using the medaka (*Oryzias latipes*) model revealed that E_2 exposure suppresses the
111 expression of vitellogenin receptors in females [27], and in cultured ovarian follicles of largemouth
112 bass (*Micropterus salmoides*), insulin, E_2 and 11-ketotestosterone (11-KT) have been also reported
113 to be involved in the complex regulation of *vtgr* expression [28].
114 Long-chain polyunsaturated fatty acids (LC-PUFA) are relevant components of Vtg and play an
115 important role during gametogenesis, oocyte maturation, embryo ontogeny and early larval
116 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

143 weight) to a sea cage of Argosaronikos Fishfarming S.A. (Salamina Island, Greece), where they
144 were reared for 2 years according to standard farming practices.
145 The fish were fed to apparent satiation every other day, using a commercial extruded broodstock
146 diet (Vitalis Cal; Skretting, SA, Norway) (see [7] for proximate and fatty acid composition), until
147 they were killed for research purposes during the three above mentioned phases of the reproductive
148 season of 2015 (N = 4 per reproductive phase). For sampling, fish were herded into a PVC
149 anesthetic bag (volume 10-15 m^3), where they were slightly anesthetized with 0.01 mL/L clove oil.
150 Then, one-by-one they were gently directed into a PVC stretcher, brought on board of a service
151 vessel and anesthetized deeply with 0.03 mL/L clove oil for sex recognition by means of gonad
152 cannulation. Subsequently, four males and four females for each sampling time were euthanized by
153 decapitation and were placed on crushed ice and transferred to the onshore farm facility for
154 processing. The remaining fish in the population were then allowed to exit from the anesthetic bag
155 and into their rearing cage, and the procedure was repeated again in the two subsequent samplings.
156 For each fish, biometric data (fork length, FL, nearest cm; body mass, BM, nearest kg; ovarian
157 mass, OM, nearest g) were recorded and ovary samples were taken. Ovarian samples destined for
158 molecular biology studies were stored in RNA later® at 4 °C and then transferred at -80 °C.
159 Ovarian samples destined for basic histological and immunohistochemical analyses were fixed in
160 Bouin's liquid for 4–6 h.
161
162 *2.2. Histology and immunohistochemistry*
163 Fixed ovary samples were dehydrated in increasing ethanol concentrations, clarified in xylene
164 and embedded in paraffin wax. The assessment of the reproductive state was performed, according
165 to Corriero et al. [44, 45], on the basis of the most advanced oocyte stage, the occurrence of post-
166 ovulatory (POFs) and atretic follicles observed in 5-μm thick, de-paraffinized sections stained with
167 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

194 total RNA to perform reverse transcription, quantification of the RNA was necessary. Quality and
195 concentrations of the RNA preparation were determined in 1x agarose- Tris-acetate buffered 1%
196 gels stained with ethidium bromide and by spectrophotometric measurements at 260, 280 and 230
197 nm using NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific Inc., MI., Italy),
198 respectively. Reverse transcription of 1μg of total RNA was performed using using SuperScript®
199 III Reverse Transcriptase as described by the manufacturer (Invitrogen). Random hexamer primers
200 were used for the first-strand cDNA synthesis. cDNA was kept at -80 °C until used in the real-time
201 PCR assay. Two microliters of cDNA were used for the PCR.
202
203 *2.4. Cloning of greater amberjack vtgr and lrp13 cDNA*
204 The complete nucleotide sequences of *vtgr* and *lrp13* were amplified from total cDNA by means
205 of overlapping PCR reactions respectively. For cloning of *lrp13*, PCR amplification was conducted
206 using primers that were designed according to the most conserved regions across Perciforms (Table
207 1). In order to clone *vtgr*, the same primer pairs used for Atlantic bluefin tuna were utilized [46]. All
208 PCRs were performed on a PCR Sprint Thermal Cycler using ~50 ng cDNA, 10 pmoles of each
209 oligonucleotide primer, 0.2 mM dNTP mix, 10× Taq polymerase buffer and 1.5 unit Taq
210 Polymerase (Eppendorf). PCR-generated DNA fragments were resolved in 1x Tris-acetate buffered
211 1.2% agarose gels and visualized by ethidium bromide staining. RACE 5 'and 3' were necessary to
212 complete the sequences of the mRNA (5'/3' RACE Kit, 2nd Generation Roche Applied Science).
213 Amplification product was excised from a 1.2% agarose gel and purified using Nucleo Spin extract
214 II (Macherey- Nagel) and ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit;
215 Invitrogen) and transformed into *Escherichia coli* competent cells (One Shot TOPO 10 chemically
216 competent cells; Invitrogen). Approximately 20 μL of purified plasmid was sent to the eurofin
217 Genomics Sequence Service (Ebersberg, Germany) for sequencing with M13 reverse and M13
218 forward primers.
219

