Deliverable Report

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OBJECTIVE: The main goal of this deliverable was to develop hormone-based treatments that will help alleviate reproductive dysfunctions in grey mullet males and females, and induce their successful breeding in captivity.

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DELIVERABLE DESCRIPTION

Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet: A protocol will be developed for the induction of spawning of captive grey mullet. The deliverable will present the procedures, dosage and timing for hormone application, which enhance synchronized gonadal development/gamete maturation within and between genders, as well as induce spawning. In addition, the deliverable will include the results from repeated trails including the following data: relative fecundity (number of total eggs obtained per kilo of female), latency period (timing of application of hormones in relation to egg collection), and egg quality parameters.

INTRODUCTION

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 and gonadotroping releasing 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.

In mammals, luteinizing hormone (LH) regulates Leydig cell sex steroid production, while follicle stimulating hormone (FSH) regulates Sertoli cell activities, including structural, nutritional, and regulatory (paracrine) support of germ cell development (Huhtaniemi and Themmen, 2005). In fish, the relative roles of FSH and LH have not been fully described. 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.

Conversely, several lines of evidence indicate that FSH-mediated steroidogenesis stimulates early stages of spermatogenesis. Plasma FSH levels, 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*).

In view of the above, to further improve gamete quality in general and sperm quality in particular, we employed yeast produced recombinant gonadotropins (r-FSH and r-LH; for additional information see D7.2) as therapeutic agents. Over two consecutive natural spawning seasons (2014 and 2015) we evaluated the effectiveness of several hormonal treatments on gonadal development and gamete maturation in captive grey mullet broodstock. In males, we studied the effects on spermiation of (i) r-FSH vs. r-LH, (ii) single vs. double r-FSH injections, and (iii) r-FSH vs. r-FSH in combination with Metoclopramide (Metoc; dopamine antagonist) and/ or MT-EVAc implants. In females, that appeared to be more susceptible to dopaminergic inhibition, we studied the effects on ovarian development and oocyte maturation of Metoclopramide (Metoc; dopamine D2 receptor antagonist) vs. Metoclopramide in combination with r-FSH or GnRHa.

MATERIALS AND METHODS

1. Broodstock source and conditioning

Grey mullet breeders, consisted of P4. IOLR hatchery-produced (G1) fish that were individually tagged and maintained in 4-m^3 or 5-m^3 tanks supplied with ambient seawater at 40-ppt salinity (Gulf of Eilat, Red Sea) and subjected to natural fluctuations of light and temperature conditions (elevation to 25°C in June, 28°C in August). Fish were fed daily at the rate of 1-1.5% of their body weight using a 30% crude protein and 4% lipid commercial feed (Raanan, Israel). Sex was predicted according to vitellogenin dotblot immunoassay as described in Aizen et al. (2005), and then validated during gametogenesis when gonadal biopsies were performed.

2. Hormonal acceleration of gonadal development

Experiment 1 (2014)- evaluated the short-term (3-weeks post treatment) effects of recombinant LH and FSH (r-LH and r-FSH, respectively) on testicular development. During the onset of the natural reproductive season (6th of August), grey mullet males (n=12 per treatment) were injected intramuscularly with either: (i) saline (control group), (ii) r-FSH (5 µg/kg BW), or (iii) 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 tissues (pituitary and gonads) were collected for further analyses, including: gonadal histology, 11- ketotestosterone (11KT) measurements, and quantification of pituitary LHβ and FSHβ mRNA levels.

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%.

LHβ and FSHβ mRNA quantification – Pituitary total RNA was extracted using Bio-Tri RNA reagent (Bio Lab Ltd., Jerusalem, Israel). The RNA pellet was re-suspended in 20 µl of ultrapure water, and kept at -80°C for further analyses. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (Synergy HT, BioTek). The purity of each sample was assessed for proteins by the 260 vs. 280 nm ratio. Total RNA samples (up to 2 µg) were treated with 2 units of RNAse-free DNase (Promega, Madison, WI, USA). Following incubation (30 min, 37°C) the DNAse was inactivated (10 min, 65°C) the reaction was stopped with 1 µl stop solution followed by cDNA synthesis using the High capacity cDNA reverse transcription kit (Applied biosystems). Real time PCR was performed using PerfeCTa® SYBR® Green FastMix®, Low ROX™ (Quanta Biosciences, Inc. MD, USA). Gene specific primers used herein (Table 1) were designed using the Primer Express 3.0 software (Applied Biosystems) and synthesized by Hy-Laboratories Ltd (Rehovot, Israel). Quantitative real-time polymerasechain reaction (qPCR) was performed (in triplicates) in a total reaction volume of 10 µl, consisting of the respective primer set (5 µM), cDNA template and PerfeCTa® SYBR® Green FastMix®, Low ROX™ (QuantaBioSciences, Inc. MD). To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR

experiments included minus-reverse transcriptase controls (i.e., PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = 2-ΔΔCt, Ct – threshold cycle (Livak and Schmittgen, 2001).

