Deliverable Report

Deliverable No:	D3.1		**Delivery Month:**	16
Deliverable Title	Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor).			
WP No:	3		**WP Lead beneficiary:**	P1. HCMR
WP Title:	Reproduction and Genetics - greater amberjack			
Task No:	3.1		**Task Lead beneficiary:**	P13. UNIBA
Task Title:	Description of the reproductive cycle of greater amberjack.			
Other beneficiaries:	P1. HCMR	P4. IOLR	P14. IFREMER	P15. ULL
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Status:	Delivered		**Expected month:**	12
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Other Scientists participating: Corriero A. (UNIBA)

Objective: Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (*Seriola dumerili*) (*i.e.*, LHβ, FSHβ, leptin, Vg and Vg receptor). The deliverable includes: (a) methods to estimate egg quality by measuring liver vitellogenin (Vg) (the yolk precursor protein) and its ovary receptor (VgR) gene expression levels through a quantitative PCR (qPCR); (b) calibrated real-time PCR assays developed and validated for the reliable mRNA quantification of sequences encoding for greater amberjack LHβ, FSHβ, leptin, Vg and Vg receptor; and (b) gene specific primer sets facilitating optimal assay sensitivity and specificity.

Description

The present task aimed at establishing quantitative PCR (qPCR) assays to measure transcript levels of chosen target genes, including those encoding for (i) gonadotropins (FSHβ and LHβ), the central regulators of gonadal development and gamete maturation, (ii) leptin, a metabolic hormone, which conveys to the brain information on the critical amount of energy stores that are necessary for activation of the brain-pituitary-gonad axis and may also influence directly gonadotropin release and gonadal steroidogenesis, (iii) vitellogenins (Vgs) and their cognate receptor (VgR), which facilitate yolk formation and accumulation in the oocyte, an important process affecting egg and larval quality in fish. Comparison of the resulting gene expression profiles in wild and captive-reared greater amberjack brooders is expected to aid in evaluating potential adverse impacts of captivity on vitellogenesis in particular.

Background

As in other vertebrates, reproduction in fish necessitates the full activation of the brain–pituitary–gonad (BPG) axis, which depends largely on the coordinated functions of the two pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both are heterodimeric, non-covalently bound glycoproteins composed of a common α-subunit and a hormone-specific β-subunit (Pierce & Parsons, 1981). While the exact physiological functions of the gonadotropins in teleosts are not entirely clear, a

growing body of evidence suggest that FSH has a dominant role during early phases of gametogenesis, while LH is considered to be responsible for the final maturational processes, including oocyte maturation and ovulation in females and spermiation in males (Rosenfeld et al., 2007; Swanson et al., 2003; Yaron &Sivan, 2006).

Reproduction is an energetically demanding process, which requires adequate energy reserves. Therefore, the central control of reproduction requires the hypothalamus to receive information regarding the energy status of an animal. The fundamental link between nutrition and reproduction was illuminated by the discovery of leptin, a 16-kDa circulating hormone member of the four-helical cytokine subfamily that also includes growth hormone, prolactin and the interleukins (Huising et al., 2006; Zhang et al., 1994). Early studies suggested that the neurons involved in regulating energy metabolism, mostly NPY neurons, can communicate with the BPG axis via interactions with gonadotropin releasing hormone (GnRH) neurons, thus operate as neuroendocrine integrators, linking energy balance to the activity of the reproductive axis (Guy et al., 1988; Sabatino et al., 1987). More recent studies demonstrated that the modulation of the hypothalamic GnRH system by leptin occurs via activation of the kisspeptin system (Castellano et al., 2005; Smith et al., 2006). Leptin was also found to act directly at the pituitary level, giving rise to gonadotropin secretion, which, in turn, affects gonadal growth and gamete maturation (Akhter et al., 2010). Likewise, *in vitro* studies with European sea bass (*Dicentrarchus labrax*; Peyron et al., 2001), and rainbow trout (*Oncorhynchus mykiss*; Weil et al., 2003) demonstrated a gonadotropic-releasing effect of heterologous leptin, by acting directly at the pituitary level.

Vitellogenin (Vg) is a high-density serum glycolipophosphoprotein synthesized by the liver under the stimulation of the 17ß-estradiol (E2) produced by the developing ovary (Kwon et al., 1993; Ng &Idler, 1983; Sumpter, 1995; Susca et al., 2001). Vitellogenin is transported via the bloodstream from the liver into the follicular layer and endocytosed by the growing oocytes, in order to form the yolk proteins (Babin et al., 2007; Lubzens et al., 2010; Sargent, 1995; Tyler et al., 1988; Wallace, 1985). Yolk content is an important determinant of egg and larval quality in fish, as it represents the major nutrient for the developing embryo/larva during the first days of endogenous feeding (Bobe & Labbé, 2009; Brooks, 1997).

