PROJECT PERIODIC REPORT

Grant Agreement number: 603121

Project acronym: DIVERSIFY

Project title: Exploring the biological and socio-economic potential of new/emerging fish species for the expansion of the European aquaculture industry

Funding Scheme: FP7=KBBE-2013-7-single-stage

Date of latest version of Annex I against which the assessment will be made: 28 March 2014 (Amendment 1)

Periodic report: 1st ☐X 2nd ☐ 3rd ☐ 4th ☐

Period covered: from 0 to 12 mo

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[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

2. Core of the report for the period:
Project objectives, work progress and achievements, project management

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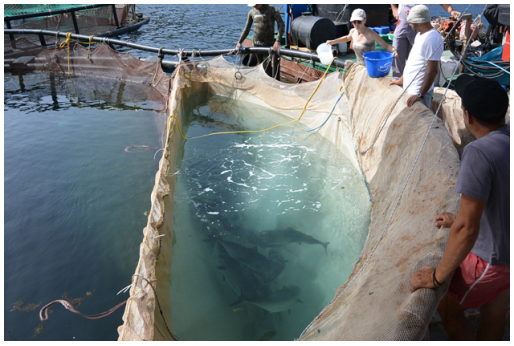
2.1 Project objectives for the period

Please provide an overview of the project objectives for the reporting period in question, as included in Annex I to the Grant Agreement. These objectives are required so that this report is a stand-alone document.

Please include a summary of the recommendations from the previous reviews (if any) and indicate how these have been taken into account.

Reproduction & Genetics



Two objectives to describe the genetic variation of captive meagre and pikeperch broodstocks were initiated and completed during the reporting period as programmed in the DOW. A total of 15 objectives were programmed to start during the period:

- experiments to improve spawning of greater amberjack, Atlantic halibut, wreckfish and grey mullet,
- sampling of tissues and blood to describe the reproductive cycle of greater amberjack and wreckfish,
- development of techniques to aid description of the reproductive cycle for greater amberjack and wreckfish,
- development of techniques to assess maturity status particularly sperm quality for greater amberjack, wreckfish and mullet,
- registration of data to document spawning in wild and F1 Atlantic halibut stocks,
- sampling to assess fecundity for Atlantic halibut; sampling and analysis to describe the genetic variation in wild pikeperch populations,
- development of SNP markers for genetic selection of meagre,
- experiments on paired spawning of meagre to produce families for genetic selection and lastly stocks were established to assess the effects of captivity on first sexual maturity in grey mullet.

One objective on cage spawning of greater amberjack was started already, even though this objective was not planed in the DOW to start during the first reporting period.

Nutrition

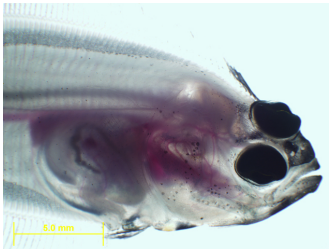
Four types of feeds are used in aquaculture, differing in nutritional and physical characteristics: enrichment products for live preys, dry feeds for weaning, grow out and broodstock diets, all differing in their formulation and production technology. The objectives of DIVERSIFY for the first reporting period focused mostly in first feeding regimes (enrichment products and weaning diets), as described in the DOW, taking advantage of the first production of larvae, whereas the juveniles are being produced this year to conduct most studies with grow-out diets in the following years of the Project. In those species with problems in reproduction, information has been obtained to formulate the first broodstock diets to improve spawning quality. Therefore the objectives this year have been focus on:

- improvement of current larval weaning feeds for meagre,
- improvement of larval enrichment products to enhance production of greater amberjack larvae,
- development of an appropriate broodstock diet to improve reproduction in greater amberjack,

- increase knowledge on the effect of nutrients essential for first feeding of pikeperch,
- determine the influence of broodstock feeds on fecundity and spawning quality of wreckfish, and
- improve enrichment products, weaning, grow out and broodstock diets for grey mullet.

Larval husbandry

In meagre the objective was determining the earliest and most cost-effective weaning period and these studies were initiated. In greater amberjack the objectives were to address the:



- Effects of different feeding strategies on larval performance in intensive systems,
- Development of feeding protocols in mesocosm and semi-intensive systems, and

In pikeperch, the objective was the improvement of larval rearing protocols by using a multifactorial approach. The work will be repeated in the next reporting period, but this meant that there was a delay in other objectives:

- Reduction of cannibalism rate to increase survival and
- Development of industrial protocol to improve larval performance during rearing.

Similarly, the initial wreckfish study was to answer the objectives:

- development of larval rearing protocol based on the most effective prey density, succession of prey type, light regime (intensity and duration), temperature and culture system, and
- description of the ontogeny of digestive system, vision, taste and smell organs in response to larval rearing methods.

In Atlantic halibut, the objective is the improvement of larval survival and quality during early development of Atlantic halibut.

In grey mullet the initial study will answer the objectives:

- investigating environmental and nutritional factors that affect larval rearing, and
- determine when to wean larvae and to feed weaning diet type according DT maturation and the shift from carnivorous to omnivorous feeding.

Grow out husbandry

For meager, the first series of experiments were related to the definition of the causes of the high size variability observed during the pre-growing phase. A second series of trials are related to the definition of the optimum environment (depth of net, light) for the cage rearing of the species.

For greater amberjack during this first period, mostly preparatory activities took place for the implementation of the various trials that will start later during the project. As the rearing of the species is not yet well-defined, larval rearing trials have been performed and methods were defined to ensure availability of the required individuals to perform the trials planned.

For pikeperch, two preliminary experiments were conducted to define better the methodological requirements of the actual trials. The objective of the first one was to determine the sensitivity of pikeperch to a single or repeated emersion stress, and to standardize the physiological and immune analyses since there is limited information on stress response for pikeperch. The second experiment was performed to define the

lethal concentration (LC50) of *Aeromonas salmonicida* that will be used for the disease tests after stress experiments.

Finally for grey mullet the objectives for the period were:

- to collect wild individuals for the implementation of the planned trials, and
- to define the formula for the "improved diet" to be tested.

Fish Health

There were several objectives during this period, to enable future deliverables to be met. For meagre this included:

- Undertaking a first diet experiment to examine whether vitamin D supplementation can ameliorate the effects of granulomatosis,
- Planning for future diet experiments in terms of availability of fish and production of the diets,
- Primer design for cloning meagre immune genes,
- Initial screens for diseases present in cultured fish,
- An experiment to characterise the ontogeny of the immune response, with samples collected at various times post-hatch, and
- First attempts to develop a challenge model.

For greater amberjack the objectives over this period were:

- Testing of a collector device as a means to control monogenean parasite infections,
- Initial screens for diseases present in cultured fish,
- Primer design for cloning amberjack immune genes,
- First attempts to develop a challenge model.

For Atlantic halibut the main objective was to make progress towards production of the VNN capsid protein for future use in vaccination studies.

Socioeconomics

The first Reporting Period included the following objectives, which have been achieved:

- Give insight in the competitive field of and market developments in the European aquaculture market with a focus on the species selected in DIVERSIFY (meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet),
- Assess the obstacles for growth in the current aquaculture production chains and for these selected species,
- Identify market opportunities for future growth of the European aquaculture sector for the selected species,
- Propose a certification framework for the species addressed in DIVERSIF, and
- To analyze and understand overall value perceptions of consumers with regard to cultured fish and the DIVERSIFY fish species in particular, and undertake a value-based segmentation study.

2.2 Work progress and achievements during the period

Please provide a concise overview of the progress of the work in line with the structure of Annex I to the Grant Agreement.

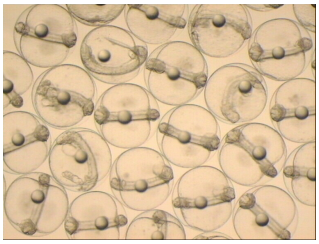
For each work package, *except project management, which will be reported in section 3.2.3, please provide the following information:*

- *A summary of progress towards objectives and details for each task;*
- *Highlight clearly significant results;*
- *If applicable, explain the reasons for deviations from Annex I and their impact on other tasks as well as on available resources and planning;*
- *If applicable, explain the reasons for failing to achieve critical objectives and/or not being on schedule and explain the impact on other tasks as well as on available resources and planning (the explanations should be coherent with the declaration by the project coordinator) ;*
- *a statement on the use of resources, in particular highlighting and explaining deviations between actual and planned person-months per work package and per beneficiary in Annex 1 (Description of Work);*
- *If applicable, propose corrective actions.*

Group Work Packages

Reproduction & Genetics

The objective to evaluate the genetic variation in the available captive broodstocks of meagre was completed (Task 2.1). A total of 432 breeders were sampled from broodstocks in 13 centers and 7 countries and studied with 18 microsatellite markers (STRI & SRTS). In general terms the broodstocks had adequate genetic variation, however, necessary strategies were proposed to avoid inbreeding. In Task 2.2, two experiments facilitated the development of protocols for paired spontaneous spawning to produce families for breeding programs. Spawning efficacy was 26 (58%) pairs spawned out of a total of 45 and egg quality was variable. Individual pairs had the potential to be induced to spawn, up to 17 times, eggs of good quality >85% mean hatching and larval survival 5 days post hatch. In task 2.5, high quality RNA has been extracted from muscle and liver and sent for sequencing.



A total of six greater amberjack broodstocks (~140 individuals) were established. Spawning experiments achieved good quality spawns (>75% mean hatching) from spontaneous natural spawning in tanks and GnRHa induced spawning in tanks and cages. These results will be improved and protocols adapted for tasks 3.2 and 3.4. In task 3.1 wild greater amberjack were sampled and work started for the description of the reproductive cycle and to develop the necessary hormonal assays.

The objective to evaluate the genetic variation in domesticated broodstocks of pikeperch was completed (Task 4.1). Over 400 breeders were sampled from 6 countries and studied with 11 microsatellite markers. In general terms the broodstocks had adequate genetic variation and were clustered by stock origin. For Task 4.2, samples from wild populations have been collected and analyzed and the results are being processed.

Work on the reproductive performance of wild-caught Atlantic halibut is being collected in Task 5.1. However, more data is needed for F1 fish that have poorer and less frequent ovulations, and more data will be collected over a second reproductive period. A preliminary study on the use of GnRHa implants, to induce spawning was made with F1 breeders. An implant of 50 µg GnRHa per kg was sufficient to induce final maturation and ovulation. The GnRHa implanted female Atlantic halibut ovulated earlier and more eggs were stripped than from sham-implanted (control) females. This trial will be scaled up and validated in 2015. In Task 5.3 samples have been taken for analysis of fecundity regulation.

Three juvenile potential wreckfish breeders were collected and acclimatized to captivity in Task 6.1. To describe the reproductive cycle for task 6.2, two captive broodstocks and >60 wild fish were sampled. Sampling mature captive fish will continue, to ensure samples relevant to different stages of gametogenesis are obtained. In Task 6.3, a total of four stocks were monitored for spawning. Two stocks matured and eggs were obtained from spontaneous natural spawning, GnRHa induced spawning and *in vitro* fertilization. However, few fertilized eggs were produced and no eggs were provided for larval culture. Work will progress aimed to ensure fertilized eggs are obtained, with a focus on improving spontaneous spawning and *in vitro* fertilization. Preliminary work described the characteristics of wreckfish sperm in Task 6.3. The wreckfish sperm had the highest reported velocity for a marine fish and duration of motility of 5 min.

The methylotrophic yeast (*Pichia pastoris*) expression system was used to produce large quantities of bioactive recombinant single-chain FSH. In an experiment, hormonal treatments (injected with recombinant FSH and metoclopramide) synchronized gonadal development within and between sexes, and increased the percentage of spermiating males and post-vitellogenic females. These breeders were used in Task 7.2 in spawning induction trials with GnRHa and metoclopramide. Successful spawning was obtained producing millions of fertilized eggs. However, two problems were encountered: (i) female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) variable fertilization ranging between 0 and 98%, underlining the need to further fine tune and optimize the hormone-based breeding protocol for captive grey mullet.

WP2 Reproduction & Genetics - meagre

WP No:	2	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Reproduction and Genetics - meagre			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P3. IRTA	P14. IFREMER
Lead Scientist preparing the Report (WP leader):	Neil Duncan			
Other Scientists participating:	Juan Manuel Afonso (P2), Costas Tsigenopoulos (P1), Christian Fauvel (P14), Constantinos Mylonas (P1)			

Objectives

1. Evaluate the genetic variation in the available captive broodstocks of meagre,
2. Genetic characterization of fast and slow growers,
3. Development of tools that facilitate the implementation of genetic selection programs,
   a. Develop protocols for the paired crossing of breeders with spontaneous spawning,
   b. Describe sperm quality and cryopreservation techniques,
   c. Develop *in vitro* fertilization protocols to provide planned genetic crosses,
   d. Develop a set of SNP markers for genetic selection and stock characterisation.

Summary of progress towards objectives and details for each task:

All tasks planned for this period have started and made good progress. **Task 2.1 Evaluation of the genetic variation in captive meagre broodstocks** has been completed with the associated deliverable D2.2. Over 432 breeders were sampled from broodstocks in 13 centres and 7 countries and studied with 18 microsatellite markers (STRI & SRTS). The broodstocks originated from 3 populations or groups. One broodstock that is held in Turkey was uniquely different from all other broodstocks. The other, 12 broodstocks originated from two populations or groups. As a whole, the combined broodstocks appear to have sufficient variation for breeding program(s). However, the majority of broodstocks appear to require an increase in the number of families for a breeding program. New families or stocks could be obtained between centres or from the wild. However, care is required as many broodstocks had the same population of origin and sample size was small from each broodstock. Further information on number of families available in each broodstock is needed to define more precisely the needs to establish breeding program(s). **Task 2.2 The development of protocols for paired crossing in spontaneous spawning** has shown that successful paired spontaneous spawning is possible. Efficacy of spawning was 26 (58%) pairs spawned out of a total of 45 and the majority, 23 of these pairs produced >100,000 hatching eggs. Four pairs that were induced repeatedly each week spawned multiple times for up to 17 weeks with high (>85%) mean hatching and larval survival 5 days post hatch. In 2015, this work will be completed with two experiments in two partners, each with 4 pairs to produce 16 families / half-sib families and aimed to increase spawning efficacy. **Task 2.5 Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers** started with the sampling of 16 individual meagre coming from 5 families (formed by 10 breeders). High quality RNA has been extracted from muscle and liver and sent for sequencing. All other tasks are programmed for later in the project as specified in the DOW.

Task 2.1 Evaluation of the genetic variation in captive meagre broodstocks (led by FCPCT, Juan Manuel Afonso).

MATERIALS AND METHODS

Samples

A total of 432 samples of meagre was collected, thanks to the collaboration of private companies and public institutions (**Fig. 2.1.1**) from Spain (SP-01 is P3.-IRTA; SP-02; SP-03 is LIMIA; SP-04 is IFAPA; SP-05 is P2.-FCPCT) Portugal (PO-01 is IPMA), France (FR-01), Italy (IT-01), Greece (GR-01; GR-02 is P1.-HCMR), Cyprus (CY-01; CY-02 is DFMR) and Turkey (TU-01). Spain was the country with the highest number of samples (174), followed by Greece (86), with a mean of 33 samples per institution. Only two localities sent samples from two consecutives generations, GR-01 and GR-02. All broodstocks have been used or acclimated as breeders to produce fingerlings.

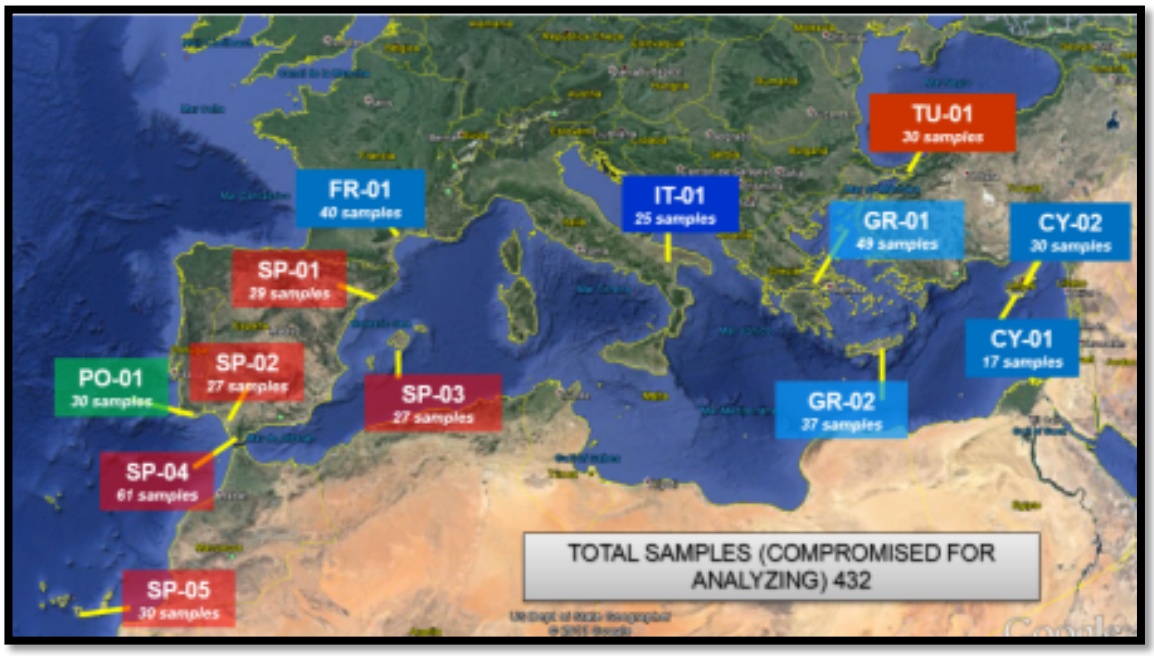


Figure 2.1.1 Geographic distribution of meagre samples in Europe, from the Canary Islands to Cyprus.

DNA extraction

DNA was extracted from the fin clips using the DNeasy kit (*Qiagen*). Some problematic DNA samples were extracted following the phenol-chloroform method described in Sambrook *et al.* (1989). DNA quality and quantity were determined using a NanoDrop 1000 spectrophotometerv.3.7 (*Thermo Fisher Scientific*). DNA integrity was checked by means of electrophoresis in a 1% agarose gel (8 v/cm) by GelRed™ staining (3X solution), and analysed with Quantity One software (*Bio-Rad Laboratories*), using Lambda Hind III as a molecular weight marker.

DNA quality was also important and when the template DNA was degraded or contained PCR inhibitors, Bovine Serum Albumin (BSA) at 0.8 µg/µl was usedsas adjuvant to promote the multiplex PCR reactions.

Microsatellites

The samples were genotyped with 18 markers that were combined in 2 multiplex reactions, one composed of 10 interspecific microsatellites (STRI) and the other of 8 specific microsatellites (STRS) (Soula *et al.,* 2011) (**Table 2.1.1**).

Table 2.1.1 *Loci* names, fluorochromes, redesigned primer sequences, primer concentration and original reference.

