Deliverable Report

Deliverable No:	D3.2		**Delivery Month:**	49
Deliverable Title	Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in the greater amberjack			
WP No:	3		**WP Lead beneficiary:**	P1. HCMR
WP Title:	Reproduction and Genetics - greater amberjack			
Task No:	3.1		**Task Lead beneficiary:**	P4. IOLR
Task Title:	Description of the reproductive cycle of greater amberjack.			
Other beneficiaries:	P1. HCMR	P13. UNIBA	P14. IFREMER	P15. ULL
	P24. ITTICAL			
Status:	Delivered		**Expected month:**	18
………..				

Lead Scientist preparing the Deliverable: Rosenfeld H. (IOLR)

Other Scientists participating:

Objective: Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack. Calibrated immuno-assays have been developed and validated for a sensitive and reliable hormone measurement. The deliverable presents: (a) yeast expression system, a means for efficient production of recombinant greater amberjack LHβ, FSHβ, leptin, (b) recombinant hormone/hormone-subunits to be used as antigen/standard, and (c) polyclonal antibodies, including anti- LHβ, anti-FSHβ, and anti-leptin, allowing for optimal immuno-assay sensitivity and specificity.

Table of Contents

DESCRIPTION 2
BACKGROUND 2
MATERIALS AND METHODS 3
SAMPLE COLLECTION 3
BLOOD AND TISSUE PROCESSING 3
PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEINS 3
GENERATION OF POLYCLONAL ANTIBODIES 4
PROTEIN GEL ELECTROPHORESIS, WESTERN BLOTTING AND IMMUNODOT BLOT 4
MASS SPECTROMETRY 4
VALIDATION OF HETEROLOGOUS ELISA TO MEASURE PITUITARY AND PLASMA LH 4
ESTABLISHMENT OF HOMOLOGOUS ELISA TO MEASURE PITUITARY AND PLASMA FSH 5
STATISTICAL ANALYSIS 5
RESULTS 5
VALIDATION OF THE STRIPED BASS LH ELISA FOR PITUITARY AND PLASMA SAMPLES 5
ESTABLISHMENT OF HOMOLOGOUS FSH ELISA FOR PITUITARY AND PLASMA SAMPLES 6
PRODUCTION OF RECOMBINANT LEPTIN AND GENERATION OF HORMONE-SPECIFIC ANTIBODIES 9
DISCUSSION 11
REFERENCES 12

Description

The present task aimed at establishing ELISAs for measuring: (i) pituitary gonadotropins, LH and FSH, the central regulators of gonadal development and gamete maturation, and (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 hypothalamic-pituitary-gonadal axis and may also influence directly gonadotropin release and gonadal steroidogenesis. Comparison of the resulting hormone profiles in wild and captive-reared greater amberjack brooders is expected to aid in evaluating potential adverse impacts of captivity on reproductive performance.

Background

As in other vertebrates, reproduction in fish necessitates the full activation of the brain–pituitary–gonadal (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 and Parsons, 1981). While the physiological functions of the gonadotropins in teleosts are not entirely clear, a growing body of evidence suggests 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 (Swanson et al., 2003; Yaron & Sivan, 2006; Rosenfeld et al., 2007).

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 (Zhang et al., 1994; Huising et al., 2006). Early studies suggested that the neurons involved in regulating energy metabolism, mostly neuropeptide Y (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 (Sabatino et al., 1987; Guy et al., 1988). 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 on 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.

Materials and Methods

Sample collection

A total of 33 (14 males and 19 females) wild and 24 (12 males and 12 females) captive-reared greater amberjack breeders were sampled at three different phases of the reproductive cycle that were determined according to the available literature (Mandich et al., 2004; Sley et al., 2014): early gametogenesis (EARLY, late April-early May), advanced gametogenesis (ADVANCED, late May-early June) and spawning (SPAWNING, late June-July) (Deliverables 3.3, 3.5 and 3.6).

Blood and tissue processing

Blood samples were centrifuged (2000 g, 10 min, 4°C), plasma collected and stored at -80°C.

Pituitaries were homogenized individually in 200 µl of cold ultra-pure H_20 and used for RNA extraction (60 µl) and gonadotropin measurements (140 µl). The latter fraction was diluted (v:v) with 140 µl of PBS (X2) aliquoted and stored at -80°C for further analyses.

Liver microsomal fractions were prepared according to Barash et al. (1983) with slight alterations. Briefly, greater amberjack liver samples (0.5 g) were cut into small pieces and homogenized in 6 ml of 25 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.3 M sucrose and 1 mM PMSF. Following a first centrifugation (11,000 g; 30 min) the supernatant was separated and re-centrifuged (100,000 g; 1 h; 4°C). The precipitates were suspended in 65µl of reaction buffer (25 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM PMSF).

Production and purification of recombinant proteins

The recombinant amberjack FSH and leptin (rFSH and rLep) were similarly produced by the *Pichia pastoris* yeast DNA expression system (Invitrogen, Carlsbad, CA). The cDNA encoding for the respective mature peptide, tagged with a chain of 6 histidine residues (6xHis), was introduced into the *P. pastoris* expression vector, pPIC9K (Invitrogen). Following linearization with SalI, each of the constructed plasmids was used to transform the host strain GS115 his4 (auxotropihic for histidine) using the Micro-Pulser-Electroporator (Bio-Rad Laboratories) adjusted to yeast cells (Voltage-2 kV; Time constant- 3.7 ms). Transformant colonies

were selected on histidine-deficient agar. Each selected colony was grown on buffered BMGY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% yeast nitrogen base; 4 X 10^{-5}% biotin; 1% glycerol) for 2 days in a shaking incubator (250 rpm; 28°C). The cells were harvested, re-suspended in buffered BMMY medium (BMGY containing 1% methanol instead of 1% glycerol) to induce the AOX1 promoter, and grown for 3 additional days. The His-tagged proteins were purified by HiTrap chelating HP column (Amersham Biosciences), quantified by the Bradford method, divided into aliquots, and kept frozen at -20°C until used to immunize rabbits and/or standardize the hormone specific ELISA.

