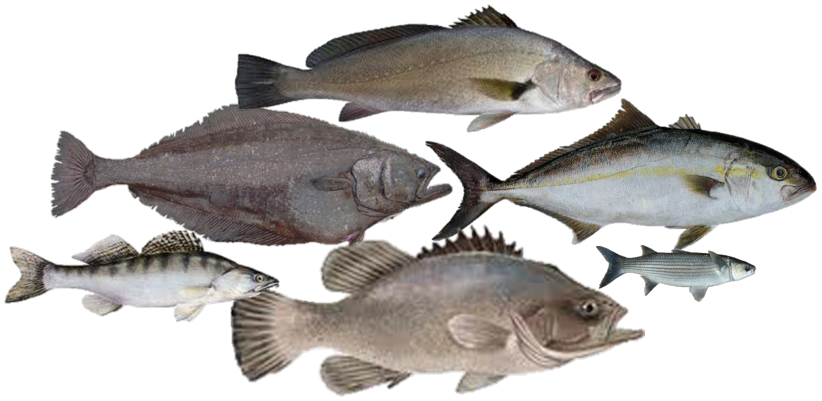
GWP
Reproduction and Genetics
WP2-7



Presenter: Neil Duncan, IRTA

2nd Annual Coordination Meeting
2-4 February 2016
Nancy, France

WP2 Reproduction and Genetics – Meagre
led by IRTA (Neil Duncan)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP2 Reproduction & genetics-meagre	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 2.1 Evaluation of the genetic variation in captive meagre broodstocks (FCPCT)				D																
Task 2.2 Development of protocols for paired crossing in spontaneous spawning IRTA)							D													
Task 2.3 Description of sperm characteristics and cryopreservation methods (IFREMER)												D								
Task 2.4 Development of in vitro fertilization methods for planned crosses (IRTA)												D								
Task 2.5 Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers (HCMR)				D						D		D								

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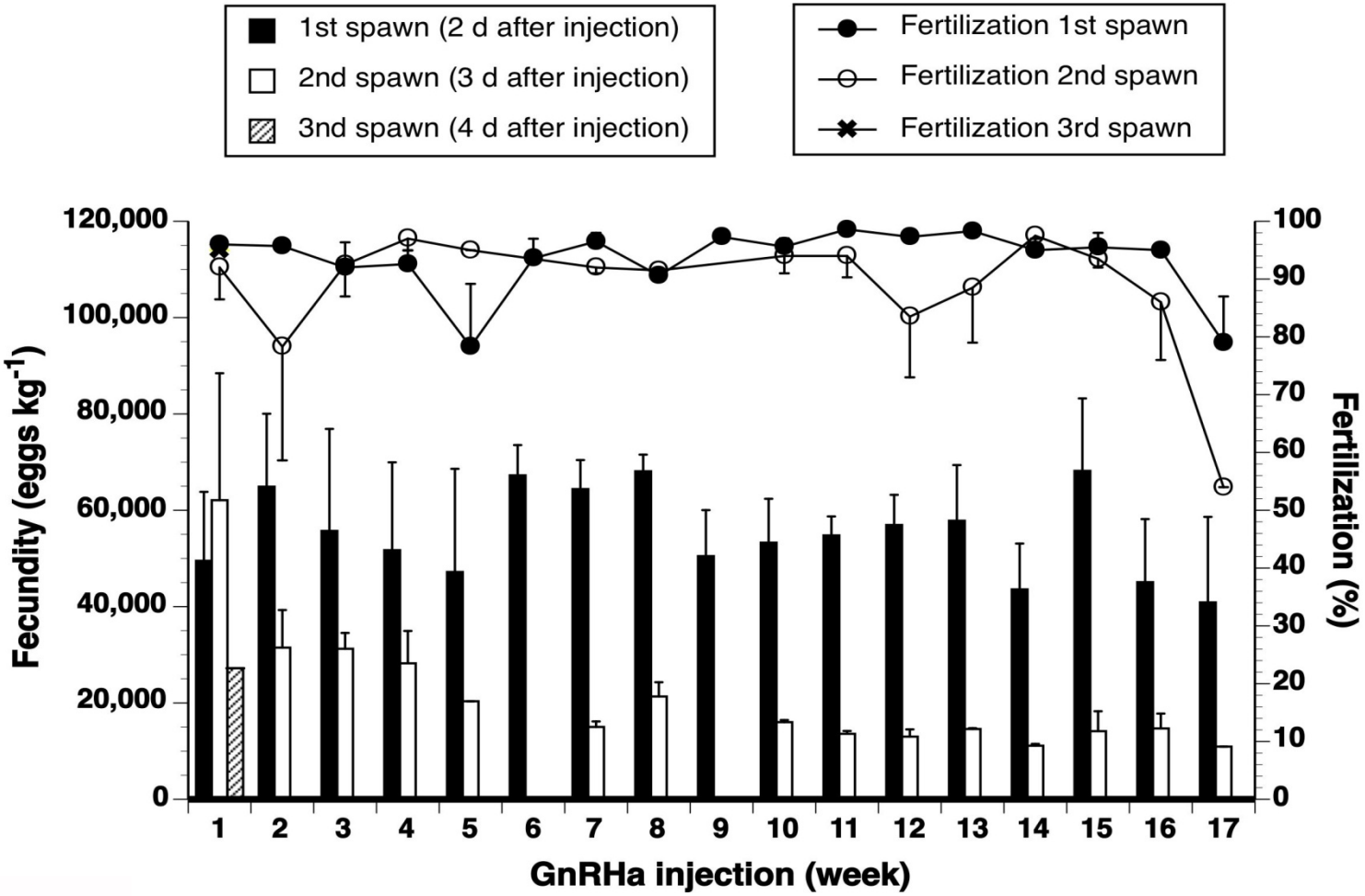
Task 2.2 Development of protocols for paired crossing in spontaneous spawning (IRTA)



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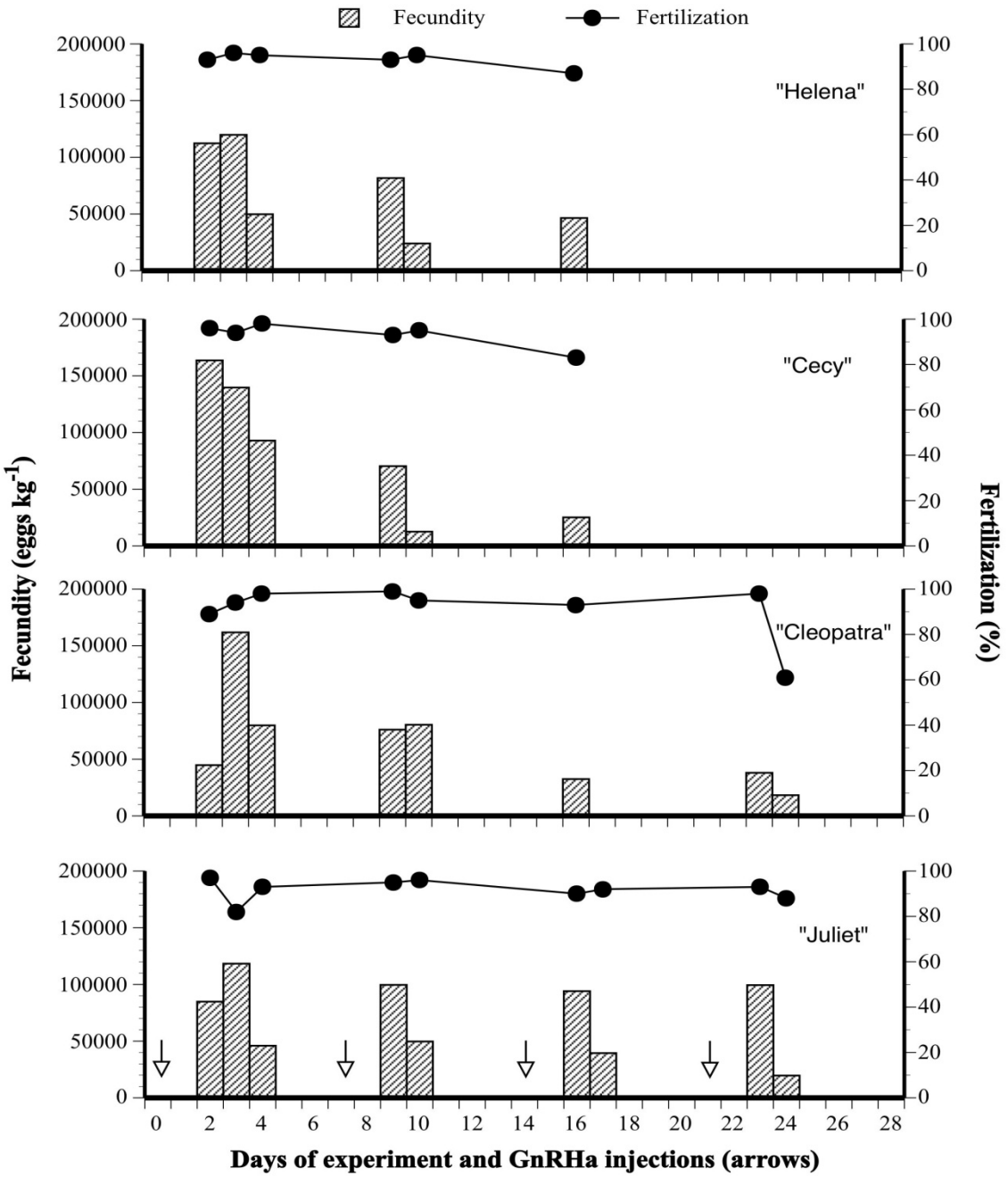
Results: Fecundity