Locus	Fluorochrome	Redesigned forward primer sequence (5'→3')	Redesigned reverse primer sequence (5'→3')	C (µM)	Original Reference
Meagre-STRI					
Cacmic14	5' 6-FAM	TGTCCTCACTCCTCTTTTTCTTTC	GTTTAAGGCGCATCTCCAGTCTC	0.02	1*
UBA054	5' 6-FAM	CCTTGTGAGAACATTAATTTGGATG	GTTTCCAAACCCTGATAGATGGATAGTT	0.02	2*
UBA050	5' 6-FAM	GCACAACTGCATCCCTTAGAT	GTTTAGAAGTGAAGACTGCGGACTG	0.05	2*
UBA053	5' VIC	TACTTCCTTCTACCCCTAAGTCTGG	GACTTTCCAGTGTAGCTGTCGTTT	0.05	2*
Soc431	5' VIC	GTGGTAGATGAAAACGTATAAAAGGAG	GTTTCATATATATAGTGTACAGCTCCAGCTTC	0.06	2*
UBA042	5' NED	TTTCTGCCTGACTAGATGTTCTTTC	GATTGTTGCTGGTTTTTCCAAT	0.05	2*
UBA853	5' NED	CAATGCTCAAGTTACAGGAAACC	GTTTGCACTCGTTCACCCTCAC	0.02	2*
UBA005	5' NED	CATCAGGATTGGCAACTAGC	GTTTCCTCCAGGTTTATTCTTCATTGAC	0.03	2*
Soc405	5' PET	AGCCTTTTGTTTAGTTTCCCTCAT	GGGGTGTAGCAGAACCACAC	0.03	2*
UBA006	5' PET	AGCACACGTAATCACACACAGAT	GTTTCCACTAGTGCAAAACGGTGGT	0.03	2*

Locus	Fluorochrome	Redesigned forward primer sequence (5'→3')	Redesigned reverse primer sequence (5'→3')	C (µM)	Original Reference
Meagre-STRS					
GCT15	5' 6-FAM	ATCCGGGCGTTACTACAGTC	GTTTCTCCACACAGTGCTTTTCAGA	0.02	3*
GA16	5' 6-FAM	CTACACAGTCTCTCTCACTCACTCG	GTTTCTGAAACAGCGCAGCATTG	0.02	3*
GA17	5' 6-FAM	CTAGAGAAATTCATCCAGGGAAGTG	GTTTAGAGCAGAGAGTTAGCGGTTGTT	0.015	3*
CA13	5' VIC	TTTTCCTTTTTCAGTAGTCTCCTTG	GTTTATAAGGAGGACGTGAGTTTGGTAG	0.035	3*
GA6	5' NED	GTCTGATGGCGACAGACAGG	GTTTCAGCCCGCTACTTTACCTACAAC	0.02	3*
CA3	5' NED	AAGTGGAGGCTCTTACATGAAAAC	GTGACAAATTGCCTTCTGTTTCTAC	0.03	3*
CA14	5' NED	ACTGAGAGTGAAGGTGGGAAACT	GTGAGTGTCTTTGTTTTTACCAACC	0.03	3*
GA2B	5' PET	AAGTGTGGCGTCATTTCCTCT	GTATTGATGGATAGCAAGTGTCAGA	0.05	3*

C (µM) = Concentration of each primer.
1* Farias *et al.,* 2006
2*Archangi *et al.,* 2009
3*Porta *et al.,* 2010

PCR conditions

Multiplex PCRs were amplified following the recommendations of Soula *et al.* (2011). The PCR conditions consisted of an initial denaturalization at 94°C for 10 min, followed by 30 cycles at 94°C for 30 s, 60°C for 1 min and 65°C for 1 min, with a final extension of 65°C for 60 min. Reactions were carried out in a final volume of 12.5 µl with the following component concentrations: 1X GeneAmp PCR Buffer II (100 mM

Tris–HCl pH 8.3, 500Mm KCl) (*Applied Biosystem*®), 3mM $MgCl_2$, 0.2 mM of each dNTP, 0.05U/µl AmpliTaq Gold DNA polymerase (*Applied Biosystem*®), 10-40 ng of DNA template and 0.01–0.06 µM of each primer. The samples were amplified with STRI and STRS multiplexes in order to genotype each locus, to test the genetic variability of all broodstocks sampled.

Before running multiplex reactions on an automatic sequencer, an aliquot of amplified products was checked on 2% agarose gel for 30 min (8 v cm^{-1}) to assess the correct amplification of amplicons. Subsequently, 1 µl of amplified products was mixed with 9.75 µl of Hi-Di formamide and 0.25 µl of GeneScan 500LIZ (*Applied Biosystem*®) size standard, and run on an ABI Prism-3730-XL Genetic Analyzer (*Applied Biosystem*®) with 50 cm capillary arrays and POP-7 polymer (*Applied Biosystem*®) (60°C, 2000v, 1500s). Electropherograms and genotypes were evaluated using GeneMapper (v4.0) (*Applied Biosystems, Inc*.) software.

Data analysis

To verify the existence of geographical subdivision of the populations studied, an AMOVA molecular variance analysis was conducted (Excoffier et al., 1992) that takes into account the frequency of the genotypes and the distance between them. With this test, the fixation index indicates how much of the total genetic variability of meagre in Europe is due to the variability between the populations analyzed. Thus, the fixation index is the ratio of the added component to the variance between analyzed populations compared to the total variance of the meagre, considering the latter as a single population. All estimates were carried out using the ARLEQUIN program (Excoffier et al., 2005).

To study the genetic variability parameters (allele number, genotypes, heterozygosities, etc.) within loci and populations, the web version of GENEPOP software was used (Raymond & Rousset, 1995; Rousset, 2008). For the biogeographic analysis or structuration of populations, GENETIX 4.05 version was used (Belkhir et al., 1996-2004).

RESULTS

Genetic diversity

In the present study, 13 European breeding populations of captive meagre were analyzed using 18 microsatellite markers (**Table 2.1.2**). In the analysis, broodstocks from GR-01 and GR-02 presented samples of two consecutive generations, which were considered separately, to assess the genetic variation between generations within populations.

Table 2.1.2 Loci and markers equivalence.

LOCUS	MARKER
1	CacMic
2	Soc 405
3	UBA50
4	UBA5
5	UBA54
6	UBA6
7	UBA853
8	UBA42
9	Soc431
10	UBA53
11	CA13
12	CA14
13	CA3
14	GCT15
15	GA16
16	GA17
17	GA6
18	GA2B

The mean number of alleles was 4 for the SP-03 population and mean observed heterozygosity was 0.56, 9.8% higher than expected heterozygosity (**Table 2.1.3**). The mean number of alleles was 5.2 for the SP-01 population, and mean heterozygosity observed was 0.59, 2.2% lower than expected heterozygosity (**Table 2.1.4**).

Table 2.1.3. Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in SP-03 population (Spain)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	54	6	0.88889	0.68903	12
2	56	3	0.42857	0.52922	8
3	56	5	0.82143	0.71429	10
4	56	3	0.82143	0.66558	4
5	54	2	0.29630	0.45283	2
6	56	8	0.92857	0.83377	16
7	56	5	0.67857	0.53442	18
8	54	3	0.29630	0.51992	6
9	46	5	0.52174	0.43575	12
11	56	6	1.00000	0.72857	20
12	56	3	0.96429	0.67273	8
13	56	3	0.07143	0.07078	8
15	50	4	0.56000	0.65551	6
16	56	5	0.78571	0.74870	12
17	56	2	0.03571	0.03571	4
18	56	8	1.00000	0.81883	16
Mean	51.667	4.000	0.56105	0.50587	9.111
s.d.	12.793	2.134	0.35707	0.27871	5.666

Table 2.1.4. Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in SP-01 population (Spain)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	58	8	0.82759	0.83061	16
2	58	4	0.72414	0.69389	10
3	58	6	0.89655	0.79734	10
4	58	4	0.65517	0.59528	6
5	58	2	0.37931	0.48699	2
6	58	7	0.68966	0.65275	18
7	58	7	0.82759	0.60980	20
8	56	3	0.17857	0.28636	6
9	58	6	0.75862	0.64489	14
10	46	6	0.39130	0.77585	18
11	56	10	0.96429	0.87662	24
12	54	4	0.29630	0.67365	10
13	56	3	0.42857	0.36039	8
14	56	2	0.17857	0.16558	3
15	28	5	0.78571	0.65608	14
16	56	4	0.60714	0.62857	10
17	56	3	0.17857	0.16818	6
18	52	10	0.76923	0.86350	22
Mean	54.444	5.222	0.58538	0.59813	12.056
s.d.	7.041	2.393	0.25625	0.21548	6.346

The mean number of alleles was 3.4 for the IT-01 population, and mean heterozygosity observed was 0.55, 9.1% higher than expected heterozygosity (**Table 2.1.5**).

Table 2.1.5 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in IT-01 population (Italy)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	50	5	0.76000	0.67918	12
2	48	3	0.54167	0.43528	10
3	48	3	0.70833	0.61082	8
4	48	3	0.29167	0.26507	4
5	50	2	0.76000	0.50694	2
6	44	5	0.81818	0.72199	14
7	48	4	0.50000	0.41755	20
8	48	2	0.41667	0.38298	6
9	46	4	0.56522	0.61932	10
10	46	3	0.39130	0.57101	18
11	50	5	0.92000	0.70122	20
12	46	4	0.21739	0.46667	10
13	50	3	0.56000	0.42204	8
14	50	2	0.16000	0.15020	3
15	12	3	0.33333	0.62121	10
16	50	4	1.00000	0.67184	10
17	50	2	0.08000	0.07837	4
18	50	5	0.84000	0.72408	8
Mean	46.333	3.444	0.54799	0.50254	9.833
s.d.	8.518	1.066	0.26371	0.18782	5.252

The mean number of alleles was 7.1 for the SP-04 population, and mean heterozygosity observed was 0.53, 11.8% lower than expected heterozygosity (**Table 2.1.6**).

Table 2.1.6. Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in SP-04 population (Spain)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	118	9	0.76271	0.78502	16
2	108	6	0.48148	0.55192	14
3	100	9	0.80000	0.85253	20
4	104	4	0.30769	0.31759	6
5	118	4	0.45763	0.53412	6
6	98	11	0.79592	0.79992	24
7	94	6	0.85106	0.58705	20
8	88	3	0.27273	0.43913	6
9	110	7	0.56364	0.63336	12
10	78	7	0.20513	0.59374	28
11	100	10	0.78000	0.81010	22
12	92	7	0.45652	0.61156	19
13	104	7	0.59615	0.49739	14
14	104	3	0.32692	0.27913	6
15	98	9	0.40816	0.76836	20
16	106	8	0.66038	0.73513	32
17	104	2	0.01923	0.01923	8
18	104	15	0.84615	0.90795	34
Mean	101.556	7.056	0.53286	0.59573	17.056
s.d.	9.488	3.153	0.24000	0.22208	8.657

The mean number of alleles was 6.3 for the SP-02 population, and mean heterozygosity observed was 0.55, 14.2% lower than expected heterozygosity (**Table 2.1.7**).

Table 2.1.7 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in SP-02 population (Spain)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	44	8	0.77273	0.78224	16
2	48	5	0.58333	0.60993	12
3	48	9	0.79167	0.82890	24
4	48	3	0.41667	0.38209	4
5	48	4	0.41667	0.52926	6
6	48	10	0.75000	0.79965	24
7	46	7	0.91304	0.70242	20
8	46	3	0.26087	0.57488	6
9	28	4	0.35714	0.71693	6
10	38	8	0.52632	0.79801	34
11	48	9	0.75000	0.82801	22
12	48	8	0.54167	0.74645	20
13	48	5	0.45833	0.39007	8
14	48	2	0.41667	0.33688	6
15	48	6	0.45833	0.66667	12
16	48	6	0.45833	0.56028	14
17	48	3	0.08333	0.08245	11
18	48	14	0.91667	0.92996	34
Mean	45.889	6.333	0.54843	0.62584	15.500
s.d.	4.965	3.000	0.22023	0.21076	9.155

The mean number of alleles was 2.8 for the CY-02 population, and mean heterozygosity observed was 0.47, 3% higher than expected heterozygosity (**Table 2.1.8**).

Table 2.1.8 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in CY-02 population (Cyprus)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	28	2	0.14286	0.47619	4
2	38	4	0.78947	0.67710	24
3	38	3	0.68421	0.56188	6
4	30	2	0.80000	0.51494	4
5	22	3	0.45455	0.64502	6
6	34	2	0.41176	0.45098	6
7	38	5	0.52632	0.55903	18
8	38	3	0.57895	0.43670	6
9	22	3	0.27273	0.25541	16
10	36	4	0.33333	0.61587	16
11	40	3	0.85000	0.59487	8
12	40	3	0.70000	0.65256	8
13	40	2	0.05000	0.05000	6
15	36	4	0.72222	0.55714	10
16	40	3	0.85000	0.67821	10
18	36	2	0.27778	0.47460	2
Mean	35.333	2.778	0.46912	0.45558	8.333
s.d.	5.774	1.030	0.28873	0.22049	6.263

The mean number of alleles was 3.1 for the CY-01 population, and mean heterozygosity observed was 0.49, 2.8% lower than expected heterozygosity (**Table 2.1.9**).

Table 2.1.9 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in CY-01 population (Cyprus)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	26	4	0.61538	0.76615	8
2	26	2	0.46154	0.51692	6
3	32	4	0.75000	0.62702	16
4	24	3	0.25000	0.23551	4
5	30	2	0.66667	0.51494	2
6	28	2	0.14286	0.13757	2
7	24	4	0.75000	0.54348	18
8	30	2	0.60000	0.43448	6
9	18	4	0.77778	0.75817	10
10	28	3	0.71429	0.55291	18
11	20	5	0.40000	0.51053	22
12	18	3	0.55556	0.69935	10
13	22	4	0.36364	0.46320	10
14	34	3	0.47059	0.60428	6
15	16	3	0.12500	0.67500	4
16	20	2	0.60000	0.44211	10
18	18	4	0.66667	0.68627	14
Mean	24.000	3.056	0.49500	0.50933	9.222
s.d.	5.375	1.026	0.22988	0.20188	6.079

The mean number of alleles was 2.6 for the TU-01 population, and mean heterozygosity observed was 0.37, 0.8% lower than expected heterozygosity (**Table 2.1.10**).

Table 2.1.10 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in TU-01 population (Turkey)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	60	4	0.03333	0.12938	6
2	60	2	0.36667	0.30452	6
3	60	4	0.53333	0.54350	8
5	54	3	0.18519	0.43396	4
6	60	5	0.96667	0.80395	10
7	60	3	0.96667	0.52373	18
8	52	2	0.23077	0.48265	6
9	54	2	0.40741	0.49825	4
11	60	2	0.60000	0.47232	8
12	58	2	0.10345	0.49909	2
13	60	3	0.90000	0.51808	12
14	60	2	0.30000	0.38136	3
15	44	3	0.04545	0.21247	6
16	60	3	0.53333	0.48418	10
18	60	3	0.53333	0.47232	16
Mean	57.889	2.556	0.37253	0.37554	6.611
s.d.	4.188	1.066	0.32370	0.21668	5.024

The mean number of alleles was 2.8 for the SP-05 population, and mean heterozygosity observed was 0.52, 16.4% higher than expected heterozygosity (**Table 2.1.11**).

Table 2.1.11 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in SP-05 population (Spain)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	60	3	0.40000	0.33672	4
2	58	3	0.93103	0.61645	10
3	58	3	0.96552	0.62613	10
4	58	2	0.44828	0.47005	4
5	60	2	0.00000	0.18305	2
6	58	3	0.44828	0.36116	8
7	58	2	0.34483	0.29038	12
8	58	2	0.44828	0.47005	6
9	60	2	0.40000	0.32542	10
10	52	2	0.38462	0.49774	4
11	60	4	0.96667	0.73503	20
12	60	3	0.96667	0.61525	10
13	60	2	0.06667	0.12655	4
15	14	5	0.57143	0.72527	16
16	60	5	0.80000	0.64633	10
17	60	2	0.40000	0.32542	4
18	60	5	0.80000	0.68136	20
Mean	56.333	2.833	0.51901	0.44624	8.556
s.d.	10.440	1.167	0.31445	0.20997	5.610

The mean number of alleles was 2.6 for the GR-01-F1 population, and mean heterozygosity observed was 0.45, 0.8% higher than expected heterozygosity (**Table 2.1.12**).

Table 2.1.12 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in GR-01-F1 population (Greece)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	28	3	0.64286	0.59524	8
2	28	3	0.71429	0.62698	10
3	28	3	0.50000	0.56878	8
4	26	2	0.38462	0.50769	4
5	26	2	0.23077	0.40923	2
6	28	2	0.42857	0.34921	6
7	28	3	1.00000	0.58201	18
8	28	2	0.28571	0.25397	6
9	26	3	0.53846	0.42769	10
10	26	3	0.30769	0.55077	12
11	28	4	0.42857	0.58466	20
12	16	2	0.12500	0.45833	8
13	28	3	0.92857	0.57407	8
15	28	3	0.42857	0.36243	4
16	28	4	0.85714	0.75397	10
18	28	2	0.35714	0.49471	2
Mean	26.889	2.556	0.45322	0.44999	7.556
s.d.	2.767	0.831	0.28232	0.19562	5.315

The mean number of alleles was 3 for the GR-01-F2 population, with a mean heterozygosity observed of 0.39, 3.8% lower than expected heterozygosity (**Table 2.1.13**).

Table 2.1.13 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in GR-01-F2 population (Greece)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	52	5	0.57692	0.54299	8
2	50	3	0.72000	0.55429	10
3	50	4	0.68000	0.51592	10
4	50	3	0.36000	0.36490	6
5	44	3	0.22727	0.66490	6
6	48	3	0.29167	0.25975	8
7	52	4	0.50000	0.45400	20
8	52	3	0.11538	0.18175	12
9	38	3	0.31579	0.28023	14
10	34	2	0.17647	0.50802	4
11	68	4	0.41176	0.45698	20
12	54	2	0.07407	0.49196	8
13	68	3	0.97059	0.54917	8
15	66	3	0.24242	0.22191	4
16	68	4	0.79412	0.72388	10
18	56	3	0.53571	0.47857	20
Mean	54.778	3.000	0.38846	0.40273	9.333
s.d.	10.459	1.000	0.27401	0.20027	5.925

A comparison of the two consecutive generations of GR-01 population shows that F2 reported a lower observed heterozygosity than F1, as normally expected (15% lower). By contrast, the number of alleles in F2 was slightly higher than F1 (17%). The mean number of alleles was 3.4 for the GR-02-F1 population, and mean heterozygosity observed was 0.47, 7.2% lower than expected heterozygosity (**Table 2.1.14**).

Table 2.1.14 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in GR-02-F1 population (Greece)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	40	6	0.70000	0.70128	12
2	38	3	0.26316	0.24324	10
3	38	3	0.57895	0.56188	8
4	38	3	0.31579	0.55619	4
5	36	2	0.50000	0.51270	2
6	38	4	0.47368	0.66714	18
7	38	3	0.78947	0.51067	18
8	38	2	0.31579	0.50071	6
9	36	3	0.38889	0.33810	10
10	38	2	0.10526	0.27312	4
11	32	5	0.68750	0.75605	20
12	22	5	0.54545	0.77922	12
13	32	2	0.68750	0.46573	8
14	32	2	0.06250	0.06250	3
15	32	3	0.62500	0.65927	4
16	32	4	0.75000	0.68347	14
17	32	2	0.12500	0.12097	4
18	30	8	0.60000	0.73333	22
Mean	34.556	3.444	0.47300	0.50698	9.944
s.d.	4.310	1.606	0.22389	0.21235	6.133

The mean number of alleles was 2.4 for the GR-02-F2 population, and mean heterozygosity observed was 0.49, 12% higher than expected heterozygosity (**Table 2.1.15**).