Table 1. Gene specific primer sets used for quantitative Real time PCR

Target gene	Forward sequence (5`→3`)	Reverse sequence (3`→5`)
FSHβ	5` ACCACCGCATGTGAAGGAAA 3`	5` CCAGTCCCCATTGCAGACTT 3`
LHβ	5` GGAGGGCTGTCCCAAGTGT 3`	5` CCGGTATGTGCAAACATGCT 3`
18S rRNA	5' AGTTGGTGGAGCGATTTGTCT 3'	5' ACGCCACTTGTCCCTCTAAGAAG 3'

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.

Experiment 2 (2014)- In a second series of trials, mullet females and males (n= 186; age: 5-year old) were treated during mid-July (2014) with r-FSH (5 µg per kg BW) combined with Metoclopramide (Metoc; dopamine antagonist) dissolved in ddH2O (15 mg/KgBW). The control fish were injected intramuscularly with saline only. The experiment was conducted in triplicates. Two weeks following the first injection (early August 2014), males received 17alpha-methyltestosterone (MT) loaded on EVAc slow-release implants, at 5 mg/kg BW produced by P1. HCMR. Upon need, additional (1 or 2) MT injections were given to enhance spermiation. The state of ovarian development, as determined by oocyte diameter, was examined, during late September and through October (natural spawning season) by obtaining ovarian biopsies using a polyethylene canula. Females were considered post-vitellogenic when mean oocyte diameter was greater than 550 µm (De Monbrison et al., 1997) and more than 50% of sampled oocytes exhibited germinal vesicle migration. In parallel, males were checked for the presence of milt by applying gentle abdominal pressure.

Experiment 3 (2015)- In a third series of trials (4 groups, each consisting of 25 specimens; age: 5-year old), mullet females were injected on July 29th (2015) with either Metoc (15mg/KgBW) alone or its combination with GnRHa- EVAc (36 µg per fish) produced by P1. HCMR. Males in both treatment groups were injected with r-FSH (5 µg per kg BW). One month later half of the males received MT-EVAc implant (5 mg/kgBW). Two additional control groups were injected intramuscularly with saline only. Gonadal biopsies were carried out at two consecutive months: September 2nd, and October 7th. The relative abundance of fully mature females, and spermiating males were recorded. Sperm quality was classified into one of four categories based on its quantity, fluidity and ability to spread in the water. Additionally, treatment effects on sperm characteristics were evaluated using CASA adapted to mullet (see deliverable D7.1).

Experiment 4 (2015)- The forth experiment tested the effect of a single r-FSH injection (5 µg/kg BW) vs. double r-FSH (2.5 µg/kg BW) injections given two weeks apart; August 5th and 19th) on sperm production over time. Eeach group consisted of 25 fish. Four weeks following the first rFSH injection, the non-spermiating specimens in each treatment group received MT-EVAc implant. The females in both treatment groups received Metoc (15 mg/KgBW) injection on August 5th. The relative abundance of fully mature females, and spermiating males were recorded, and sperm quality was evaluated as above.

3. Spawning induction trials

Spawning induction trial were carried out during 2014 and 2015. Once identified, a reproductively mature female was stocked with either two or three spermiating males (unless specified otherwise) in a 1-m^3 tank supplied with seawater at 24-27°C. The selected fish were treated with GnRHa combined with Metoc. Each treatment consisted of priming (GnRHa 10 µg/kg; Metoc 15mg/kg) and resolving injections (GnRHa 20 µg/kg; Metoc 15 mg/kg) given 22.5 h apart.

RESULTS

Effects of recombinant LH and FSH on testicular development (2014)

During the onset of the reproductive season (early August), 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, 11-KT circulating levels, and pituitary expression of the LH and FSH β-subunit genes were evaluated. Our results indicate no significant difference in BW across all treatment groups (**Table 2**). Nonetheless, the r-FSH- treated males exhibited significantly ($P < 0.05$) higher GSI values (**Table 2**) as well as advanced stages of spermatogenic cells (**Fig. 1**) when compared to those treated with saline only.

Table 2. 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).