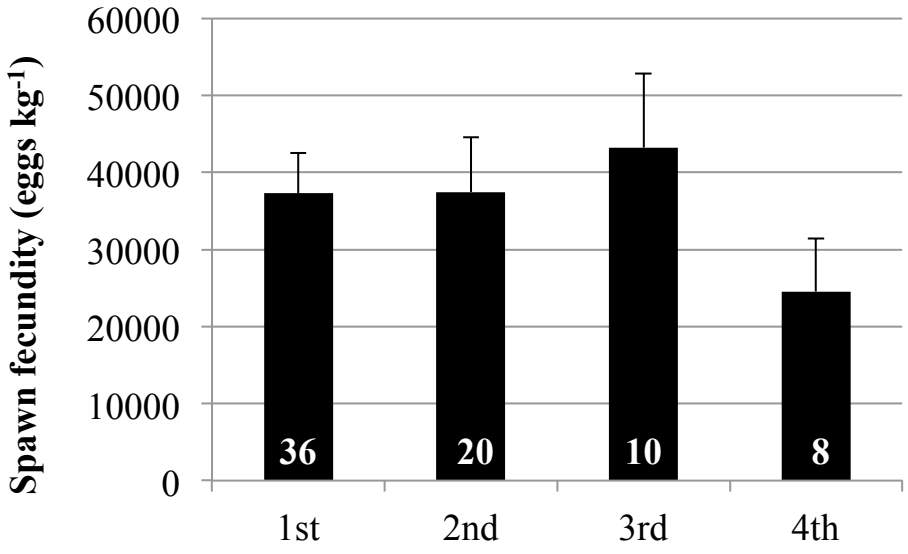
Two pairs did not spawn after 4th injection of GnRHa

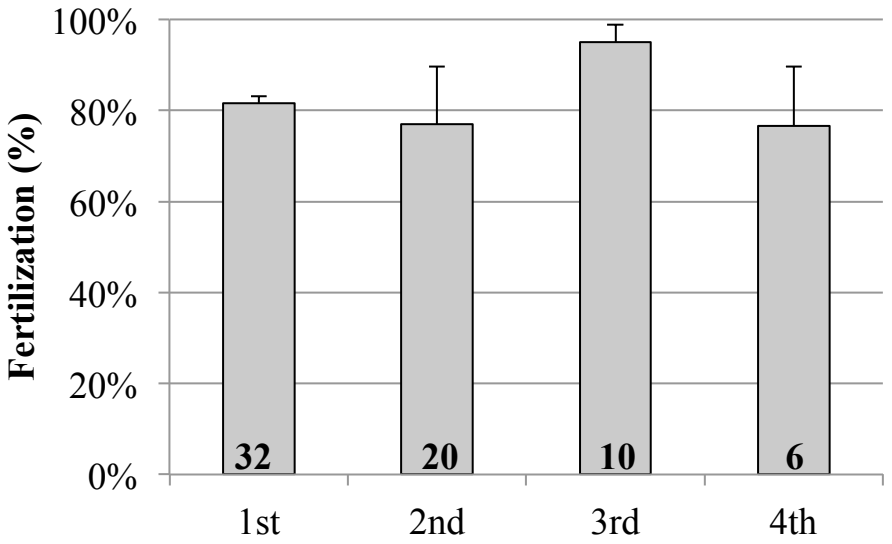
Apparent decline in fecundity



Task 2.2 Development of protocols for paired crossing in spontaneous spawning

Results: Fecundity
Significant decline in fecundity with successive injections of GnRHa





GnRHa injection for female

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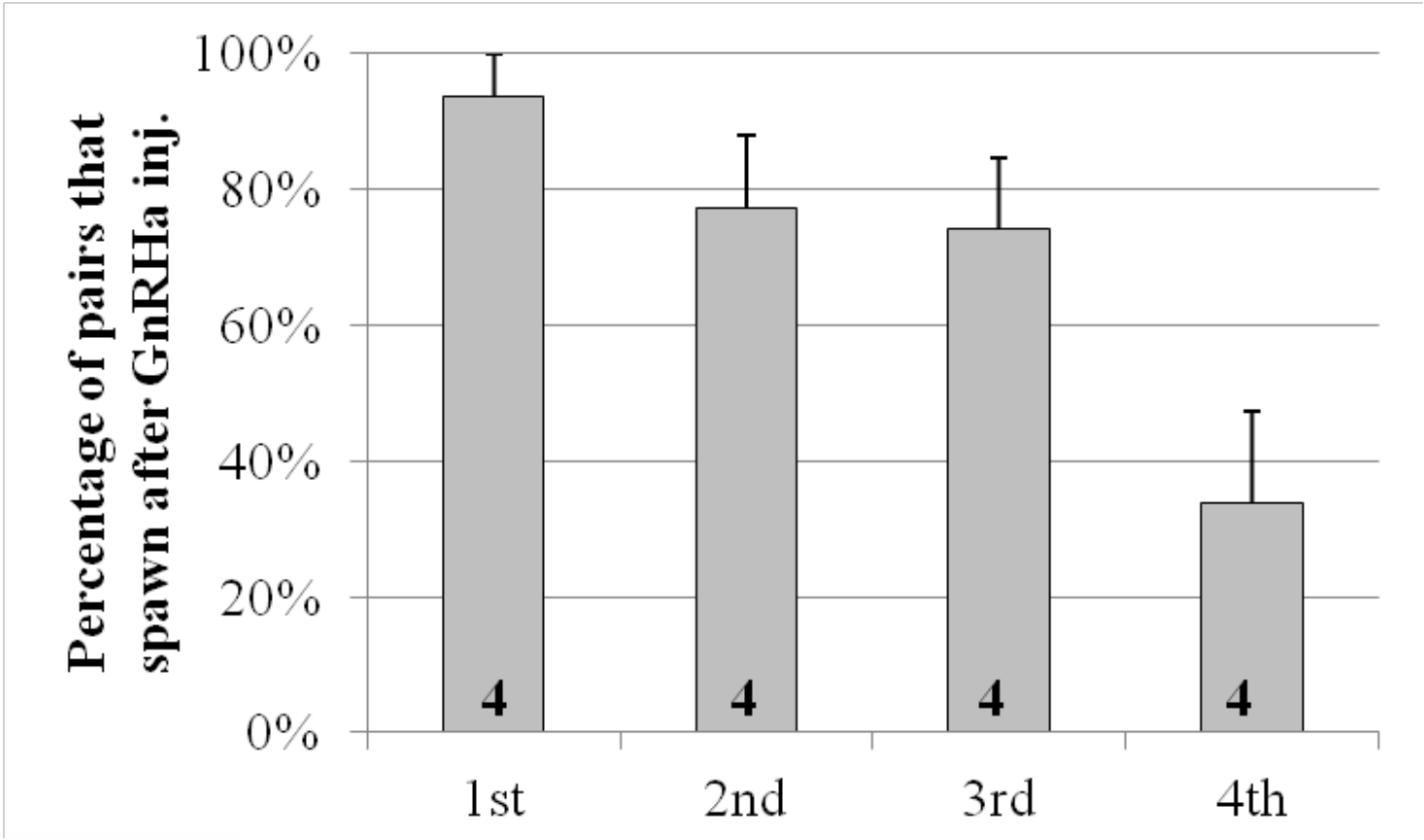
Task 2.2 Development of protocols for paired crossing in spontaneous spawning

Results: Spawning success

Decline in percentage of pairs that spawn with successive injections of GnRHa



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Task 2.3 Description of sperm characteristics and cryopreservation methods (IFREMER)

Species	meagre
Main scope	*controlled crosses*
Motility evaluation (CASA)	running
Concentration evaluation	OK
Chilled storage	running
Cryopreservation	running
In vitro fertilization	running
Experiments	running
Data analysis	
Deliverables	End 2016

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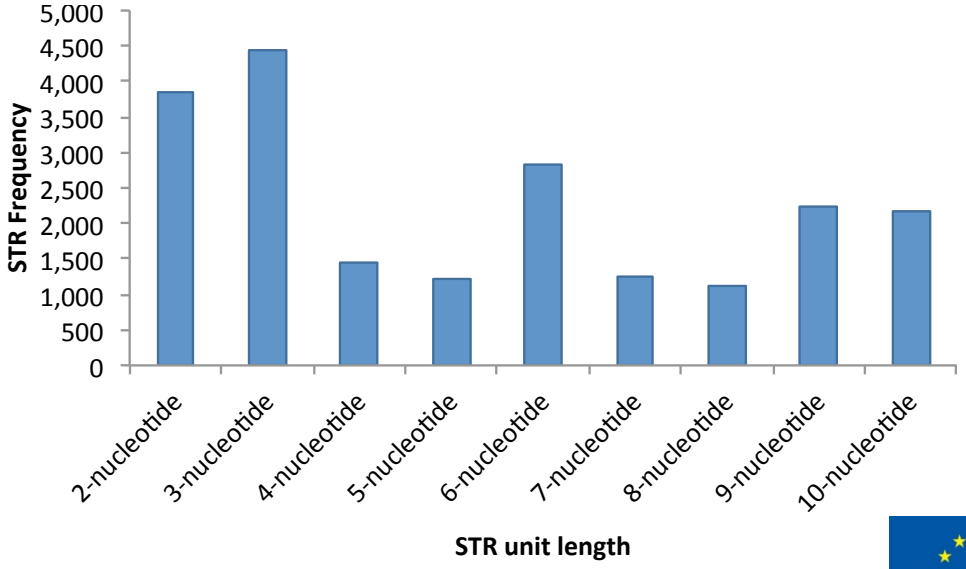
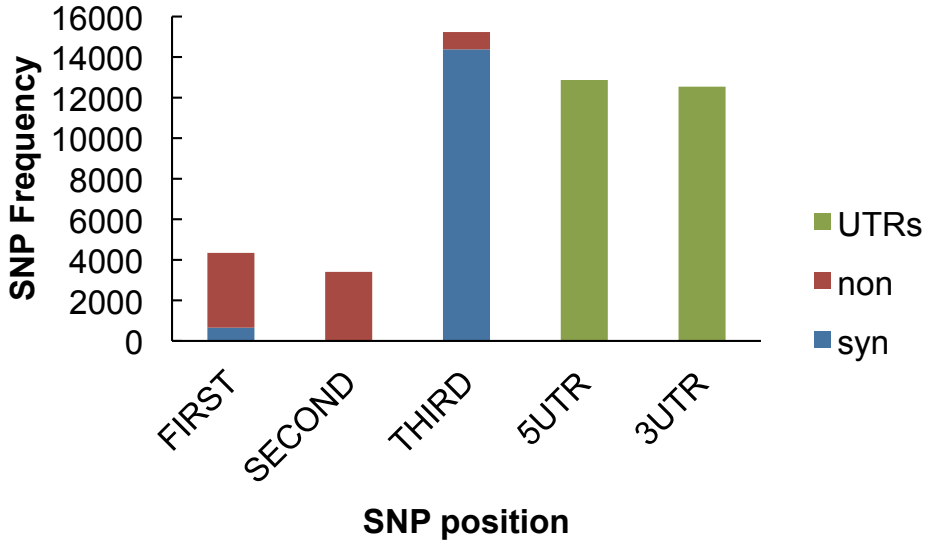
Task 2.5 Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers (HCMR)

Our search for microsatellites revealed 20,582 total markers located in 16,517 transcripts belonging to 12,565 genes.