Table 2.1.15 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in GR-02-F2 population (Greece)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	28	4	0.85714	0.75926	12
2	32	3	0.25000	0.28427	10
3	32	2	0.25000	0.22581	6
4	30	2	0.46667	0.50805	4
5	32	2	0.43750	0.49798	2
6	30	4	0.73333	0.68276	18
7	32	2	1.00000	0.51613	18
8	32	2	0.06250	0.51411	6
9	26	3	0.61538	0.64308	10
10	34	2	0.23529	0.21390	4
11	28	3	0.57143	0.50000	20
13	32	2	1.00000	0.51613	8
15	24	4	1.00000	0.68841	6
16	32	3	0.56250	0.59879	10
18	24	4	0.83333	0.77174	18
Mean	28.111	2.444	0.49306	0.44002	8.556
s.d.	7.438	1.117	0.35008	0.25053	6.282

A comparison of the two consecutive generations of the same population shows that F2 unexpectedly reported a higher observed heterozygosity than F1 (4.2% lower). By contrast, the number of alleles in F2 was lower than F1 (40%), as expected.

The mean number of alleles was 5.3 for the FR-01 population, and mean heterozygosity observed was 0.43, 17% lower than expected heterozygosity (**Table 2.1.16**).

Table 2.1.16 Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in FR-01 population (France)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	56	7	0.85714	0.77078	14
2	56	4	0.67857	0.54026	10
3	58	8	0.58621	0.73382	26
4	56	3	0.46429	0.53247	4
5	50	6	0.40000	0.56980	12
6	60	8	0.63333	0.58814	20
7	60	5	0.23333	0.21921	18
8	58	4	0.13793	0.50393	9
9	22	6	0.27273	0.73160	37
10	48	6	0.41667	0.73050	18
11	80	8	0.80000	0.75570	20
12	70	5	0.57143	0.57888	14
13	78	4	0.28205	0.25175	8
14	78	3	0.05128	0.05095	6
16	80	4	0.72500	0.73323	10
17	80	2	0.02500	0.02500	4
18	78	12	0.66667	0.81785	34
Mean	59.556	5.333	0.43342	0.50744	14.667
s.d.	19.928	2.560	0.26614	0.26811	9.792

The mean number of alleles was 1.9 for the SP-03 population, and mean heterozygosity observed was 0.26, 1.1% higher than expected heterozygosity (**Table 2.1.17**).

Table 2.1.17. Number of alleles, allelic range and heterozygosities (observed and expected) on *loci*, in PO-01 population (Portugal)

Locus#	Num. gene copies	Num. alleles	Obs. Het.	Exp. Het	Allelic range
1	60	6	0.93333	0.80056	12
2	58	3	0.48276	0.56564	8
3	60	7	0.93333	0.81695	28
4	58	2	0.20690	0.24198	4
5	56	2	0.17857	0.22273	2
6	60	6	0.66667	0.60960	14
7	58	3	0.55172	0.43981	18
8	60	2	0.20000	0.47232	6
10	32	3	0.56250	0.50202	10
Mean	27.889	1.889	0.26199	0.25953	6.667
s.d.	28.530	2.307	0.32658	0.29478	7.087

Seven populations presented exclusive alleles, SP-04 and SP-02 being the populations with the most exclusive alleles, 6 and 4 respectively, followed by FR-01 with 3 alleles, CY-01 and SP-03 with 2 alleles and PO-01 and SP-05 with only 1 allele (**Table 2.1.18**). On the other hand, SP-02 and SP-04 also presented exclusive alleles versus the remaining of populations for *loci*: SOC405 (116), UBA50 (141), UBA6 (140, also shared with PO-01), CA14 (103, also shared with FR-01), GCT15 (79, also shared with FR-01), GA16 (126), GA17 (78), GA6 (120), GA2B (78).

Table 2.1.18 Alleles exclusive per population and *locus*

LOCUS	MARKER	SP-02	SP-04	CY-01	FR-01		PO-01	SP-03	SP-05
1	CacMic								
2	Soc 405								
3	UBA50	131	135	139	129		127		
4	UBA5								
5	UBA54				66	68			
6	UBA6								
7	UBA853								
8	UBA42								
9	Soc431								
10	UBA53	88	86						
11	CA13	99	95	91					
12	CA14		100						
13	CA3								
14	GCT15								
15	GA16		110						112
16	GA17							80	
17	GA6								
18	GA2B							88	

Mean alleles ranged from 2 to 7, for PO-01 and SO-04, respectively. Close to SP-04 was SP-02, with 6 alleles. As heterozygosities, both arithmetic and weighted estimates were the same (0.48) for observed heterozygosity, while the values were similar for expected heterozygosity, 0.48 and 0.49, respectively.

Table 2.1.19 Total averages for number of gene copies, alleles and observed and expected heterozygosities

	AVERAGES			
POPULATION	Nº GENE COPIES	Nº ALLELES	HET.OBS.	HET.EXP.
PO-01	27.8	1.8	0.261	0.259
SP-01	54.4	5.2	0.585	0.598
SP-02	45.8	6.3	0.548	0.625
SP-03	51.6	4	0.561	0.505
SP-04	101.5	7.1	0.532	0.595
SP-05	56.3	2.8	0.519	0.446
FR-01	59.5	5.3	0.433	0.507
IT-01	46.3	3.4	0.547	0.502
GR-01-F1	26.88	2.5	0.453	0.449
GR-01-F2	54.7	3	0.388	0.402
GR-02-FI	34.5	3.4	0.473	0.506
GR-02-F2	28.1	2.4	0.493	0.44
TU-01	57.8	2.5	0.375	0.375
CY-01	24	3	0.495	0.509
CY-02	35.3	2.7	0.469	0.455
Arithmetic mean		3.69	0.48	0.48
Weighted mean		4.13	0.48	0.49

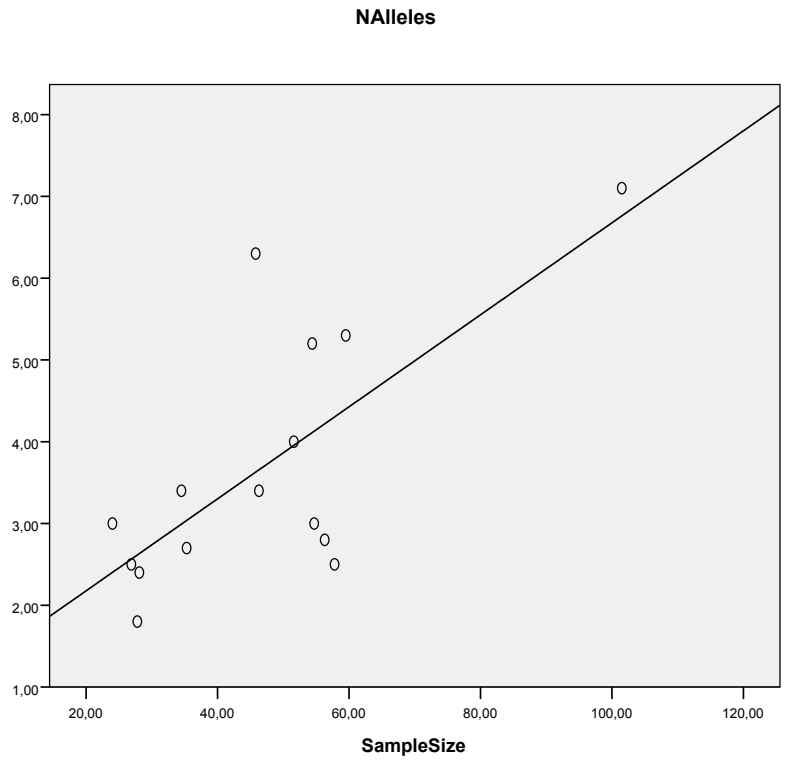


Figure 2.1.3 Relationship between number of alleles and sample size per population studied.

Molecular analysis of Variance (AMOVA)

The AMOVA was run for *loci* 1-8, and it revealed that 18.19% of the variation was found among studied populations, while the remaining 81.82% was located within populations. This partition of variance was highly significant (P<0.0001), both jointly (**Table 2.1.20**) and separately (**Table 2.1.21**), denoting a high differentiation among populations studied and a low gene flow. This fixation index was independent of *loci* (**Table 2.1.21**) and ranged from 0.094 to 0.307 for loci 7 and 4, respectively.

Table 2.1.20 AMOVA for European populations of meagre

Source of variation	Sum of squares	Variance components	Percentage variation
Among populations	358.311	0.47282	18.19220
Within populations	1567.290	2.12622	81.80780
Total	1925.601	2.59905	

Average F-Statistics over all loci
Fixation Indices
FST : 0.18192

Table 2.1.21 AMOVAs in all populations for loci 1-8.

	Among Populations:				Within Populations:				Fixation indices:	
Locus	SSD	d.f.	Va	% variation	SSD	d.f.	Vb	% variation	FST	P-value
1	59.64035	14	0.07853	19.53170	241.68904	747	0.32355	80.46830	0.19532	0.00000
2	72.44379	14	0.09767	27.13830	195.88823	747	0.26223	72.86170	0.27138	0.00000
3	35.87438	14	0.04407	11.59604	251.61908	749	0.33594	88.40396	0.11596	0.00000
4	63.25199	14	0.08802	30.69286	144.88645	729	0.19875	69.30714	0.30693	0.00000
5	31.02726	14	0.04086	14.85683	169.30878	723	0.23418	85.14317	0.14857	0.00000
6	37.82815	14	0.04842	13.55425	226.34431	733	0.30879	86.44575	0.13554	0.00000
7	21.30366	14	0.02671	9.44332	181.19101	735	0.24652	90.55668	0.09443	0.00000
8	36.94136	14	0.04955	18.64083	156.36382	723	0.21627	81.35917	0.18641	0.00000

Effective population size

To estimate the effective size of each population (*Ne*), a mean of several scenarios was considered, in terms of two different mutation rates per generation of microsatellite markers, 4.5 10^{-3} from 8.5 10^{-3} in fish and 6 10^{-4} in higher vertebrates, respectively. Thus, *Ne* estimations (**Table 2.1.22**) were realised from *Theta* estimations for each population, using the stepwise mutation model.

Table 2.1.22 Effective size (Ne) and Theta values (H) per population.

POPULATION	Theta (H)	Ne
PO-01	2,10178	115,48
SP-01	1,58012	86,82
SP-02	1,63525	89,85
SP-03	1,50028	82,43
SP-04	1,57611	86,60
SP-05	1,52339	83,70
FR-01	1,50044	82,44
IT-01	1,50005	82,42
GR-01-F1	1,52021	83,53
GR-01-F2	1,57866	86,74
GR-02-F1	1,50039	82,44
GR-02-F2	1,5292	84,02
TU-02	1,6321	89,68
CY-01	1,5007	82,46
CY-02	1,51591	83,29

The populations of meagre analysed presented a mean effective size (*Ne*) of 86.79, i.e., under an ideal population, there would be 43.4 males and the same number of females. This was higher than the minimum recommended to minimize inbreeding depression (50), but lower than the minimum suggested for maintaining sufficient evolution capacity (500). The coefficient of variation between populations was 3.1%.

Geographic structure

The Factorial Correspondence Analysis (FCA) shows how populations were grouped in three clusters (**Fig. 2.1.4**). Within *group-a*, there are three exclusive populations (SP-03, SP-04, PO-01) from the southern Iberian peninsula. In *group-b*, there are six exclusive populations (SP-5, IT-01, GR-01, GR-02, CY-01, CY-02). *Group*-c, was constituted only by the TU-01 population. Fr-01, SP-01 and SP-02, were presented in *groups a* and *b*. The FCA grouping was in concordance with the geographic distribution of the European meagre populations.

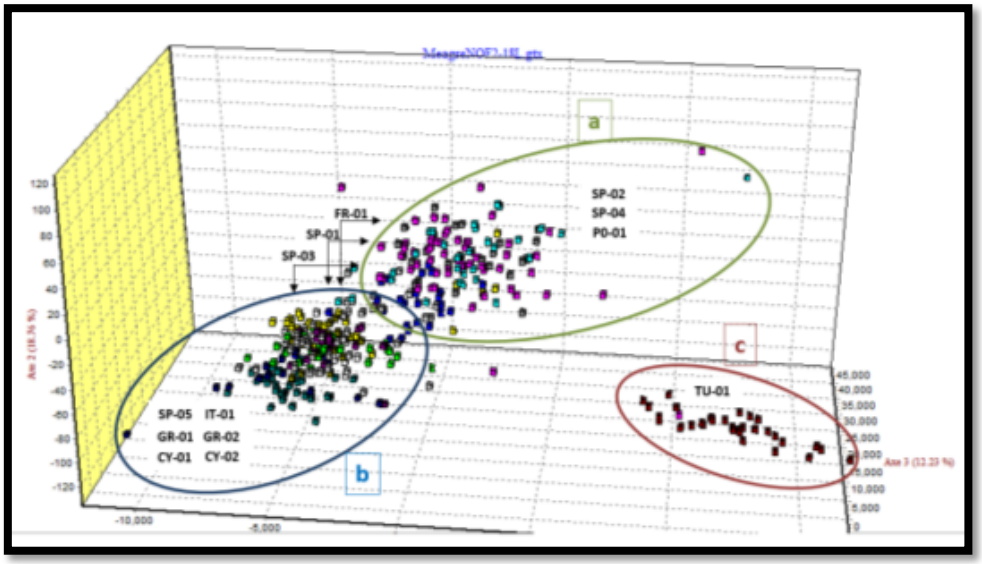


Figure 2.1.4 Graph of Factorial Correspondence Analysis from 18 loci and 376 fish (no F2) distributed in 13 Mediterranean populations of meagre.

Task 2.2 Development of protocols for paired crossing in spontaneous spawning (led by IRTA, Neil Duncan).

Experiment 1 (P3. IRTA) An experiment was set up as described in the DOW, with a total of six pairs selected, females that had ovaries with vitellogenic oocytes >500 µm and males with flowing milt index of 2 or 3 (where 0 = no sperm, 1 = sperm present but not flowing, 2 = sperm flows and 3 = sperm flows easily and abundantly). Breeders were selected from a stock of wild and captivity-bred fish and females had a mean weight of 21.24±3.69 kg and males had a mean weight 16.12 ±2.61 kg. To induce spawning GnRHa (des-Gly10, [D-Ala6]-gonadotropin releasing hormone, Sigma, Spain), was administered to the selected breeders at doses of 15 µg kg^{-1} to females and 7.5 µg kg^{-1} to males. The pairs of breeders were placed in separate tanks 10-16 m^3 with >400 % water exchange, natural photoperiod and temperature (16-21ºC maintained <21ºC) and a surface water egg collector. Every 7-10 days the breeders were sampled, maturity status was determined, breeders in advanced stages of maturity were induced as above and males were exchanged to form a different pair for each induced spawning. Breeders were induced to spawn on 8 dates between the 7th April and the 2nd June 2014. Breeders that did not have advanced stages of maturity were replaced with new breeders and breeders that did not spawn over 2-3 induced spawning dates were replaced. On the 29th April, 4 females and 2 males were replaced and on the 23rd May all females and three males were rejected and three females were introduced to form just 3 pairs. On the 2nd June one pair was rejected to leave just two pairs. On the 9th June the last two pairs were rejected.

Eggs were collected every morning into a 10-l bucket and the number (fecundity) and percentage fertilization was evaluated at the same time by examining each of the eggs in this 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope. In order to monitor embryo and larval survival, eggs from each spawn were incubated in a 30-l egg incubator and placed individually in 96-well plates (in duplicates). The 96-well plates were then placed in a controlled-temperature incubator (17-19ºC) and maintained until all larvae had died. Hatching rate (plates and incubator) and survival at 3 and 5 days post hatch were recorded. Oocyte size varied from 506±96 to 630±63 µm and sperm index from 2 to 3 with 60±20 to 92±11 % motility and motility duration from 1.15 min to 3.5 min. Efficacy of spawning was 22 (53%) pairs spawned out of a total of 41 pairs that were induced. The quantity and quality of eggs amongst the different pairs was highly variable (**Fig. 2.2.1** and **2.2.2**).

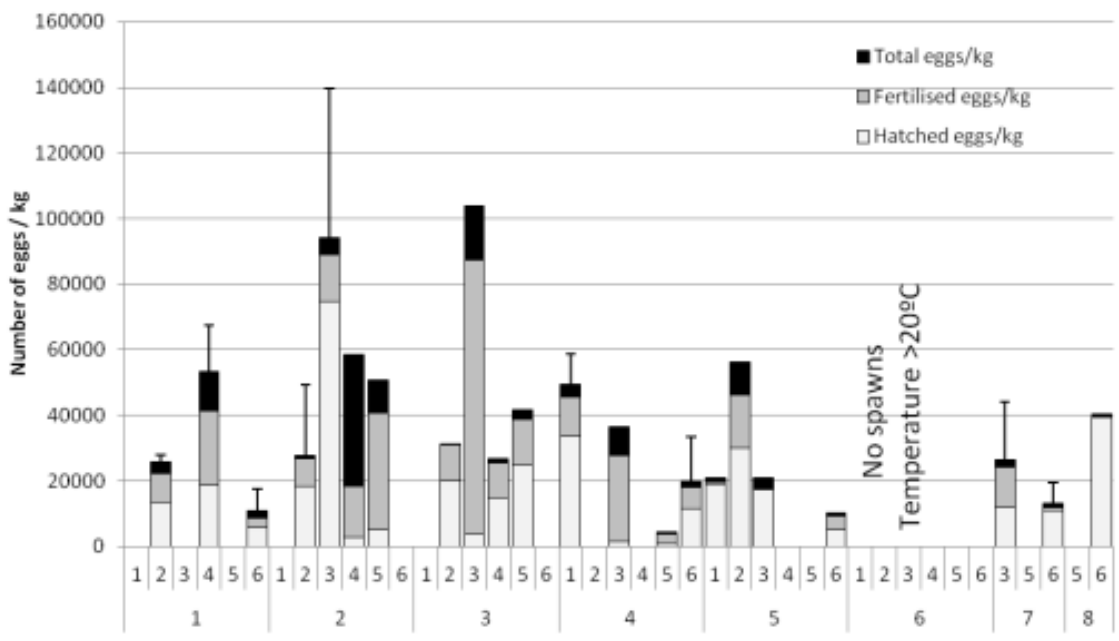


Figure 2.2.1 Mean (±SD) daily number of eggs per kg. Total number of eggs was multiplied by percentage fertilization and hatch to determine number of fertilized eggs and eggs that would hatch.

The success of pairs to produce hatching eggs was 10 (24%) pairs produced a total of >500,000 eggs that hatched, 16 (39%) pairs produced >250,000 eggs that hatched and 19 (46%) pairs produced >100,000 eggs that hatched. Correlations were poor amongst the following parameters a) oocyte size or sperm quality and egg quality parameters, fertilization, hatching and larval survival, b) number of spawn, number of cumulative spawns or number of inductions and egg quality parameters, fertilization, hatching and larval survival. These poor correlations indicated that poor spawning was not caused by maturity status or repeated spawning / inductions.