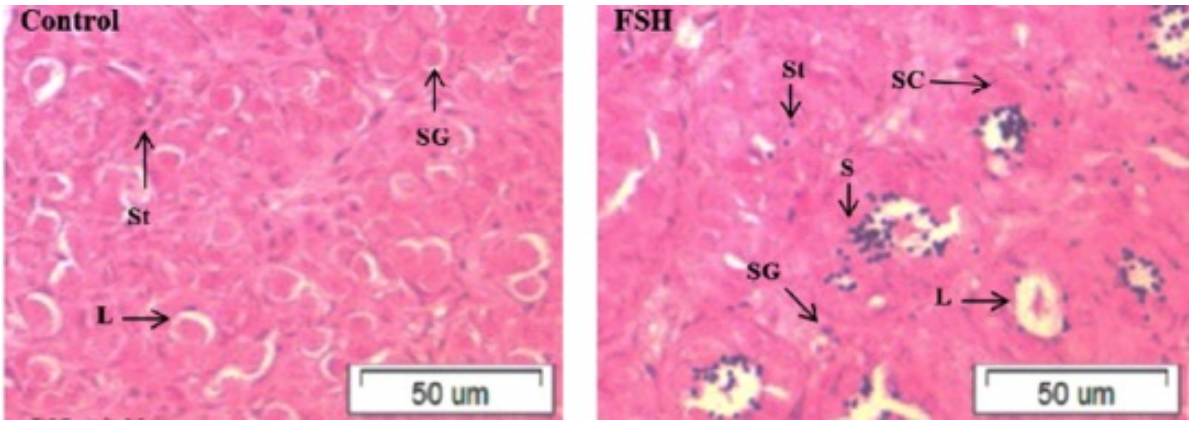

Figure 1. ***In vivo effect of rFSH on spermatogenic development in captive grey mullet***. Coronal section of gonads, H&E staining. Organ is composed of mainly undifferentiated gonocytes. Black triangle indicates early features of male differentiation of the gonad as appearance of a clustered organization (spermatocysts). The holes in the images indicate early lumen (L) formation. St = Spermatids; SG= Spermatogonias; SC= Spermatocytes

The plasma 11-KT levels were higher in the r-FSH treated-group than in the control (90.36 ± 20.21 and 54.24 ± 8.7 pg/ml, respectively; **Fig. 2**). Lowest levels of 11-KT were measured in the r-LH treated males (43.38 ± 5.1 pg/ml).

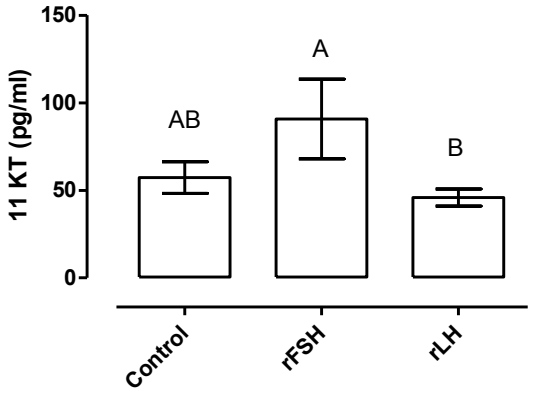

Figure 2. ***Plasma levels of 11-KT in control, rFSH or rLH treated grey mullet males.*** 11-KT Values are expressed as mean ± SEM (*n*=11). Different letters indicates significantly different means (P < 0.05).

Pituitary FSHβ and LHβ transcript levels in control groups sampled at the beginning of the experiment (C0; early-August, 2014) and 3-weeks later (C1; mid-September, 2014) indicate 3-fold and 5-fold increased expression over time for the FSHβ and LHβ genes, respectively (**Fig. 3**). Nonetheless, the initial levels of LHβ were approximately 3-fold lower as compared to the FSHβ ones.

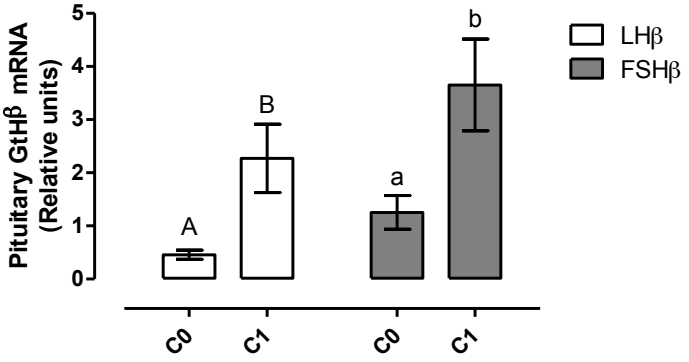

Figure 3. ***Pituitary expression levels of FSHβ and LHβ genes in grey mullet males at early stages of the reproductive season.*** The pituitary mRNA levels of *FSHβ* and *LHβ* were measured using relative Real time PCR method (RQ) at the onset of the reproductive season (C0; early August 2014) and 3-weeks later (C1; mid-September). Levels (Mean ± SEM) are expressed as relative units, normalized to the amount of 18S rRNA. Means with different letters were significantly different (P < 0.05).

Treatment effect on endogenous LHβ and FSHβ transcript levels was observed only in the r-FSH-treated fish (**Fig. 4**). These fish expressed significantly (P < 0.05) higher LHβ mRNA levels as compared to the r-LH and saline treated groups, which did not significantly (P > 0.05) differ from one another.