Filtering resulted in ~50,000 SNPs located in 18,000 transcripts belonging to ~16,000 meagre loci.

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WP3 Reproduction and Genetics - Greater amberjack

led by UNIBA (Aldo Corriero)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP3 Reproduction & genetics-greater amberjack	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 3.1 Description of the reproductive cycle of greater amberjack (UNIBA)				D		D		D			D					2D				
Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (HCMR)																		D		
Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic (FCPCT)																D				
Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic (IEO)																		D		
Task 3.5 Spawning induction of greater amberjack and egg collection in cages (HCMR)																		D		

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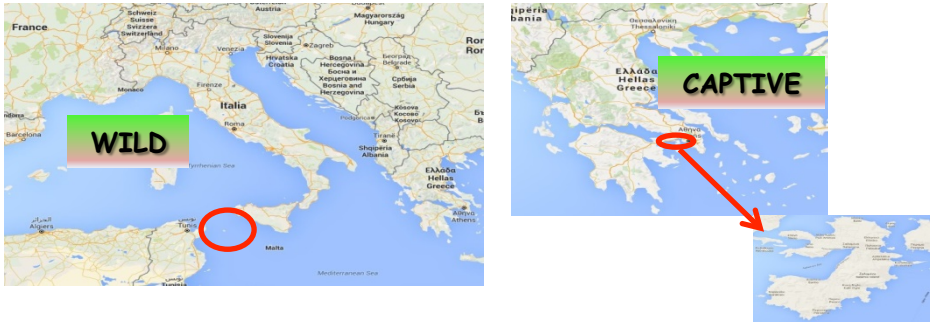
Task 3.1_Objective 1: Description of the reproductive cycle of greater amberjack

SAMPLING AREAS



SAMPLING PERIODS (2014-2015)

EARLY GAMETOGENESIS (EG)
(late April-early May)

ADVANCES GAMETOGENESIS (AG)
(late May-early June)

SPAWNING (SP)
(late June-early July)

WILD *vs* CAPTIVE COMPARATIVE ANALYSES

.....accomplished

- ✓ biometrics
- ✓ routine histological analyses
- ✓ liver vitellogenins (Vgs) and gonad vitellogenin receptor (VgR) gene expression
- ✓ oocyte yolk accumulation
- ✓ male germ cell proliferation and apoptosis

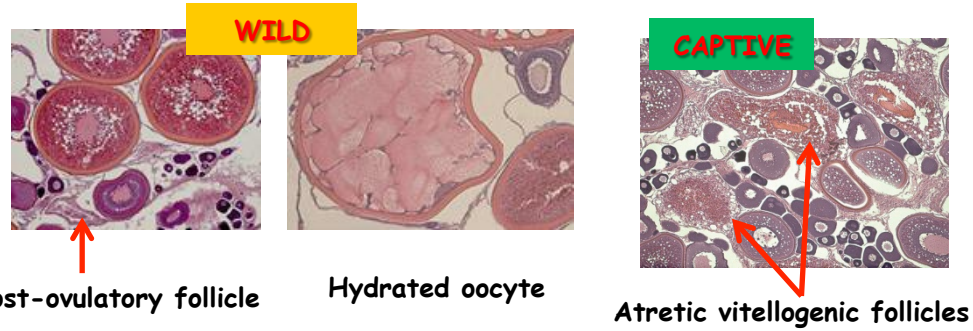
.....ongoing

- ➢ plasma vitellogenin
- ➢ FSH and LH pituitary gene expression and plasma levels
- ➢ sex hormone plasma levels
- ➢ sperm analysis

Task 3.1

CAPTIVE-REARED FEMALES

- lower gonado-somatic index
- extensive atresia of vitellogenic oocytes
- lower Vgs liver expression
- normal oocyte yolk content



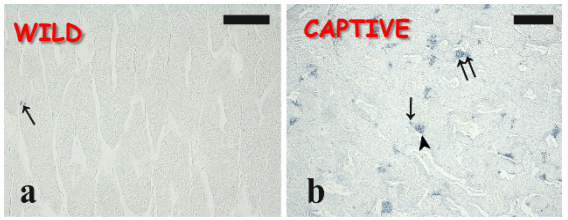
Post-ovulatory follicle

Hydrated oocyte

Atretic vitellogenic follicles

CAPTIVE-REARED MALES

- lower gonado-somatic index
- lower seminiferous lobule diameter
- very high germ cell apoptosis during early gametogenesis
- earlier decrease of germ cell proliferation
- precocious cessation of spermatogenic activity
- progressive T and 11-KT decrease from April to July

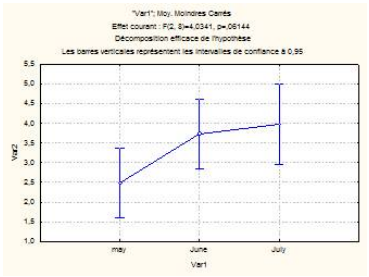


Apoptotic germ cells

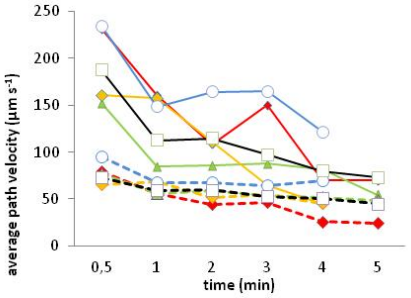
CONCLUSIONS

Impaired oogenesis and spermatogenesis in captivity with increased germ cell apoptosis/ atresia and reduced proliferation

Sperm analysis (ongoing)



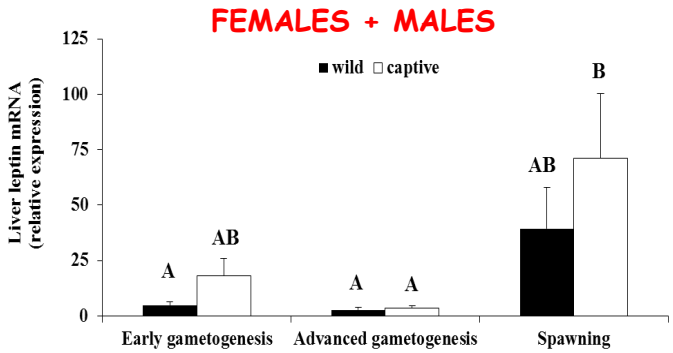
Sperm concentration



Sperm motility

Task 3.1_Objective 2: Description of greater amberjack broodstock nutritional status

Liver **leptin** gene expression analysis



Higher transcript profiles in captivity

Dramatic increase during spawning period

Early Gamet (EG)

Advanced Gamet (AG)

Spawning (SP)

- Total Protein and Moisture (AOAC, 2002)
- Total Carotenoids (Barua et al., 1993)

- Total Lipid (Christie, 1982)
- Lipid Clases Profile (Olsen y Henderson , 1984)
- Fatty Acid Composition (Christie, 1982)

1. Significant differences in total polar lipids, individual lipid classes and essential fatty acids (EFA) between wild and captive-reared fish gonads.
2. 30-40% lower contents of DHA and ARA, and lower DHA/EPA and ARA/EPA ratios in captive fish (all crucial factors for reproductive success and sperm, egg and larval quality).

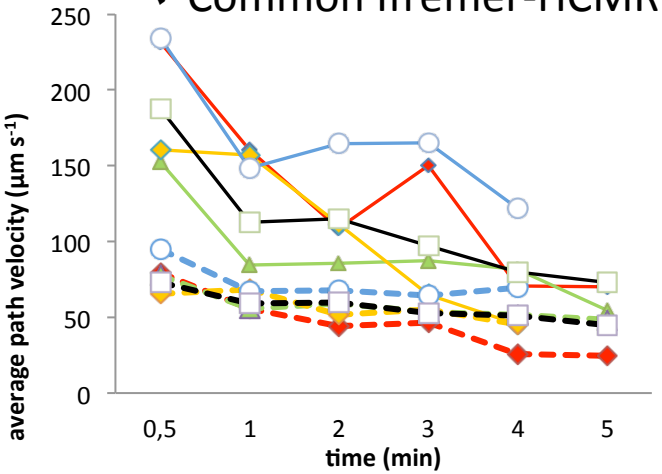
DHA=docosahexaenoic acid; ARA=arachidonic acid; EPA= eicosapentaenoic acid

Task 3.1_Objective 2: Description of greater amberjack broodstock

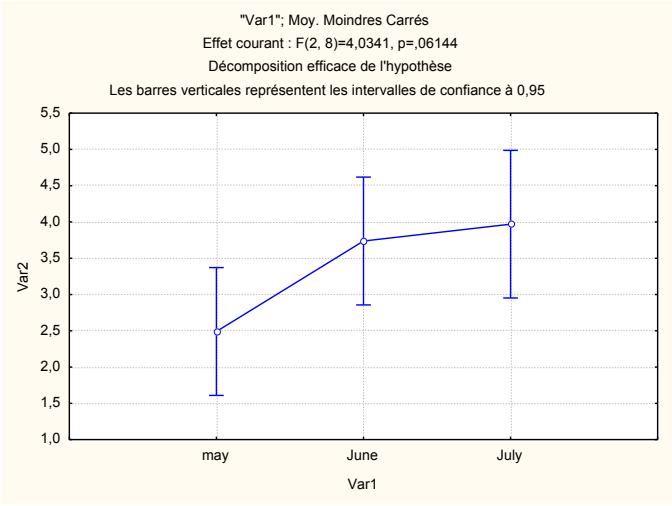
Seriola dumerilii sperm quality evaluation

Goal: to better know about reproductive cycle of captive amberjack

- ✓1 training in Heraklion on 4 males
- ✓3 sampling travels to Salamina (4 males each)
- ✓Common HCMR-IFREMER benchwork
- ✓Common Ifremer-HCMR analyses (ongoing)



Motility decrease in sperm of 5 seriola from HCMR laboratory in may 2015 full line : max imum , dotted line mean



Mean concentration of Seriola sperm along the reproductive season in Salamina (Greece): There are no significant differences

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Tasks 3.2 & 3.5_Objectives:

1) Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (Task 3.2)