Evidence of the existence of multiple Vg genes in many different teleost species has been obtained through molecular cloning of cDNAs encoding two distinct Vgs, named VgA (or VgI) and VgB (or VgII) (Babin et al., 2007; LaFleur et al., 1995a, 1995b, 2005; Sawaguchi et al., 2005, 2006; Pousis et al., 2011). Both VgA and VgB genes encode proteins that undergo proteolytic cleavage during oocyte maturation, in order to generate a pool of free amino acids and lipids for oocyte hydration (Cerdá et al., 2007) and for allocation of specific types of nutrients to the developing embryo (Sawaguchi et al., 2006). A third Vg gene (VgC) and protein has also been identified in fish (Finn et al., 2009; Sawaguchi et al., 2005, 2006), but this Vg does not undergo proteolytic cleavage during oocyte maturation and probably gives rise to nutrients at a later stage of embryonic development (Sawaguchi et al., 2006). In our laboratory, we sequenced all the three Vg forms of the Atlantic bluefin tuna (*Thunnus thynnus*) and compared VgA and VgB expression in wild and captive-reared individuals during different phases of the reproductive cycle (Pousis et al., 2011).

Vitellogenesis is a hormonally controlled and regulated process. In fish, the primarily exogenous synthesis of vitellogenin is initiated by gonadotropins and regulated by estrogens. Vitellogenin is a species-specific protein synthesized by hepatocytes, released into the bloodstream and actively sequestered by maturing oocytes. Vitellogenin is specifically incorporated in the oocytes by receptor-mediated endocytosis through receptors belonging to the Low Density Lipoprotein Receptor (LDLR) family, which have been named Very Low Density Lipoprotein Receptors (VLDLR), Vg Receptors (VgR) or LR8, due to the presence of eight Ligand-binding Repeats (Bujo et al., 1994; Hiramatsu et al., 2004). In the last years, the structural characteristics of this gene family had been described in several teleosts (Agulleiro et al., 2007; Davail et al., 1998; Hiramatsu et al., 2004; Li et al., 2003). The Atlantic bluefin tuna expresses two forms of VgR mRNA, which are generated by differential splicing of an exon called the O-linked sugar domain corresponding to a 20-amino acid sequence in the mature protein (Pousis et al., 2012). This exon, which is characteristic in some lipoprotein receptors, encodes a serine and threonine-rich glycopeptide motif (Li et al., 2003). The O-linked form (VgR+) is dominant in somatic tissues, whereas the non-O-linked form (VgR-) is principally

expressed in ovary (Hiramatsu et al., 2003). No functional differences were found between the two VgR proteins in terms of ligand binding (Hiramatsu et al., 2004).

In light of the above, comparative analysis of the related gene expression profiles in wild and captive-reared greater amberjack will signify if the vitellogenic process is impaired in individuals reared in captivity.

Methods and Results

Sample collection

A total of 11 wild adult female greater amberjack (mean body mass 13.7 ± 3.8 kg) were sampled on 31 May 2014 (n=2) and 30 June 2014 (n=9) in the waters around Lampedusa Island (Mediterranean Sea, Sicily, Italy) onboard a commercial purse seine vessel. Soon after capture, fish pituitary, liver and gonads were dissected and stored in RNA later®.

RNA extraction and reverse transcription

Total RNA was obtained from pituitary and liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 µl of RNase free water and stored at –80°C. The cDNA was prepared from 1.2 µg total RNA. Random hexamer primers were used for the cDNA synthesis using U SuperScript III Reverse Transcriptase (Invitrogen).

cDNA cloning and sequencing

The partial cDNA sequences of vitellogenins (VgA, VgB, VgC), vitellogenin receptor (VgR) and β-actin were amplified from total cDNA by means of several overlapping PCR reactions (**Figs. 1 and 2**). For that purpose degenerate primer pairs were designed against conserved sequences from various Perciform species (Pousis et al., 2011; Pousis et al. 2012). All PCRs were performed on a PCR Sprint Thermal Cycler using ~120 ng cDNA, 10 pmoles of each oligonucleotide primer, 0.2 mM dNTP mix, 10X Taq polymerase buffer and 1.5 unit Taq Polymerase (Eppendorf). The amplification products were analyzed for size on 1.2% agarose gels containing ethidium bromide. The band of interest was excised from the gel, purified using Nucleo Spin extract II (Macherey-Nagel) and ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit; Invitrogen). *Escherichia coli* competent cells (Invitrogen) were used for transformation. Approximately 20 µl of the recombinant plasmid was sent to the Primm Sequence Service (Primm Srl, Italy) for sequencing with M13 reverse and M13 forward primers.