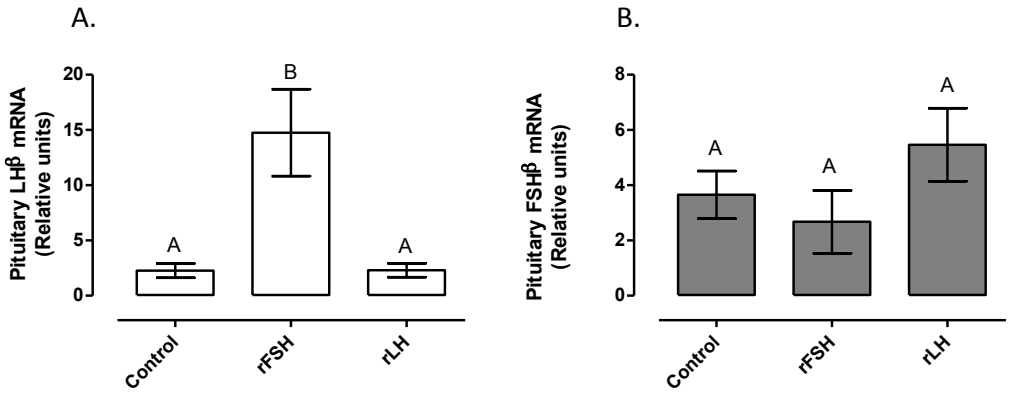

Figure 4. ***Effect of recombinant FSH and LH (r-FSH and r-LH, respectively) treatments on endogenous pituitary FSHβ and LHβ mRNA levels in grey mullet males.*** The pituitary mRNA levels of *FSHβ* and *LHβ* were measured using relative Real time PCR method (RQ). Levels (Mean ± SEM) are expressed as relative units, normalized to the amount of 18S rRNA. Means with different letters differ significantly ($P < 0.05$) from one another.

Effects of recombinant FSH and dopamine antagonist on gonadal development in grey mullet females and males (2014).

The relative abundance of fully mature grey mullet females and spermiating males in hormonally treated and control groups, at early- and mid-spawning season (mid-September and mid-October, respectively) are summarized in **Table 3**. The hormonal treatment consisting of r-FSH and Metoc, appears to synchronize gonadal maturation in both females and males, giving rise to consistently higher percentages of fully mature specimens as compared to control groups. The treatment effect was more pronounced in females (up to 4-fold higher frequencies of fully developed specimens compared to controls) than in males (1.2-1.3 fold higher frequencies compared to controls).

Table 3. Relative abundance of post-vitellogenic grey mullet females and spermiating males at early- and mid-spawning season (mid-September and mid-October, 2014) in control and hormonally treated fish.

	Control		Treatment	
	Mid September	Mid October	Mid September	Mid October
Fully mature females (%)	29	20	91	75
Spermiating males (%)	70	50	86	67

Effects of dopamine antagonist and GnRH agonist on ovarian development (2015)

The added potential of GnRHa-EVAc was tested to complement the Metoc-stimulatory effect on captive grey mullet ovarian development. During the early phase of the spawning period (September 2015) higher frequencies of females exhibiting advanced stages of vitellogenesis (i.e., oocyte diameter greater than 300 µm) were found in both treatment groups compared to controls (**Fig. 5A**). Nonetheless, the combined treatment, consisting of Metoc and GnRH-EVAc, appeared to be more effective than the Metoc only. The

Metoc and GnRH-EVAc combined treatment synchronized and accelerated oocyte development, giving rise to over 70% vitellogenic females during September (Fig. 5A) and 50% post- vitellogenic females one month later, as the spawning season progressed (Fig. 5B). Interestingly, relatively high frequencies of vitellogenic females were observed in the control groups (up to 50%) during September (Fig. 5A), however, only a minority (less than 18%) reached the post-vitelogenic stage (i.e., oocyte diameter greater than 550 µm) during October (**Fig. 5B**).

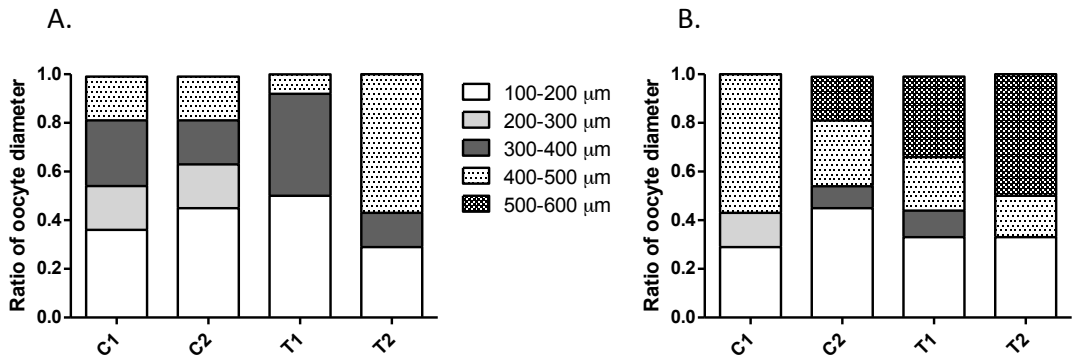

Figure 5. ***Effects of Metoc and GnRH-EVAct on ovarian development in captive grey mullet females (July–October 2015).*** Oocyte diameter profiles in control (C1, C2), Metoc (T1) and Metoc+GnRH-EVAc (T2) treatment groups (n=25), during September (A) and October (B).