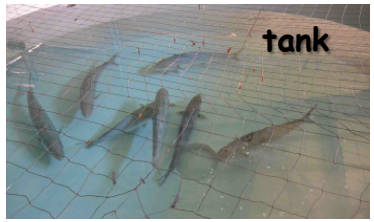
2) Spawning induction of greater amberjack and egg collection in cages (Task 3.5)

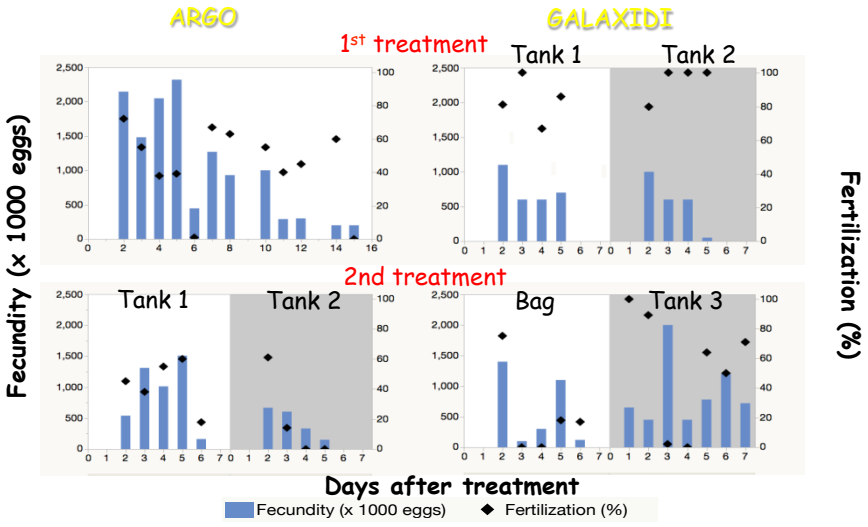
ARGO

GALAXIDI

HCMR cage

2 treatments

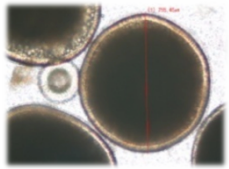


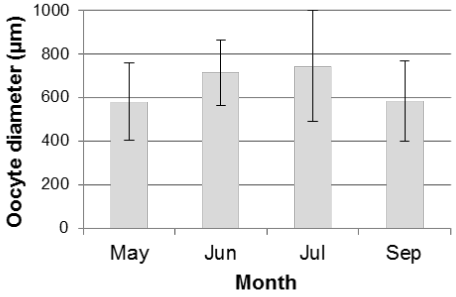


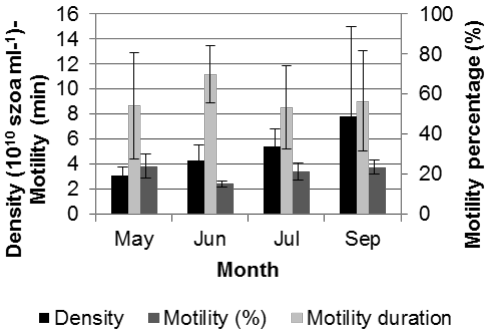
CONCLUSIONS

1. Good sperm quality
2. Females in cages in better reproductive stage than in tanks
3. Water quality issues or tank volume may affect vitellogenesis of fish in tanks
4. Fish mature even in lower temperature than 20°C
5. Different management of breeders seems to be effective: keep them in cages and transfer in tanks for spawning after induction
6. Test with anaesthetizing bag for egg collection was successful

Task 3.4_Objective:
Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic





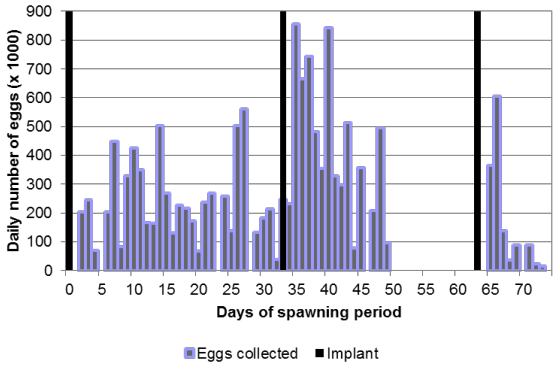


15 fish selected and placed in an outdoor covered 500 m^3 tank under natural T and photoperiod

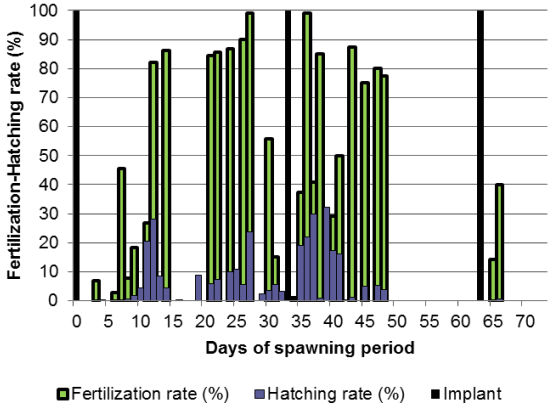
Biopsy samples (oocyte diameter, sperm analysis)

3 GnRHa implantations (May, June and July)

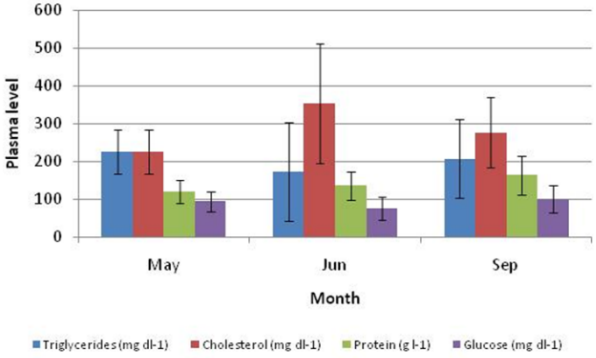
RESULTS



52 spawning and 14.9 x 10^6 eggs obtained
29 spawning obtained in 31 days period



Mean fertilization 41.9 ± 37.1 %
Hatching rate 6.0 ± 8.8 %
Highest fertilization values from June to July



Hematological and biochemical parameters evaluation

WP4 Reproduction and Genetics - Pikeperch

led by HCMR (Costas Tsigenopoulos)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP4 Reproduction & genetics-pike perch	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 2.3.1 Evaluation of the genetic variation in available domesticated broodstocks of pike perch				D																
Task 2.3.2 Evaluation of the genetic variation in non-domesticated broodstocks of pike perch						D														

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Conclusions

- In general, the mean heterozygosity estimates and the count of the number of alleles per population indicate that domesticated samples do not suffer from inbreeding.
- There are few domesticated populations that either due to their small sample size or their *a priori* known use as 'selected' fish, which indicates the notion of some level of inbreeding
- Interestingly, the number of alleles in domesticated samples is slightly higher than that in the wild (2.63 vs 2.58), whereas the unbiased heterozygosity is slightly lower (0.553 vs 0.573)

Conclusions

- Evidence that pikeperch populations in Europe are part of at least two genetically differentiated groups.
  - The first is found in northern Europe from Netherlands/Denmark to the West and Poland (at least) to the East to the North of Finland. This is the group probably referred also as “Baltic Sea” stock by Björklund et al. (2007) and Poulet et al. (2009).
  - The second group comprises all remaining populations in Central Europe to as south as

Conclusions

- In the second stock, the Hungarian populations are having a key-position being different from those found geographically near, *e.g.*, from Czech Rep. and Germany.
  - It might be another stock associated with Hungarian lakes, as opposed to all other populations that probably dispersed through the Danube River west-and southwards (see also Kohlmann *et al*., 2013)
- Most populations analyzed seemed to contain fish of a single origin with very few exceptions

WP5 Reproduction and Genetics - Halibut

led by IMR (Kristin Hamre, NIFES)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP5 Reproduction & genetics-Atlantic halibut	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut (IMR)										D										
Task 5.2 GnRH implant therapy as a means to improve spawning performance (HCMR)										D										
Task 5.3 Fecundity regulation (IMR)												D								

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Task 5.1 Spawning performance

	Domesticated	Farmed
n	3 (4)	5
length (cm)	142-154	109-118
weight (kg)	40-53	17-22
number of batches/female	7-8	8-12
spawning interval (hours)	82.2 ± 8.4	72.4 ±22.9
batch volume (litres)	1-4	0.25-1.5
total fecundity (l)	12.8-22.8	4.9-8.3
fecundity (l/kg)	0.35 ± 0.09	0.35 ± 0.09
average fertilization	89 ± 7%	61±29%

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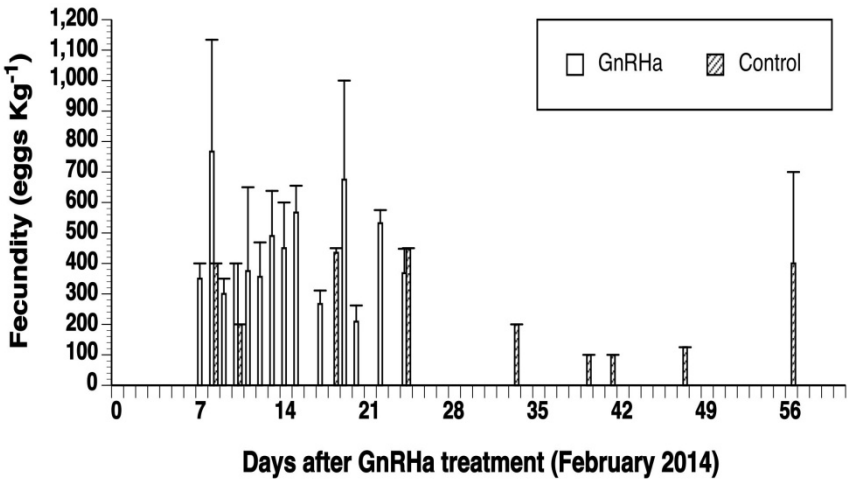
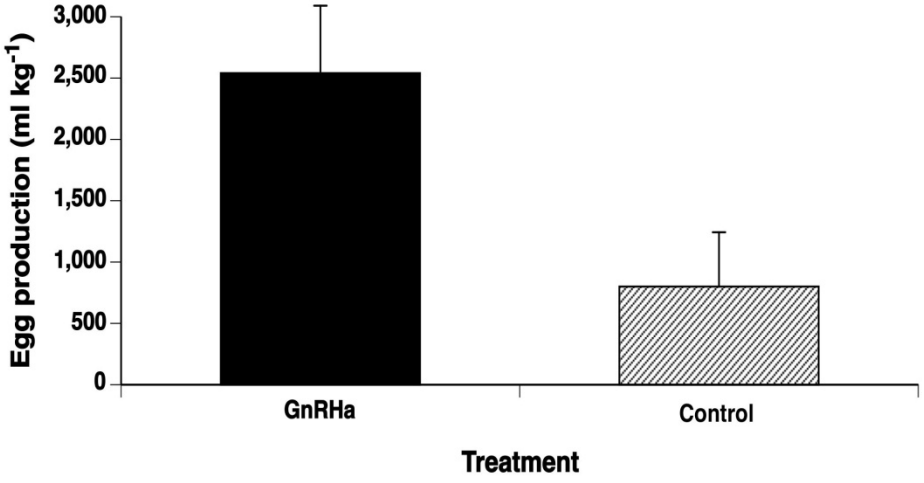
Task 5.2 Effect of GnRH implantation on spawning time and fecundity