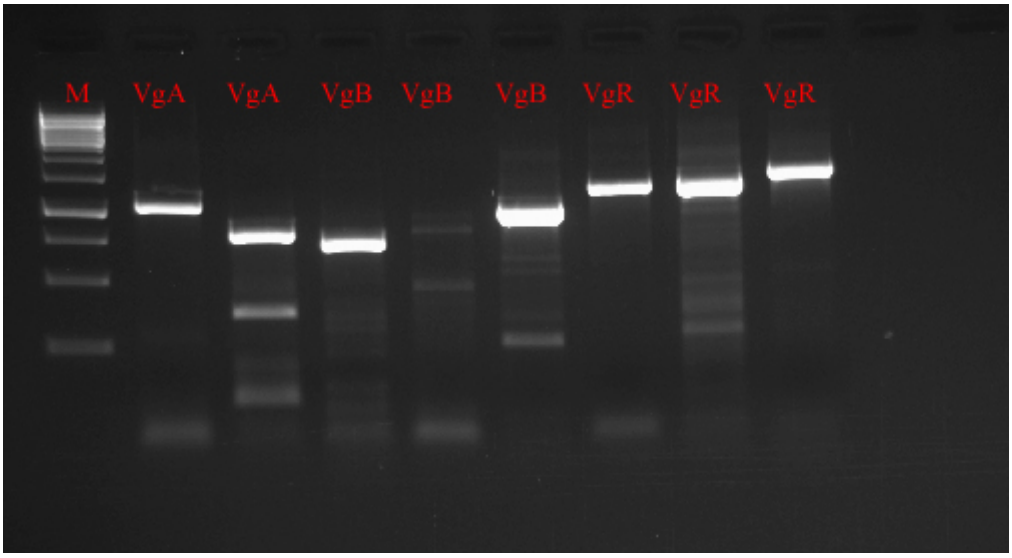

Figure 1. Agarose gel electrophoretic analysis of Vitellogenin A (VgA), Vitellogenin B (Vgb) and Vitellogenin Receptor (VgR) RT-PCR products. mRNA at 125 ng from greater amberjack liver and ovary was used as template for reverse transcription, followed by amplification with two specific primers pairs. Aliquots of 8 µl were loaded onto the agarose gel together with 1 kbp DNA marker (M).

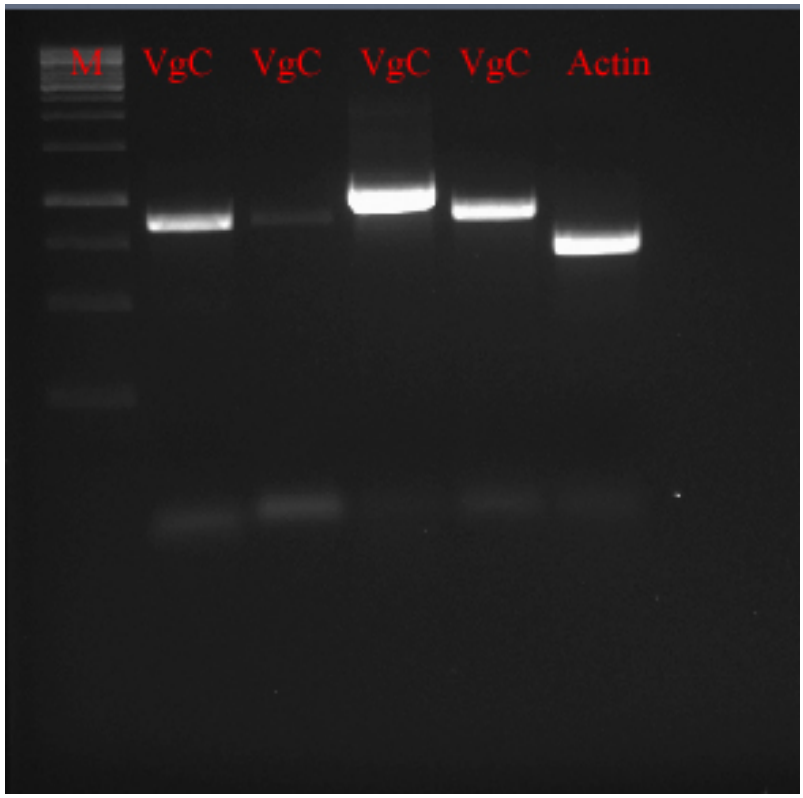

Figure 2. Agarose gel electrophoretic analysis of Vitellogenin C (VgC), and β-actin RT-PCR products. mRNA at 125 ng from greater amberjack liver was used as template for reverse transcription, followed by amplification with two specific primers pairs. Aliquots of 8 µl were loaded onto the agarose gel together with 1 kbp DNA marker (M).