Effects of r-FSH and MT-EVAc implants on sperm production (2015)

In parallel, experimentation with the grey mullet males evaluated the effect of r-FSH as a sole therapeutic agent vs. r-FSH use to prime the fish prior to the administration of MT-EVAc implants. The r-FSH treatment showed no stimulatory effect on sperm production compared to controls neither during September (**Fig. 6A**) nor during October (**Fig. 6B**). However, all males that were primed with r-FSH and then subjected to MT-EVAc implantation produced sperm (Fig. 6B). Moreover, the latter group exhibited relatively higher percentages (66%) of fully spermiating males compared to rFSH-treated (33%) and control groups (24%).

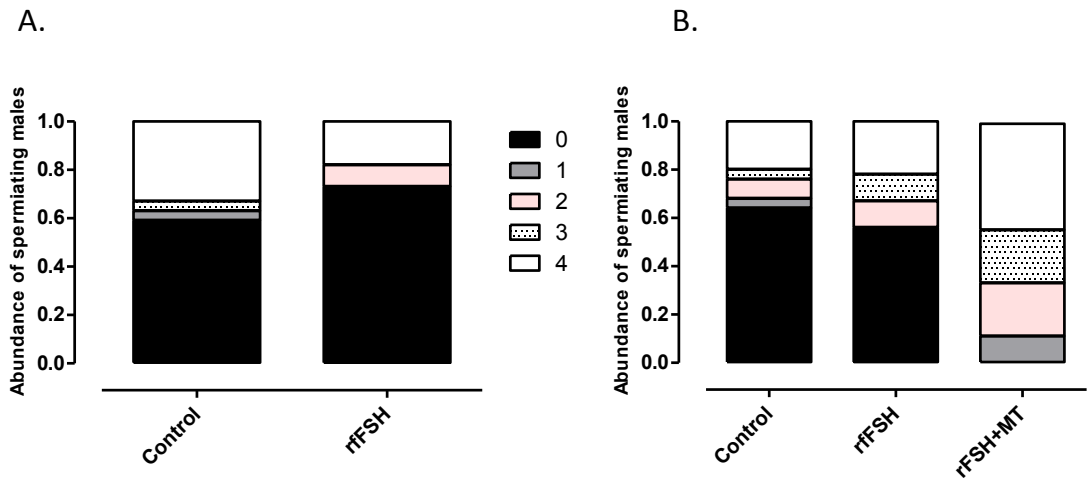

Figure 6. ***Effects of rFSH and MT-EVAc on sperm production in captive grey mullet males (July–October 2015).*** (A) Abundance of spermiating males in r-FSH treated and control groups (n=25) 4 weeks post treatment (September 2015). (B) Abundance of spermiating males in control, r-FSH, and r-FSH+MT treated groups (n=25) 4-weeks after the MT-EVAc implantation (October 2015). 0- no milt, 1- traces of viscous milt, 2-relatively small amounts of white milt, 3- fluid milt 4- flowing fluid milt, easily spread in the water.

CASA analysis revealed no significant ($P > 0.05$) treatment-effect on spermatozoa concentrations. Regardless of the treatment group, all counts ranged between 50 to70 million cells per ml (**Fig. 7**). However, males in the rFSH-treated group exhibited sperm with relatively prolonged motility compared to those in the rFSH+MT and control groups (**Fig. 8**).

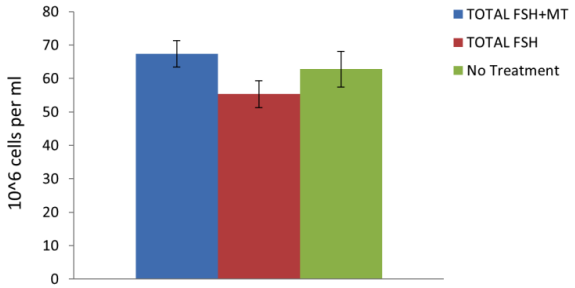

Figure 7. ***Effects of hormonal treatment (rFSH and rFSH+MT) on spermatozoa concentrations (n=27).***

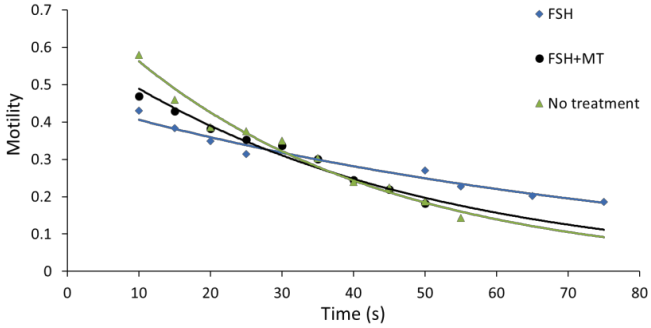

Figure 8. Effects of hormonal treatment (rFSH and rFSH+MT) on **spermatozoa motility (n=27).**

In addition, this study evaluated the effectiveness of a fixed r-FSH dose (5 µg/kg BW) administrated as a single injection or divided into two injections given 2-weeks apart. Although of a preliminary nature, it appears that treatment based on two- r-FSH injections is more effective giving rise to prolonged spermiation over time compared with a single injection (**Fig. 9**).