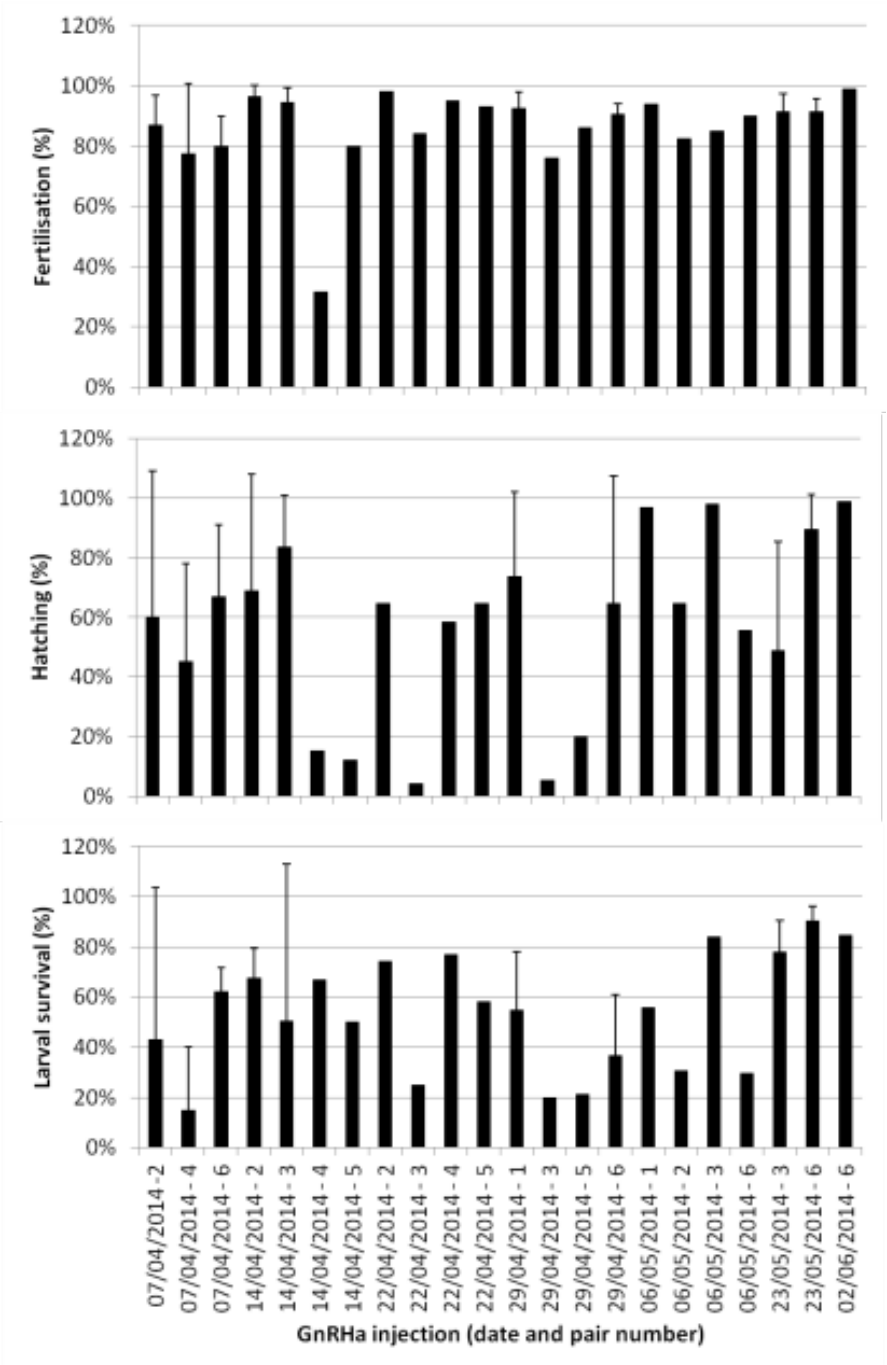


Figure 2.2.2 Mean (±SD) percentage fertilization, hatching and larval survival (5 days post hatching) for eggs obtained from pairs of meagre induced to spawn on 8 dates.

Different individuals had clear differences in production as fecundity and egg quality. One female produced 12 spawns from 5 different inductions with 5 different males. Three females produced significantly higher ($P<0.05$) volumes of eggs and two of these females also had significantly higher ($P<0.05$) survival of larvae 5 days after hatch. Similarly, one male fertilised 13 spawns in six different inductions with six different females and this male with two other males fertilised significantly higher ($P<0.05$) number of eggs than other males. Apart from females and males that produced significantly less eggs of lower quality, one female with oocyte diameters of 561±65 µm did not spawn during three inductions with three different males and one male with a sperm index of 2, percentage motility of 60±20 % and duration of motility of 2 min 2 sec ± 37 sec did not spawn during three inductions with three different females. In addition to this individual variation the efficacy, fecundity and quality varied with time in relation to date or temperature. Mean latency period was negatively correlated (R=-0.76) with mean temperature ranging from 111 ± 31 hours at 16.6 ± 0.3ºC to 48 hours at 18.2ºC. The first spawning induction (7th April) and the last spawning induction (02/06/2014) had an efficacy of 50% successful spawning. On the 15th May when the temperature was > 20ºC there was no successful spawning and when the females were assessed for maturity one week after the induction all females had a low level of maturity with <500 µm oocyte diameter and were replaced. If these three dates and the pairs that involved the non-spawning female and male are removed from the paired spawning experiment with the efficacy of spawning rises to 18 (78%) from 23 pairs.

Experiment 2 (P1. HCMR) An experiment was added to the planned work of Task 2.2, in order to obtain more data on the potential of repeated injections to induce spawning in meagre. The objective of this additional work was to determine how many successful spawns individual females can produce in response to consecutive weekly injections.

Single pairs of fish (one male and one female) were transferred to 5,000-l tanks under simulated natural photoperiod, but controlled temperature ranging between 19 and 20°C. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 µm. Male fish were considered eligible for spawning induction, if they were releasing substantial amounts of sperm upon application of gentle abdominal pressure. Injections of GnRHa were administered once a week (every Monday) between 7 May and 28 August 2014 using four pairs of fish per treatment (n=4). Females (mean ±SD body weight 9.7 ± 1.0 kg) were treated with a GnRHa injection of 15 µg kg^{-1}. Four males (7.9 ± 1.0 kg body weight) were treated at the start of the experiment with 43–57 µg kg^{-1} using a 450-500 µg GnRHa implant for an effective dose of ~50 µg kg^{-1} GnRHa, in order to enhance spermiation. GnRHa implantation of males was repeated at subsequent samplings (at the time of the GnRHa injection of the females), if sperm production was considered low. After treatment with GnRHa, fish were placed in tanks connected to overflow egg collectors and were allowed to spawn. If a female failed to spawn in response to 2 consecutive injections, it was removed and was not considered for the remaining experiment. When a cumulative total of two females (*i.e.*, 50%) failed to spawn in response to 2 consecutive injections, the experiment was concluded, and no further injections were given.

Eggs were collected every morning into a 10-l bucket and their number (fecundity) and fertilization success was evaluated at the same time by examining each of the eggs in this 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope. In order to monitor embryo and larval survival, eggs from each spawn were placed individually in 96-well microtiter plates (in duplicates). The microtiter plates were then placed in a controlled-temperature incubator and maintained for 5 days at 19 ± 0.5°C. Using a stereoscope, embryonic and early larval development was evaluated once a day, recording the number of live embryos 24 h after egg collection (or ~36 h after spawning), hatched larvae (examined ~60 h after spawning) and viable larvae on day 5 after egg collection (~ yolk sack absorption). At 18-20°C, hatching of meagre eggs takes place in 44-56 h.

Mean (± SEM) oocyte diameters at the onset of the study were 590 ± 10 µm, and throughout the study ranged between 550 ± 9 µm and 620 ± 6 µm, with some small, but statistically significant variations (ANOVA, P=0.04). Large numbers of vitellogenic oocytes, could be seen in the biopsies of all four females until week 5, of three females until week 16 and of two females until week 18 --one week after the last

GnRHa injection. One female failed to spawn in response to GnRHa injections on weeks 6 and 7, and was thus not used again for the study. Another female failed to spawn in response to GnRHa injections on weeks 16 and 17, at which time the experiment was concluded.

The GnRHa injected females spawned for up to 17 consecutive weeks, most of the times spawning both on the 2nd and 3rd day after each weekly injection (Fig. 2.2.3). The first spawns obtained 2 d after each injection had significantly higher fecundity compared to the second spawns obtained 3 d after each injection (ANOVA, $P < 0.001$). Overall, there was no significant effect of injection number on mean fecundity (ANOVA, P=0.83), but there was a slight negative linear correlation (n=32, R^2=0.38, $P < 0.01$, not shown) between 2nd spawn fecundity values and GnRHa injection number. Fertilization success was high during the experiment (**Fig. 2.2.3**), without any significant effect of either GnRHa injection week (ANOVA, P= 0.16) or spawn number after each injection (ANOVA, P=0.21), and with the exception of the 2nd spawn of the last GnRHa injection (54%) it was always >80% and most of the times >90%.

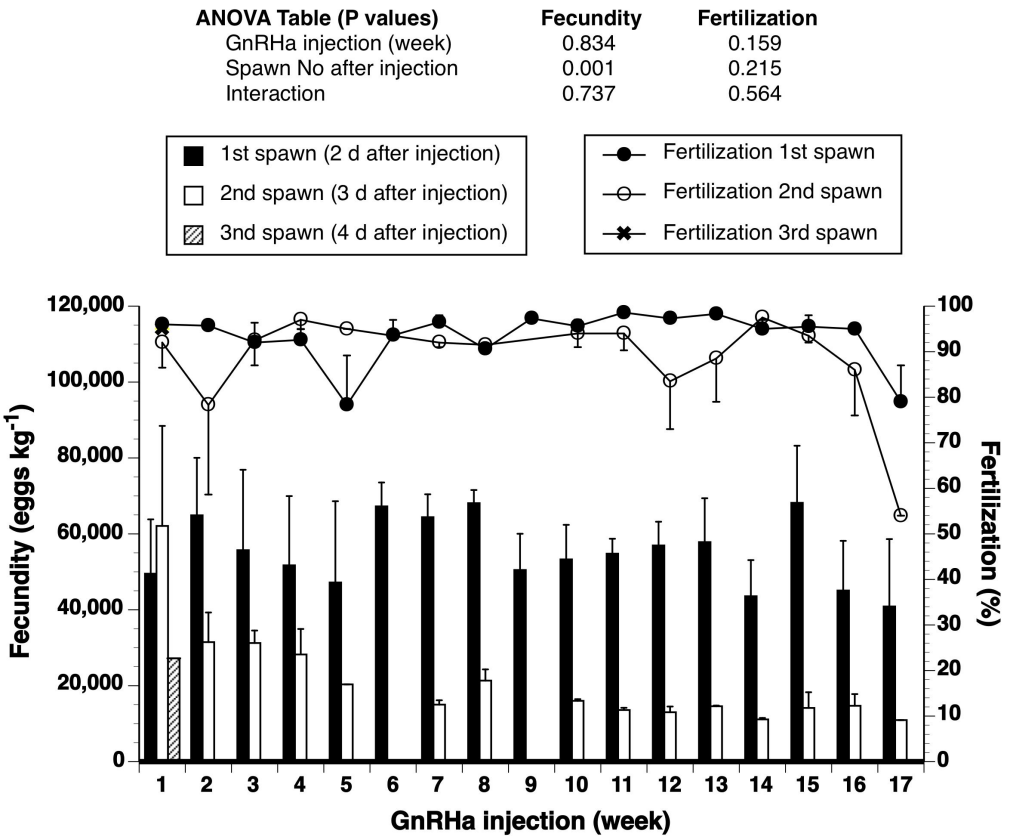


Figure 2.2.3 Mean (±SEM) daily batch fecundity and fertilization of meagre (n=1-4) induced to spawn with GnRHa injections (n=17, once every week) during 2014. The two-way ANOVA (GnRHa injection number vs Spawn number after each injection) indicated the existence of a significant interaction (P=0.001) in fecundity only, while the two main factors did not have any significant effect (either in fecundity or fertilization). Linear regression analysis indicated the existence of a significant negative relation between GnRHa injection number and fecundity for the 2nd spawn data (n=32, R^2=0.37, P =0.001, data not shown).

Embryonic development was very high overall and did not seem to differ significantly between eggs obtained in the first or second spawn after the GnRHa injections (data not shown), in terms of 24-h embryo survival (ANOVA, P=0.54), hatching (ANOVA, P=0.50) or 5-d larval survival (ANOVA, P=0.80). Similarly, there were no significant differences over the course of the study in response to the consecutive GnRHa injections, in terms of 24-h embryo survival (ANOVA, P=0.99), hatching (ANOVA, P=0.88) or 5-d larval survival (ANOVA, P=0.33), even after 17 weekly injections of GnRHa (**Fig. 2.2.4**).

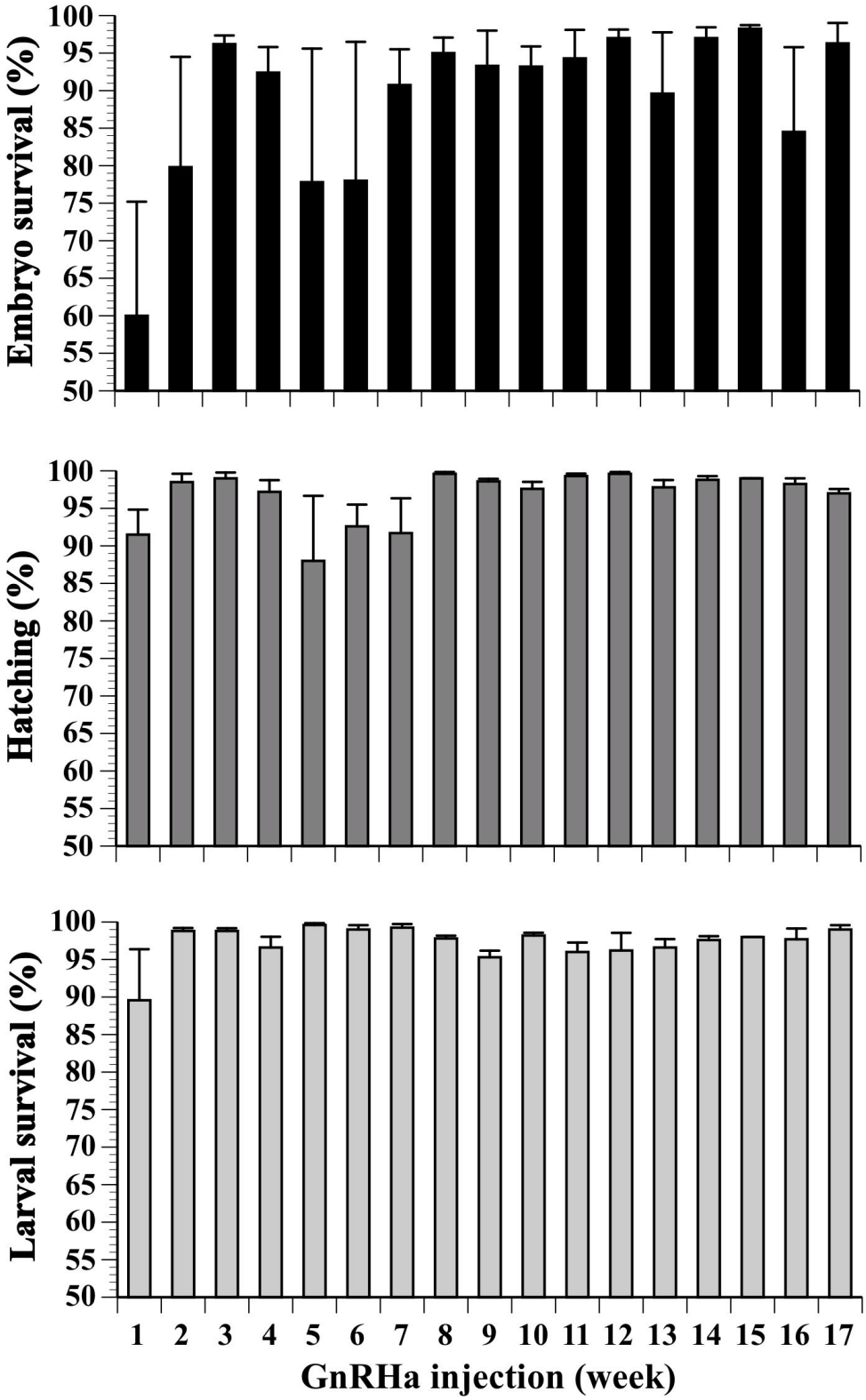


Figure 2.2.4 Embryonic development and larval survival eggs obtained from females (n=4) treated with consecutive weekly GnRHa injections (n=17) for a period of 125 days between May and September 2014. There were no significant differences over the course of the study in response to the consecutive GnRHa injections, either in 24-h embryo survival (ANOVA, P=0.99), hatching (ANOVA, P=0.88) or 5-d larval survival (ANOVA, P=0.33).

This experiment demonstrated that meagre are able to spawn multiple times and in a predictable fashion for up to 17 weeks during the reproductive season to produce large numbers of high quality eggs. Together these two experiments have shown that paired spawning of meagre was possible for the production of known families from parents with known phenotypes. The production of desired families forms the bases of a genetic improvement program and this has been a bottleneck in setting up programmes in some marine species such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). The present studies demonstrate that this is not a bottleneck for meagre and provide a tool for the production of families for a genetic improvement programs. In 2015, this work will be completed with two more experiments each with 4 pairs to produce 16 families / half-sib families. In order to ensure high or improved (in IRTA) efficacy of spawning in the experiments, fish with a proven spawning record will be selected, the trial stared in May and temperature will be maintained between 18-20ºC.

Task 2.3 Description of sperm characteristics and cryopreservation methods (led by IFREMER, Christian Fauvel).

This task will be started in 2015. Deliverable D2.6 due month 36, work is on schedule.

Task 2.4 Development of in vitro fertilization methods for planned crosses (led by IRTA, Neil Duncan).

This task will be started in 2015. Deliverable D2.7 due month 36, work is on schedule.

Task 2.5 Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers (led by HCMR, Costas Tsigenopoulos).

The genetic variation and the identification of Single Nucleotide Polymorphisms (SNPs) were based on sixteen meagre individuals that were selected from four groups of fish (Lset1, Lset2, Sset1 and Sset2, see Table 2.5.1). The groups were from two spawning dates that were one week apart, set 1 consisted of two families spawned on the 24/04/2014 and set2 of three families spawned on the 01/05/2014. The two sets were graded and fish for RNA extraction were selected from the largest (groups "L") and the medium grades (groups "M") in order to have the highest chances to sample from 5 families (1 & 4 plus 2, 5 & 6, respectively). Selection was based on the expected kinship of individuals targeting on fish that are theoretically not closely related. For this purpose, we selected fish from the four groups that contained five different meagre crosses (families) that resulted mostly from wild outbred parents (**Table 2.5.2**). Muscle and liver tissues were dissected and preserved in RNAlater (IRTA, Spain).