220 *2.5. Real Time PCR*

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

225 Total RNA was obtained from ovary using the RNeasy Mini Kit (Qiagen) as described by the
226 manufacturer. Reverse transcription of 1.500 μg of total RNA was performed using SuperScript III
227 Reverse Transcriptase (Invitrogen®) and diluted cDNA (1:10) was used in all following qPCR
228 reactions. The qRT-PCR experiments were carried out in triplicate using the QuantStudio™ 7 Flex
229 System (Applied Biosystems®, Thermo Fisher SCIENTIFIC, Milan, Italy) using 1 μL of diluted
230 (10^{-1}) cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). Thermal
231 cycling conditions included an initial heat-denaturing step at 95 °C for 15 s, 40 cycles at 95 °C for
232 15 s, 60 °C for 30 s and at 95 °C for 15 s. Following the amplification, melting curves of the PCR
233 products were determined from 60 to 95 °C to ascertain the specificity of the amplification. No
234 template controls were included as negative controls for each primer pair. The quantification of the
235 *β-actin* gene was used as the endogenous control to normalize initial RNA levels. Real-time PCR
236 Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied
237 Biosystems®, Thermo Fisher SCIENTIFIC) and analyzed through the spreadsheet working DART-
238 PCR Excel version 1.0. This method of analyzing real-time PCR converts raw fluorescence data
239 into R0 values, based upon the theory that fluorescence is proportional to DNA concentration. This
240 allows an automatic calculation of amplification kinetics, as well as performing the subsequent
241 calculations for the relative quantification and calculation of assay variability giving a final estimate
242 of the efficiency of amplification of the primer pairs used in real-time reactions [47].

243 Amplification efficiency values (E) for each amplicon were used to correct Ct values before
244 analysing these data by the ΔCt method to compare relative expression results. For all PCRs gene

245 expression levels and the cycle threshold values were processed with the “Delta delta method” and
246 were calculated by relative expression = 2-ΔΔCt [48].
247
248 *2.6. Sequence analyses*
249 The molecular weight (Mw) and the isoelectric point (pI) were predicted using the Compute pI/Mw
250 tool [49-51]. The signal peptide sequence of the deduced protein was predicted using the SignalP
251 5.0 prediction tool (http://www.cbs.dtu.dk/services/SignalP) [52]. Comparisons of nucleotide and
252 amino acid sequences with those of other fish, were performed using the CLUSTALW
253 (https://www.ebi.ac.uk/Tools/msa/clustalo) program [53] and BLASTP 2.2.24+
254 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [54, 55].
255
256 *2.7. Fatty acid analysis of lipid classes*
257 Total lipid (TL) was extracted by sample homogenization in chloroform/methanol (2:1, v/v)
258 according to the method of Folch et al. [56]. The organic solvent was evaporated under a stream of
259 nitrogen and the lipid content was determined gravimetrically and stored in chloroform/methanol
260 (2:1), containing 0.01% butylated hydroxytoluene (BHT) [57].
261 Individual phospholipids and total neutral lipid (TNL) were separated by thin layer
262 chromatography (TLC) in 20 x 20 cm silica plates (Merk, Darmstadt, Germany) using methyl
263 acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (25: 25: 25: 10: 9, by volume) as
264 developing solvent system. After development, the plate was dried under vacuum and sprayed with
265 2′,7′-dichlorofluorescein. The bands corresponding to individual lipid classes and TNL were
266 scraped off the plate and directly subjected to acid-catalysed transmethylation with 1% sulphuric
267 acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted with
268 isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic
269 acid (90:10:1, by volume) as developing system. Fatty acid methyl esters were separated and
270 quantified through gas chromatography analysis as described by Zupa et al. [7].

271

272 *2.8. Statistical analysis*

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

275 Ovarian *vtgr* and *lrp13* trascription levels were expressed as medians, interquartile range and
276 range. Differences in *vtgr* and *lrp13* expression among the different groups were assessed through
277 the Kruskall-Wallis test for non-parametric comparison; the multiple comparison Dunn's test was
278 then used to assess differences in *vtgr* and *lrp13* between the following pairs of groups: wild
279 specimens sampled in consecutive phases of the reproductive cycle; captive-reared specimens
280 sampled in consecutive phases of the reproductive cycle; wild vs captive-reared specimens sampled
281 in the same phase of the reproductive cycle.

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

284

285 **3. Results**

286 *3.1. Histological and immunohistochemical analysis of the ovaries*

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

294 All the fish from the wild sampled in the advanced phase of the reproductive cycle (AW group)
295 showed oocytes in advanced vitellogenesis (Fig. 2a) along with POFs. Oocytes in late vitellogenesis
296 were present in all captive-reared females sampled in the same period (AC group); however, in