Generation of polyclonal antibodies

Polyclonal antibodies, anti-rFSH and anti-rLep were generated in rabbits following immunization with the respective purified recombinant proteins. Two rabbits were immunized at Adar-Biotech (Ness Ziyona, Israel); 4 subsequent immunizations with a total of 2 mg antigen (either rFSH or rLep). The sera were harvested and direct validation of the antibody specificity was carried out by means of Western blotting and/or ELISA.

Protein gel electrophoresis, Western blotting and immunodot blot

Yeast (*P. pastoris*) produced recombinant proteins and/or proteins extracted from greater amberjack liver were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using the miniVE vertical electrophoresis system (Amersham Biosciences, Piscataway, NJ). Denaturated protein samples were separated by fixed (15%) or gradient (10-20%) SDS–PAGE in parallel to commercial size markers (Bio-Rad). Following separation the proteins were either visualized by a Coomassie staining or electro-transferred from the polyacrylamide gel to a nitrocellulose membrane (0.2 µm Protran Nitrocellulose membrane; Thermo Fisher Scientific, Victoria, Australia) using the Semi-Dry electro-blotting system (Thermo Fisher Scientific). After blocking with 5% skim milk blots were incubated with the hormone-specific antibody. A horseradish peroxidase conjugated to goat-anti-rabbit-IgG (Bio-Rad Laboratories) was used as secondary antibody. Signals were visualized by an ECL detection system (Amersham Biosciences). The ECL detected signals were imaged using the G-Box CHEMI HR (SynGene, Frederick, MD).

An immunodot blot assay employing polyclonal anti-rLep and purified recombinant greater amberjack standards was conducted to visualize native leptin in greater amberjack liver samples. Accordingly, 4 dilutions of each sample were spotted in duplicate on a nitrocellulose membrane in comparison to serial dilutions of rLep. Once air-dried the membrane was subjected to Western blotting procedures as specified above. The ECL detected signals were imaged using the G-Box CHEMI HR (SynGene, Frederick, MD).

Mass spectrometry

For recombinant protein identification, the visualized bands on the Coomassie-stained gel were excised and trypsinized in-gel (37°C, overnight) using 30 µl of Trypsin solution (15 lg/ml in 25 mM NH_4CO_3, pH 8.0; Promega). The resulting peptides were extracted, resolved by reversed phase capillary chromatography, and analysed on-line by electrospray tandem mass spectrometry (MS/MS) at the Smoler Proteomics Center (Technion – Israel Institute of Technology, Haifa, Israel). The identities of the peptides were confirmed by searching amino acid sequence databases (SwissProt) with tandem mass spectra using the SEQUEST algorithm (ThermoFinnigan, San Jose, CA) or MS/MS search program from Matrix science (Mascot, http://www.matrixscience.com) using the NCBInr database.

Validation of heterologous ELISA to measure pituitary and plasma LH

In order to expedite the evaluation of the reproductive potential of captive-reared greater amberjack broodstock, the current study examined whether an heterologous assay that was developed to measure

striped bass (*Morone saxatilis*) LH (stbLH; Mañanós et al, 1997) and was modified for tuna species (Rosenfeld et al., 2012, Berkovich et al., 2013) is suitable for monitoring LH profiles in the greater amberjack. For that purpose, pituitary extract derived from a captive amberjack female (specimen C111) was assayed at four serial dilutions (1:50, 1:100, 1:200 and 1:400) and plasma samples of two wild caught fish (specimens W19 and W27) were assayed at three serial dilutions (1:8, 1:16, 1:32).

Ninety-six well polystyrene plates were coated with recombinant LH (rLH; 2.4 ng per well) and incubated overnight at 4°C. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl per well) for 0.5 h at 37°C. The primary antibody (anti-stbLH) was diluted 1:80,000 in PBST containing 2% normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4°C. Then the content in each tube was dispensed into the antigen-coated wells (100 µl per well in duplicate). Following an incubation (overnight at 4°C), AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch laboratories, inc.) in 1% NGS-PBS T was added (100µl per well) for 0.5 h at 37°C. The wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 µl per well). The reaction was stopped after 20 to 40 min at RT by the addition of 100 µl of 1N phosphoric acid and the absorbance was read at 450 nm.

Establishment of homologous ELISA to measure pituitary and plasma FSH

The homologous ELISA for measuring amberjack FSH consists of standard antigen, the recombinant greater amberjack FSH (rFSH), and polyclonal antibodies, anti- rFSH. The standard curve ranged from 100 ng/ml to 0.19 ng/ml. The assay detection limit is 0.78 ng/ml. The intra-assay coefficient of variation (CV) for a standard of 10 ng/ml in the same plate was 3.0% (n = 10). The inter-assay CV for the same plasma sample on different plates was 9.6% (n = 7).

Statistical analysis

Data calculations in the ELISA were performed using sigmoid curves, which were linearized by the logit transformation: $\mathrm{logit}(B/B\mathrm{o})=\log\,[r/(1-r)]$, where $r=B/B\mathrm{o}$, B represents the binding at each point, and Bo the maximum binding.

Results

Validation of the striped bass LH ELISA for pituitary and plasma samples

In order to test the possibility of using the stbLH ELISA for LH measurement in greater amberjack, displacement curves obtained with serial dilutions of pituitary and plasma extracts from wild and captive amberjack were compared with the rLH standard curve (**Fig. 1**). A clear linearity was obtained in the dilution of the pituitary of the captive amberjack female (**Fig. 1A**) and the plasma samples derived from wild fish (**Fig. 1B**). Moreover, the dilution curves exhibited parallelism with the standard rLH enabling the determination of LH in this species. The sensitivity of the assay was 0.65 ng/ml and the respective inter- and intra- assay coefficients of variation were 8% and 15%.