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Task 5.2
Industry implementation

- Pilot experiment in 2015
- Inconclusive results
  - Timing
  - Choice of individuals
- Larger scale study started January 2016

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Tasks 5.2 and 5.3
Spawning performance and fecundity regulation in wild-captured, domesticated and farmed halibut

- Domesticated, wild-captured females and farmed females were closely monitored during the spawning season 2015
- Eggs were stripped and batch interval, volume and fertilisation rate recorded
- Egg content of testosterone and cortisol analysed
- Samples taken for metabolomics
- Cell division symmetry, other morphological parameters and hatching rate will be documented in 2016.
- Blood samples will be taken monthly from (newly caught) wild and cultured females starting in August 2016.

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WP6 Reproduction and Genetics - Wreckfish

led by IEO (Jose Benito (Tito) Peleteiro)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP6 Reproduction & genetics-wreckfish	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 6.1 Collect wild fish to establish new broodstocks (CMRM)												D								
Task 6.2 Describe reproductive cycle (IEO)															2D					
Task 6.3 Development of spawning induction procedures (IEO)												D						D		
Task 6.4 Evaluation of sperm characteristics and cryopreservation protocols (IFREMER)							2D													

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1) **6.1 Collect wild fish to establish new broodstocks.**

Code	Lt	Ls	Perím	Weight
68521	62	55	48.5	4.86
68372	27	26.5	31.5	0.94

Two live fish were captured in the Atlantic coast (NW of Spain) and were maintained in quarantined at the "Acuario de El Grove". Prior to sampling, animals were anesthetized with phenoxiethanol (3.5 ml/l of seawater). Total and standard length, perimeter and weight were determined. A sample from the fin was also taken, for genetic analysis. These two animals will be maintained separated from the existent stock at the IEO, until they become adults.

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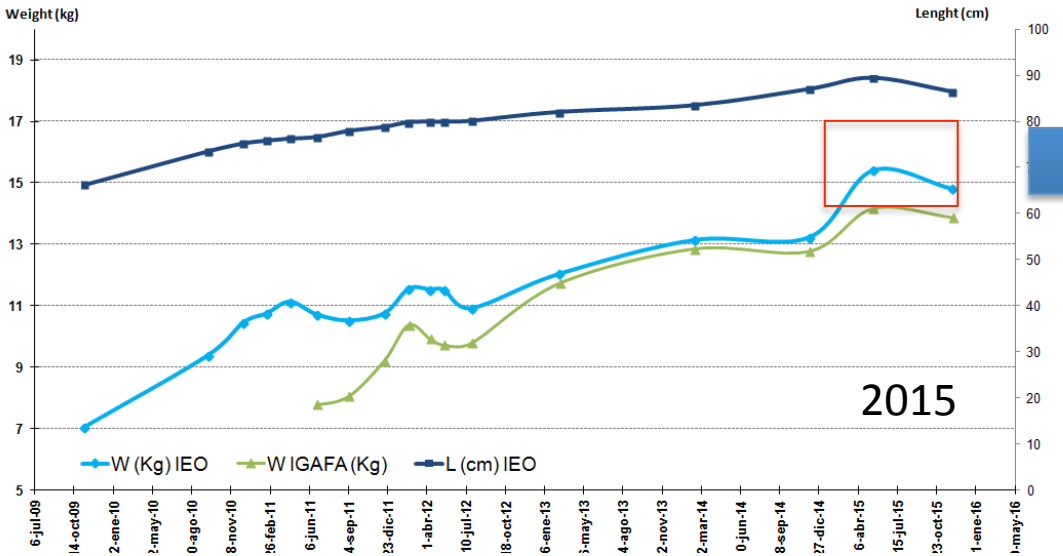
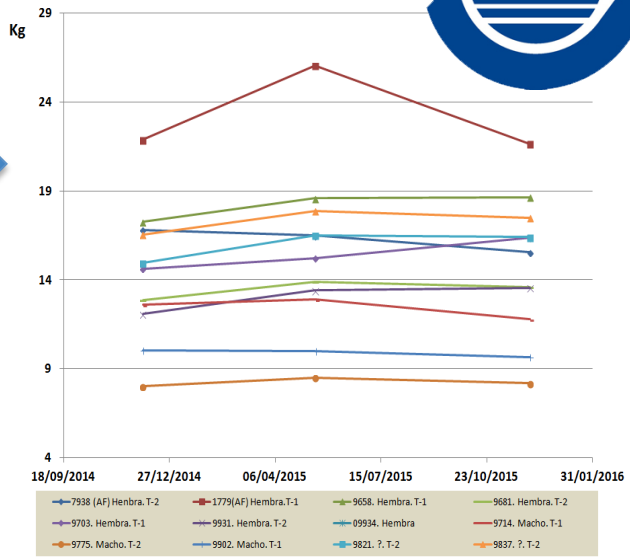
6.2. Describe the reproductive cycle in captivity

- **HCMR: n=5**
  Simulated natural photoperiod and constant temperature (16°C).
- **IEO: n=11 + 1**
  Natural temperature and photoperiod
- **MC2: n=27**
  Natural temperature and simulated natural photoperiod
- **CMRM: n=10**
  Natural temperature and photoperiod

- **Totaling 31 ♀, 18 ♂ and 5 undetermined.**

6.2. Describe the reproductive cycle in captivity





All stocks were sampled every month, since February until June or July, and bi-monthly until December 2015.

This study on morphometric parameters indicates that animals stabilized their growth (size and weight), with weight increase during the breeding season.

Blood was extracted from some animals in each stock to determine steroid levels (HCMR).

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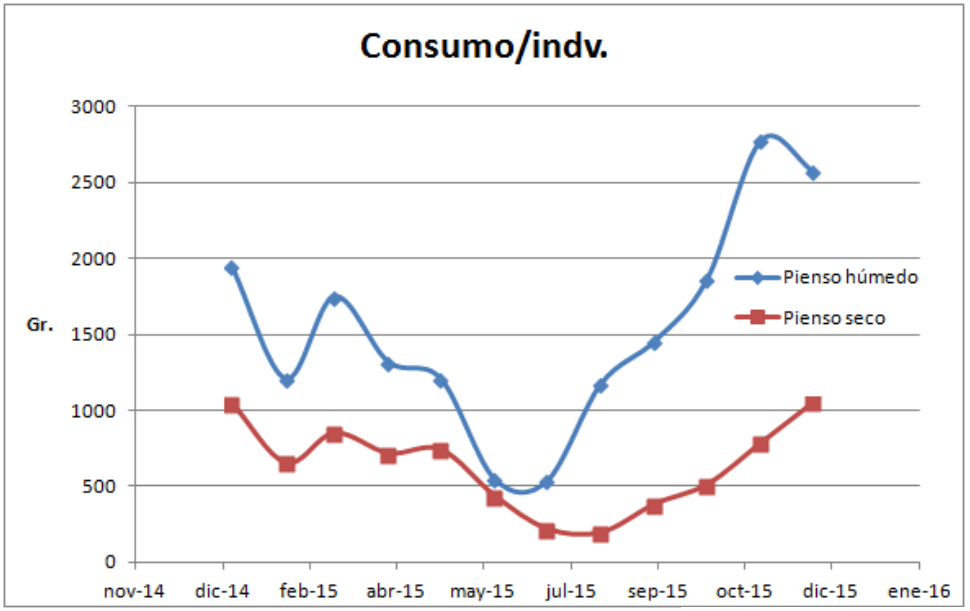
6.2. Describe the reproductive cycle in captivity

Ingestion rate varied between 0.2 y 0.5 % for fish fed the semi-moist diet, and between 1 and 1.8 % those fed dry pellets.
Low feeding rates were recorded during Summer, and high feeding rates occurred during spawning, for both diets.



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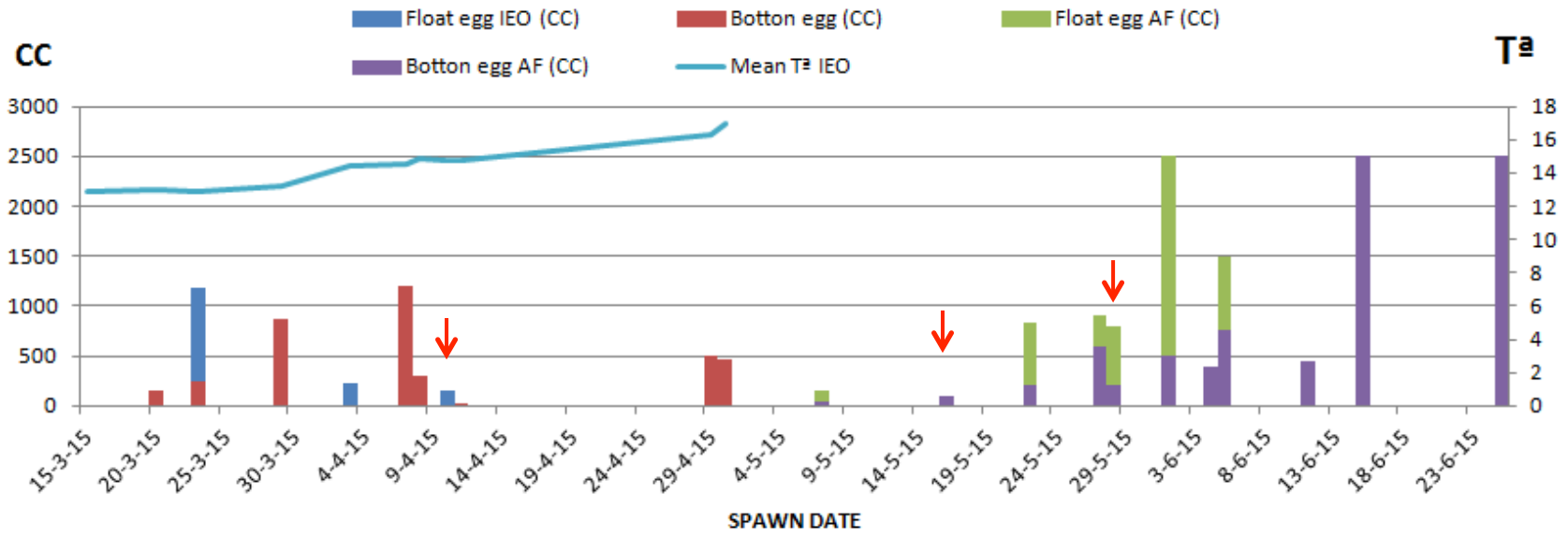
6.2. Describe the reproductive cycle in captivity