297 three of them the majority of the oocytes were atretic (Fig 2b). All the fish sampled in the wild
298 during the spawning period (SW group) were in spawning condition showing either POFs or
299 hydrated oocytes (Fig. 2c). Captive-reared specimens sampled in the same period were in regressed
300 condition, showing primary growth oocytes and extensive atresia of vitellogenic oocytes. The
301 occurrence of late vitellogenesis follicles in the ovary was significantly higher in wild than in
302 captive-reared greater amberjack females (5.6 ± 0.3 *vs* 2.9 ± 0.4 oocytes / mm^2 ovary section;
303 $P<0.05$).
304
305 *3.2. Molecular characterization of greater amberjack vtgr and lrp13*
306 The complete greater amberjack *vtgr* cDNA was amplified from total cDNA by means of
307 overlapping PCR reactions and deposited in Genbank with the accession number (MK111068). The
308 nucleotide sequence of greater amberjack *vtgr* cDNA clone contained an open reading frame of
309 2532 bp encoding 844 amino acid residues. The amino acid sequence homology analysis showed
310 high identities among Vtgr deduced amino acid sequences of greater amberjack and those of the
311 European seabass (*Dicentrarchus labrax,* GenBank: AAO92396.1) (97%), largemouth bass
312 (*Micropterus salmoides,* GenBank: HQ326241.1) (97%), Atlantic bluefin tuna (GenBank:
313 HQ675023.1) (96%). Greater amberjack *vtgr* showed 100% identity to that of the greater amberjack
314 *vldlr* derived from the genomic sequence (GenBank: XM022744486.1; [58]). Moreover, greater
315 amberjack Vtgr protein has the same structural characteristics of the Atlantic bluefin tuna Vtgr-
316 protein [46] and it is not further described in the present paper.
317 The complete *lrp13* cDNA was amplified from total cDNA by means of overlapping PCR
318 reactions. Full-length cDNA sequence encoding greater amberjack *lrp13* was deposited in
319 GenBank with the accession number MH651044. The nucleotide sequence of greater amberjack
320 *lrp13* cDNA clone contained an open reading frame of 4098 bp encoding 1365 amino acid residues
321 (Fig. 3). The 5' untranslated region (5'UTR) of the transcripts, extended from nucleotide 1 to 30
322 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 Table 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 ($P<0.05$) 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.

349 *3.4. Fatty acid profiles of lipid classes.*

350 There were significant differences between wild and captive-reared greater amberjack in the
351 fatty acid profiles of five different lipid fractions (PC, PS, PI, PE and TNL) in the ovaries of fish
352 during the three analyzed periods of the reproductive cycle (Table 5). Other relevant fatty acids
353 were oleic acid (18:1n-9), which varied in a similar way along the three periods in all lipid classes
354 from both fish groups, being particularly abundant in the TNL fraction (20-30%), and linoleic acid
355 (18:2n-6), also highly abundant in (Supplementary file 2) TNL from captive fish, ranging from 9.6
356 to 12.4% compared to values of 1.4 to 2.7% in wild specimens.

357 Remarkable differences in the fatty acid composition between ovaries of wild and captive
358 individuals were found during early gametogenesis for each single lipid fraction analyzed (Table 5).
359 At this period, all phospholipids of captive fish displayed dramatic increases of saturates (16:0 and
360 18:0) and decreases of ARA and DHA compared to their wild counterpart. Specifically, notable
361 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
362 *vs* 8.8±0.9, respectively). TNL also displayed this reduction in ARA and DHA levels, although their
363 content of saturates remained stable. It is also worth highlighting the constant proportion of EPA at
364 this stage, decreasing exclusively in the PC of reared specimens (5.6±0.7 *vs* 1.2±0.0).

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

369 At spawning, the proportion of DHA in ovaries of captive fish was lower in PC (22.7±0.9 *vs*
370 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
371 fraction (19.3±0.4 *vs* 16.1±1.0) whereas EPA levels increased in PS and PI.

372

373

374

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

401 is in agreement with previous observations in the Atlantic bluefin tuna [46], rainbow trout
402 (*Oncorhynchus mykiss*) [63], white perch [21] and eel (*Anguilla anguilla*) [64], as well as with
403 findings by Reading et al. [20], who found by *in situ* hybridization *lrp13* transcripts in striped bass
404 previtellogenic oocytes and suggested that vitellogenin receptor transcripts were synthesized in the
405 early phase of gametogenesis by previtellogenic oocytes and remained in the cell mRNA pool
406 during all the oocyte previtellogenic arrest, being finally translated into functionally active proteins
407 only when oocytes resumed meiosis and entered on their secondary growth.

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

419 The reduced capacity of captive-reared greater amberjack to transcribe vitellogenin receptor
420 genes during previtellogenic growth was associated with a reduced number of vitellogenic oocytes
421 during the following phase of the reproductive cycle. We reported previously that the vitellogenic
422 process does not seem to be altered in greater amberjack under rearing conditions, since both *vtg*
423 expression in the liver and oocyte yolk accumulation were similar to those of wild specimens [8].
424 However, in the present study we found that rearing in captivity together with handling during the
425 early reproductive season was associated with a reduced transcription of vitellogenin receptor genes
426 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

