Deliverable Report

Deliverable No:	D7.2	**Delivery Month:**		18
Deliverable Title	Production of recombinant bioactive LH and FSH assay for grey mullet			
WP No:	7	**WP Lead beneficiary:**		P4. IOLR
WP Title:	Reproduction and Genetics – grey mullet			
Task No:	7.1	**Task Lead beneficiary:**		P4. IOLR
Task Title:	Evaluation of the effectiveness of hormone-based treatment on synchronizing gonadal development and improving gamete quality in mature grey mullet			
Other beneficiaries:	P1. HCMR	P3. IRTA	P4. IOLR	P13. UNIBA
P14. IFREMER	P15. ULL	P24. ITTICAL	P25. DOR	
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Objective: The main goal of this deliverable was to produce recombinant fish gonadotropins (r-FSH and r-LH) and assess their bio-potency and ability to enhance spermatogenesis in captive grey mullet male brooders.

Background

The grey mullet (*Mugil cephalus)* does not reproduce spontaneously in captivity. Previous studies suggested that dopaminergic inhibition is a major barrier along the reproductive axis that arrests spontaneous spawning in captivity (Aizen et al., 2005). Consequently, a practical technique using dopamine antagonists (DA) and gonadotroping realeasing hormone agonists (GnRHa) was developed. Nonetheless, the latter hormone-based treatment was found to enhance vitellogenesis in females, but fell short in stimulating spermatogenesis in males. To augment testicular development, grey mullet males were treated early on in the reproductive season with methyl testosterone (MT) administered *via* Ethylene-Vinyl acetate (EVAc) slow-release implants (Aizen et al., 2005). Despite the reported success, in most cases the treated males produced a very small volume of semen, which was highly viscous and failed to fertilize the eggs. Therefore, to further enhance mullet sperm quality, herein we produced and characterized fish recombinant gonadotropins, FSH and LH, the most important pituitary hormones regulating testicular physiology.

In mammals, luteinizing hormone (LH) regulates Leydig cell sex steroid production, while follicle stimulating hormone (FSH) regulates Sertoli cell activities, such as the structural, nutritional, and regulatory (paracrine) support of germ cell development (Huhtaniemi and Themmen, 2005). In fish, the relative roles of FSH and LH are not yet conclusive. In various species, plasma LH levels are very low or undetectable during the onset of testis development, become detectable when germ cells enter meiosis, and peak close to the spawning season. Plasma FSH levels, on the other hand, show a transient increase in association with spermatogonial proliferation, then re-increase with spermiation, and decrease before the spawning season starts. Campbell et al. (2003) reported that elevated circulating levels of androgens and FSH coincided in male chinook salmon (*Oncorhynchus tshawytscha*) with active spermatogonial proliferation and Ohta et al. (2007) demonstrated that suppressing the steroidogenic activity of FSH abolished the stimulatory effect of

FSH on spermatogonial proliferation in Japanese eel (*Anguilla japonica*). Hence, the present knowledge in fish indicates that FSH-mediated steroidogenesis stimulates early stages of spermatogenesis.

Both FSH and LH are glycoproteins composed of two subunits, α and β. Within a given animal species the α-subunit is common to other glycoprotein hormones, including chorionic gonadotropin (CG), and thyroid-stimulating hormone (TSH), whereas the β-subunit is hormone specific. Both subunits exhibit high content of cysteine residues (10 and 12 in the α and β, respectively) forming multiple intramolecular disulfide bonds known to determine the tertiary structure of the molecule. In addition, each subunit contains N- linked glycosylation sequons, which are highly important for structural as well as functional characteristics of the molecules (*i.e.*, folding, subunit assembly, heterodimer secretion, interaction with the specific receptor, and metabolic clearance rate).