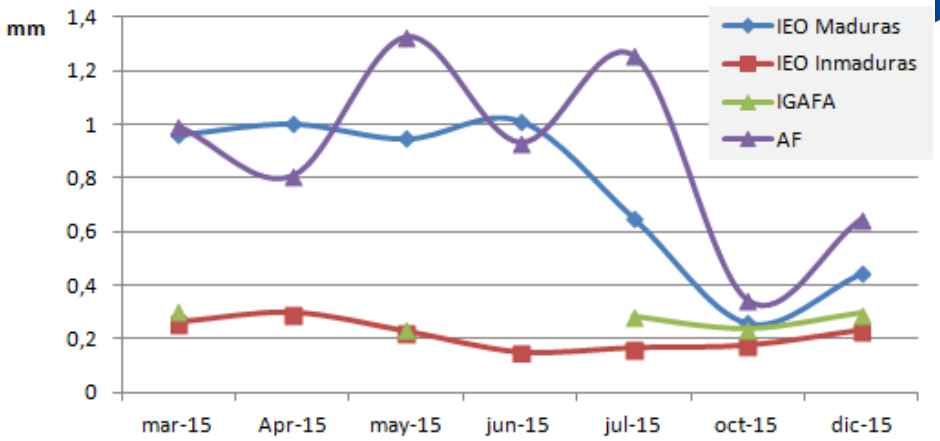
The majority of spawns were spontaneous, except one at the IEO (10/04/2015) and two from "Acuario" (16 and 28/05/2015)

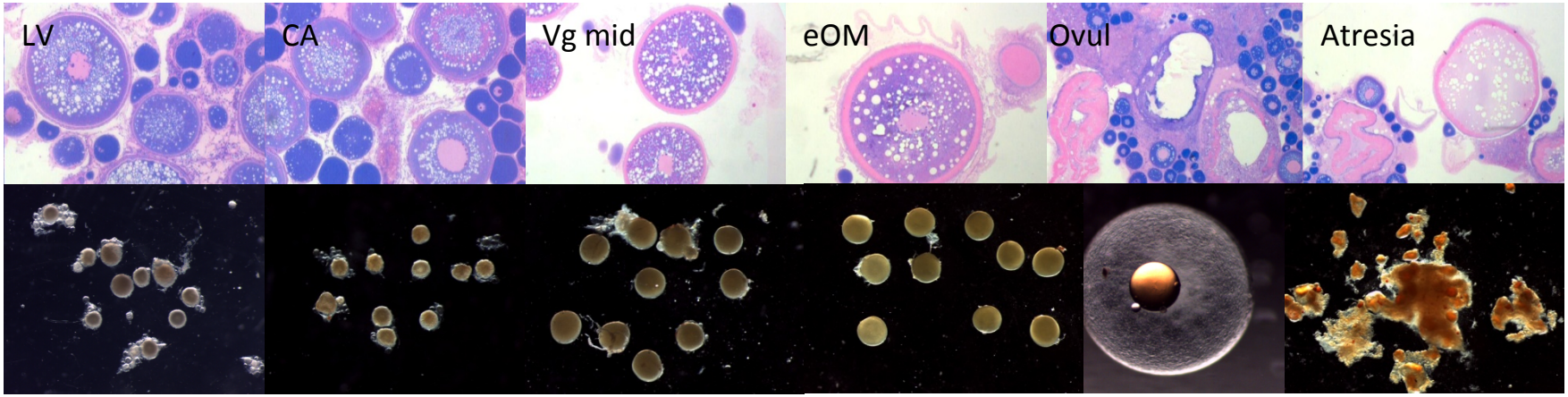


2015 Spawns Wreckfish broodstocks (IEO, MC2)

6.2. Describe the reproductive cycle in captivity

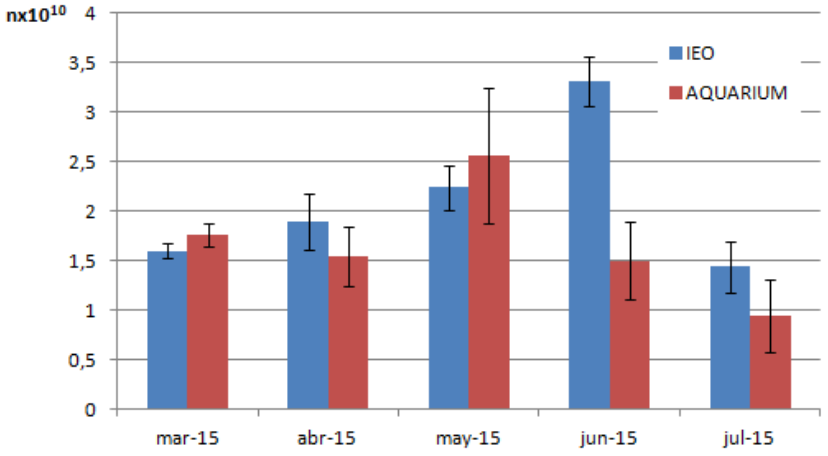
Oocytes average size throughout the year was within expected values, with highs during the spawning season. Biopsies from females that did not spawn had oocytes with similar size al year.

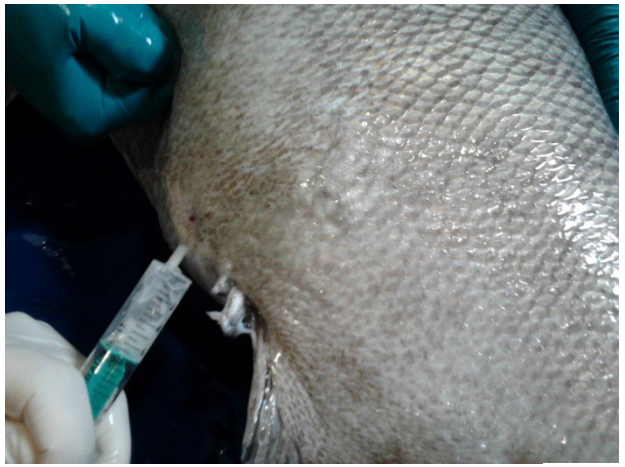




6.2. Describe the reproductive cycle in captivity

- Sperm quality parameters were evaluated: sperm concentration, motility and time of sperm motility.
- Results demonstrated that males produce large volumes of good quality sperm for a very long period of time in captivity.
- Blood samples were taken to determine the relation between sexual steroids and sperm quality.
- Sperm concentration during breeding season was1.33 $X10^{10}$ a 5.5 $X10^{10}$





6.3. Development of spawning induction procedures

- On May 2015, two females from the "Acuarium Finisterrae" weighning 27.35 y 33.15 Kg were implanted (*500 µg GnRHa implant*). Oocyte size were 1.121 y 1.092 mm respectively. A female from the IEO (16.5 Kg) with oocyte diameters of 0.95 mm was also implanted. None of these implanted females spawned.

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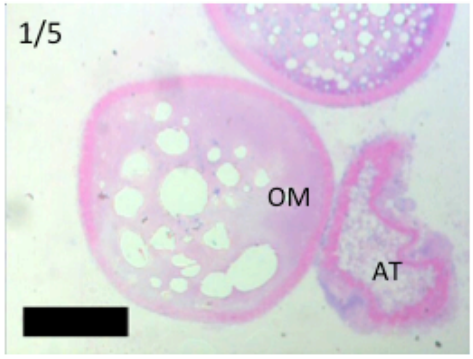
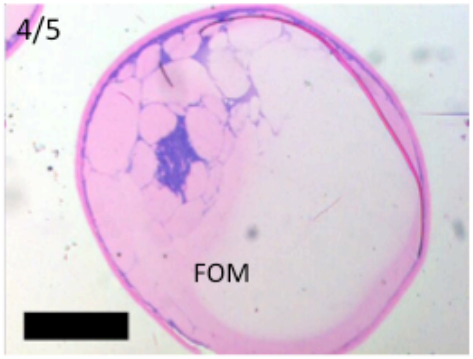
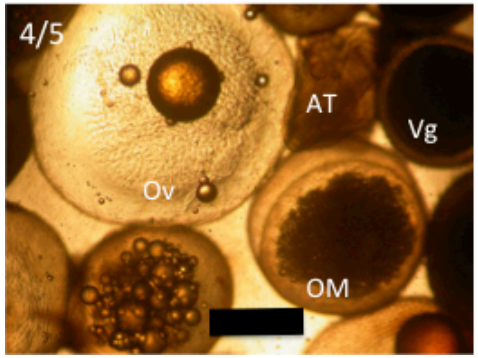
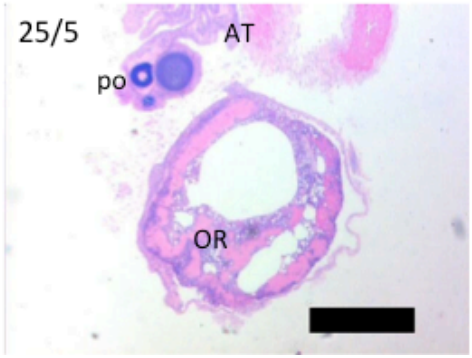
Spawning induction…