453 are required for oocyte growth and maturation and for the production of good quality eggs [17]. In
454 the regulation of vitellogenesis, cues signaling inadequate nutritional status or specific fat reserves
455 may constrain vitellogenesis processes including signaling for vitellogenin receptors building.
456 Dietary ARA (20:4n-6) is preferentially accumulated in the gonad of fish species [29, Baeza et al.
457 doi: 10.1016/j.aquaculture.2014.10.016] and may provide the material base for regulating maturation
458 of the gonads, being more important for immature than for mature females in tongue sole
459 (*Cynoglossus semilaevis*) [29]. Miura et al. [70] proposed that ARA-derived progestins have a role
460 together with estrogens in the regulation of early stages of oogenesis in fish. Although the role for
461 progestins in early ovarian development is not clear, it has been also suggested that they regulate
462 gene transcription in early ovarian follicles [71].
463 A reduced transcription of greater amberjack vitellogenin receptor genes and a diminished
464 capacity of oocytes to enter into secondary growth coincided in time with the strong imbalances of
465 fatty acids (higher saturates and lower ARA and DHA contents) found in the structural lipids of the
466 ovaries from captive-reared fish. The relative proportions of fatty acids of each molecular species of
467 phospholipids is of great importance, because cell membrane competency is highly dependent on
468 fluidity, ion transport, enzyme function and protein-bound membrane interactions regulated by fatty
469 acids [72].
470 Omega-3 fatty acids, and also ARA have specific functions based on their structural
471 characteristics. These functions include serving as ligands for several receptors, and components of
472 membrane glycerophospholipids (GPLs). Since ω-3 FAs (especially DHA) are highly flexible, the
473 levels of DHA of GPLs may affect membrane biophysical properties such as fluidity, flexibility,
474 and thickness [73]. Arachidonic acid bounds specifically PI and once it becomes free through the
475 action of a phospholipase A, it also modulates the function of ion channels, and several receptors
476 and enzymes, via activation as well as inhibition of complex pathways [74]. All phospholipids
477 analyzed at early gametogenesis of captive greater amberjack in the present study were deficient in
478 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 [75] 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 [76]. 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.

Source of funding

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY) to AC, CCM and CR.

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.

Acknowledgments

Thanks are due to Mr Peppe, Giovanni and Vincenzo Billeci, and all the crew of the purse-seine fishing vessel 'Graziella' for their hospitality on board and assistance during wild greater amberjack sampling. Special thanks are due to Mr Tasos Raftopoulos of Argosaronikos Fishfarming S.A. (Greece) for the hospitality in his farm, and the maintenance and sampling of captive-reared greater amberjack broodstock. We thank two anonymous reviewers whose comments helped us improve the quality of the manuscript.

References

[1] Bauchot ML. Poisson osseux, Familie Carangidae. In: Fischer W, Bauchot ML, Schneider M, editors. Fiches FAO d'identification des espèces pour les besoins de la pêche. Méditerranée et mar Noire. Zone de pêche 37. Vertébrés. Rome: FAO; 1987, p.1009-30.

[2] Mylonas C, Katharios P, Grigorakis K, Papandroulakis N, Robles R, Corriero A, et al. Advances in greater amberjack (*Seriola dumerili*) research: the DIVERSIFY project, Aquaculture Europe. Belgium: European Aquaculture Society; 2016. p. 12-9.

[3] Mylonas CC, Zohar Y. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev Fish Biol Fish 2001;10:463-91. https://doi.org/10.1023/A:1012279814708

[4] Marino G, Mandich A, Massari A, Andaloro F, Porrello S, Finoia MG, et al. Aspects of reproductive biology of the Mediterranean amberjack (*Seriola dumerilii* Risso) during the spawning period. J Appl Ichthyol 1995;11:9-24. https://doi.org/10.1111/j.1439-0426.1995.tb00002.x

[5] Díaz MV, García A, Agulleiro B. Caracteristicas histologicas del ovario de la seriola mediterránea (*Seriola dumerilii*, Risso) mantenida en cautividad, durante su ciclo reproductivo annual. Proceedings of the VI Congresso National de Acuicultura; Cartagena, Spain. Ministerio de Agricultura, Pesca y Alimentacion; 1997, p. 389-94.

[6] Micale V, Maricchiolo G, Genovese L. The reproductive biology of the amberjack, *Seriola dumerilii* (Risso 1810): I. Oocyte development in captivity. Aquac Res 1999;30:349-55. https://doi.org/10.1046/j.1365-2109.1999.00336.x

[7] Zupa R, Rodríguez C, Mylonas CC, Rosenfeld H, Fakriadis I, Papadaki M, et al. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLOS ONE 2017;12: e0169645. https://doi.org/10.1371/journal.pone.0169645