Table 2.5.1 Details for the *A. regius* fish used for RNA extraction and transcriptome sequencing from the six families initially formed and their stocking sets (rearing tanks) after size-grading for small (S) , medium (M) and large (L) fish.

		Proportion (%) of each family in each group					
Group	**Number of fish**	**1**	**2**	**3**	**4**	**5**	**6**
(L-set1)	12	38	0	0	62	0	0
(M-set1)	46	61	0	0	39	0	0
(S-set1)	224	51	14	0	33	0	3
(L-set2)	19	0	26	0	0	74	0
(M-set2)	49	0	83	0	0	0	17
(S-set2)	66	0	83	0	0	0	17

Table 2.5.2 Characteristics of the six families formed in *A. regius*, spawning dates, ID number of breeder and origin (wild or cultured) of the breeders used.

Family	Tank (Spawning Date)	Female	Male
1	V8-1 (24/04/2014)	5wild	19wild
2	V8-1 (01/05/2014)	5wild	20wild
3	V8-2 (01/05/2014)	1wild	19wild
4	C2 (24/04/2014)	16cultured	21wild
5	C1 (01/05/2014)	2wild	22wild
6	V6 (01/05/2014)	13cultured	17wild

RNA extraction protocols have been completed for 16 liver and 16 muscle samples. For both tissue types, RNA was extracted after grinding the tissue with liquid nitrogen using pestle and mortar. In the case of liver tissues, total RNA was extracted with Qiagen's RNeasy Plus extraction kit, while muscle tissues were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.) and RNA was isolated according to the manufacturer's instructions. The quantity of the isolated total RNA was measured spectrophotometrically with NanoDrop® ND-1000 (Thermo Scientific), and quality was tested on an agarose gel (electrophoresis in 1.5% w/v) (**Fig. 2.5.1**)

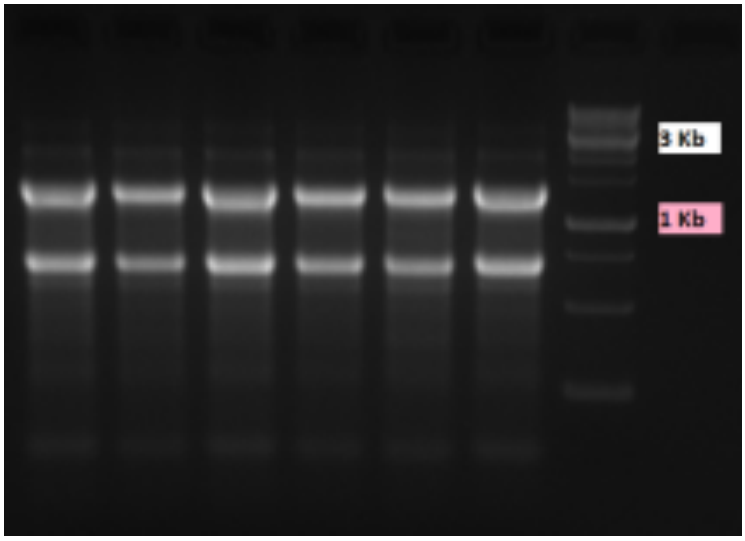


Figure 2.5.1 Total RNA extraction profile from meagre liver tissues. The size marker on the right side of the gel is the 1Kb DNA ladder RTU from Nippon Genetics GmbH.

Following extraction, RNA from different individuals will be pooled and a normalised library will be constructed and sequenced per tissue (one for muscle and one for liver) to increase the efficiency of marker discovery. The library normalisation will lead to a better representation of rare transcripts in the RNA pool allowing for an improved marker discovery. Sequencing will take place onto one lane of a HiSeq2500 instrument (2x100bp) and is expected to result in ~400 million paired reads. Sequencing results are expected to be generated by the end of November the latest, and the subsequent analysis will lead to the discovery of thousands of SNPs and microsatellites in time before Deliverable 2.5 (Month 18). In the meantime, the

bioinformatics pipeline has been set to build the transcriptome of meagre and identify markers. For the transcriptome assembly, the *de novo* assembler Trinity will be employed, while for SNP discovery we will use the well-known package SAMTOOLs. The pipeline set up has taken place at the computer cluster of HCMR.

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Deviations from Annex I and their impact:

An experiment was added to the planned work of Task 2.2, in order to obtain more data on the potential of repeated injections to induce spawning in meagre. The task was undertaken **at no extra cost for DIVERSIFY**, taking advantage of the maintenance during the project of a broodstock by P1. HCMR, which is used to produce eggs and fingerlings for the Larval and Grow out husbandry, and Fish health WPs. The objective of this additional work was to determine how may successful spawns individual females can produce in response to consecutive weekly injections. The results obtained were very encouraging.

WP No:	3	**WP Lead beneficiary:**		P13. UNIBA
WP Title (from DOW):	Reproduction and Genetics – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P4. IOLR	P8. IEO
P14. IFREMER	P15. ULL	P23. ARGO	P24. ITTICAL	
Lead Scientist preparing the Report (WP leader):	Aldo Corriero			
Other Scientists participating:	Constantinos Mylonas (P1), Hipolito Fernandez Palacios (P2), Hanna Rosenfeld (P4), Salvador Jerez Herrera (P8), Christian Fauvel (P14), Covadonga Rodriguez (P15), Tasos Raftopoulos (P23), Fulvio Cepollaro (P24)			

Objectives

1. Describe the endocrine control of reproduction in captive broodstocks, and the nutritional status of fish during the reproductive season,
2. Assess reproductive potential of wild vs. captive amberjack broodstocks and identify possible reproductive/metabolic dysfunctions during gametogenesis,
3. Develop spawning induction methods for captive-reared and F1 broodstocks of both the Mediterranean and Atlantic stocks,
4. Apply the developed spawning induction methods for broodstocks maintained in cages, and examine the efficiency of an egg collector to obtain fertilized eggs,
5. Develop a Computer Assisted Sperm Analysis method (CASA) for the evaluation of greater amberjack sperm during the reproductive season, and evaluate the possible effects of captivity.

Summary of progress towards objectives and details for each task.

Significant progress has been made this first year of the project. Firstly, a large stock of mature wild-caught broodstock has been acquired by three partners (P1. HCMR, P23. ARGO, P24. ITTICAL), resulting in the establishment of six stocks numbering ~140 individuals. Two stocks were maintained in cages (P1. HCMR, P23. ARGO) and three stocks in tanks (P1. HCMR, P23. ARGO, P24. ITTICAL). Even though one stock was eventually lost due to a disease outbreak (P24. ITTICAL), the remaining stocks allowed the implementation of the preliminary spawning induction experiments in the Mediterranean (**Task 3.2**), the egg collection in sea cages (**Task 3.5**) and the supply of eggs to WP 15 Larval husbandry – greater amberjack. In addition, the inclusion of an existing broodstock from P27. FORKYS and the establishment of a collaboration with a commercial producer from outside the consortium (Galaxidi Marine Farms S.A., Greece), has provided us with access to 2 more stocks of ~80 mature individuals for the above experiments.

Excellent progress has been achieved in **Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic**, with both natural and hormone induced spawnings providing large numbers of eggs for WP 15 Larval husbandry – greater amberjack. In **Task 3.5 Spawning induction of greater amberjack and egg collection in cages**, it was shown that it is possible to induce maturation and collect fertilized eggs of high quality from greater amberjack maintained in sea cages in two facilities. Moreover, in **Task 3.1 Description of the reproductive cycle of greater amberjack**, the sampling of wild greater amberjack provided preliminary information on the reproductive cycle of wild

greater amberjack. Full set of samples have been collected and will be delivered to the Partners involved in this task. In **Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean**, preliminary spawning induction experiments underlined main bottlenecks in the control of reproduction of captive-reared greater amberjack, providing precious information for Y2 experiments. In **Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic**, spontaneous and induced spawns from groups formed with females and males F1 greater amberjack broodstock were obtained, even if eggs were not fertilized.

Task 3.1 Description of the reproductive cycle of greater amberjack (led by P13. UNIBA).

Broodstock acquisition. The main purpose of the present task is to study the reproductive cycle of greater amberjack in captivity, in order to understand if confinement could impair reproductive performance of this species. During Y1, a total of 47 wild-caught fish (5-7 kg) were purchased from a farm in Greece (Asterias S.A., Astakos) and transferred to Italy where they were placed in a 30-m^3 tank at P24. ITTICAL (Lesina, Apulia), on 5 April 2014 (36 fish) and 26 April (11 fish). During the first days of adaptation, live European sea bass fingerlings were offered for feeding. After about one month, when fish appeared to be adapted to the new rearing conditions and started feeding regularly, they were fed fresh sardines and squids. Unfortunately, an infestation of the parasite *Amylodinium ocellatum* affected the stock in the farm and, despite the administration of copper-based therapies, all fish died between 30 May and 01 June 2014.

In order to reconstitute the greater amberjack stock, P24. ITTICAL recruited a professional fishing vessel equipped for the capture of pelagic fish in the central Adriatic Sea. On 8 and 25 August 2014, two fishing sets were carried out and a total of 112 adult alive greater amberjack were captured, with body weight ranging between 20 and 30 kg. Part of the fish were harvested, maintained in an oxygenated tank and transported from the place of capture (40 miles from the cost) to the port. Due to the stress of the capture, handling and the long permanence in the tank, at landing the fish appeared to be agonizing and it was not possible to transport them to the farm. Further efforts undertaken by P24. ITTICAL to reconstitute the lost great amberjack broodstock, were not met with success. As an alternative, an additional stock at P23. ARGO will be used for this Task, and will be sampled during the reproductive season in 2015. The stock has been from the same source as the P24. ITTICAL stock, and by 2015 will have been maintained in captivity, under broodstock conditions for >20 months (see further details in Deviations).

Wild greater amberjack sampling. During Y1, wild adult greater amberjack individuals were sampled for comparative studies on reproductive activity with captive-reared fish. On 31 May and 30 June 2014, a total of 17 (11 females and 6 males) greater amberjack were caught with a purse seine by a professional fishing vessels operating in the water around Lampedusa Island, Italy (35°30′58.23″ N, 12°34′40.33″ E; Pelagic Islands, Sicily, Italy) (**Fig. 3.1.1a,b,c**). All the biological samples of wild greater amberjack were collected directly on-board by P13. UNIBA staff. Soon after death, for each fish blood was immediately collected, centrifuged and stocked in dry ice. Subsequently, biometric data (fork length, FL, and total body mass, BM) were recorded, gonads were dissected and weighted (gonad mass, GM in g) and the relative gonadal mass (gonadosomatic index, GSI) calculated as $GSI=100\ GM\ BM^{-1}$ (**Table 3.1.1**).

For each fish, small slices of gonad, liver and muscle as well as brain and pituitary, spines, vertebrae and otoliths were collected and stored following different protocols depending on the analyses for which they were destined (**Fig. 3.1.1d,e,f**). Subsequently, all the material collected was transported to P13.UNIBA laboratories. The samples have been delivered to P1. HCMR and P15. ULL during the Annual Coordination Meeting at the beginning of November 2014. Due to the current restrictions in air transportation, it was agreed to deliver to P4. IOLR only one sample (brain and pituitary from only one greater amberjack specimen) that will be used for cDNA sequencing. The whole sample set will be shipped in specific liquid nitrogen containers using an *ad hoc* procedure.

Figure 3.1.1 Pictures showing wild greater amberjack sampling carried out in May and June 2014 in the Mediterranean Sea (Lampedusa, Sicily, Italy). (a) Fishing vessel targeting greater amberjack. (b) and (c) purse seine used for wild greater amberjack capture. (d), (e) and (f) Biological samples collected on-board.

Table 3.1.1 Biometric data of wild adult greater amberjack specimens caught in May and June 2014 by a purse seine vessel in Mediterranean Sea.

Sampling Area	SST* (C°)	Sampling Date	Fish code	Sex	Fork Lenght (FL; cm)	Total Body Mass (BM; kg)	Visceral Mass (VM; g)	Gonad Mass (GM; g)	Gonado-somatic Index (GSI; %)
Lampedusa - Pantelleria	19.3	31/05/2014	WP3_W1	f	117	21.6	3100	1650	7.64
			WP3_W2	m	124	22.4	2800	1900	8.48
			WP3_W3	f	114	21.0	2900	1600	7.62
			WP3_W4	m	102	13.0	1200	650	5.00
			WP3_W5	m	115	19.2	3000	2200	11.45
			WP3_W6	m	99	14.4	1750	1150	7.98
10 miles east from Lampedusa	23.4	30/06/2014	WP3_W7	f	99	11.0	1000	500	4.54
			WP3_W8	f	100	11.8	900	490	4.15
			WP3_W9	m	100	10.5	1100	400	3.80
			WP3_W10	f	97	11.6	1000	450	3.89
			WP3_W11	f	100	11.8	850	400	3.37
			WP3_W12	f	98	12.0	1270	500	4.15
			WP3_W13	f	96	11.5	850	390	3.40
			WP3_W14	f	102	13.0	1300	600	4.61
			WP3_W15	m	99	10.9	1090	577	5.29
			WP3_W16	f	104	13.6	1650	950	7.01
			WP3_W17	f	95	11.7	1220	450	3.85

*SST= mean Sea Surface Temperature

Biometric data and gonado-somatic index

Mean FL and BM were calculated for wild female and male fish sampled in May and June, and mean GSI was compared by sex in the two sampling month by using one-way ANOVA. Statistical significance was accepted at $P \leq 0.05$ and results presented as mean ± sd.

Females caught in May were 115.5 ± 2.1 cm in mean FL and 21.3 ± 0.4 kg in mean BM, whereas in June they were 99.0 ± 2.9 cm and 12.0 ± 0.8 kg in mean FL and BM, respectively. In May, males were 110.0 ± 11.6 cm in mean FL and had a mean BM of 17.3 ± 4.3 kg; in June they were 99.5 ± 0.7 cm and 10.7 ± 0.3 kg in mean FL and BM, respectively. Mean GSI of females was significantly higher in May than in June ($P \leq 0.05$); a GSI decline, although not statistically significant, was also observed in males between the two months (**Fig. 3.1.2**).

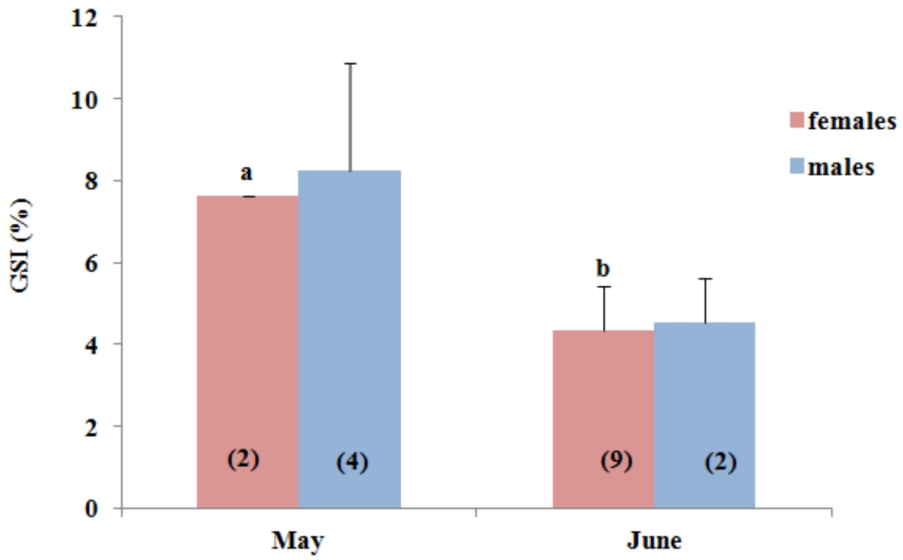

Figure 3.1.2 Mean gonado-somatic index (GSI) in wild female and male greater amberjack sampled in May and June 2014 in the Mediterranean Sea (Lampedusa, Sicily, Italy). In brackets: number of sampled individuals; different letters above column indicate statistical significance (ANOVA, $P \leq 0.05$).

Histological evaluation of reproductive state in wild greater amberjack

In order to evaluate the reproductive condition of wild greater amberjack sampled in May and June, slices of ovaries and testes were cut on-board, fixed in Bouin's solution and transported to P13.UNIBA laboratories for histological analyses. Gonad samples were then dehydrated in increasing ethanol concentrations, cleared in xylene and embedded in paraffin wax. Five-µm-thick sections were cut and stained with haematoxylin and eosin.

Females

The reproductive state of female was assessed by recording the most advanced oocyte stage in the ovary, as reported by Corriero et al. (2007). Minimum and maximum diameters were measured for each oocyte developmental stage. All the measurements were carried out on digital fields photographed with a 5X objective using a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), and performed using an image-analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK).

In the examined sections, oogonia along with the following oocyte developmental stages were observed: chromatin-nucleolus, perinucleolar, lipid, cortical alveoli, early vitellogenesis, late vitellogenesis, hydrated.

Oogonia (**Fig. 3.1.3a**) (diameter 8-13 µm), often found in small clusters, were rounded cells with a large central euchromatic nucleus. Chromatin-nucleolus stage oocytes (**Fig. 3.1.3a**) (diameter 15-30 µm), had a slightly basophilic ooplasm and a large eccentric nucleus showing chromatin strands. Squamous follicular cells were associated to oocytes at this stage. Perinucleolar stage oocytes (**Fig. 3.1.3b**) (diameter 30-120 µm) where characterized by the presence of several nucleoli adjoining the nuclear envelope. Ooplasm basophily was strong in smaller perinucleolar oocytes and decreased with oocyte development. Flat follicular cells surrounded the oocytes at this stage. Oocytes at lipid/cortical alveoli stage (**Fig. 3.1.3c**) (diameter 120-200 µm) showed a further reduction of ooplasm basophily, small lipid droplets and the appearance of a thin *zona radiata*. Oocyte growth was associated to the increase of lipid globules, the appearance of cortical alveoli and zona radiata thickening. Early vitellogenic oocytes (**Fig. 3.1.3d**) (diameter 200-400 µm) were characterized by the appearance of eosinophilic yolk globules in the peripheral ooplasm, increase of *zona radiata* thickness. Follicular cells surrounding oocytes at this stage became cubic. In late vitellogenic oocytes (**Fig. 3.1.3e**) (diameter 400-550 µm) yolk granules progressively filled the entire ooplasm and the zona radiata further increased in thickness.

Hydrated oocytes (**Fig. 3.1.3f**) (diameter ≈ 800 µm) were characterized a remarkable size increase, by an irregular in shape due to the dehydration caused by the paraffin embedding procedure, by the coalescence of yolk granules and lipids and by the detachment of the follicular layer. Atretic vitellogenic follicles (**Fig.3.1.3g**) displayed *zona radiata* and fragmentation, coalescence of yolk globule and nucleus disintegration. Post-ovulatory follicles (**Fig. 3.1.3h**) were convolute structures constituted of hypertrophic follicular cells surrounded an irregular lumen.

The two females sampled in May showed late vitellogenic oocytes follicles as the most advanced stage in the ovaries as well as the presence of post-ovulatory follicles (POFs) and atretic vitellogenic oocytes (**Fig. 3.1.4a**). Seven females sampled in June showed oocytes in late vitellogenesis stage together with POFs, and two of them had also atretic vitellogenic follicles. The remaining two females sampled in June showed hydrated oocytes, sign of imminent spawning (**Fig. 3.1.4b**).