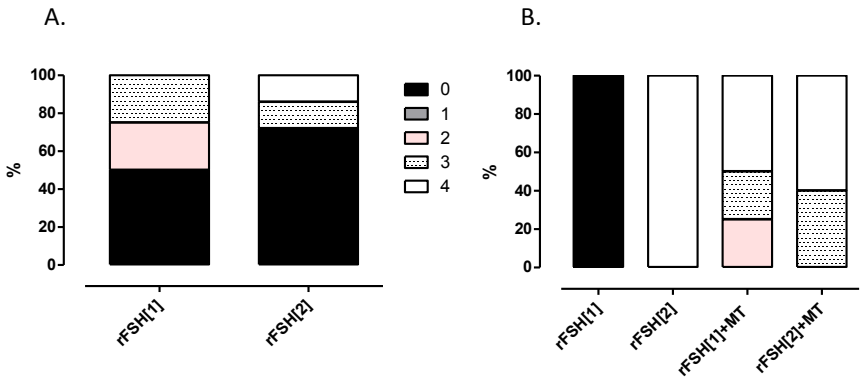

Figure 9. ***Effects of rFSH and MT-EVAc on sperm production in captive grey mullet males (July–October 2015).*** (A) Abundance of spermiating males in groups (n=13 per group) treated with a fixed rFSH dose given either via a single or double injections (r-FSH[1] and r-FSH[2], respectively) 4 weeks after first injection (September 2015). (B) Abundance of spermiating males in r-FSH, and r-FSH+MT treated groups (n= 6 per group) 4-weeks after the MT-EVAc implantation (October 2015). 0- no milt, 1- traces of viscous milt, 2-relatively small amounts of white milt, 3- fluid milt 4- flowing fluid milt, easily spread in the water.

Spawning induction trials

Spawning trials were carried out from Mid-September throughout November during 2014 and 2015. The spawning data obtained from hormonally induced grey mullet brooders during 2014 is summarized in **Table 4**. Both hormonally primed (Metoc + FSH) and control (saline injected) groups gave rise to candidates (14 and 17, respectively; **Table 4**) fulfilling prerequisite criteria for the spawning induction trials. However, spawning successes was improved in the pre-treated groups compared to controls (42.9% and 29.4%, respectively). The overall successful spawns produced 42 million eggs in total. Based on numbers of floating eggs a total of 75% of the spawned eggs were considered viable.

Nonetheless, despite of the successful spawning season, two major problems were highlighted: (i) female's failure to ovulate (70% and 57% in control and hormonally-treated groups, respectively) and (ii) varible fertilization rate ranging between 0 to 100%.

Table 4. Summary of the spawning data obtained from hormonally induced grey mullet females during September-November 2014. Relative fecundity is expressed as means ± SEM. Spawning ratio signifies the number of females that ovulated after injection, divided by the total number of injected females. Relative fecundity stands for total number of eggs per kg body weight of treated females.

Date	Control				Treatment			
	No. of induction trials	Spawning success (%)	Fecundity (million eggs /KgBW)	Fertilization rate (%)	No. of induction trials	Spawning success (%)	Fecundity (million eggs /KgBW)	Fertilization rate (%)
10.9.14	2	0	0	0	2	0	0	0
29.9.14	1	0	0	0	3	100	2.6 ± 0.55	0-98
6.10.14	0	0	0	0	2	0	0	0
22.10.14					3	66	2.1±0.39	0-80
25.10.14					1	100	0.6	30
30-31.10.1	6	50	1.99±0.9	50-100				
5.11.14	5	0	0	0	2	0	0	0
14.11.14					1	0	0	0
21.11.14	1	100	1.64	0				
22.11.14	2	50	2.75	90				
	17	29.4			14	42.9		

The spawning data obtained from hormonally induced grey mullet brooders during 2015 is summarized in Table 5. Twenty three females were induced to spawn resulting with the production of about 35 million eggs. However, the vast majority of the eggs were non fertile. It seems that the conserved spawning induction treatments, consisting of GnRH and Metoc were most effective in inducing final oocyte maturation and ovulation. Following the priming and to a greater extent the resolving injection, the ovulating females can be easily recognized by their swollen belly (**Fig. 10A**). However, many of the females with swollen bellies (over 40% during the 2015 spawning induction trials) did not advance to spontaneously release the eggs, and frequently (30%) died (**Fig. 10B**). Altogether, the female´s failured to spawn the eggs and the low fertilization rate suggest that there were male to female communication problems during the spawning event. Indeed, during 2015 preliminary trials have been carried out to study potential effects of group structure on incidence of successful spawning. So far, a spawning unit consisting of 1 female and 3 males appears to imporve spawning success, however, further experimentation is still needed to understand the grey mullet spawning behavior.