557 [8] Pousis C, Mylonas CC, De Virgilio C, Gadaleta G, Santamaria N, Passantino L, et al. The observed
558 oogenesis impairment in greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is
559 not related to an insufficient liver transcription or oocyte uptake of vitellogenin. Aquac Res
560 2018;49:243-52. https://doi.org/10.1111/are.13453
561 [9] Zupa R, Fauvel C, Mylonas CC, Pousis C, Santamaria N, Papadaki M, et al. Rearing in captivity
562 affects spermatogenesis and sperm quality in greater amberjack *Seriola dumerili* (Risso, 1810).
563 J Anim Sci 2017;95:4085-100. https://doi.org/10.2527/jas.2017.1708
564 [10] Fakriadis I, Lisi F, Sigelaki,I, Papadaki M, Mylonas CC. Spawning kinetics and egg/larval quality
565 of greater amberjack (*Seriola dumerili*) in response to multiple GnRHa injections or implants.
566 Gen Comp Endocrinol 2019;279:78-87. https://doi.org/10.1016/j.ygcen.2018.12.007.
567 [11] Jerez S, Fakriadis I, Papadaki M, Martín MV, Cejas JR, Mylonas CC. Spawning Induction of
568 first-generation (F1) greater amberjack *Seriola dumerili* in the Canary Islands, Spain using
569 GnRHa delivery systems. Fishes 2018;3:35. doi:10.3390/fishes3030035.
570 https://doi.org/10.3390/fishes3030035
571 [12] Sarih S, Djellata A, La Barbera A, Fernández-Palacios Vallejo H, Roo J, Izquierdo M, et al. High-
572 quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its
573 comparison with GnRHa implants or injections. Aquac Res 2018;49:3442-50.
574 https://doi.org/10.1111/are.13808
575 [13] Jerez S, Samper M, Santamaría FJ, Villamandos JE, Cejas JR, Felipe BC. Natural spawning of
576 greater amberjack (*Seriola dumerili*) kept in captivity in the Canary Islands. Aquaculture
577 2006;252:199-207. https://doi.org/10.1016/j.aquaculture.2005.06.031
578 [14] Stifani S, Barber DL, Nimpf J, Schneider WJ. A single chicken oocyte plasma membrane protein
579 mediates uptake of very low density lipoprotein and vitellogenin. Proc Natl Acad Sci USA
580 1990;87:1955-9.
581 [15] Mizuta H, Luo W, Ito Y, Mushirobira Y, Todo T, Hara A, et al. Ovarian expression and
582 localization of a vitellogenin receptor with eight ligand binding repeats in the cutthroat trout

583 (*Oncorhynchus clarki*). Comp Biochem Physiol B 2013;166(1):81-90.
584 https://doi.org/10.1016/j.cbpb.2013.07.005
585 [16] Reading BJ, Hiramatsu N, Sawaguchi S, Matsubara T, Hara A, Lively MO, et al. Conserved and
586 variant molecular and functional features of multiple egg yolk precursor proteins
587 (vitellogenins) in white perch (*Morone americana*) and other teleosts. Mar Biotechnol
588 2009;11:169-87. https://doi.org/10.1007/s10126-008-9133-6
589 [17] Reading BJ, Andersen LK, Ryu YW, Mushirobira Y, Todo T, Hiramatsu N. Oogenesis and egg
590 quality in finfish: Yolk formation and other factors influencing female fertility. Fishes
591 2018;3:45. https://doi.org/10.3390/fishes3040045
592 [18] Tyler CR, Lubberink K. Identification of four ovarian receptor proteins that bind vitellogenin but
593 not other homologous plasma lipoproteins in the rainbow trout, *Oncorhynchus mykiss*. J Comp
594 Physiol B 1996;166:11-20. https://doi.org/10.1007/BF00264634
595 [19] Reading BJ, Hiramatsu N, Sullivan CV. Disparate binding of three types of vitellogenin to
596 multiple forms of vitellogenin receptor in white perch. Biol Reprod 2011;84:392-9.
597 https://doi.org/10.1095/biolreprod.110.087981
598 [20] Reading BJ, Hiramatsu N, Schilling J, Molloy KT, Glassbrook N, Mizuta H. Lrp13 is a novel
599 vertebrate lipoprotein receptor that binds vitellogenins in teleost fishes. J Lipid Res
600 2014;55:2287-95. https://doi.org/10.1194/jlr.M050286
601 [21] Hiramatsu N, Chapman RW, Lindzey JK, Haynes MR, Sullivan CV. Molecular characterization
602 and expression of vitellogenin receptor from white perch (*Morone americana*). Biol Reprod
603 2004;70:720-30. https://doi.org/10.1095/biolreprod.103.023655
604 [22] Bujo H, Lindstedt KA, Hermann M, Dalmau LM, Nimpf J, Schneider WJ. Chicken oocytes and
605 somatic cells express different splice variants of a multifunctional receptor. J Biol Chem
606 1995;270:23546-51. https://doi.org/10.1074/jbc.270.40.23546

607 [23] Bujo H, Yamamoto T, Hayashi K, Hermann M, Nimpf J, Schneider WJ. Mutant oocytic low
608 density lipoprotein receptor gene family member causes atherosclerosis and female sterility.
609 Proc Natl Acad Sci USA 1995;92:9905-99. https://doi.org/10.1073/pnas.92.21.9905
610 [24] Mushirobira Y, Mizuta H, Luo W, Todo T, Hara A, Reading BJ, et al. Molecular cloning and
611 partial characterization of a low-density lipoprotein receptor-related protein 13 (Lrp13)
612 involved in vitellogenin uptake in the cutthroat trout (*Oncorhynchus clarki*). Mol Reprod Dev
613 2015;82:986-1000. https://doi.org/10.1002/mrd.22579
614 [25] Schneider WJ, Nimpf J, Brandes C, Drexler M. Low-density lipoprotein receptor family: genetics,
615 function, and evolution. Curr Atheroscler Rep 1999;1:115-22. https://doi.org/10.1007/s11883-
616 999-0007-9
617 [26] Schneider WJ, Nimpf J. LDL receptor relatives at the crossroad of endocytosis and signaling. Cell
618 Mol Life Sci 2003;60:892-903. https://doi.org/10.1007/s00018-003-2183-Z
619 [27] Chakraborty T, Katsu Y, Zhou LY, Miyagawa S, Nagahama Y, Iguchi T. Estrogen receptors in
620 medaka (*Oryzias latipes*) and estrogenic environmental contaminants: an in vitro-in vivo
621 correlation. J Steroid Biochem Mol Biol 2011;123:115-21.
622 https://doi.org/10.1016/j.jsbmb.2010.11.015
623 [28] Dominguez GA, Quattro JM, Denslow ND, Kroll KJ, Prucha MS, Porak WF, et al. Identification
624 and transcriptional modulation of the largemouth bass *Micropterus salmoides*, vitellogenin
625 receptor during oocyte development by insulin and sex steroids. Biol Reprod 2012;87(3):67.
626 https://doi.org/10.1095/biolreprod.112.099812
627 [29] Xu HG, Cao L, Zhang YQ, Johnson RB, Wei YL, Zheng KK, et al. Dietary arachidonic acid
628 differentially regulates the gonadal steroidogenesis in the marine teleost, tongue sole
629 (*Cynoglossus semilaevis*), depending on fish gender and maturation stage. Aquaculture
630 2017;468(1):378-85. https://doi.org/10.1016/j.aquaculture.2016.11.002
631 [30] Silversand C, Haux C. Fatty acid composition of vitellogenin from four teleost species. J Comp
632 Physiol B 1995;164(8):593-9. https://doi.org/10.1007/BF00389799