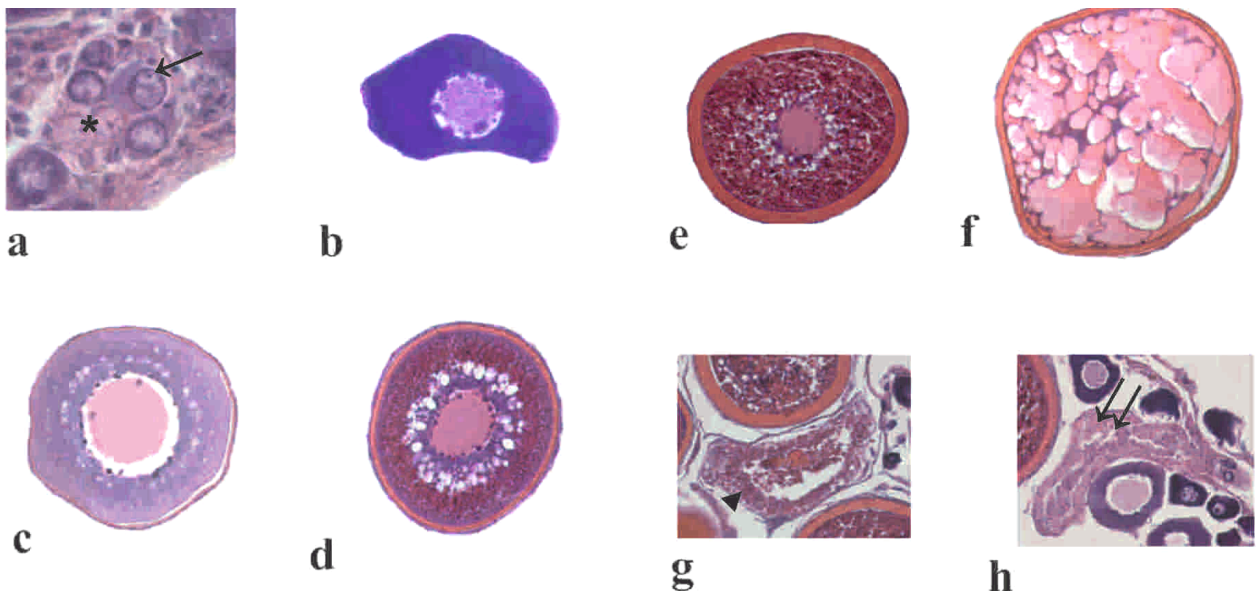

Figure 3.1.3 Micrographs from ovary sections of different wild adult greater amberjack specimens showing germ cells in different developmental stages. a) Oogonia (asterisk) and chromatin-nucleolus stage oocyte (arrow). b) Perinucleolar stage oocyte. c) Cortical alveoli stage oocyte. d) Early vitellogenic oocyte. e) Late vitellogenic oocyte. f) Hydrated oocyte. g) Atretic vitellogenic follicle (arrowhead). h) Post-ovulatory follicle (double arrows).

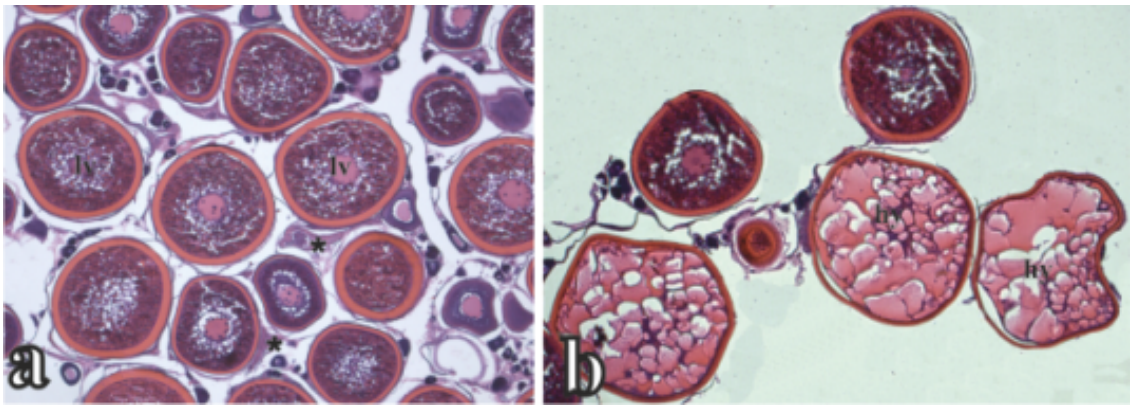

Figure 3.1.4 Micrographs showing ovaries of wild greater amberjack specimens caught in the Mediterranean Sea. (a) Ovary section of a female caught in May showing late vitellogenic oocytes as the most advanced oocyte stage as well as post ovulatory follicles. (b) Ovary section of a spawning specimen sampled in June with hydrated oocytes. Asterisk: post ovulatory follicle; hy: hydrated oocyte; lv = late vitellogenic oocyte.

Males

Male greater amberjack reproductive state was evaluated on the basis of the spermatogenic cyst types and the amount of spermatozoa in the seminiferous lobule lumina, according to Corriero et al. (2007). Seminiferous lobule diameter was measured by using the same equipment described for females. Mean values of seminiferous tubule diameter of specimens sampled in May and June were compared by one-way ANOVA. Statistical significance was accepted at $P \leq 0.05$ and results presented as mean ± sd.

All wild male grater amberjack individuals sampled in May (n = 4) released spontaneously large amount of sperm when they were brought on-board (**Fig. 3.1.5a**). The histological analysis of the testes of these fish showed the presence of all the stages of spermatogenesis in the germinal epithelium as well as a massive presence of spermatozoa in the lumen of seminiferous lobules (**Fig. 3.1.5b**). Males sampled in June (n = 2) released sperm after abdominal pressure. Their testes showed all the spermatogenic stages in the germinal epithelium and a reduced amount of spermatozoa in the lumen of seminiferous lobules.

The diameter of seminiferous lobules was significantly higher in wild fish sampled in May compared to fish caught in June ($P \leq 0.05$) (**Fig. 3.1.6**).

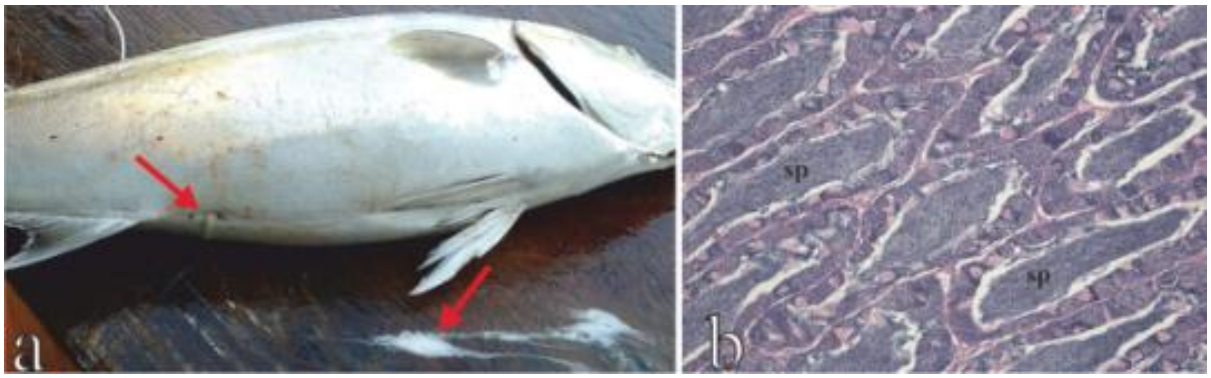

Figure 3.1.5 (a) Wild male greater amberjack caught in the Mediterranean Sea in May 2014 realising sperm spontaneously. (b) Micrograph of a testicular section from a wild male greater amberjack caught in May, showing the presence of all stages of spermatogenesis, as well as spermatozoa in the lumen of seminiferous lobules. Red arrows: sperm; sz: spermatozoa in the lumen of seminiferous lobules.

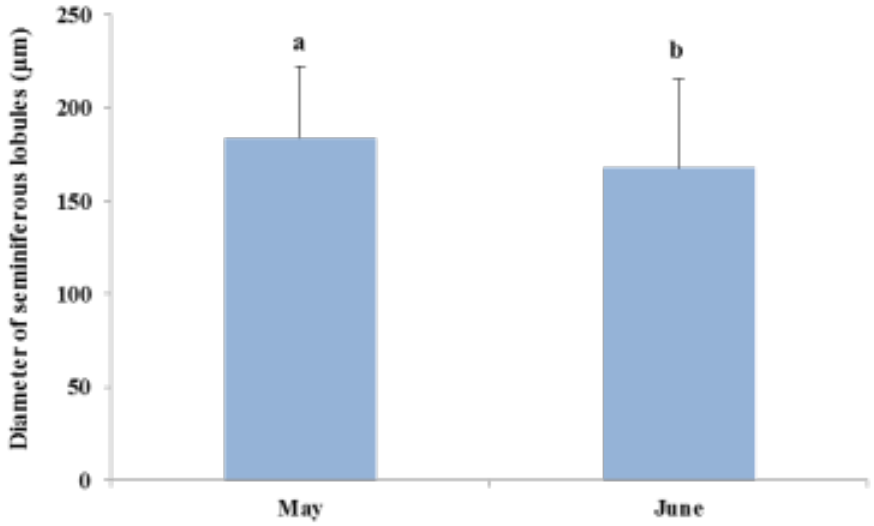

Figure 3.1.6 Mean diameter of seminiferous lobules in wild male greater amberjack sampled in May and June 2014 in the Mediterranean Sea (Lampedusa Island, Italy). Different letters above column indicate statistical significance (ANOVA, $P \leq 0.05$).

The histological analysis of the gonads of wild greater amberjack showed that between the end of May and the end of June this species is at the peak of its the reproductive season. In particular, at the end of May females had high GSI mean values and fully vitellogenic oocytes together with POFs, signs of recent spawning activity. The presence of hydrated oocytes in the specimens caught during the sampling campaign carried out in June, indicates that the fish are reproductively active until the end of this month. Wild male greater amberjack sampled at the end of May showed large amount of sperm in their seminiferous tubules and active spermatogenesis in the germinal epithelium. The spermatogenetic activity persisted until the end of June when meiotic and spermatidic cysts were still present in the germinal epithelium along with a moderate amount of sperm in the lumina of seminiferous lobules.

In 2015, in order to complete the study of the reproductive cycle of wild greater amberjack, it will be necessary to carry out new sampling campaigns in late April-early May and, possibly, after the cessation of the spawning season.

Identification of proliferation and apoptotic germ cells in wild male greater amberjack

Spermatogenesis is a developmental process during which spermatogonia proliferate and, throughout consecutive events involving mitosis and meiosis, produce a large number of highly differentiated spermatozoa. Apoptosis (or programmed cell death) is a highly conserved process that plays a major role during the normal development and homeostasis of multicellular organism. In mammals as well as in fish, apoptosis of germ cells is an integral component of normal testicular function and has been hypothesized to limit the germ cell population and prevent maturation of aberrant germ cells. Moreover, during spermatogenesis, germ cell apoptosis plays an important role in determining sperm output.

During Y1, the detection of proliferating and apoptotic germ cells was carried out on wild greater amberjack testis, as a preliminary analysis to verify if confinement could affect greater amberjack normal spermatogenesis in terms of proliferation and/or apoptosis of germ cells (Deliverable 3.3). Proliferating germ cells were identified by immune-histochemical localization of the proliferating cell nuclear antigen (PCNA), a polymerase delta accessory protein that is synthesized in late G1 and S phases of the cell cycle (Zupa et al., 2013). Apoptotic germ cells were detected throughout the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labelling (TUNEL) method, and apoptotic cell labelling was obtained by using an In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany).

The immune-staining of greater amberjack testes with anti-PCNA antibodies labelled single primary spermatogonia as well as cysts containing late spermatogonia (spermatogonial cyst), primary and secondary spermatocytes (spermatocyte cyst) (**Fig. 3.1.7a**). The TUNEL reaction labelled mainly spermatogonia and primary spermatocytes (**Fig. 3.1.7b**).

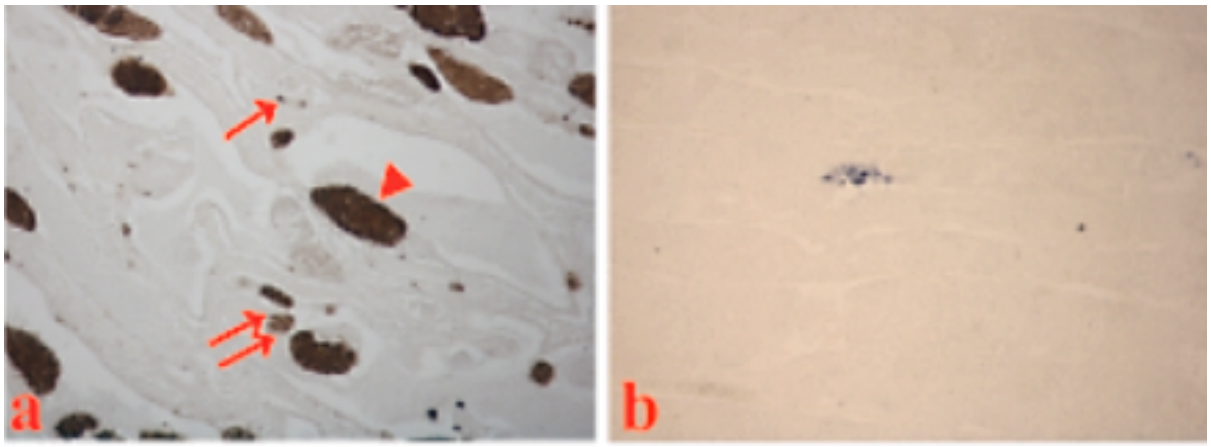

Figure 3.1.7 Micrographs of wild greater ambrejack testis showing (a) proliferating germ cells immunolabelled with antibodies against proliferating cell nuclear antigen (PCNA) and (b) apoptotic germ cells stained (dark blue) with TUNEL method. Arrow indicates single primary spermatogonium; double arrows show spermatogonial cysts; arrowhead indicate spermatocyte cysts.

In Y2, a comparative analysis of germ cell proliferation and apoptosis will be carried out between wild and captive male greater amberjack in the different phases of the reproductive cycle.

Establishment of quantitative PCR essay to measure transcript levels of vitellogenin and vitellogenin-receptor genes

Vitellogenesis is the process through which vitellogenin (Vg), a high density glycolipophosphoprotein synthesized by the liver, is transported via the bloodstream to the follicular layer and incorporated in the oocytes by receptor-mediated (VgR) endocytosis, in order to form yolk proteins. Yolk content is a key determinant of egg and larval quality in fish and, in order to verify if a normal vitellogenesis occurs in greater amberjack reared in captivity, a comparison of liver Vg and ovary VgR gene expression will be carried out (Deliverable 3.5).

During Y1, the determination of Vg gene sequence for greater amberjack was implemented, according the methodology reported by Pousis et al. (2011). Small liver samples of wild female greater amberjack sampled in May and June were stored in RNA later® solution and kept at 4°C. Subsequently, total RNA was extracted by using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 µl of RNase free water and stored at −80°C. The cDNA was prepared from 1.2 µg total RNA. In order to amplify the complete cDNA of Vg and actin (endogenous control) sequences from total cDNA by means of PCR, primer pairs for each gene were designed against conserved sequences from various Perciform species. PCR was performed on a PCR Sprint Thermal Cycler using ~250 ng cDNA, 15 moles of each oligonucleotide primer, 0.2mM dNTP mix, 5× Taq polymerase buffer and 1.5 unit Taq Polymerase (Promega). The amplification product was analyzed for size on 1.5% agarose gels containing ethidium bromide. The band (**Fig. 3.1.8**) will be excised from the gel, purified and cloned. The recombinant plasmid will be send to the Primm Sequence Service (Primm Srl, Italy) for sequencing.

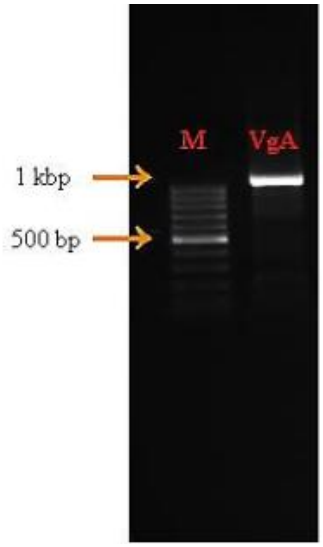

Figure 3.1.8 Agarose gel showing Vitellogenin A band (VgA) in a wild female greater amberjack. M = marker.

Task 3.2. Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (led by P1.HCMR).

According to the DOW, during Y1 of the project wild fish would be caught and acclimatized to captivity conditions, in order to be used for spawning induction experiment during the following years. This was planned in this way, because (a) we were not sure that all captured fish would be reproductively mature, (b) we were not sure how many months before the reproductive season we would be able to capture wild fish and (c) we considered it important to allow fish to acclimatize in captivity for about a year before expecting them to undergo gametogenesis normally in captivity. Fortunately, we were able to obtain a large number of fish already in September 2013 (8 months prior to the reproductive season) and some partners (P1. HCMR, P24. ITTICAL, FORKYS) already had some mature fish in their facilities for some years, so this allowed us to make some preliminary experiments already in Y1 of the project, in preparation for larger scale experiments in the following years. In addition, we made an agreement with another commercial company (Galaxidi Marine Farms, S.A., GALAXIDI) that is not a partner in the consortium, to utilize their recently captured broodstock for our experiments. The agreement to incorporate these stocks in the Task (at no extra charge to DIVERSIFY) enhances greatly our ability to carry out different experiments and at commercial settings, thus increasing our chances for success and speeding up the dissemination of the results to the aquaculture industry. Table 3.2.1 summarizes all the greater amberjack broodstocks available for the experiments in this task and also Task 3.5 (see later).

Broodstock acquisition

A total of 90 wild-caught greater amberjack (5-9 kg) were purchased from a farm in Greece (Asterias S.A., Astakos) in September 2013, and 58 individuals were transferred to P23. ARGO (September 2013) and 32 individuals to P1. HCMR (April 2014). At P23. ARGO, the fish were placed in two 40-m perimeter cages, while at P1. HCMR 24 individuals were placed in two 35-m tanks and 8 individuals were transferred to a 40-m perimeter cage at the Souda Bay pilot cage farm of P1. HCMR, joining existing populations of 3-4 individuals. Another stock of wild-caught individuals obtained 2 years earlier was maintained at the Panittica Pugliese facility of P24. ITTICAL. Apart from the above, two additional stocks of greater amberjacks were included in this Task. One stock was maintained by GALAXIDI (not a partner) and another stock by FORKYS (which is a partner of DIVERSIFY, but not a beneficiary in this WP3). For identification purposes, all fish were tagged with Passive Integrated Transponder (PIT) tags.

Table 3.2.1 Description of the various broodstocks maintained for this task.