Description

Thus far, therapeutic preparations of FSH and LH are available only for the treatment of human infertility. Hence, in the present study, recombinant fish gonadotropins were produced using the *Pichia pastoris* (methylotrophic yeast) expression system, which was proved to be an attractive system for expressing mammalian (Fidler et al., 1998; Gupta and Dighe, 1999, 2000; Richard et al., 1998), as well as teleost (Kamei et al., 2001) recombinant gonadotropins. The *P. pastoris* system is distinguished for its: i) high-level expression of heterologous proteins, which are secreted into a simple, defined medium, ii) capacity to carry out post-translational modifications resembling those of vertebrates cells (i.e. glycosylation), and iii) genetic stability.

Materials and Methods

1. Production, purification and characterization of recombinant proteins

The cDNA encoding for the gonadotropin subunits (LHβ, FSHβ and GPα) were cloned and their sequence will be publicly available at the GenBank (NIH genetic sequence database) concomitant with manuscript publication. Based on the isolated sequences, synthetic fused forms of grey mullet FSHβα or LHβα were synthesized (GenScript, USA) and introduced into the *P. pastoris* expression vector, pPIC9K (Invitrogene), as an EcoRI / NotI insertion, 5` flunked by the sequence coding for the yeast mating factor-α secretion signal (S). Each of the latter chimeras was transcriptionally induced by the 5` promoter of the alcohol oxidase gene (AOX1) in the presence of methanol as a sole carbon source, giving rise to a yoked protein tagged with a 6-histidin tail. The constructed plasmids (5μg) were linearized with SalI and used to transform the host strain GS115 his4 (auxotrophic for histidine) by electroporation. The procedure was carried out by the Gene Pulser II Electroporation System (Bio-Rad) using the pulse parameters of 1.5 kV and 400 ohm, as established by transformation efficiency tests. Following selection on histidine-deficient agar plates, Geneticin hyper-resistance transformants were picked for further expression analysis.

The selected yeast clones were incubated in 10 ml buffered complex glycerol medium (BMGY), at 29°C in a shaking incubator. The following day, the medium was divided into 5 Erlenmeyer flasks, each containing 200 ml BMGY and incubated (29°C, 250 rpm, over night) for generating biomass. The cells were then harvested following centrifugation (2000 g, 8 min, 16°C) and the cell-pellet was re-suspended in an induction medium (Buffered Methanol-complex Medium, BMMY) supplemented with 1% methanol as the sole carbon source. The culture was incubated for 2 days at 29°C in a shaking incubator (250 rpm). Inductions were repeated every 24 h with 100% methanol at a final concentration of 0.5%. At the end of the incubation period the induced culture supernatant was collected following centrifugation (10,000 g, 25 min, 4°C). The 6XHis-Taged r-LH and r-FSH were isolated on HiTrapTM Chelating HP affinity chromatography (Amersham Pharmacia Biotech), according to manufacturer's guidelines. Incubation media (600 ml) containing the related recombinant hormones were loaded onto HisTrap affinity columns (HisTrap FF 5 ml, GE Healthcare), with the presence of 20 mM Imidazole. Elution and recovery of captured His-tagged r-FSH or r-LH from the immobilized nickel affinity column was accomplished with a high concentration of

imidazole (500 mM). The eluted fraction was dialyzed against 1:10 PBS buffer, (pH =7.4) in 4°C for 24h, and then stored in (-20°C) until use. Proteins were lyophilized and rehydrated in water prior injections.

2. ***In vitro*** **assay**

The *in vitro* bioactivity of r-FSH and r-LH was examined by their capacity to stimulate 11 keto testosterone (11-KT) secretion from grey mullet testicular fragments. For this purpose, grey mullet males were anesthetized in 0.07% clove oil and killed by decapitation. Gonads were rapidly removed and placed in a cold incubation medium (75% Leibovitz L-15 medium with L-glutamine, and 0.1 g/ml gentamycine, pH 7.4). Then, uniformly sized pieces (n=16, per treatment) (average of 100 ± 5 mg/piece) were pre-incubated using 24-well culture plate containing 1.5 ml of ice-cold incubation medium. Following three consecutive washes to eliminate endogenous steroids, the testicular fragments were challenged (16 h; 18°C) with fresh ice-cold medium containing graded doses of either r-FSH or r-LH. When the experiment ended, media were collected, steroids were extracted twice with ethyl ether, and the 11-KT levels were measured by the specific enzyme-linked immunosorbent assay (ELISA) as described below.