633 [31] Sorbera LA, Asturiano JF, Carrillo M, Zanuy S. Effects of polyunsaturated fatty acids and
634 prostaglandins on oocyte maturation in a marine teleost, the European sea bass (*Dicentrarchus*
635 *labrax*). Biol Reprod 2001;64:382-9. https://doi.org/10.1095/biolreprod64.1.382
636 [32] Khajeh M, Rahbarghazi R, Nouri M, Darabi M. Potential role of polyunsaturated fatty acids, with
637 particular regard to the signaling pathways of arachidonic acid and its derivatives in the process
638 of maturation of the oocytes: Contemporary review. Biomed Pharmacother 2017;94:458-67.
639 https://doi.org/10.1016/j.biopha.2017.07.140
640 [33] Tong X, Yang X, Bao C, Wang, J, Tang X, Jiang D, et al. Changes of biochemical compositions
641 during development of eggs and yolk-sac larvae of turbot *Scophthalmus maximus*. Aquaculture
642 2017;473:317-26. https://doi.org/10.1016/j.aquaculture.2017.02.024
643 [34] Sargent JR, Tocher DR, Bell GJ. The lipids. In: Halver JE, Hardy RW, editors. Fish Nutrition. 3rd
644 ed. San Diego: Academic Press; 2002, p. 181-257.
645 [35] Rodríguez-Barreto D, Jerez S, Cejas JR, Martin MV, Acosta NG, Bolaños A, et al. Comparative
646 study of lipid and fatty acid composition in different tissues of wild and cultured female
647 broodstock of greater amberjack (*Seriola dumerili*). Aquaculture 2012;360-361:1-9.
648 https://doi.org/10.1016/j.aquaculture.2012.07.013
649 [36] Rodríguez-Barreto D, Jerez S, Cejas JR, Martin MV, Acosta NG, Bolaños A, et al. Ovary and
650 egg fatty acid composition of greater amberjack broodstock (*Seriola dumerili*) fed different
651 dietary fatty acids profiles. Eur J Lipid Sci Technol 2014;116:584-95.
652 https://doi.org/10.1002/ejlt.201300462
653 [37] Fernández-Palacios H, Izquierdo MS, Robaina L, Valencia A, Salhi M, Vergara JM. Effect of n−3
654 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.).
655 Aquaculture 1995;132:325-37. https://doi.org/10.1016/0044-8486(94)00345-O
656 [38] Navas JM, Bruce M, Thrush M, Farndale BM, Bromage N, Zanuy S. The impact of seasonal
657 alteration in the lipid composition of broodstock diets on egg quality in the European sea bass.
658 J Fish Biol 1997;51:760-73. https://doi.org/10.1111/j.1095-8649.1997.tb01997.x