However, it should be noted that regardless of the treatment effects, the 2015 spawning season have faced some abnormal climate conditions including massive floods, which adversely affected the water quality in

the rearing tanks. Relatively high mortality rate was recorded among grey mullet brooders most likely due to stress and opportunistic pathogens.

Table 5. Summary of the spawning data obtained from hormonally induced grey mullet females during September-November 2015. Priming treatment refers to hormonal treatments administered prior to or during gametogenesis to accelerate gonadal development in captive grey mullet females and males. Group structure indicates the number of female and males in each spawning unit.

Date	Priming treatment		Group structure		Remarks	Total volume of eggs (ml)	Fertilization rate	Hatching rate
	Females	Males	#Females	#Males				
9.9.2015	Metoc+GnRH	FSH + MT	1	2		640	no	
9.9.2015	Metoc+GnRH	FSH + MT	1	2	The female has died	0		
10.10.2015	Metoc	FSH + MT	1	3		930		
10.10.2015	Control	Control	1	3		650	no	
10.10.2015	Control	Control	1	3	The female was stripped	350	10%	
14.10.2015	Metoc	FSH + MT	1	4	The female has died	0		
14.10.2015	Metoc	FSH + MT	1	3		370		
14.10.2015	Metoc	FSH + MT	1	3		650	20%	11.60%
14.10.2015	Metoc	FSH + MT	4	5	2 out of 4 females spawned	520	20%	
14.10.2015	Metoc	FSH + MT	1	3		0		
21.10.2015	Metoc	FSH + MT	1	3		1930	5%	0.20%
31.10.2015	Metoc	FSH + MT	2	3		1105		
31.10.2015	Metoc	FSH + MT	3	4	1 female died and 1 spawned	1900	15%	
31.10.2015	Metoc	FSH + MT	1	3		0		
31.10.2015	Metoc	FSH + MT	2	3		710		
11.11.2015	Metoc	FSH + MT	1	1				
		Total number of induction trials	23		Total volume/ number of spawned eggs	9755 ml = 35.12 million eggs		

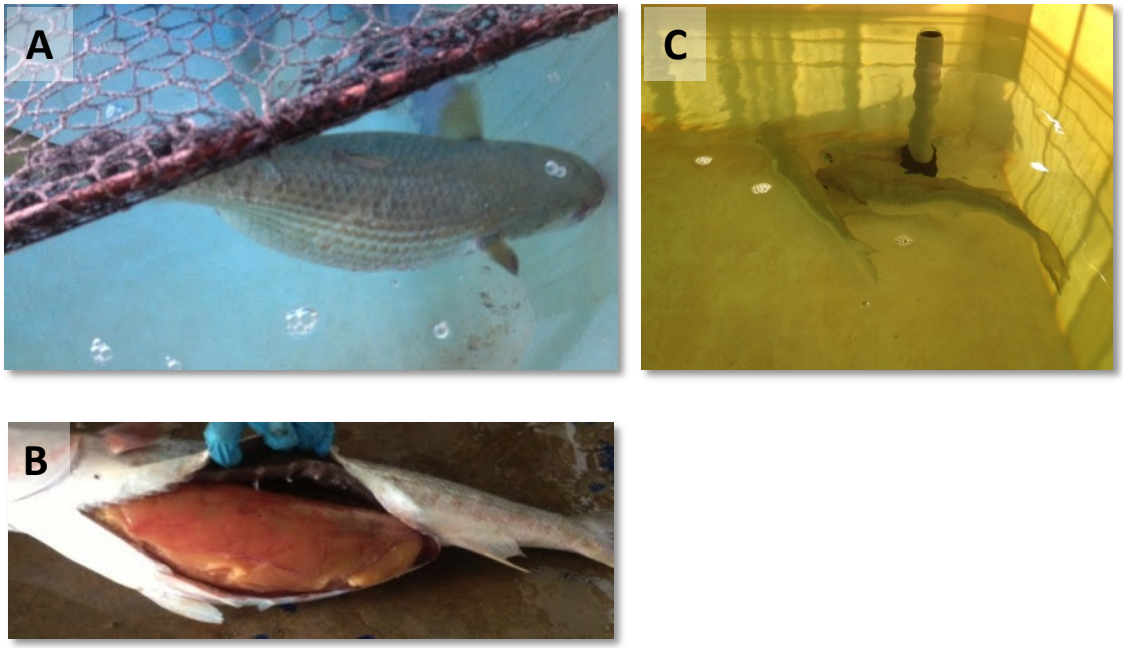

Figure 10. Spawning of captive grey mullet. (A) Hormonally- stimulated grey mullet female exhibiting swollen belly prior to egg release. (B) Hormonally-stimulated female that failed to release the eggs and died. (C) Grey mullet female and male (big and small specimens, respectively) courtship prior to spawning.

DISCUSSION AND CONCLUSIONS

During two consecutive spawning seasons vast progress was made in optimizing hormonal treatments for alleviating reproductive dysfunctions among captive grey mullet broodstocks.