3. *In vivo* assay

Grey mullet males (BW 0.917 ± 0.03 kg) (n=12 per treatment) were maintained outdoors in three 4-m^3 fiberglass tanks (7.9 kg/m^3, n=25) supplied with seawater at 40-ppt salinity from the Gulf of Eilat, Israel and subjected to natural conditions of light and water temperature. Fish were fed daily at the rate of 1.5% of their body weight using a 35% crude protein and 7.2% lipid feed formulation prepared at the National Center for Mariculture (Eilat, Israel). On the 6th of August, each group was injected intramuscularly with saline (control group), r-FSH (5 µg/kg BW) or r-LH (5 µg/kg BW). Three weeks later all fish were sampled. Body and gonad mass were recorded and the respective gonadosomatic index (GSI: gonad weight/body weight*100) values were calculated. Blood and gonad samples were collected for hormonal and histological analyses, respectively.

Histological sections of gonads- Gonad samples were fixed in natural buffered formalin (NBF), transferred to 70% ethanol after overnight at room temperature, and then dehydrated by increasing ethanol concentrations, clarified in xylene substitute – K-clear (kaltek, Italy) and embedded in paraffin wax. Five µm thick sections were cut and stained by Eosin–Hematoxylin.

Sex steroid measurement - Blood samples of grey mullet were collected using heparinized syringes and transferred into tubes containing 0.1 mM Complete (Roche, Mannheim, Germany) to prevent proteolysis. The blood was centrifuged (1500 g for 30 min at 4°C) and plasma was recovered. Steroids were extracted twice with ethyl ether. The 11-KT levels were determined by specific ELISA, according to Cuisset et al. (1994) and Nash et al. (2000), using acetylcholinesterase as a label. All samples were analyzed in duplicate, and for each ELISA plate, a separate standard curve was run. The lower limits of detection were 3.9 pg/ml for 11-KT. The intra- and inter-assay coefficients of variance were less than 7%.

4. Statistical analysis

The results for each treatment are expressed as means ± SEM. Statistical analyses were performed using Prism 4 (Graph-Pad Software, San Diego, CA). Homogeneity of variance was assessed by Bartlett's test and data were compared by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple-range test or by non-parametric comparison. The data was transformed to fulfill the assumptions of normality. Unless specified, the significance of differences between groups' means was determined by ANOVA followed by Tukey's post-hoc tests. For data calculations in the ELISA, sigmoid curves were linearized using the logit transformation, where logit (B/Bo) = log [r/(1-r)], where r = B/Bo, B represents the binding at each point, and Bo the maximum binding.

Results

1. Recombinant FSH and LH production and purification

The recombinant gonadotropins rLH and rFSH were produced utilizing the yeast, *P. pastoris*, expression system. *Pichia pastoris* transformants each encompasses a translational fusion (=a single chain) of the gonadotropin alpha- and respective hormone specific beta- (*i.e.*, βLH and βFSH) subunits. The employed methylotrophic yeast *P. pastoris* expression system was found to produce up to 4 mg recombinant gonadotropins per liter of culture supernatant. Once produced the purity and integrity of the ~ 37 kDa recombinant proteins were verified by SDS-PAGE and Western blot analyses (**Fig. 1**).