- Treatment of female induced maturation
- Ovulation of females seems to be problematic (latency period not well defined, small batch number)
- Fertilized eggs produced both spontaneously and with strip-spawning after spawning induction

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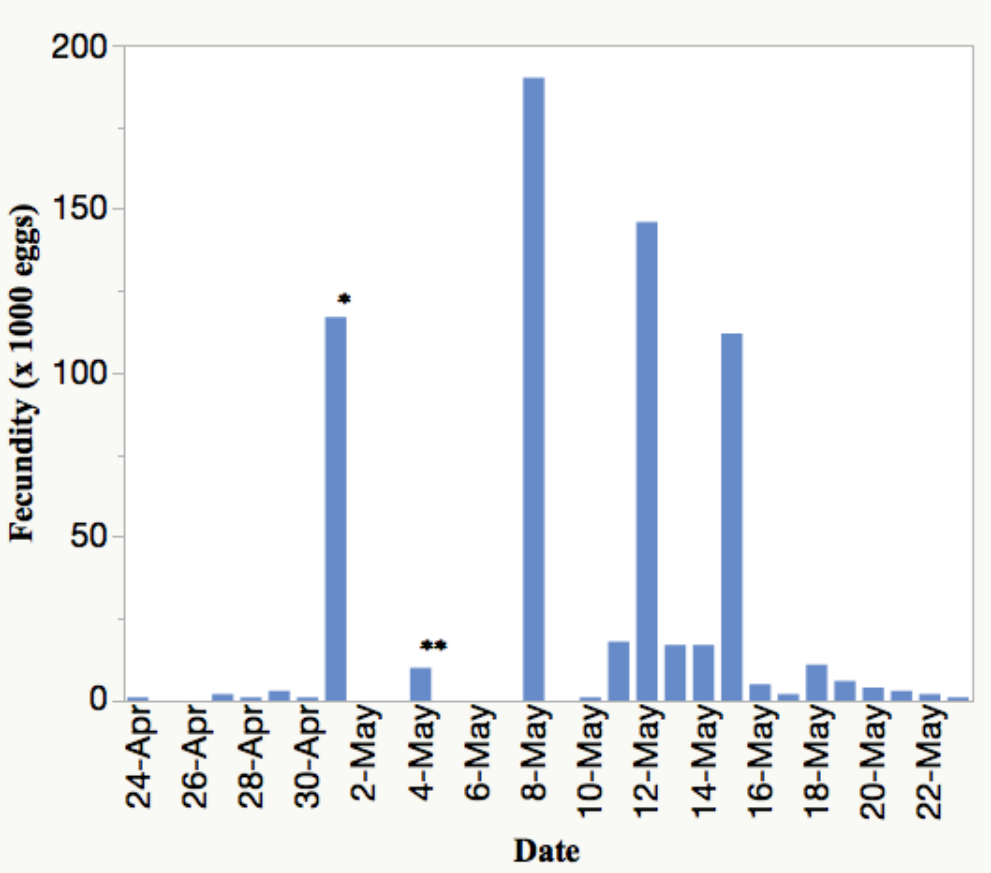
Egg production…



- Fertilized eggs from spontaneous spawning only on 1-May and 8-May
- 4-May eggs from stripped spawning

* Fecundity on 1-May was the sum of spontaneous and strip-spawning produced eggs.
** Eggs on 4-May produced with strip-spawning.

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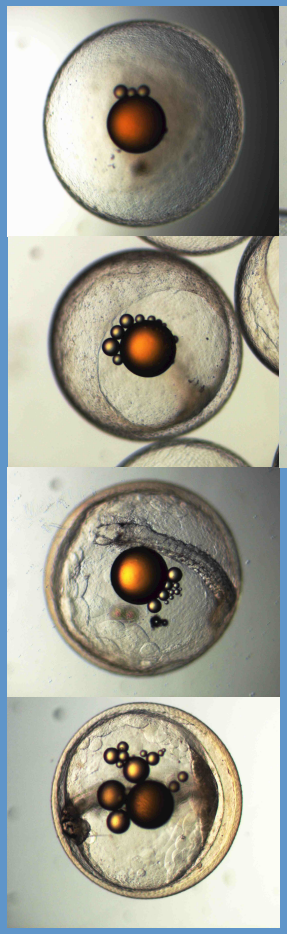
Natural

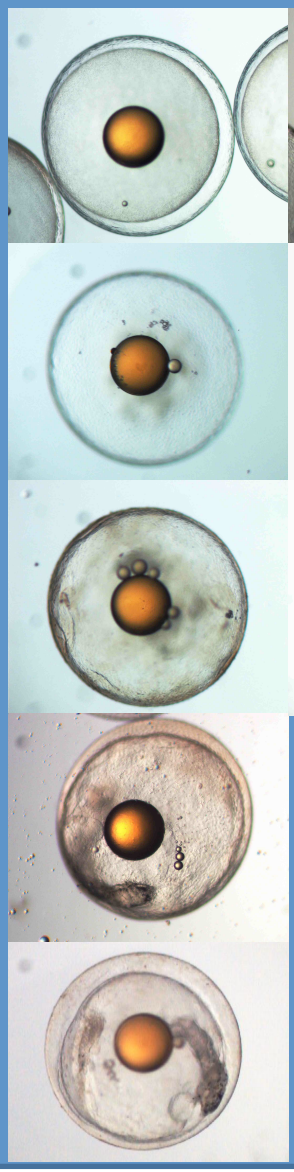
Spontaneous



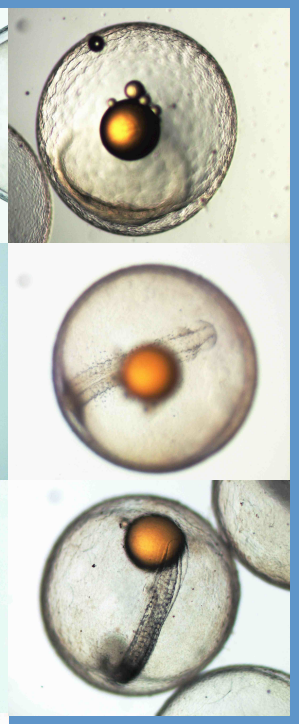
Stripped

Induced

Spontaneous



Stripped



Transferred to larval rearing

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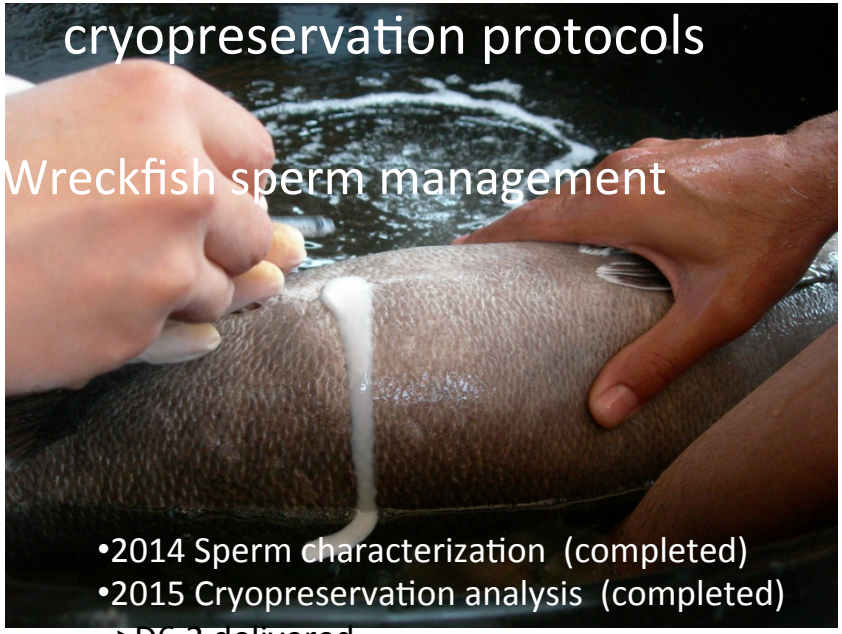
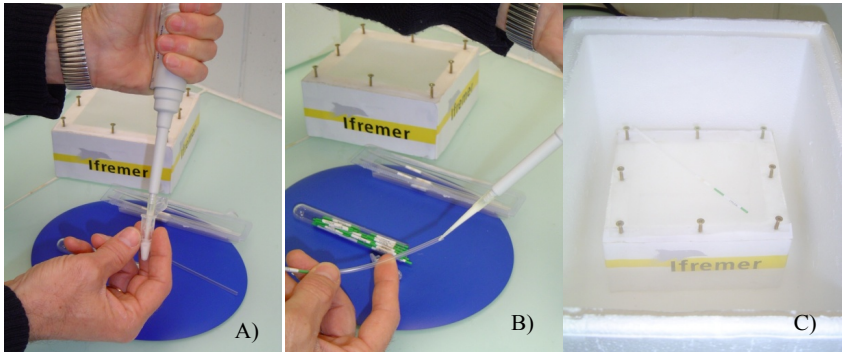
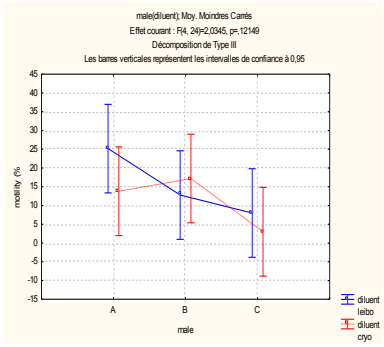
4) 6.4. Evaluation of sperm characteristics and cryopreservation protocols