In the grey mullet males, we employed yeast produced recombinant gonadotropins (r-FSH and r-LH) as therapeutic agents to improve sperm production and quality. The r-FSH treatment was a potent steroidogenic hormone during the early stages of spermatogenesis. The r-FSH treatment stimulated both gonadal growth and steroidogenic activity, while the comparable r-LH-treatment had no significant effects. The recognized differences between the two treatments seem to be attributed to r-FSH ability to stimulate, at this critical time window, circulating 11-ketotestosterone levels and consequently induce expression of pituitary LH. Such steroidal positive feedback triggering the production of pituitary gonadotropins has been shown previously in various fish species (Crim and Evans, 1979; Lin et al., 1991; Schmitz et al., 2005).

The combination of r-FSH injection and EVAc implant for sustained release of Methyltestosterone (MT), was the treatment that both induced a further advance in spermatogenesis and a higher percentage of breeders to advance to spermiation among captive grey mullet males. A previous study demonstrated that sole MT-EVAc treatment stimulates circulating levels of 11-KT, however in most cases the volume of the produced milt was relatively low (Aizen et al., 2005). In this regard, the value-added of the r-FSH-priming treatment administered as a single and to a greater extent double injections, seem to be attributed to the hormone's unique capabilities to regulate Sertoli cell activities (Schulz et al., 2010). Indeed, accumulated data on gonadotropin 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 (Schulz et al., 2010). The FSH main activities in mammals include stimulation of Sertoli cell proliferation during the pre-pubertal 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. 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, in-as-much as the number of these cells increases in association with the mitotic expansion of spermatogonial cysts in tilapia and African *Clarias gariepinus* (Schulz et al., 2005). Altogether, r-FSH seems to be a potent initiator of gonadal growth and germ cell proliferation/differentiation, however, further study is needed to fine-tune the optimal dose and time-window for its administration.

Grey mullet, like the European eel *Anguilla anguilla* (Dufour et al., 1988), are prone to dopaminergic inhibition during early stages of gonadal growth. To release the latter inhibition in the grey mullet, a single injection of domperidone (an antagonist of dopamine D2 receptors) was sufficient (Aizen et al., 2005), whereas in the eel, a triple treatment consisting of GnRHa, testosterone, and pimozide (an antagonist of dopamine D2 receptors) was necessary (Vidal et al., 2004). Aiming to further improve the onset and progression of vitellogenesis among captive grey mullet, in the current study we tested the effects of twined combination of Metoclopramide (Metoc; an antagonist of dopamine D2 receptors) with either rFSH or GnRH-EVAc implants. The Metoc+r-FSH treatment enhanced and synchronized ovarian development in captive grey mullet females, giving rise to 91% post-vitellogenic females within the treatment-group. However, as we suspect that the 2015 spawning season has encountered some abnormal environmental conditions that could have stress the fish and adversely affect their reproductive state, caution should be taken when comparing results of the 2014 and 2015 trials.

Interestingly, relatively higher abundance of fully mature females and males (50-70%) could be found in untreated groups, compared to the relatively low percentages (10-20%) that were reported previously (Aizen et al., 2005). We tend to think that the enhanced spontaneous maturational process among captive grey mullet population also relates to the fact that all experimental fish in the current study were hatchery-produced G1 broodstock, which have begun the process towards domestication.

In grey mullet, as in many other teleosts (Dufour et al., 2005), dopamine is involved in the control of spermiation, ovulation, and spawning. So far, regardless of the broodstock origin (i.e., wild- caught or G1) no spontaneous spawning have been documented in captivity. It is well established that dopamine can inhibit basal and GnRH-induced LH release (Peter et al., 1986, 1991; De Leeuw et al., 1986; Yu and Peter, 1992, Yaron et al., 2003), and also may modulate pituitary sensitivity to GnRH by decreasing the number of GnRH-receptors (De Leeuw et al., 1986, 1988; Omeljaniuk et al., 1989; Levavi-Sivan et al., 2004). Therefore, to induce grey mullet spawning, fully mature females and males were treated with two consecutive injections consisting of GnRHa combination with Metoc. The rationale behind this treatment was to stop the strong dopaminergic inhibition with the dopamine antagonist, and promote LH release from the pituitary through a GnRHa injection (reviewed by Zohar and Mylonas, 2001). Results obtained so far, indicate up to 50% spawning successes following a treatment with priming (GnRHa 10 µg/kg; Met 15mg/kg) and resolving (GnRHa 20 µg/kg; Met 15mg/kg) injections given 22.5-h apart to selected fully mature females and males. The first series of spawning induction trials (September- November, 2014) gave rise to high quality eggs and larvae and later on to large numbers of robust juveniles. Nonetheless, the employment of similar treatment during the following spawning season (September- November, 2015) was less productive. These differences can be explained by external effectors (i.e., climate conditions and water quality) that seem to stress the fish and negatively affect their overall physiological state, including reproductive development. However, the accumulating data from the two spawning seasons highlight female's failure to release the eggs and variable fertilization rate, as two major problems that should be addressed in future experiments.

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No deviations exist in the Deliverable

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