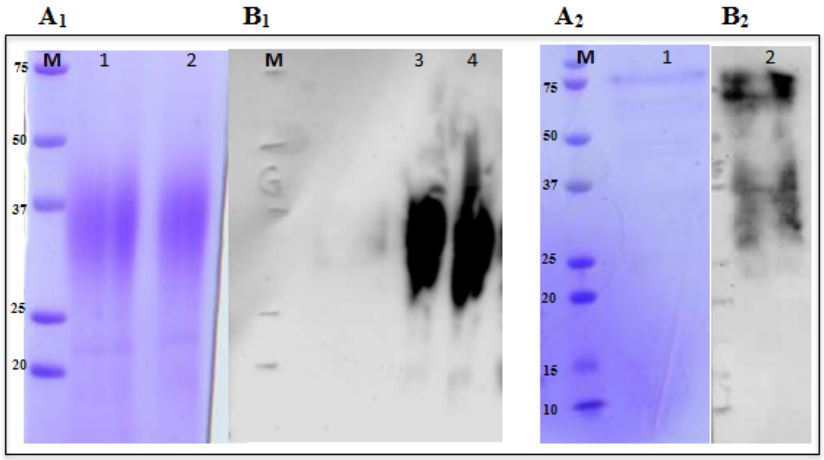

Figure 1. Production of recombinant FSH (A) and LH (B). The integrity and purity of the yeast (*Pichia pastoris*) produced recombinant hormones were verified by SDS PAGE (left panel) and Western blot (right panel) analyses. The immuno-detection was conducted with hormone specific polyclonal anti-FSH and anti-LH. M- Molecular size marker.

The purified gonadotropins were quantified by dot-blot analysis using the specific anti-FSH and anti-LH (**Fig. 2**), as described in Berkovich et al. (2013).

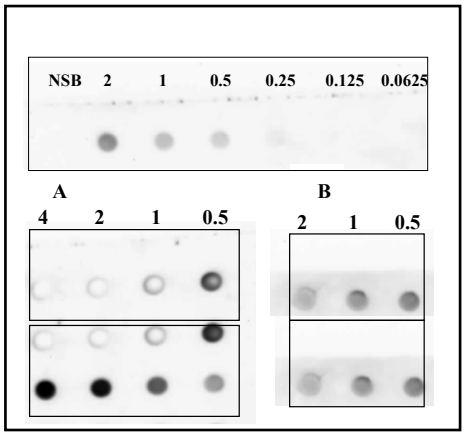

Figure 2. Dot-blot immuno-detection of r-LH (A) and r-FSH (B). The upper panel illustrates a serial dilution of the recombinant hormone that was used for standardization. Rows depict concentration loaded onto wells in duplicates. Different hormone batches are distinguished by separated frames.

2. *In vitro* functional characterization of r-FSH and r-LH

The *in vitro* bioactivity of the produced r-FSH and r-LH, was examined by their capacity to stimulate 11-ketotestosterone (11KT) secretion from grey mullet testicular fragments (Fig. 3). Both r-FSH and r-LH stimulated 11-KT secretion 4-folds higher than the controls. Nevertheless, the r-FSH effective dose (0.5 ng/ml; **Fig. 3A**) was 200-folds lower than that of r-LH (100 ng/ml), indicating higher potency of the former hormone.

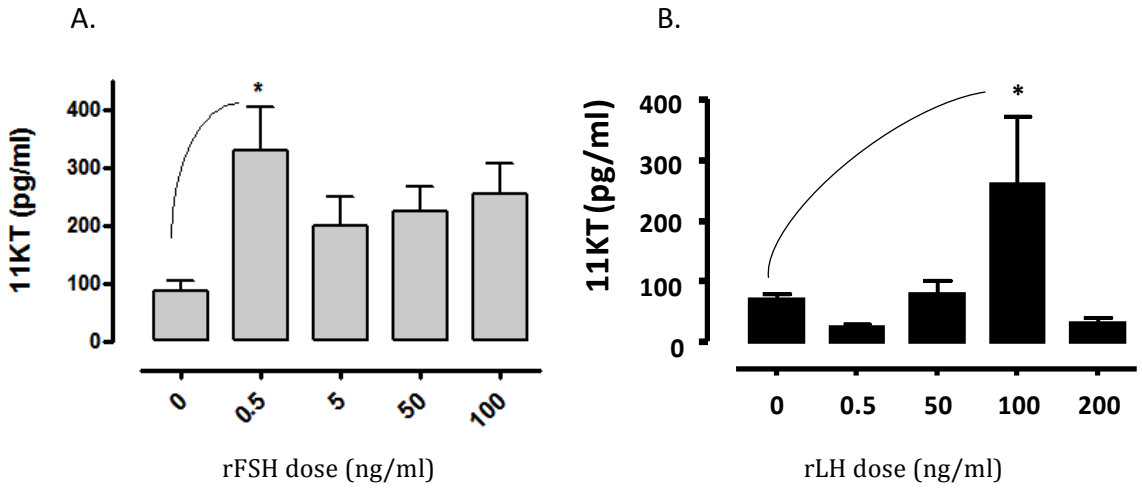

Figure 3. *In vitro* effect of r-FSH (A) and r-LH (B) on 11-ketotestosterone (11-KT) secretion from testicular fragments. Results are shown as means ± SEM (n=16). An asterisk indicates significantly different means ($P < 0.05$, Student's t-test).