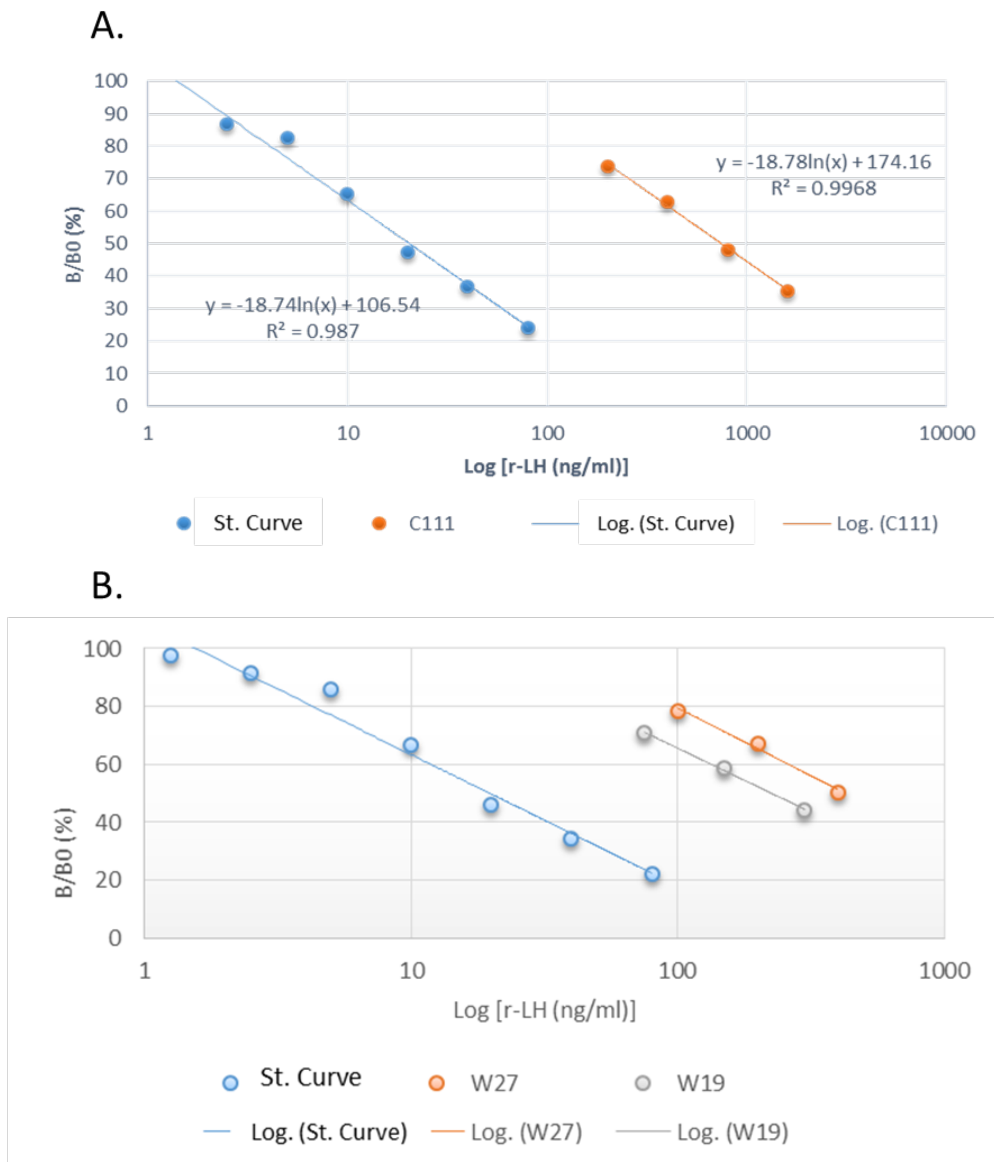

Figure 1. Displacement curves for standard rLH and serial dilutions of pituitary extract (A) and plasma (B) samples from captive (C111) and wild (W19 and W27) greater amberjack. The LOGIT function was utilized to transform standard curve to a linear plot. Each point is the mean of two determinations.

Establishment of homologous FSH ELISA for pituitary and plasma samples

The greater amberjack rFSH was produced using the yeast, *P. pastoris* expression system.

Following SDS–PAGE separation of the purified protein, a Coomassie blue stain (**Fig. 2**) showed a single protein band of the expected size (23 kDa) confirming the purity of the produced recombinant protein and its molecular weight resemblance to those obtained from the Mediterranean yellowtail (García-Hernández et al., 1997) and other teleosts (Suzuki et al., 1988a; Swanson et al., 1991; Van der Kraak et al., 1992; Banerjee et al., 1993; Koide et al., 1993; Okada et al., 1994). The stained band was excised from the gel and analyzed by Mass spectrometry (MS/MS). The MS/MS analysis of the corresponding excised spot identified greater amberjack FSH as the sole protein.