In order to identify and clone the cDNA sequences encoding for FSHβ, LHβ and leptin, total RNA from target tissues (i.e., pituitary and liver) was extracted by the guanidiniumthiocyanate–phenol–chloroform extraction method using Bio-Tri RNA reagent (Bio Lab Ltd., Jerusalem, Israel). One microgram of DNAse treated total RNA was reverse transcribed with random primers using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Branchburg, NJ, USA) according to manufacturer's protocol. The PCR amplification was conducted using degenerate primers (**Table 1**) that were designed according to the most conserved regions across Perciforms (see **Table 2**).

Table 1. Primer list used to clone greater amberjack FSHβ, LHβ

Target gene	sequence
Seriola LH F1	CACTGCATCACCAAGGACC
Seriola LH R1	TGGGCTGAAGACGTACTTAC
Seriola LH R2	CCGGGACTTGTATTACAGG
Seriola FSH F1	CCGTCGTCAACATCTCTGTGGA
Seriola FSH R1	AACATGTTCTGTGGGCGCTTCCT
Seriola FSH R2	CGCTCCACCATATGTGCAGGA
Seriola Lep F1	GAAATCAAAAGTGAAATGGATGG
Seriola Lep F3	CCAGGTCCCTCCTGGCCTGAC
Seriola Lep R3	TTGACCTGRGWGACYCCRTY

Table 2. Comparable sequences from closely related species for primer design

Species	Target gene	Accession number
Seriola quinqueradiata	FSH	KF719237.1
Seriola quinqueradiata	LH	KF719238.1
Seriola lalandi	FSH	HQ449731.1
Seriola lalandi	LH	HQ449732.1
Atlantic bluefin tuna	Leptin	HQ288053

The amplified PCR products were electrophoretically separated upon 1.5% Agarose gel (**Fig. 3**). The desired amplicons were purified using the QIAquick Gel Extraction Kit (QIAGENE, Hilden, Germany) and cloned into the pGEM®-T Easy Vector (Promega) containing T7 and SP6 RNA polymerase promoters. Plasmids were extracted employing Qiagen Mini-prep columns (Qiagen, Inc.) and then subjected to sequence analysis at the, Hy- labs molecular laboratory (Rehovot, Israel). Gene identity was confirmed by comparing the obtained sequences with those available at the Genebank (http://www.ncbi.nlm.nih.gov/Genbank/) and using the BLAST algorithm (Blastn) of National Center for Biotechnology Information.

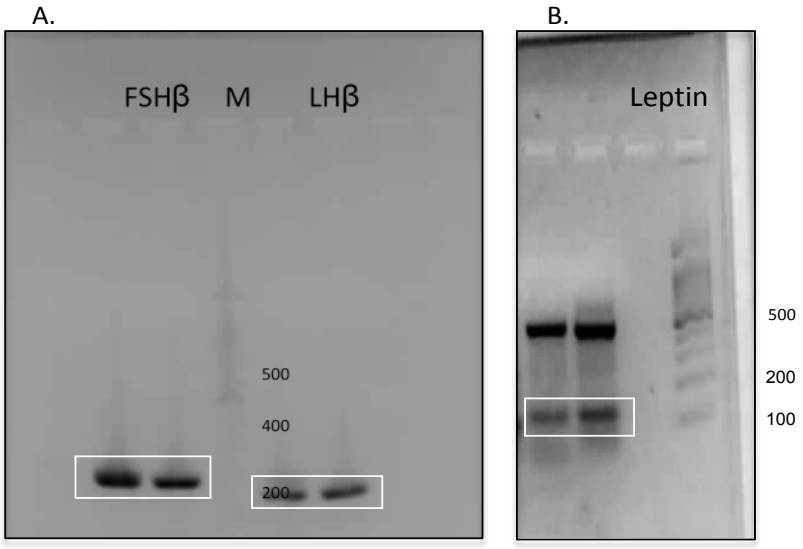

Figure 3. PCR amplification of greater amberjack FSHβ, LHβ and leptin partial cDNA sequences. PCR amplifications on (A) greater amberjack pituitary and (B) liver cDNA using degenerate primers. All PCR products were fractionated by electrophoresis through 1.5% agarose gel in parallel to a molecular size marker (M).