3. *In vivo* functional characterization of r-FSH and r-LH

During the onset of the reproductive season (early August, 2014), the grey mullet males received a single injection containing either: r-FSH, r-LH or saline (control). Three weeks later, the treatment effects on BW, GSI, testicular development and circulating levels of 11-KT, were evaluated. Our results indicate no significant difference in BW across all treatment groups (**Table 1**). Nonetheless the r-FSH- treated males exhibited significantly ($P < 0.05$) higher GSI values (**Table 1**) as well as advanced stages of spermatogenic cells (**Fig. 4**) when compared to those treated with saline.

Table 1. Treatment effect on body weight (BW) and Gonadosomatic Index (GSI).

Treatment Group	BW (g)	GSI (%)
C	950 ± 49	0.045 ± 0.010^{a}
r-FSH	863 ± 41	0.088 ± 0.012^{b}
r-LH	890 ± 52	0.072 ± 0.014^{ab}

*Values are expressed as mean ± SEM, (n=12). Different letters indicates significantly different means ($P < 0.05$, Student's t-test).

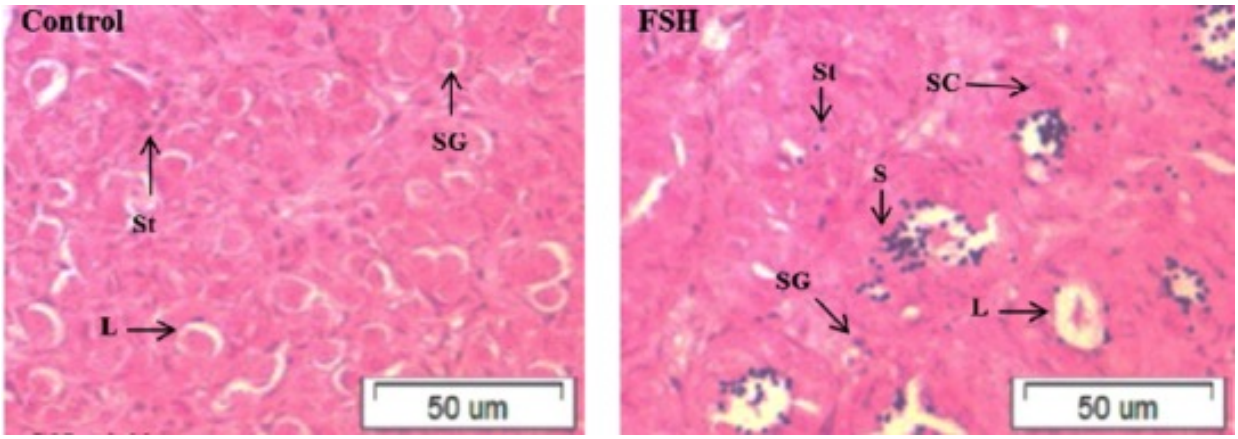

Figure 4. *In vivo* effect of r-FSH on spermatogenic development in captive grey mullet. Coronal section of gonads, H&E staining. Organ is composed of mainly undifferentiated gonocytes. Black triangle indicates early features of male differentiation of the gonad as appearance of a clustered organization (spermatocysts). The holes in the images indicate early lumen (L) formation. St = Spermatids; SG= Spermatogonias; SC= Spermatocytes

The r-FSH treatment also increased plasma 11-KT levels, which were found to be higher compare to the control group fish (90.36±20.21 and 54.24±8.7 pg/ml, respectively; **Fig. 5**). Lowest levels of 11-KT were measured in the r-LH treated males (43.38±5.1 pg/ml).