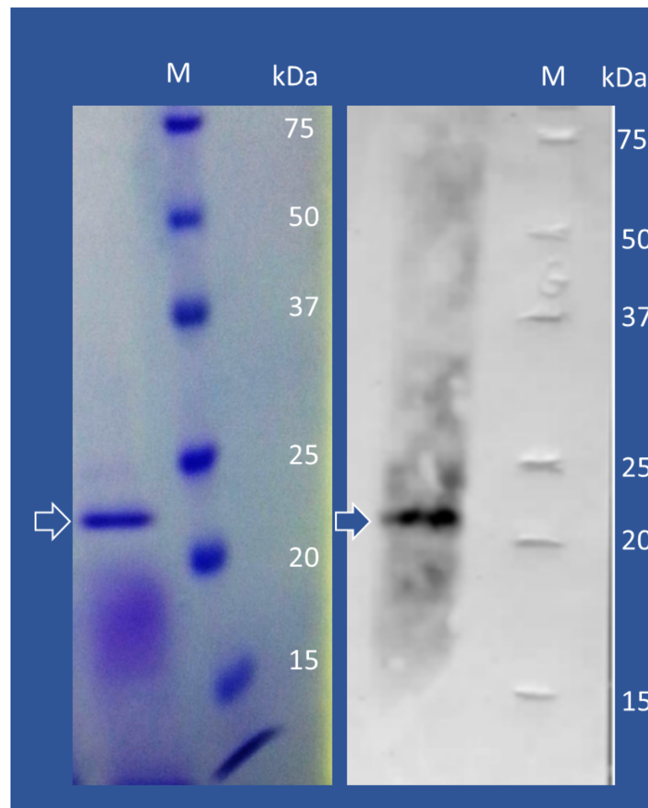

Figure 2. SDS–PAGE (left) and Western blot (right) analyses of purified recombinant greater amberjack FSH. Recombinant greater amberjack FSH eluted with PBS containing 500 mM Imidazole was separated on 12% SDS-PAGE gel in parallel to standard pre-stained molecular weight markers [M]. Western blot analysis was carried out using polyclonal antibodies generated in rabbits using the purified rFSH. An arrow points to the visualized rFSH at an estimated size of 23 kDa.

The purified rFSH was used to generate specific polyclonal antibodies (anti-rFSH) through rabbit immunizations. Western blot analysis confirmed the anti-rFSH specificity (**Fig. 2**).

A homologous competitive ELISA was developed for FSH determination in pituitary and plasma samples, using rFSH for standardization, and the polyclonal anti-rFSH for immunodetection. A series of tests were performed to optimize the ELISA protocol by studying the effects of varying parameters (i.e., antibody's dilution rate, coating concentration and incubation conditions) on the performance of the standard curve. Under optimized conditions, the standard curve ranged from 100 ng/ml to 0.19 ng/ml. The assay detection limit was 0.78 ng/ml. The intra-assay coefficients of variation (CV) for a standard of 10 ng/ml in the same plate were 3.0% (n = 10). The inter-assay CV for the same plasma sample on different plates was 9.6% (n = 7).

Validation of the assay for the determination of greater amberjack FSH was performed by testing the parallelism between the standard curves and displacement curves obtained by serial dilutions of pooled pituitary extracts and plasma samples derived from wild caught greater amberjack (specimens W19 and W24). A clear linearity was obtained in the dilution of the amberjack pituitary (**Fig. 3A**) and plasma samples (**Fig. 3B**). To further rule out cross reactivity with LH, displacement curve obtained by serial dilutions of rLH was compared to the rFSH standard curve (**Fig. 4**). No immunoreaction was detected for the rLH even at relatively high concentrations (500 ng/ml).

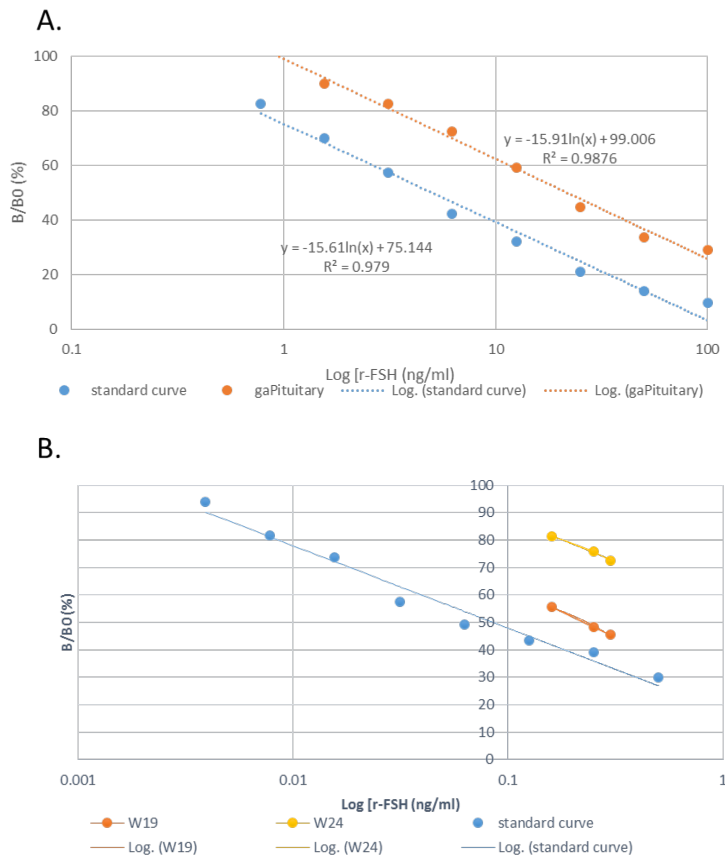

Figure 3. Displacement curves for standard rFSH and serial dilutions of pituitary extract (A) and plasma (B) samples from wild (W19 and W24) greater amberjack. The LOGIT function was utilized to transform standard curve to a linear plot. Each point is the mean of two determinations.

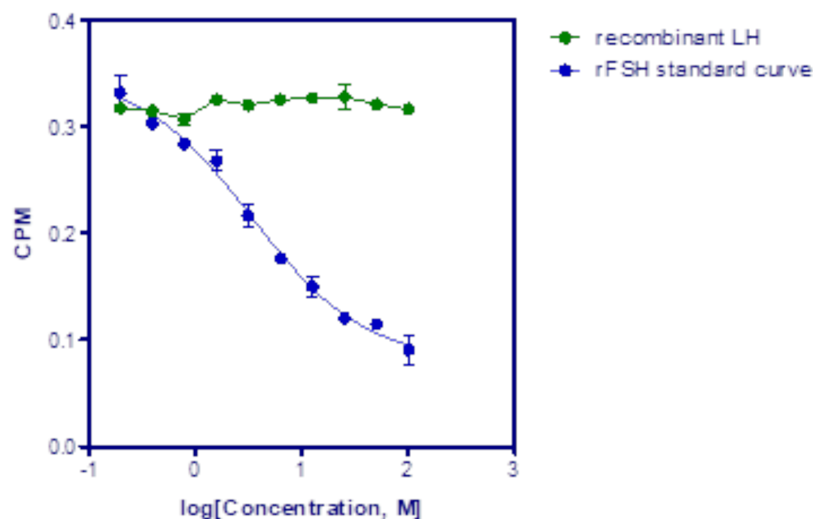

Figure 4. Displacement curves for standard rFSH and a serial dilution of rLH. Each point is the mean of two determinations.