659 [39] Bruce M, Oyen F, Bell G, Asturiano JF, Farndale B, Carrillo M., et al. Development of
660 broodstock diets for the European sea bass (*Dicentrarchus labrax*) with special emphasis on the
661 importance of n-3 and n-6 HUFA to reproductive performance. Aquaculture 1999;177:85-98.
662 https://doi.org/10.1016/S0044-8486(99)00071-X
663 [40] Li K, Olsen Y. Effect of enrichment time and dietary DHA and non-highly unsaturated fatty acid
664 composition on the efficiency of DHA enrichment in phospholipid of rotifer (*Brachionus*
665 Cayman). Aquaculture 2015;446:310-7. https://doi.org/10.1016/j.aquaculture.2015.05.005
666 [41] Watanabe T, Vassallo-Agius R. Broodstock nutrition research on marine finfish in Japan.
667 Aquaculture 2003;227:35-61. https://doi.org/10.1016/S0044-8486(03)00494-0
668 [42] Mandich A, Massari A, Bottero S, Pizzicori P, Goos H, Marino G. Plasma sex steroid and
669 vitellogenin profiles during gonad development in wild Mediterranean amberjack (*Seriola*
670 *dumerilii*). Mar Biol 2004;144:127-38. https://doi.org/10.1007/s00227-003-1185-6
671 [43] Sley A, Hadj Taeib A, Jarboui O, Ghorbel M, Bouain A. Reproductive biology of greater
672 amberjack *Seriola dumerili* (Risso, 1810) from the Eastern Mediterranean Sea (Tunisia, Gulf of
673 Gabes). Cah Biol Mar 2014;55:421-30. https://doi.org/10.21411/CBM.A.21508B77
674 [44] Corriero A, Desantis S, Deflorio M, Acone F, Bridges CR, de la Serna JM, et al. Histological
675 investigation on the ovarian cycle of the bluefin tuna in the western and central Mediterranean.
676 J Fish Biol 2003;63:108-19. https://doi.org/10.1046/j.1095-8649.2003.00132.x
677 [45] Corriero A, Medina A, Mylonas CC, Abascal FJ, Deflorio M, Aragón L, et al. Histological study
678 of the effects of treatment with gonadotropin-releasing hormone agonist (GnRHa) on the
679 reproductive maturation of captive-reared Atlantic bluefin tuna (*Thunnus thynnus* L.).
680 Aquaculture 2007;272:675-86. https://doi.org/10.1016/j.aquaculture.2007.08.030
681 [46] Pousis C, Santamaria N, Zupa R, De Giorgi C, Mylonas CC,. Bridges CR, et al. Expression of
682 vitellogenin receptor gene in the ovary of wild and captive Atlantic bluefin tuna (*Thunnus*
683 *thynnus*). Anim Reprod Sci 2012;132:101-10. https://doi.org/10.1016/j.anireprosci.2012.03.014

[47] Peirson SN, Butler JN, Foster RG. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 2003;31(14):e73. https://doi.org/10.1093/nar/gng073

[48] Livak KJ, Schmittgen, TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−(ΔΔCt) method. Methods 2001;25:402-8. https://doi.org/10.1006/meth.2001.1262

[49] Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, et al. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 1993;14(10):1023-31. https://doi.org/10.1002/elps.11501401163

[50] Bjellqvist B, Basse B, Olsen E, Celis JE. Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 1994;15:529-39. https://doi.org/10.1002/elps.1150150171

[51] Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Wilkins MR, et al. Protein Identification and Analysis Tools on the ExPASy Server. In: Walker JM, editor. The Proteomics Protocols Handbook. Humana Press; 2005, p. 571-607.

[52] Almagro Armenteros JJ,Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019;37:420-23. https://doi.org/10.1038/s41587-019-0036-z.

[53] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80. https://doi.org/10.1093/nar/22.22.4673

[54] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402. https://doi.org/10.1093/nar/25.17.3389

710 [55] Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, et al. Protein
711 database searches using compositionally adjusted substitution matrices. FEBS J
712 2005;272:5101-9. https://doi.org/10.1111/j.1742-4658.2005.04945.x
713 [56] Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total
714 lipids from animal tissues. J Biol Chem 1957;226:497-509.
715 [57] Christie WW. Lipid analysis. 2nd ed. Oxford: Pergamon Press; 1982.
716 [58] Araki K, Aokic J, Kawase J, Hamada K, Ozaki A, Fujimoto H, et al. Whole genome sequencing
717 of greater amberjack (*Seriola dumerili*) for SNP identification on aligned scaffolds and genome
718 structural variation analysis using parallel resequencing. Int J Genomics 2018;2018:1-12.
719 https://doi.org/10.1155/2018/7984292
720 [59] Bujo H, Hermann M, Kaderli MO, Jacobsen L, Sugawara S, Nimpf J, et al. Chicken oocyte
721 growth is mediated by an eight ligand binding repeat member of the LDL receptor family.
722 EMBO J 1994;13:5165-75.
723 [60] Davail B, Pakdel F, Bujo H, Perazzolo LM, Waclawek M, Schneider W, et al. Evolution of
724 oogenesis: the receptor for vitellogenin from the rainbow trout. J Lipid Res 1998;39:1929-37.
725 [61] Li A, Sadasivam M, Ding JL. Receptor–ligand interaction between vitellogenin receptor (VtgR)
726 and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein
727 B/E. The first three ligand-binding repeats of VtgR interact with the amino-terminal region of
728 Vtg. J Biol Chem 2003;278:2799-806. https://doi.org/10.1074/jbc.M205067200
729 [62] Agulleiro MJ, André M, Morais S, Cerdà J, Babin PJ. High transcript level of fatty acid-binding
730 protein 11 but not of very low-density lipoprotein receptor is correlated to ovarian follicle
731 atresia in a teleost fish (*Solea senegalensis*). Biol Reprod 2007; 77:504-16.
732 https://doi.org/10.1095/biolreprod.107.061598
733 [63] Perazzolo LM, Coward K, Davail B, Normand E, Tyler CR, Pakdel F, et al. Expression and
734 localization of messenger ribonucleic acid for the vitellogenin receptor in ovarian follicles