Establishment of real-time PCR assays

Once the greater amberjack homologous sequences were obtained, gene specific primers were designed (Error! Reference source not found.**3**) using either the Primer Express software (V2.0; Applied Biosystems) or the Primer3 software (Rozen & Skaletsky, 2000) and their specificity was checked with both the UCSC "In-Silico PCR" and the NCBI Primer-BLAST tools. For gene expression analysis qRT-PCR experiments were carried out in triplicate using the ABI PRISM 7900HT platform (Applied Biosystems®, Life TechnologiesTM) using 1µl of diluted cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). No template controls were included as negative controls for each primer pair. Amplification parameters were as follows: hot start at 95°C for 15 min; 40 amplification cycles (94°C for 15 sec, 62°C for 30 sec, 72°C for 30 sec); dissociation curve step (95° C for 15 sec, 60°C for 15 sec, 95°C for 15 sec). Fluorescence raw data were exported from the SDS 2.4 software (Applied Biosystems®, Life TechnologiesTM) and analyzed with the DART-PCR Excel workbook (Peirson et al., 2003). Actual amplification efficiency values (E) for each amplicon were used to correct Cq values before analyzing these data by the ΔCq method to compare relative expression results.

The real time PCR method for Vgs and VgR expression does not need any validation of primers (Pousis et al., 2011). As reported for the Atlantic bluefin tuna (Pousis et al., 2011), a wide range of samples from basal level to peak expression is required to calibrate the method. After the completion of the sampling planned for Deliverable 3.3, when samples of individuals in different phases of the reproductive cycle will be available, the real time PCR method will be calibrated using, as internal calibration group, individuals expressing basal transcript levels of Vgs and VgR.

Table 3. Primers for greater amberjack real-time PCR.

VGA FOR	TCTGTACCTGAACACATTGCAG
VGA REV	GAGGATCTTGACAAACTAGCTG
VgR FOR	GAGGACGAGGTCAACTGTGG
VgR REV	ATGCACGATGAGTTCCCACAC
VGB FOR	TGAGAATGCTGACAGGCTCAC
VGB REV	GCCTTGAGCATAAACTGCAGG
β-actin FOR	GAGCGTGCCTACACCTAGAC
β-actin rev	GGCAGGTCGATCCTCTTCTCC
FSHβ FOR	TGTAACGGGGACTGGTCCTT
FSHβ REV	AGGAAGCGCCCACAGAACA
LHβ FOR	ACCTACCCGGTGGCTTTGAG
LHβ REV	TCATTCATGCAGAAGTCGG
leptin FOR	CCGTTAAGGGTGTCAGAGA
leptin REV	TTCCAGGTCCCTGTTGGTC

Conclusions

According to the original plan we cloned partial cDNAs encoding for the greater amberjack pituitary gonadotropin β-subunits (FSHβ and LHβ), liver leptin and Vgs, and ovary *VgR* and were able to establish the respective gene specific qRT-PCR.

Greater amberjack VgA, VgB and VgC partial amino acid sequences have high similarities compared to those of other teleost fishes, thus confirming the high conservation of structure and function for these molecules.

The *in silico* analysis showed that greater amberjack VgR, like other piscine VgRs, is highly homologous to amphibian and bird VgRs and to mammal LDLR/VLDLR.

Liver Vg gene expression and Vg protein synthesis are stimulated by circulating E_2 (Ng & Idler, 1983; Sawaguchi et al., 2006; Susca et al., 2001). Female greater amberjack sampled in the waters around Lampedusa in May and June were in spawning conditions, showing fully yolked oocytes along with hydrated oocytes and/or post-ovulatory follicles (see Y1 scientific report) and Vgs and VgR gene expression was detected in all of them. A comparative analysis of Vg and VgR gene expression in different periods of the reproductive cycle of wild and captive-reared greater amberjack will be performed when the sampling programme will be finalised (Deliverable 3.3 due on Month 24).

Deviations: The deliverable is submitted 4 months later that indicated in the DOW due to the severe restrictions in air transportation of biological samples (in dry ice or liquid nitrogen) to Israel that caused a delay in sample delivery to P4. IOLR.

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