Production of recombinant leptin and generation of hormone-specific antibodies

Similar to the greater amberjack rFSH, recombinant leptin (rLep) was produced using the *P. pastoris* expression system. Following SDS–PAGE separation of the purified protein, a Coomassie blue stain (**Fig. 5**) showed a single protein band of the expected size (17 kDa) supporting the purity of the produced recombinant protein. The visualized band was excised from the gel for further mass spectrometry analysis, which confirmed the identity of the protein as greater amberjack leptin. The purified rLep was used to generate specific polyclonal antibodies (anti-rLep) through rabbit immunizations.

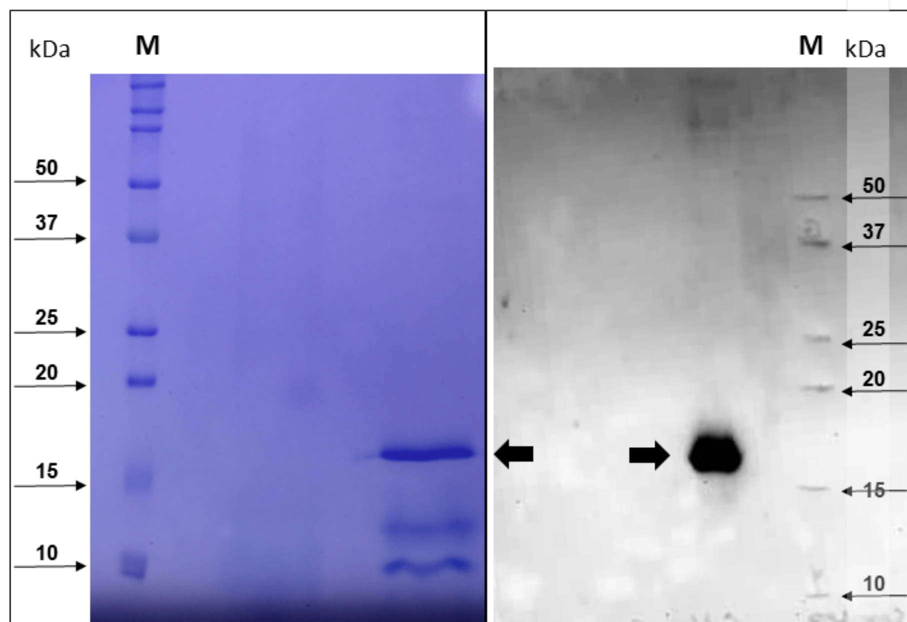

Figure 5. SDS–PAGE (left) and Western blot (right) analyses of purified rLep. Eluted rLep (PBS containing 500 mM Imidazole) was separated on 10-20% SDS-PAGE gradient gel in parallel to standard pre-stained molecular weight markers [M]. Western blot analysis was carried out using the polyclonal anti-rLep. An arrow points to the visualized rLep at approximately 17 kDa.

The anti-rLep specificity was tested and confirmed in a series of Western blot analyses (**Fig. 5**). To further set a positive control other than the rLep, we employed commercially available human leptin and its homologous antibodies (anti-human leptin). A Western blot analysis compared the immune-recognition of the human leptin by the homologous antibodies vs. heterologous anti-rLep. The homologous antibodies recognised a major and a minor band at 14 and 28 kDa, reflecting the monomer and dimer forms of human leptin, respectively (**Fig. 6A**). The immunoreaction with the anti-rLep was less intense, yet, resulted in a single band at 14 kDa, corresponding to the human leptin monomer (**Fig. 6B**). These results points to structural similarities between the human and fish leptins despite the fact that the hormone amino acid sequences exhibit high variation among vertebrate lineages.

Next, we verified immune-recognition of the native amberjack leptin. Since liver is the major site for leptin expression in fish, we screened microsomal fractions (supernatant and pellet) derived from greater amberjack liver samples by Western blotting and immunodot- blot analyses providing denaturing and non-denaturing reaction conditions, respectively (**Figs. 7 and 8**, respectively). In these trials the anti-rLep revealed specific (**Fig. 7B**) and stoichiometric (**Fig. 8**) immuno-detection of the rLep. Nonetheless, no such identification was

obtained with the greater amberjack liver samples, the potential source for native leptin. The lack of immuno-reactive signals was apparent under both denaturing (**Fig. 7**) and non-denaturing (**Fig. 8**) conditions. It is not yet clear what the causes are: a failure of our anti-rLep to detect native form of the hormone and/or scarcity of this hormone in the examined liver samples. These results hindered our ability to keep progressing with establishment and validation of the respective ELISA.

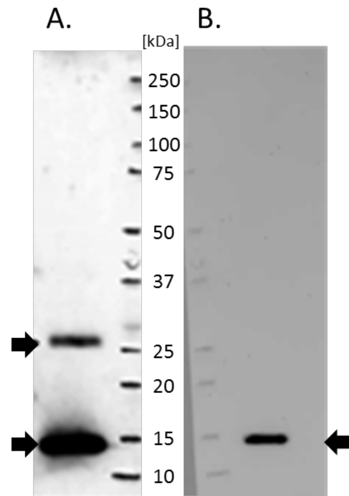

Figure 6. Western blot analysis of commercially available recombinant human leptin. The analysis was carried out with homologous anti-human leptin (A) and heterologous anti-rLep (B). M- Standard pre-stained molecular weight markers. Arrows point to the immuno-detected human leptin monomer (14 kDa) and dimer (28 kDa) forms.