735 throughout oogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biol Reprod 1999;60:1057-
736 68. https://doi.org/10.1095/biolreprod60.5.1057
737 [64] Morini M, Vílchez MC, Lafont AG, Peñaranda DS, Gallego V, Dufour S, Asturiano JF, Pérez L
738 Influence of temperature regime on the vitellogenesis of the European eel (*Anguilla anguilla*).
739 Aquaculture Europe 2014, 15-17 October, 2014. Donostia–San Sebastián, Spain. pp. 848-849.
740 [65] Fakriadis I, Raftopoulos A, Iakovopoulos G, Papandroulakis N, Papadaki M, Siggelaki I, et al.
741 Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared
742 in tanks and sea cages. PLoS ONE 2019 (in preparation).
743 [66] Lubzens E, Young G, Bobe J, Cerdà J. Oogenesis in teleosts: How fish eggs are formed. Gen
744 Comp Endocrinol 2010;165:367-89. https://doi.org/10.1016/j.ygcen.2009.05.022
745 [67] Luckenbach JA, Iliev DB, Goetz FW, Swanson P. Identification of differentially expressed
746 ovarian genes during primary and secondary oocyte growth in coho salmon, *Oncorhynchus*
747 *kisutch*. Reprod Biol Endocrinol 2008;6:2-16. https://doi.org/10.1186/1477-7827-6-2
748 [68] Luckenbach JA, Goetz FW, Swanson P. Expressed sequence tags (ESTs) of follicle/interstitial
749 cell enriched ovarian tissue from previtellogenic coho salmon. Cybium 2008;32:142-4.
750 [69] Borges P, Medale F, Dias J, Valente LMP. Protein utilisation and intermediary metabolism of
751 Senegalese sole (*Solea senegalensis*) as a function of protein:lipid ratio. Br J Nutr
752 2013;109:1373-81. https://doi.org/10.1017/S0007114512003418
753 [70] Miura C, Higashino T, Miura T. A progestin and an estrogen regulate early stages of oogenesis in
754 fish. Biol Reprod 2007;77:822-8. https://doi.org/10.1095/biolreprod.107.061408
755 [71] Zapater C, Chauvigné F, Scott AP, Gómez A,, Katsiadaki I, Cerdà J. Piscine follicle-stimulating
756 hormone triggers progestin production in gilthead seabream primary ovarian follicles. Biol
757 Reprod 2012;87:111. https://doi.org/10.1095/biolreprod.112.102533
758 [72] Arts MT, Kohler CC. Health and condition in fish: the influence of lipids on membrane
759 competency and immune response. In: Kainz M, Brett M, Arts M, editors. Lipids in Aquatic
760 Ecosystems, New York: Springer; 2009.

761 [73] Hishikawa D, Valentine WJ, Iizuka-Hishikawa Y, Shindou H, Shimizu T. Metabolism and
762 functions of docosahexaenoic acid-containing membrane glycerophospholipids. FEBS Letters
763 2017;591:2730-44. https://doi.org/10.1002/1873-3468.12825
764 [74] Tallima H, El Ridi R. Arachidonic acid: Physiological roles and potential health benefits – A
765 review. J Adv Res 2018;11:33-41. https://doi.org/10.1016/j.jare.2017.11.004
766 [75] Lancaster PM, Tyler CR. Developmental expression and modulation of the vitellogenin receptor
767 in ovarian follicles of the rainbow trout, *Oncorhynchus mykiss*. J Exp Zool 1994;269(5):458-
768 66. https://doi.org/10.1002/jez.1402690509
769 [76] Gawrisch K, Soubias O, Mihailescu M. Insights from biophysical studies on the role of
770 polyunsaturated fatty acids for function of G-protein coupled membrane receptors. Prostag
771 Leukotr Ess 2008;79(3-5):131-4. https://doi.org/10.1016/j.plefa.2008.09.002
772

773 **Figure Captions**

774

775 **Fig. 1**. Micrographs of ovary sections from greater amberjack individuals sampled during the early
776 phase of the reproductive cycle. (a) Ovary section of a wild female showing cortical alveoli and
777 early vitellogenic oocytes. Haematoxylin-eosin staining. (b) Ovary section from a captive-reared
778 female with an early vitellogenic oocyte showing anti-vitellogenin immunostaining in the peripheral
779 ooplasm and in some granulosa cells. Immunostaining with purified IgG fraction from rabbits
780 immunised with the portion from amino acid 764 to amino acid 1025 of the Atlantic bluefin tuna
781 vitellogenin sequence. (c) Higher magnification of part of the early vitellogenic oocyte shown in
782 (b). Arrow, anti-vitellogenin positive ooplasm; arrowhead, anti-vitellogenin positive granulosa cell;
783 dashed arrow, cortical alveoli oocyte; double arrow, early vitellogenic oocyte. Magnification bar =
784 200 µm in (a), 50 µm in (b) and 10 µm in (c).

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

792 **Fig. 3.** Greater amberjack Lrp13 amino acid sequence numbered from the initiator, methionine.
793 Arrows above the sequence indicate cysteine binding repeats, epidermal growth factor (EGF)
794 homologue repeats sequences (A, B, C, D, E), transmembrane and cytosolic domains. Cysteine
795 repeats, likely involved in protein folding, are numbered from I to XI and evidenced in violet. The
796 (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.