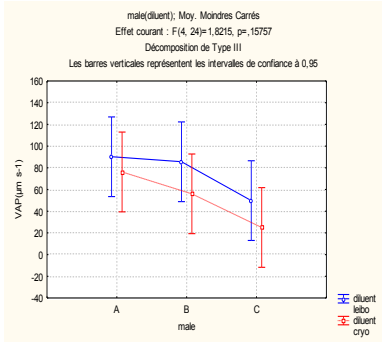


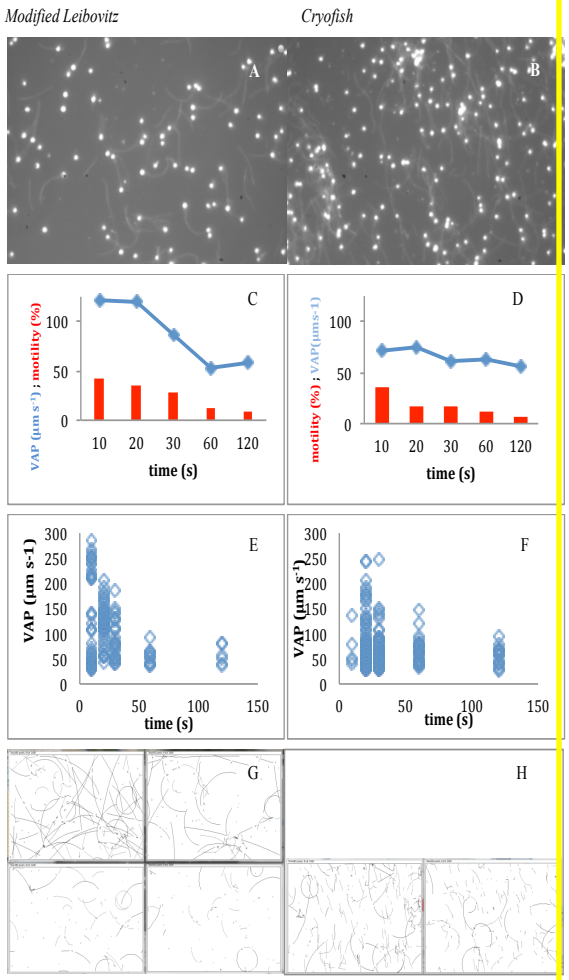


Test of 2 diluents

- Patented Cryofish
- modified Leibovitz L15 (published formula)







Conclusions :

- Cryopreservation-> efficient with acceptable impairment of motility parameters to be compensated by optimized sperm to egg ratio at fertilization, no significant diff between media.
- Chilled storage-> to be improved

WP7 Reproduction and Genetics – Grey mullet
led by IOLR (Hanna Rosenfeld)

	Year 1 (2014)				Year 2 (2015)				Year 3 (2016)				Year 4 (2017)				Year 5 (2018)			
	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No	Fe	Ma	Au	No
WP7 Reproduction & genetics-grey mullet	3	6	9	12	15	18	21	24	27	30	33	36	39	42	44	48	51	54	57	60
Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development (IOLR)				D		D		D												
Task 7.2 Development of hormone-based treatments for inducing spawning (IOLR)								D												
Task 7.3 Optimization and scale-up a breeding protocol for grey mullet in captivity (IOLR)																				D
Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish (IOLR)																		D		
Task 7.5 Establish a shipping protocol for grey mullet eggs (DOR)								D												

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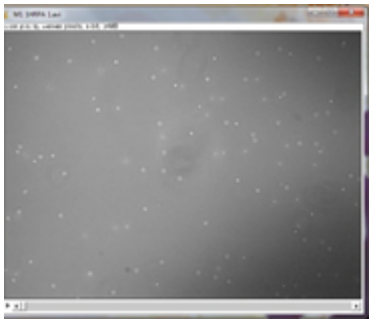
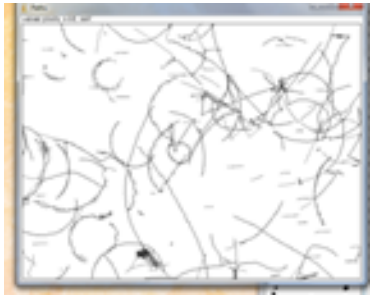
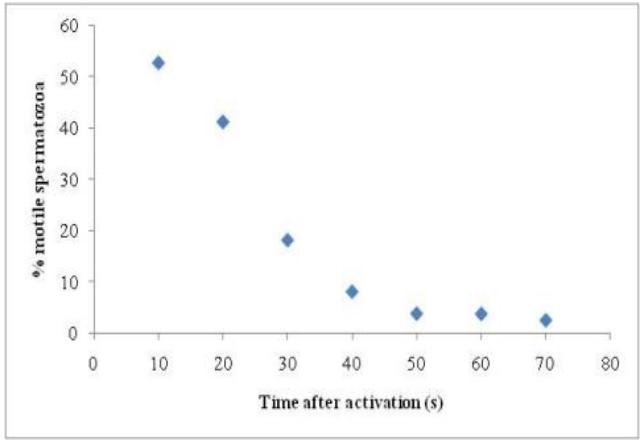
DIVERSIFY

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development

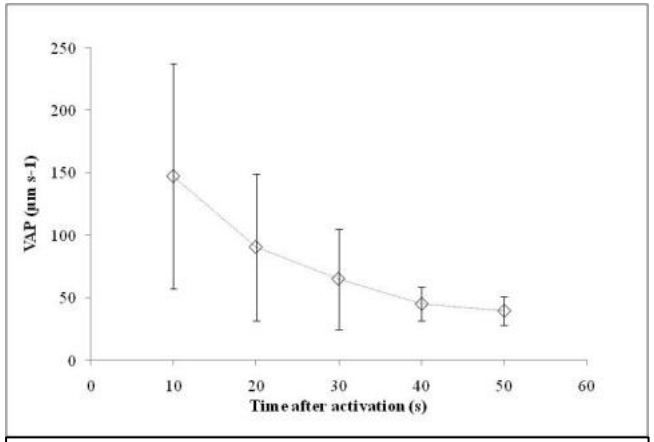
Objective 1- Establishment of a Computer Assisted Sperm Analysis (CASA) for an objective evaluation of sperm quality of male grey mullet sperm subjected to different hormone-based treatments.







Sperm motility variation assessed by % of motile sperm with time after activation.



Modification of Average Path Velocity (VAP)

Results so far, indicate that mullet sperm does not present a pattern of motility similar to that of seabass or rainbow trout in terms of velocity as well as duration which lasts less than one minute.

✓ D7.1- Month 12

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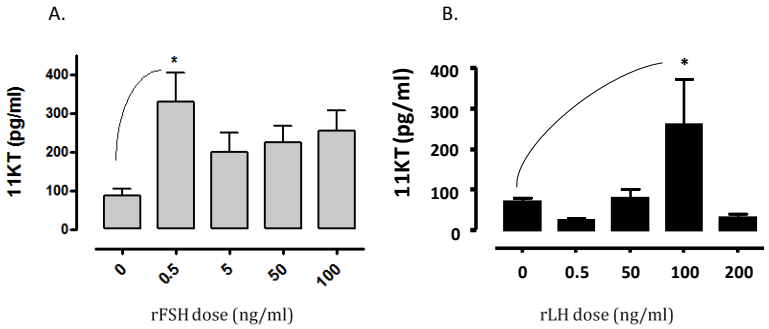
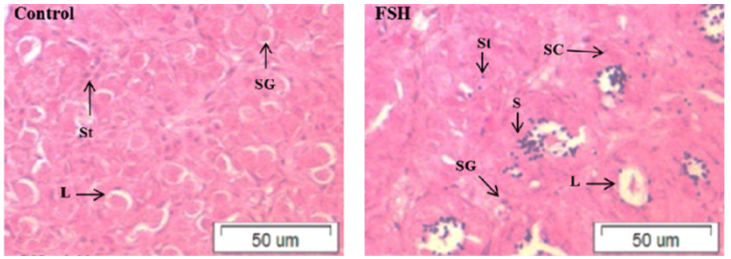
Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development

Objective 2- Production of recombinant bioactive LH and FSH as therapeutic agents to enhance gametogenesis in captive grey mullet broodstock

- Recombinant LH and FSH have been produced using the yeast (*Pichia pastoris*) expression system, and purified on the Ni-NTA affinity column.
- *in vitro*: both, r-FSH and r-LH stimulated 11-KT secretion. Yet, the r-FSH was found to be more effective than the r-LH.
- *in vivo*: 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





✓ D7.2- Month 18

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DIVERSIFY

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development

Objective 3- Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet

Relative abundance of post-vitellogenic females

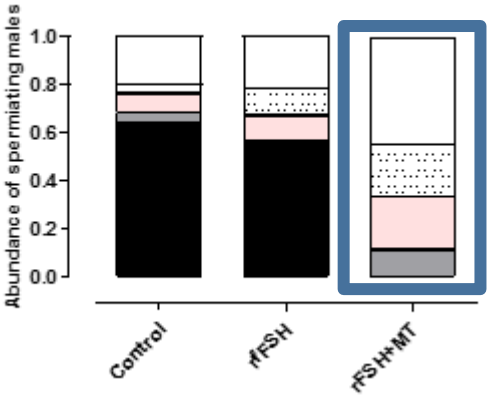
	Control	DA	DA+GnRH	DA+rFSH
Exp. I	27%	58%	71%	
Exp. II	29%			91%

DA-Dopamine Antagonist
rFSH- recombinant FSH

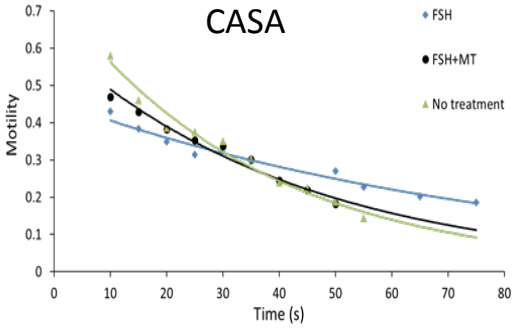
Treatment effect on sperm production

0- no milt,
1- only traces
2- small amounts
3- fluid milt
4- flowing fluid, easily spread in the water

MT- EVAc implant for sustained release of methyltestosterone



Interestingly, relatively high abundance of fully mature females and males (50-70%) could be found in untreated G1 (Control). Probably these broodstocks have begun the process towards domestication.



✓ D7.3- Month 24

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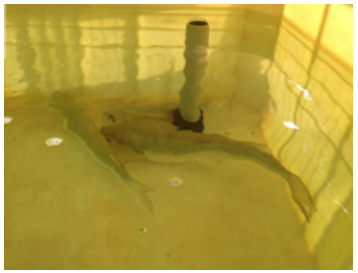
DIVERSIFY

Task 7.2 Development of hormone-based treatments for inducing spawning

Spawning induction protocol: Two consecutive injections consisting of GnRH & DA given 22.5 h apart.

Date	None-primed				Primed			
	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		



- Spawning successes is improved in the pre-treated vs. control groups (42.9% and 29.4%, respectively).
- The overall successful spawns produced 42 million eggs in total.
- Nonetheless, two major problems were highlighted: (i) female's failure to ovulate (70% and 57% in control and hormonally-treated groups, respectively) and (ii) variable fertilization rate ranging between 0 to 100%.

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THE END

Thank for your attention