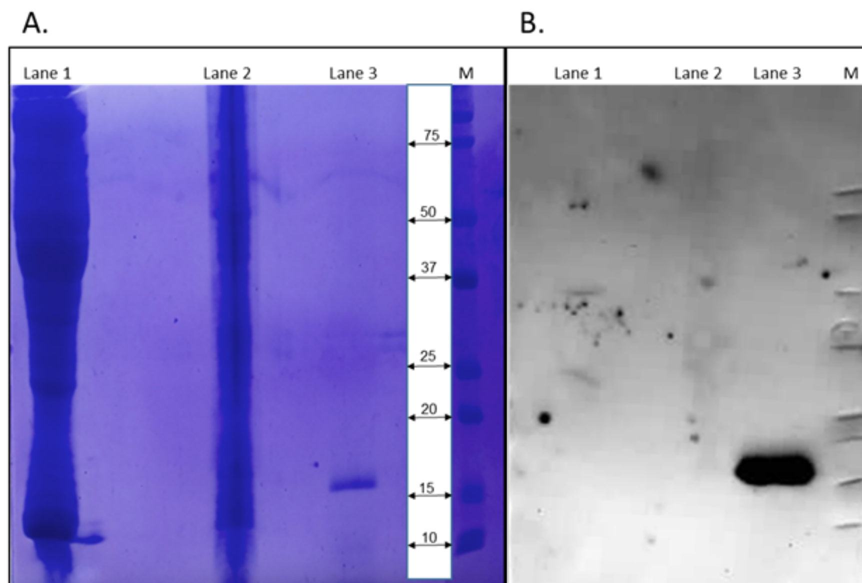

Figure 1. SDS PAGE (A) and Western blot (B) analysis to verify immuno-recognition of native greater amberjack leptin by the homologous antibodies. Greater amberjack liver microsomal fractions (Lane 1- supernatant; Lane 2- pellet) were run in parallel to rLep (Lane 3) and molecular size marker [M]. The analysis was carried out using anti-rLep.

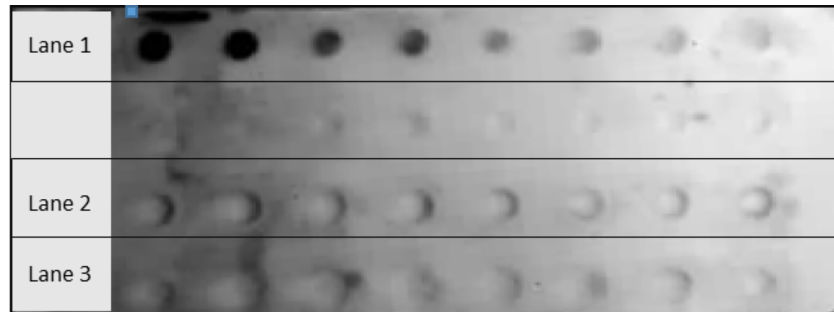

Figure 8. Immunodot blot analysis to verify recognition of native greater amberjack leptin by the homologous antibodies. Lanes represent serial dilutions of rLep (Lane 1) and greater amberjack liver microsomal fractions (Lane 2- supernatant; Lane 3-pellet).

Discussion

According to the original plan we established sensitive and specific ELISAs to measure both gonadotropins, LH and FSH, in greater amberjack. The employed methylotrophic yeast *Pichia pastoris* expression system was found to produce a satisfying yield of recombinant hormones, which in turn were used as antigens to generate specific antibodies and to standardize the immunoassay.

The successful adaptation of the striped bass LH ELISA to monitor LH in the greater amberjack is not surprising inasmuch as this assay was found to be highly generic among perciform species, including the gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), white grouper (*Epinephelus aeneus*) and bluefin tuna (*Thunnus thynnus*). This could be explained by the slow basal rate of evolution of LH β-subunit among all teleost lineages (Rosenfeld et al., 2007), which is altogether different from the episodes of rapid change of LH in mammals, especially in Perissodactyla, Artiodactyla and Primates (Wallis, 2001). In contrast, the low conservation of the FSH β-subunit in the evolution of teleosts (Rosenfeld et al., 2007) hampered the establishment of generic immunoassay to monitor FSH in various fishes, and in most cases necessitates the development of the species-specific ones.

Until recently, most of the immunoassays developed to measure gonadotropins in fish involved the purification of native FSH and LH from vast amount of pituitaries. The availability of sufficient quantity of pituitaries, on one hand, and the complex purification procedure that in most cases resulted in poor quantities and qualities of the native hormones, on the other hand, have limited the establishment of immunoassays to monitor gonadotropins and to a greater extent FSH levels in fish. Therefore, assays to monitor both FSH and LH, were restricted to salmonid species (Govoroun et al., 1998; Suzuki et al., 1988b), while in other fish species, the assay was developed merely for LH. Consequently, for many years, studies aiming to elucidate the relative role of LH and FSH in fish were incomplete being limited to the gene expression level. In that respect, the ability to produce recombinant hormone, which maintains typifying characteristics of the native one, provides a good alternative to gain yields of highly purified protein that have no cross-contamination with other related glycoproteins, thereby expedite the development of hormone-specific ELISAs. Indeed, during the past few years, this approach has led to the development of several ELISAs to measure LH and FSH in several fish species including tilapia (*Oreochromis niloticus*) (Aizen et al., 2007), European sea bass (Molés et al., 2012), killifish (*Fundulus heteroclitus*) (Shimizu et al., 2012), Senegalese sole (*Solea senegalensis*) (Chauvigné et al., 2015), and herein, the greater amberjack. It is important to note that parallel to our success, Nyuji and his colleagues (2016) have similarly established independent ELISAs to measure LH and FSH in this species.

Our amberjack LH- and FSH- ELISAs were found to be highly specific and sensitive. The detection limits of the greater amberjack LH and FSH ELISAs (0.65 and 0.78 ng/ml, respectively) are comparable to those reported for fish gonadotropin immunoassays (Suzuki et al., 1988b; Swanson et al., 1989; Salbert et al., 1990; Tanaka et al., 1993; Prat et al., 1996; Manaños et al., 1997; Govoroun et al., 1998; Santos et al., 2001; Mateos et al., 2006) that range between 0.2 to 2.34 ng /ml, with the exception of the Nile tilapia FSH (0.24 pg/ml; Aizen et al., 2007) and Senegalese sole FSH (10 pg/ml; Chauvigné et al., 2015).