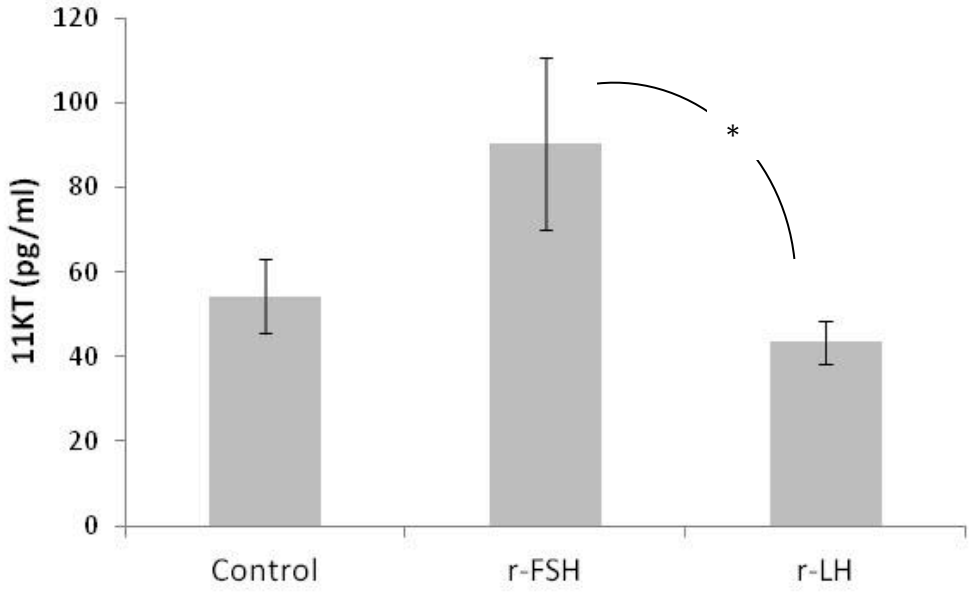

Figure 5. Plasma levels of 11-KT in control, r-FSH or r-LH treated grey mullet males. 11-KT Values are expressed as mean ± SEM (n=11). Statistically significant difference between r-FSH and r-LH treated plasma is marked by asterisk (*P < 0.05).

Discussion and conclusions

In summary, the employed methylotrophic yeast *P. pastoris* expression system was proved to produce a satisfying yield of recombinant gonadotropins. Both r-FSH and r-LH were able to stimulate grey mullet gonadal steroidogenesis *in vitro*, although with different biopotencies. Nonetheless, at the early stages of the reproductive season r-FSH but not r-LH enhanced steroidogenesis, as well as somatic and germ cell proliferation in captive grey mullet males. Indeed, accumulated data on gonadotropins' receptor pharmacology and localization in various fishes suggest that Leydig cell steroidogenesis is directly regulated by LH and by FSH, while Sertoli cell functions are predominantly regulated by FSH, although high LH concentrations, such as during the spawning season, might cross activate the FSH receptor (FSHR) (Schulz et al., 2010). The FSH main activities in mammals include stimulation of Sertoli cell proliferation during the prepubertal period; induction of terminal differentiation of Sertoli cells together with androgens and thyroid hormones during puberty; regulation of growth factor release, which in turn modulates germ cell proliferation/differentiation. However, little information on FSH effects on fish spermatogenesis that is not related to steroidogenesis is available so far. Based on the current results, it seems that elevated FSH plasma levels during the start of spermatogonial proliferation may be functionally related to proliferation of Sertoli cells, inasmuch as the number of these cells increases in association with the mitotic expansion of spermatogonial cysts in tilapia and African catfish (Schulz et al., 2005).

Altogether, r-FSH seems to be a potent initiator of gonadal growth and germ cell proliferation/differentiation, therefore its timing administration may solve the observed reproductive dysfunctions in male grey mullet giving rise to successful captive breeding of this species.

References

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Deviations: There was no deviation from the DOW.

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