Stock	Location	Number of Individuals	Size at sampling (range in kg)	Feeding
HCMR	tanks	27	6.5-23.8	raw fish, squid
HCMR	cages	12	7.4-14.8	moist pellet
ARGO	tanks	9	8.1-11.1	live, raw fish
ARGO	cages	49	7.1-16.0	live, raw fish
FORKYS	tanks	22	7.7-10.3	raw fish, squid
GALAXIDI	cages	28	6.3-15-6	live fish
ITTICAL	tanks	20	20-30	raw fish, squid

Evaluation of reproductive stage

Evaluation of reproductive stage begun in mid May in the various broodstocks, based on the ambient temperature (the different stocks were maintained at different geographical locations). For the evaluation of the reproductive stage, fish were fully anaesthetized and:

- Gonadal biopsy was taken from female fish to evaluate the reproductive stage under an optical microscope, and a portion of the biopsy was stored in fixative solution for histological evaluation.
- Sperm sample was taken from male fish using a catheter (since it is difficult to obtain sperm with abdominal pressure) to estimate quality parameters such as motility percentage, motility duration and density.

13/5/2014 P23.ARGO cage: The initial sampling for evaluation of reproductive maturation was done in one of the two cages (cage A). All sampled males at P23.ARGO produced sperm, which was accessible only with a catheter; hence it was called intra-testicular sperm (IT sperm). The abdominal musculature of greater amberjack is very thick, making it very difficult to "strip" sperm as it is done in other fishes. The sperm was motile upon activation with seawater, having initial motility of 10-80%. We believe that the low values could be the result of collecting the sperm with a catheter, as opposed to stripping the sperm after drying thoroughly the genital pore. The catheter might have introduced some water into the sperm sample, thus affecting spermatozoa motility. Almost all females were in vitellogenesis (Vg) with oocytes of 450-650 μm in diameter.

14/5/2014 P1.HCMR tank: Six fish from tank L2 were sampled, with the single male producing sperm, which was accessible only with a catheter; hence it was called intra-testicular sperm (IT sperm). Of the females, two had oocytes in Vg with a diameter of 450-480 μm, one was in early Vg (eVg) with oocytes at 280 μm in diameter and the others had only primary oocytes (po, **Fig. 3.2.1A and B**).

15/5/2014 FORKYS tank: Six females and four males were sampled. Few females were in Vg with oocytes of 400-580 μm in diameter, while others were immature (containing only po) or in eVg having oocytes with a diameter of 250-350 μm. Males produced IT sperm, but motility was not evaluated.

13/6/2014 P1.HCMR tank: Eighteen fish from tank S2 were sampled, with the males having IT sperm of good quality parameters (**Fig. 3.2.2**) and some of the females having oocytes in Vg with a diameter of 450-670 μm, but with a percentage of cells in atresia/apoptosis (at), while the rest having only primary oocytes. One female had a small number of post-ovulated eggs in its ovaries, indicating that it matured and ovulated spontaneously in the previous days (**Fig. 3.2.1C**).

18/6/2014 P1.HCMR tank: Nine fish were sampled from tank S1, with the females being in various stages of oocyte development (**Fig. 3.2.1D**) and one female having ovulated eggs in its ovaries, indicating that it matured and ovulated spontaneously in the previous days (**Fig. 3.2.1C**). Males had IT sperm with good quality parameters (**Fig. 3.2.2**).

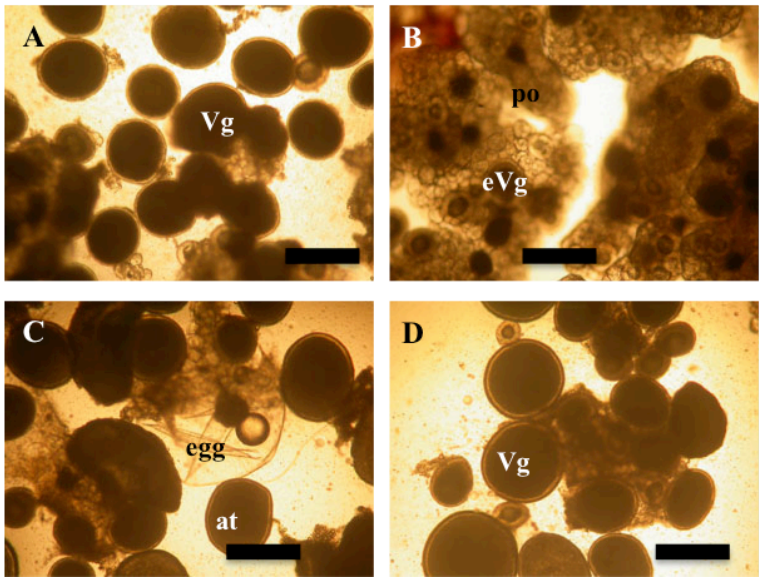

Figure 3.2.1 Wet mount photographs of greater amberjack oocytes obtained from the P1.HCMR broodstock maintained in tanks during the study. (A and B) Females on 14/5/2014, being in full vitellogenesis or in early vitellogenesis with a large number of primary oocytes. (C and D) Females on 13-18/6/2014 at the P1.HCMR tank, being in full vitellogenesis with a small number of post-ovulated eggs in the ovaries, and some signs of apoptosis/atresia. at = atresia/apoptosis, eVg = early Vg, , po = primary oocytes, Vg = vitellogenic oocytes. Bar = 500 μm.

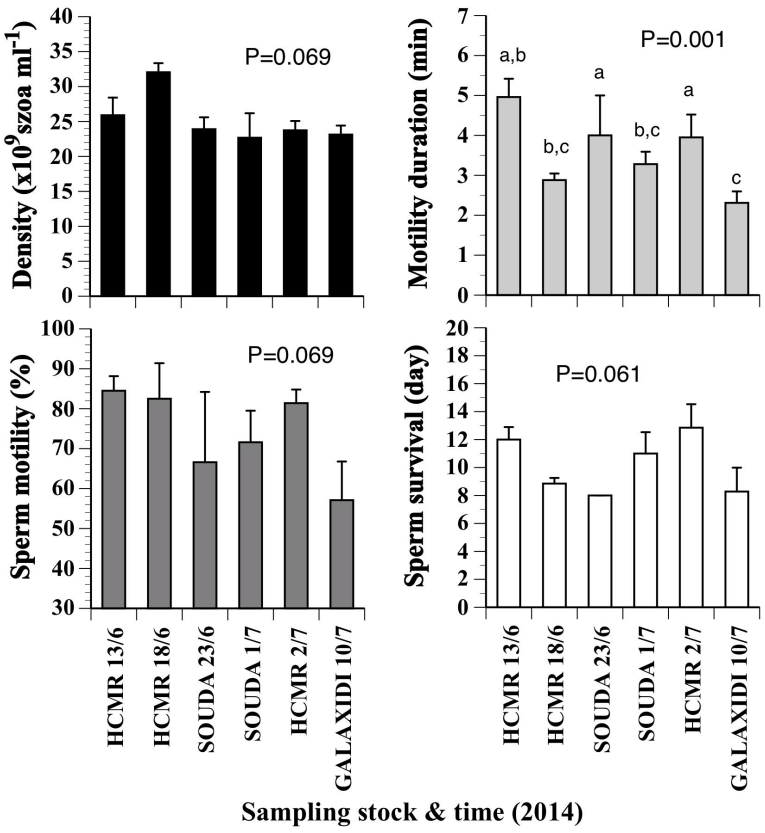

Figure 3.2.2 Sperm quality parameters from various stocks of greater amberjack sampled during the 2014 reproductive season. Statistical analysis (one-way ANOVA, DNMR, P≤0.05) indicated the existence of significant differences among stocks/sampling times only for motility duration.

20/6/2014 FORKYS tank: All fish were evaluated. Two females were in Vg but with relatively small oocyte diameters (470-500 μm), while the rest were immature having only po. Males produced IT sperm, but motility was not evaluated.

23/6/2014 P1.HCMR Souda cage: The three sampled females were in Vg with a significant number of oocytes in early oocyte maturation (OM) with oocytes of 680-700 μm in diameter (**Fig. 3.2.3A and B**). All sampled males produced IT sperm with initial motility of 70-100%, motility duration was 2.4 – 4.5 min and density was 10 - 31 x 10^9 szoa ml^{-1} (**Fig. 3.2.2**).

25/6/2014 P23.ARGO cage: At this time the sampling was done using fish of the second cage (Cage B). Males had IT sperm, while females were in all stages of development including po, eVg, Vg, as well as one female having ovulated oocytes in its ovary, indicating that it ovulated spontaneously. However, in almost all females there was a high occurrence of AT. Sperm motility ranged between 0-100%.

26/6/2014 GALAXIDI cage: All females were at Vg with oocyte diameters of 500-700 μm and very little occurrence of AT (**Fig. 3.2.3C**). In addition, some females were found to contain oocytes in advanced OM (**Fig. 3.2.4**) and some that had already ovulated spontaneously (**Fig. 3.2.3D**). Male fish produced IT sperm with initial motility of 60-100%.

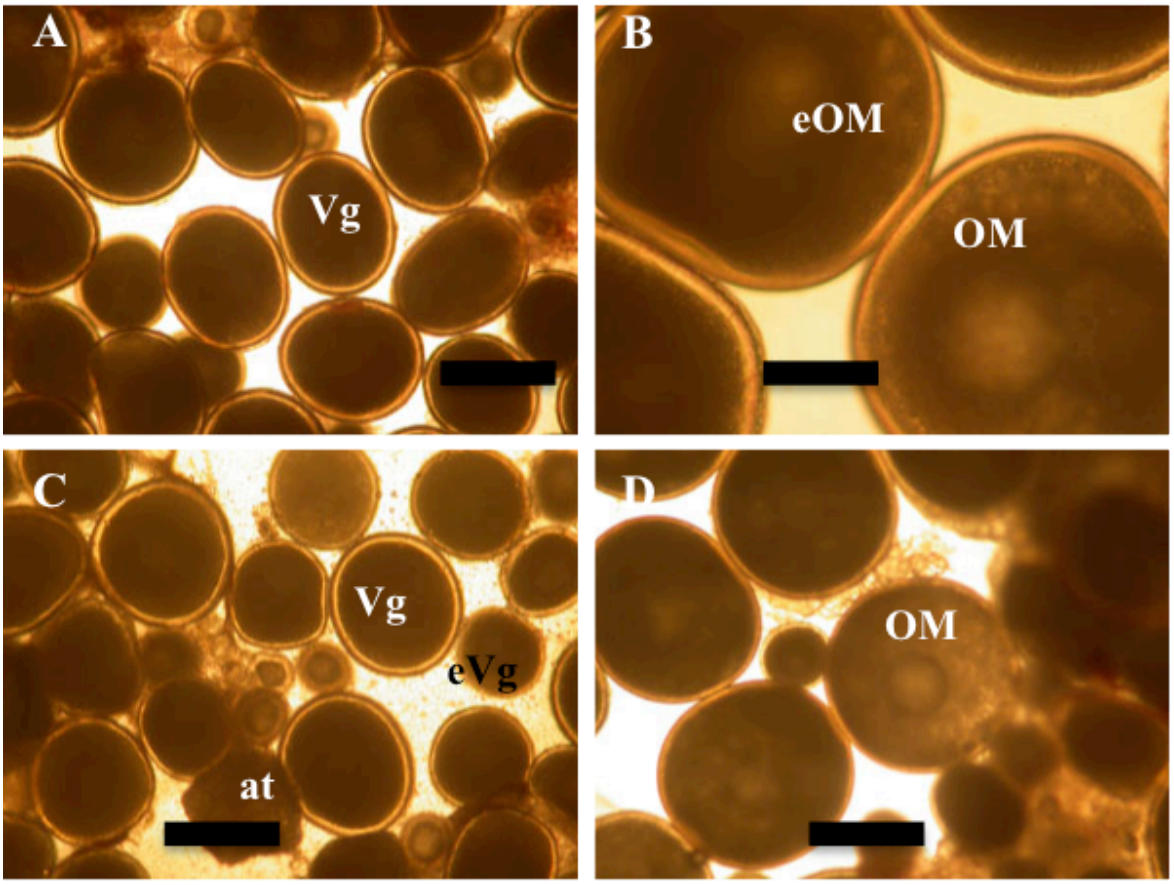

Figure 3.2.3 Wet mount photographs of greater amberjack oocytes obtained from the P1.HCMR and GALAXIDI broodstocks maintained in sea cages during the study. (A and B) Females on 23/6/2014 at the P1.HCMR Souda Bay cages, being in full vitellogenesis or in oocyte maturation. (C and D) Females on 26/6/2014 at the GALAXIDI cage, being in full vitellogenesis with some females in oocyte maturation, and some signs of apoptosis/atresia. at = atresia/apoptosis, eOM = early OM, OM = oocyte maturation, Vg = vitellogenic oocytes. Bar = 200 μm.

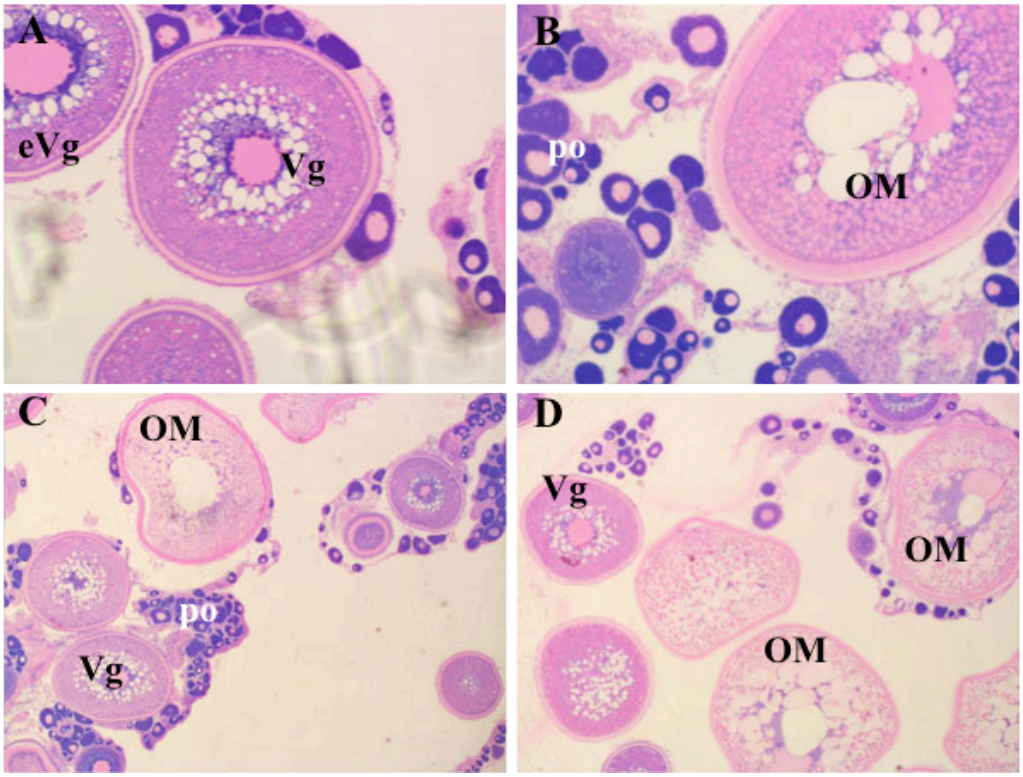

Figure 3.2.4 Histological sections of greater amberjack oocytes obtained from the P1.HCMR and GALAXIDI broodstocks maintained in sea cages during the study, showing the various stages of oogenesis and oocyte maturation. at = atresia/apoptosis, eVg = early vitellogenic oocytes OM = oocyte maturation, po = primary oocyte, Vg = vitellogenic oocytes.

Overall from the monitoring of the above stocks during the period of the expected spawning season, it was possible to draw some conclusions as to the reproductive capacity of greater amberjack in captivity:

1. We found that reproductive maturation may occur in fish as small as ~6 kg in body weight. This particular individual (from GALAXIDI) had oocytes at an advanced stage of maturation (germinal vesicle breakdown).
2. The majority of sampled females in May-June appeared to undergo vitellogenesis, but not all of them succeeded in reaching post vitellogenesis (oocyte diameter of 700-800 μm) and many entered atresia (apoptosis) when supposedly "spawning" temperatures (23°C) were reached in the second half of June and in July.
3. All sampled males were producing sperm, but probably due to the muscular nature of the abdominal wall of this species, it was not possible to collect sperm with "stripping". The obtained IT sperm, however, showed good motility and sperm parameter characteristics.
4. Broodstock feeding did not seem to be a significant factor in the reproductive maturation of greater amberjack, and both live or raw fish, produced comparable results with re-moistened commercial extruded feeds.
5. Fish maintained in cages had a better degree of reproductive maturation, compared to fish maintained in tanks.
6. A small percentage of both fish maintained in tanks and in cages have the capacity of undergoing maturation and ovulation spontaneously, without the use of any hormones.
7. Wild-caught greater amberjack (at least a percentage of them) have the potential of undergoing gametogenesis and completing vitellogenesis to the stage that could be induced to spawn with hormonal therapies (see below for results).

Given the above and the fact that (a) most of the broodstocks described above were in their first reproductive season (based on their size at the time of capture in past years), and (b) the fish were transferred to P1. HCMR tanks on 1 April 2014 (too close to the reproductive season) and were probably affected negatively by this transfer from sea cages to tanks, we feel that it is reasonable to expect that gametogenesis will proceed better in the coming years, allowing the implementation of all planned experiments, and the production of the necessary amounts of eggs for the larval rearing activities of the project.

To facilitate reproductive function and based on the excellent results obtained from the P1. HCMR Souda Bay cage, it was decided to train all broodstocks to feed on commercial broodstock feed, and a protocol was developed that succeeded in switching wild-caught fish from feeding on live/raw fish to re-moistened commercial extruded feed. The written protocol together with a short video has been uploaded in the project's website (http://www.diversifyfish.eu/greater-amberjack.html) and it is accessible for all interested parties, both within and from outside the consortium.

Spawning induction

As this year the experiments were considered preliminary, a single dose of GnRHa controlled-release delivery systems (implants) were used, chosen based on previous experiments with greater amberjack, but also other marine fish (Mylonas et al., 2004, 2010). When female fish were in the appropriate stage of oocyte development (**Fig. 3.2.3** and **3.2.4**), they were administered with a combination of GnRHa implants of 750 and/or 500 mg GnRHa, depending on their size, to obtain an effective dose of ~50 μg GnRHa kg^{-1} body weight. Similarly, males received implants of 500 or 750 mg GnRHa to obtain an effective dose of ~30 μg GnRHa kg^{-1} body weight. Tanks were fitted with passive egg collectors, which were monitored for eggs every day. Fecundity and fertilization success were estimated after transferring the eggs in a 10 l bucket, and taking a sub sample of 10 ml. Also, the stage of egg development was determined. A sample of eggs was transferred to microtiter plates for estimating the hatching and larval survival percentage as described in Panini et al. (2001).

Of the stocks examined for their reproductive stage (see section above), a number of females reached a stage that was appropriate to be given a hormonal therapy to induce maturation, ovulation and spawning. So individuals from all maintained stocks were given a hormonal therapy, although expectations were high only in the P1. HCMR Souda Bay cage and in the GALAXIDI cage broodstocks, which contained females with large Vg oocytes as well as some females with oocytes in early or even advanced stage of maturation. Fertilized eggs were obtained only from these two stocks, so we are presenting only these results. Also, in both stocks an evaluation after the first hormonal therapy, lead to the decision to treat some of the fish again, as described below.