The developed assays enabled us to profile FSH and LH levels, during the reproductive season, in pituitary and plasma samples derived from captive reared greater amberjack broodstock and compare them with similar profiles monitored in wild-caught greater amberjack undergoing natural reproductive development. Our results pointed to impaired accumulation and release of FSH and LH in the captive broodstocks as major dysfunctions preventing these fish from exploiting their maximal reproductive potential (D3.5 and D3.6).

Nonetheless, a similar approach to establish the greater amberjack leptin ELISA was less successful. Recombinant greater amberjack leptin was produced using the yeast *P. pastoris* expression system and homologous polyclonal antibodies were generated. These antibodies detected both greater amberjack and human recombinant leptins with high specificity, further attesting the notion that despite the low amino sequence conservation, leptin preserved throughout evolution the tertiary structure common to all vertebrate class I helical cytokines (Huising et al., 2006). Even so, the anti-rLep failed to detect the native hormone in liver extracts, which adversely affected our ability to further establish and validate the respective ELISA.

Deviations: According to the original plan relatively high quantities of recombinant leptin were produced and purified, which enabled the generation of specific polyclonal antibodies. These antibodies were found to be highly specific to the recombinant antigen, yet, failed to detect native greater amberjack leptin. While it could be interesting to compare circulating leptin profiles during the reproductive cycle in wild vs. captive reared greater amberjack, the impact on the deliverable is negligible, particularly as we were able to follow the expression levels of the leptin gene and highlight a time window during which it appears to play a significant role (D3.5, D3.6; Zupa et al., 2017).

References

Aizen, J., Kasuto, H., and Levavi-Sivan, B. (2007). Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins. Gen. Comp. Endocrinol. *153*, 323–332.

Akhter, N., Crane, C., and Childs, G.V. (2010). Pituitary leptin-A paracrine regulator of gonadotropes: A review. Open Neuroendocr. J. *4*, 22-24.

Banerjee, P.P., Banerjee, S., Shen, S.T., Kao, Y.H., and Yu, J.Y.L. (1993). Studies on isolation and properties of two isoforms of pituitary gonadotropin from black silver carp, *Aristichthys nobilis*. Comp. Biochem. Physiol. *104B*, 241–253.

Barash, I., Madar, Z., and Gertler, A. (1983). Down-regulation of prolactin receptors in the liver, mammary gland and kidney of female virgin rat, infused with ovine prolactin or human growth hormone. Biochem. Biophys. Res. Commun. *116(2)*, 644-650.

Berkovich, N., Corriero, A., Santamaria, N., Mylonas, C.C., Vassallo-Aguis, R., de la Gándara, F., Meiri-Ashkenazi, I., Zlatnikov, V., Gordin, H., Bridges, C.R., and Rosenfeld H. (2013). Intra-pituitary relationship of follicle stimulating hormone and luteinizing hormone during pubertal development in Atlantic bluefin tuna (*Thunnus thynnus*). Gen. Comp. Endocrinol. *194*, 10-23.

Castellano, J., Navarro, V., Fernandez-Fernandez, R., Nogueiras, R., Tovar, S., Roa, J. M., Vazquez, J., Vigo, E., Casanueva, F.F., Aguilar, E., Pinilla, L., Dieguez, C., and Tena-Sempere, M. (2005). Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptinin undernutrition. Endocrinol. 146(9), 3917-3925.

Chauvigné, F., Verdura, S., Mazón, M. J., Boj, M., Zanuy, S., Gómez, A., Cerdà, J. (2015). Development of a flatfish-specific enzyme-linked immunosorbent assay for Fsh using a recombinant chimeric gonadotropin. Gen. Comp. Endocrinol. *221*, 75-85.

García-Hernández, M.P., Koide, Y., Díaz, M.V., and Kawauchi, H. (1997). Isolation and characterization of two distinct gonadotropins from the pituitary gland of Mediterranean yellowtail, *Seriola dumerilii* (Risso, 1810). Gen. Comp. Endocrinol. *106(3)*, 389-399.

Govoroun, M., Chyb, J., and Breton, B. (1998). Immunological cross-reactivity between rainbow trout GTH I and GTH II and their a and b subunits: Application to the development of specific radioimmunoassays. Gen. Comp. Endocrinol. *111*, 28–37.

Guy, J., Li, S., and Pelletier, G. (1988). Studies on the physiological role and mechanism of action of neuropeptide Y in the regulation of luteinizing hormone secretionin the rat. Regul. Pept. *23(2)*, 209-216.

Huising, M.O., Kruiswijk, C.P., and Flik, G. (2006). Phylogeny and evolution of class-I helical cytokines. J. Endocrinol. *189(1)*, 1-25.

Koide, Y., Itoh, H., and Kawauchi, H. (1993). Isolation and characterization of two distinct gonadotropins, GTHI and GTHII, from bonito (*Katsuwonus plelamis*) pituitary glands. Int. J. Pept. Protein Res. *41*, 52–65.

Mañanós, E. L., Swanson, P., Stubblefield, J., and Zohar, Y. (1997). Purification of gonadotropin II from a teleost fish, the hybrid striped bass, and development of a specific enzyme-linked immunosorbent assay. Gen. Comp. Endocrinol. *108*, 209–222.

Mandich, A., Massari, A., Bottero, S., Pizzicori, P., Goos, H., and Marino, G. (2004). Plasma sex steroid and vitellogenin profiles during gonad development in wild Mediterranean amberjack (*Seriola dumerilii*). Mar. Biol. *144*,127-138.

Mateos, J., Mañanós, E., Swanson, P., Carrillo, M., and Zanuy, S. (2006). Purification of luteinizing hormone (LH) in the sea bass (*Dicentrarchus labrax*) and development of a specific immunoassay. Cienc. *32*, 271-283.

Molés, G., Gómez, A., Carrillo, M., and Zanuy, S. (2012). Development of a homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males. Gen. Comp. Endocrinol. *176 (1)*, 70-78.

Nyuji, M., Kazeto, Y., Izumida, D., Tani, K., Suzuki, H., Hamada, K., Mekuchi, M., Gen, K., Soyano, K., and Okuzawa, K. (2016). Greater amberjack Fsh, Lh, and their receptors: Plasma and mRNA profiles during ovarian development. Gen. Comp. Endocrinol. *225*, 224-234

Okada, T., Kawazoe, I., Kimura, S., Sasamoto, Y., Aida, K., and Kawauchi, H. (1994). Purification and characterization of gonadotropin I and II from pituitary glands of tuna (*Thunnus obesus*). Int. J. Pept. Protein Res. *43*, 69–80.

Peyron, P., Zanuy, S., and Carrillo, M. (2001). Action of leptin on *in vitro* luteinizing hormone release in the European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 65(5), 1573-1578.

Pierce, J.G., and Parsons, T.F. (1981). Glycoprotein hormones: structure and function. Annu. Rev. Biochem. *50*, 465–495.

Prat, F., Sumpter, J.P., Tyler, C.R. (1996). Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod. *54*, 1375-1382.

Rosenfeld, H., Meiri, I., and Elizur, A. (2007). Gonadotropic regulation of oocyte development. In: Babin, P.J., Cerdá, J., and Lubzens, E. (Eds.), The Fish Oocyte: from Basic Studies to Biotechnological Applications. Vol. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 175–202.

Rosenfeld, H., Mylonas, C.C., Bridges, C.R., Heinisch, G., Corriero, A., Vassallo-Aguis, R., Medina, A., Belmonte, A., Garcia, A., De la Gándara, F., Fauvel, C., De Metrio, G., Meiri-Ashkenazi, I., Gordin, H., and Zohar, Y. (2012). GnRHa-mediated stimulation of the reproductive endocrine axis in captive Atlantic bluefin tuna, *Thunnus thynnus*. Gen. Comp. Endocrinol. *175*, 55–64

Sabatino, F.D., Murnane, J.M., Hoffman, R.A., and McDonald, J.K. (1987). Distribution of neuropetide Y-like immunoreactivity in the hypothalamus of the adult golden hamster. J. Comp. Neurol. *257(1)*, 93-104.

Salbert, G., Bailhache, T., Zohar, Y., Breton, B., and Jego, P. (1990). A Rapid and Sensitive ELISA for Rainbow-Trout Maturational Gonadotropin (tGtH II): Validation on Biological Samples; in vivo and in vitro Responses to GnRH. Gen. Comp. Endocrinol. *78*, 110-122.

Santos, E.M., Rand-Weaver, M., and Tyler, C.R. (2001). Follicle-stimulating hormone and its alpha and beta subunits in rainbow trout (*Oncorhynchus mykiss*): purification, characterization, development of specific radioimmunoassays, and their seasonal plasma and pituitary concentrations in females. Biol. Reprod. *65*, 288-294.

Shimizu, A., Ohkubo, M., and Hamaguchi, M. (2012). Development of non-competitive enzyme-linked immunosorbent assays for mummichog *Fundulus heteroclitus* gonadotropins – examining seasonal variations in plasma FSH and LH levels in both sexes. Gen. Comp. Endocrinol. *178*, 463-469

Sley, A., Hadj Taeib, A., Jarboui, O., Ghorbel, M., and Bouain, A. (2014). Reproductive biology of greater amberjack *Seriola dumerili* (Risso, 1810) from the Eastern Mediterranean Sea (Tunisia, Gulf of Gabes). Cah. Biol. Mar. *55*, 421-430.

Smith, J. T., Acohido, B. V., Clifton, D. K., and Steiner, R.A. (2006). KiSS-1 neurones are direct targets for leptin in the *ob/ob* mouse. J. Neuroendocrinol. *18(4)*, 298-303.

Suzuki, K., Kanamori, A., Nagahama, Y., and Kawauchi, H. (1988b). Development of salmon GTH I and GTH II radioimmunoassays. Gen. Comp. Endocrinol. *71*, 459–467.

Suzuki, K., Kawauchi, H., and Nagahama, Y. (1988a). Isolation and characterization of subunits from two distinct salmon gonadotropins. Gen. Comp. Endocrinol. *71*, 302–306.

Swanson, P., Bernard, M., Nozaki, M., Suzuki, K., Kawauchi, H., and Dickhoff, W.W. (1989). Gonadotropin-I and Gonadotropin-II in Juvenile Coho Salmon. Fish Physiol. Biochem. *7*, 169-176.

Swanson, P., Dickey, J., and Campbell, B. (2003). Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. *28(1)*, 53-59.

Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W.W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol. Reprod. *44*, 29–38.

Tanaka, H., Kagawa, H., Okuzawa, K., and Hirose, K. (1993). Purification of gonadotropins (Pmgth-I and II) from red seabream (*Pagrus Major*) and development of a homologous radioimmunoassay for Pmgth-II Fish Physiol. Biochem. *10*, 409-418.

Van der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. *85*, 217–229.

Wallis, M. (2001). Episodic evolution of protein hormones in mammals. J. Mol. Evol. *53*, 10-18

Weil, C., Le Bail, P., Sabin, N., and Le Gac, F. (2003). *In vitro* action of leptin on FSH and LH production in rainbow trout (*Onchorynchus mykiss*) at different stages of the sexual cycle. Gen. Comp. Endocrinol. *130(1)*, 2-12.

Yaron, Z., and Levavi-Sivan, B. (2011). Endocrine Regulation of Fish Reproduction. Farrell A.P., (ed.), Encyclopedia of Fish Physiology: From Genome to Environment, 2(San Diego: Academic Press), 1500–1508 .

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature, *372(6505)*, 425-432.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., and Corriero, A. (2017). Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PloS ONE *12*: e0169645.

Co-funded by the Seventh Framework Programme of the European Union