23/6/2014 P1.HCMR Souda cage: The three sampled females were in Vg with a significant number of oocytes in early oocyte maturation (OM) with oocytes of 680-700 μm in diameter (**Fig. 3.2.3 and 3.2.4**). All sampled males produced IT sperm with good quality parameters (**Fig. 3.2.2**). Four males and three females were implanted GnRHa implants (**Fig. 3.2.5**). Fish started spawning after 48 h, and they were spawning for 6 days (3 spawns) after implantation (**Table 3.2.2**). The fertilization success ranged between 67-90%.

1/7/2014 P1.HCMR Souda cage: Upon re-examination of the fish when spawning appeared to stop, it was found that the four females sampled continued to contain post-Vg oocytes of a diameter of 630-700 μm, together with oocytes in early OM as well as post ovulated oocytes (from the spawning of two days before) (**Fig. 3.2.6**). So, it was decided to treat the fish with another GnRHa implants. Females received the same dose of GnRHa implants as in the 1st treatment, while the males were not given any hormonal treatment, as they appeared to be spermiating well.

At this time it was much easier that the first time to obtain the sperm using the catheter, as the genital pore was enlarged and sperm could be taken with the slightest aspiration. At this time, sperm motility was 70-90%, motility duration 2.0-6.2 min and density 18-28 x 10^9 szoa ml^{-1} (**Fig. 3.2.2**). In response to this second GnRHa implantation of the females, a single spawning was collected (**Table 3.2.2**).

Figure 3.2.5 Anesthetizing, evaluating oocyte stage of development and treating greater amberjack maintained in sea cages with GnRHa implants for the induction of spawning.

Table 3.2.2 Spawning results from greater amberjack stock maintained at the P1.HCMR Souda Bay sea cage facility, induced with GnRHa implants.

Stock	Days after treatment	Number of females	Fecundity (eggs)	Fertilization (%)
HCMR cage	2 (1st)	3	450,000	67.5
HCMR cage	3	"	48,000	90.0
HCMR cage	6	"	88,000	73.0
HCMR cage	2 (2nd)*	4	284,000	41.5

* A second hormone therapy was given once spawning ceased after the first treatment.

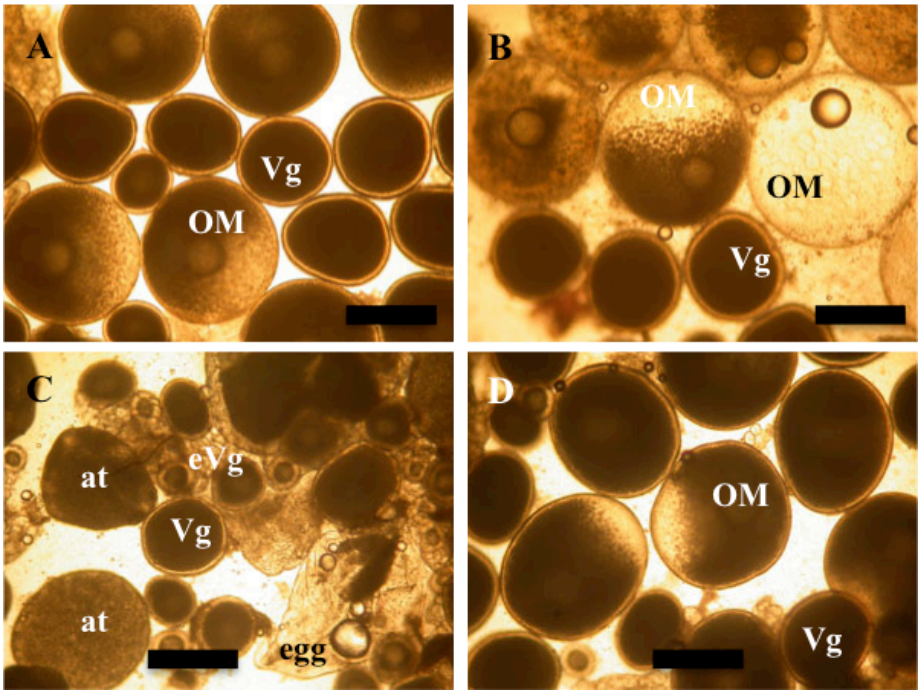

Figure 3.2.6 Wet mount photographs of greater amberjack oocytes obtained from the P1. HCMR and GALAXIDI broodstocks maintained in sea cages during the study, at the time of the second GnRHa implantation. (A and B) Females on 1/7/2014 at the P1.HCMR Souda Bay cages, being in full vitellogenesis or various stages of oocyte maturation. (C) Females on 9/7/2014 at the GALAXIDI cage, containing oocytes at various stages of development (including post-ovulated eggs), and some signs of apoptosis/atresia. (D) A female with oocytes in OM. at = atresia/apoptosis, eVg = early vitellogenic, OM = oocyte maturation, vg = vitellogenic oocytes. Bar = 200 μm.

26/6/2014 GALAXIDI cage: As mentioned earlier, all females were at Vg with oocyte diameters of 500-700 μm and very little occurrence of AT. In addition, some females were found to contain oocytes in advanced OM (Figs. 3.2.3 and 3.2.5) and some that had already ovulated spontaneously (Fig. 3.2.1). Male fish produced IT sperm with initial motility of 60-100%. So, it was considered that the fish were in an appropriate stage of reproductive maturation to be treated with GnRHa implants to induce spawning. Ten males and 14 females received GnRHa implants and were allowed to spawn (Table 3.2.3). To examine the potential of transferring fish from the cages to a tank for spawning (given that we had a large number of maturing females), a group of 3 females and 2 males was moved to a tank of about 25 m^3 in volume and was allowed to spawn (Table 3.2.3).

10/7/2014 GALAXIDI cage: Upon re-examination of the fish when spawning appeared to decrease, it was found that the females sampled continued to contain post-Vg oocytes of a diameter of 650-800 μm, together with oocytes in early OM or advanced OM, as well as post ovulated oocytes (Fig. 3.2.6). So, it was decided to treat the fish with another GnRHa implant (Table 3.2.3). Male fish produced IT sperm and were implanted again with GnRHa. Six females received GnRHa implants of 750 mg and 8 males of 450 mg GnRHa. Sperm motility was 60-100%, motility duration 2.6 – 7.3 min and spermatozoa density 5.7-35.7 x 10^9 szoa ml^{-1} (Fig. 3.2.2). On the contrary, the three females that were moved from the sea cage and were placed in the tank for spawning contained only post ovulated eggs (remnants of previous spawns) and vitellogenic oocytes in advanced atresia at this time, suggesting that the transfer to the tank had negative effects on the maturation fate of less developed batches of vitellogenic oocytes.

Table 3.2.3 Spawning results from greater amberjack stocks maintained at Galaxidi Marine Farms sea cage facility, induced with GnRHa implants. Some fish were transferred from the rearing cage to a land-based tank for spawning, after the hormone therapy.

Stock	Days after treatment	Number of females	Fecundity (eggs)	Fertilization (%)
GALAXIDI cage	3	11	10,000	100
GALAXIDI cage	4	"	30,000	100
GALAXIDI cage	5	"	30,000	100
GALAXIDI cage	6	"	60,000	66.7
GALAXIDI cage	8	"	25,000	100
GALAXIDI cage	9	"	10,000	100
GALAXIDI cage	10	"	5,000	100
GALAXIDI cage	11	"	30,000	66.7
GALAXIDI cage	2 (2^{nd})*	6	no eggs were obtained from the cage	
GALAXIDI tank 1	2**	3	350,000	85.7
GALAXIDI tank 1	3	"	660,000	38.1
GALAXIDI tank 1	4	"	185,000	43.7
GALAXIDI tank 1	5	"	150,000	33.3
GALAXIDI tank 2	6	"	265,000	15.1

* A second hormone therapy was given once spawning ceased after the first treatment.
** Fish were treated with GnRHa implants and transferred to a land-based tank for spawning.

The preliminary spawning results underlined the difficulties encountered with maturation, ovulation and spawning in captive greater amberjack broodstocks, but also provide some promising insights into the development of methods for the control of egg production:

1. Males produce sperm of good quality during the spawning period, and although it was not investigated if GnRHa treatment is absolutely necessary, males treated with GnRHa maintained good spermiation and quality parameters even after repeated spawning and production of fertilized eggs.
2. Females maintained in cages during gametogenesis had a better response to the GnRHa treatment, producing eggs of higher fecundity and most importantly of better fertilization success, compared to

females reared in tanks. This was expected based on the achievement of better degree of reproductive maturation at the time of treatment (See previous section), compared to fish maintained in tanks.

3. Egg collection is possible from broodstocks maintained in cages, but the fecundity achieved is much less than from stocks spawning in tanks. This is probably due to significant loses of eggs from the cage, and more work needs to be done to optimize the egg collection process (See later in Task 3.5).
4. An alternative broodstock management method resulting from the experience of this first year could be the maintenance of the broodstock in sea cages during the year (gametogenesis) and their transfer to land-based tanks for spawning after GnRHa treatment. This ensures collection of all spawned eggs and thus higher effective fecundities. However, transfer to the tank affected the "long-term" reproductive condition of the females, thus shortening their reproductive season, since at the second re-evaluation all fish were undergoing atresia in their ovaries. Still, this method may be an effective approach for commercial production, producing large numbers of eggs within a short period of time.

In the following years, the experience acquired from the above experiments will be used to implement the planed work in order to optimize the spawning induction protocol in terms of time of application, method of hormone administration and effective dose of GnRHa.

Preliminary spawning induction experiments in Panittica Pugliese facility (P24. ITTICAL)

The wild broodstock of greater amberjack (n=20) used for this GnRHa induction experiment belonged to Panittica Pugliese, a company controlled by P24. ITTICAL located in Torre Canne di Fasano (Brindisi, Apulia, Italy) and derived from a previous research project. The fish (body weight 20-30 kg) were maintained for two years in a 35 m^3 concrete tank under natural photoperiod conditions and with a constant 19°C sea-well water renovation. Starting from the second half of June 2014 the tank was supplied with surface sea-water to gradually increase the temperature to 24-25°C.

On 16th July the animals were anesthetized and cannulated in an attempt to determine the sex and evaluate their reproductive state. Unfortunately it was not possible to obtain any biopsy as the gonad ducts were found to be inaccessible to the biopsy probe. Eight fish were administrated 500 μg GnRHa implants, corresponding to a dose of 25-30 μg GnRHa kg^{-1} body weight. The hormonal induction was not successful possible because it was carried out late in the reproductive season.

Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic (led by P2. FCPCT).

Twenty-two greater amberjack (average weight: females, 3.41 ± 1.12 kg; males, 2.37 ± 1.07 kg), originally captured in the South-western coast of Gran Canaria (Islas Canarias, España) in May 2011, were used in this experiment. Fish were kept in a 10-m^3 tank, located in Planta Piloto de Producion of Alevines (PPPA) of the Grupo de Investigation en Acuicultura (GIA), located in the facilities of P2. FCPCT of the Universidad de las Palmas de Gran Canaria (ULPGC) (Telde, Gran Canaria, Spain) (**Fig. 3.3.1**).

Figure 3.3.1 Transfer of the captured greater amberjack broodstock to land facilities ay P2. FCPCT.

In May 2012, since broodstock biomass duplicated (females, 6.3 ± 1.4 kg; males, 6.00 ± 1.4 kg), the fish were divided in two 10 m^3 tanks. In January 2013, fish weight was further increased to to 8.3 kg (females) and 8.1 kg (males) and they were transferred to the new broodstock station of the FCPCT. They were kept in three 40-m^3 tanks under natural photoperiod conditions, salinity and temperature, with 600% daily saltwater renewal. Fish were fed twice per week with 13 mm pellets (Vitalis CAL, Skretting) at a rate of 1% biomass, and once a week with Atlantic mackerel (*Scomber scombrus*) at a rate of 2% biomass (**Fig. 3.3.2**).

Figure 3.3.2 PCTM broodstock station.

At the beginning of June 2013, the sex of the broodstock was determined by means of abdominal pressure. Eleven fish that released sperm were classified as males; eleven fish that did not emit sperm, were cannulised: 10 were found to be mature females and the other one was recorded as immature female. After sex determination, fish were allocated into 3 x 40 m^3 tanks to be treated as follows: Tank (T)A (natural spawning): 4♂; 3♀ (2 mature and 1 immature), TB (GnRHa injection): 4♂; 4♀, TC (GnRHa implant): 3♂; 4♀

On 27 May 2014, the 22 fish were cannulated (**Fig. 3.3.3**) and 12 of them were classified as males (average weight = 10.77 ± 2.33 kg). To assess the maturity state of the 10 females (average weight = 10.72 ± 1.22 kg), oocyte diameters were measured: TA: 5♂; 2♀ (mean oocyte diameter = 837 ± 166 µm); TB: 4♂; 4♀ (mean oocyte diameter = 689 ± 99 µm); TC: 3 ♂; 4 ♀ (mean oocyte diameter = 648 ± 58 µm).

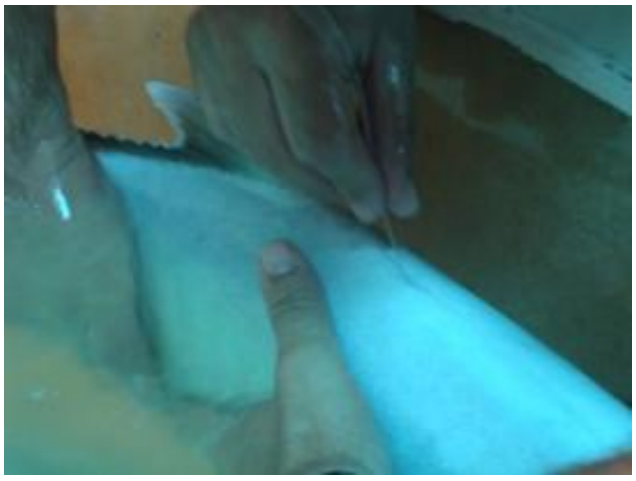

Figure 3.3.3 Greater amberjack broodstock cannulation.

On 15 June 2014 one of the males of TA (natural spawn group) jumped out of the tank and died. Three males and three females from TB were selected and were treated with an intramuscular injection of GnRHa, (des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg kg^{-1} body weight, according to the dose reported by Fernandez-Palacios *et al.*, 2013) (**Fig. 3.3.4**). These hormonal treatments were applied twice a week starting on 5 June 2014 (every Tuesday and Friday) alternating the broodstock couples so that each couple was treated every 10-11 days.

Figure 3.3.4 Greater amberjack broodstock injection with GnRHa.

Three males and three females selected in TC were induced with EV-500µg GnRHa implants (provided by the P1. HCMR). Each female was administrated an entire implant and each male was treated with 1/2 implant. Implants were given subcutaneously, about three rows of scales down from the posterior end of the dorsal fin. The interval between consecutive inductions varied according to the number of spawns obtained per treatment. Females and males were induced in pairs (one female and one male per induction), starting from 20 June 2014. The data reported in the present report were recorded between 01 June and 30 September 2014, although the spawning continued even after that period.

To test induction efficiency (Fernández-Palacios *et al*., 2013), the following parameters were determined: latency period (time from the injection to the first spawn; for this purpose egg collectors were monitored every 15-20 minutes all day long), number of spawn events and spawns obtained per induction. Moreover, the quality of each spawn was determined by the total number of eggs released, number of eggs released per each spawn and percentage of fertilization, viable eggs at 24 hours, hatching and larval survival at 4 and 8 days post hatching, following the instructions for egg collection and evaluation of quality established by DIVERSIFY (www.diversifyfish.eu/INTRA/Protocols), using 96-well microtiter plates (**Fig. 3.3.5**).

Figure 3.3.5 Spawning quality control using microtiter plate for egg incubation and larval rearing.

Eggs and larvae from natural and induced spawns were measured. The larvae were measured after being anesthetized with natural clove oil solution at 1% in salt water. The measurements were done using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan). Water temperature ranged between 21.5 ± 0.58ºC in June and 24.79 ± 0.52ºC in September. **Table 3.3.1** shows the results of hormonal inductions and natural spawns. All induced females spawned. The total number of treatments was 34, 22 of which were carried out by injection and 12 by implants. The total number of natural spawns obtained was 19, and 22 with injections and 36 with implants. The mean number of spawns obtained per implant was of 3.0 ± 1.7, significantly higher than those obtained by injection (0.8 ± 0.8).

The latency period was similar with both treatments (injection and implants). The natural spawn took place at 5.38 ± 1.65 a.m. The number of eggs obtained in natural spawn, was nearly triple than those obtained by injection and almost five times higher than those obtained by implants. Significant differences were found between the three treatments. The trend of fecundity during the experimental period for the three treatments is shown in **Fig. 3.3.6, 3.3.7** and **3.3.8**.

Table 3.3.1 Efficiency of induced and natural spawning

Treatment	Nº females that spawned	Nº Inductions	Nº spawns	Spawns/ Induction P < 0.01	Spawn hour Latency period (h):	Nº eggs/spawn P < 0.01
Natural	-	-	19	-	5.38±1.65	1,151,610±339,375[a]
Injected	3	29	22	0.79±0.49[b]	43.14±2.36	448,483±265,552[b]
Implanted	3	12	36	3.0±1.65[a]	45.35±8.65	256,454±283,554[c]

*Different superscripts in the same column indicate significant differences.

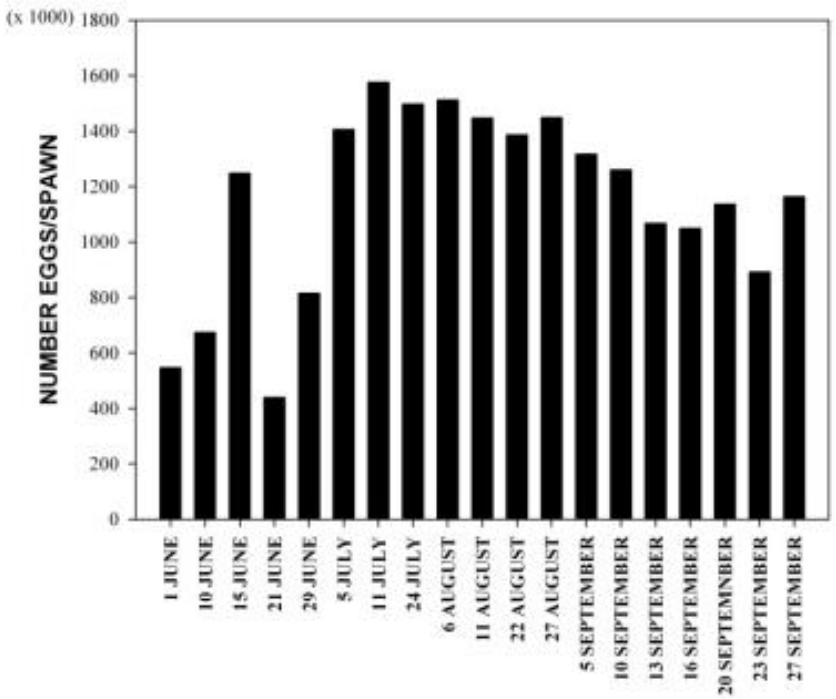

Figure 3.3.6 Trend of fecundity in females with natural spawning.

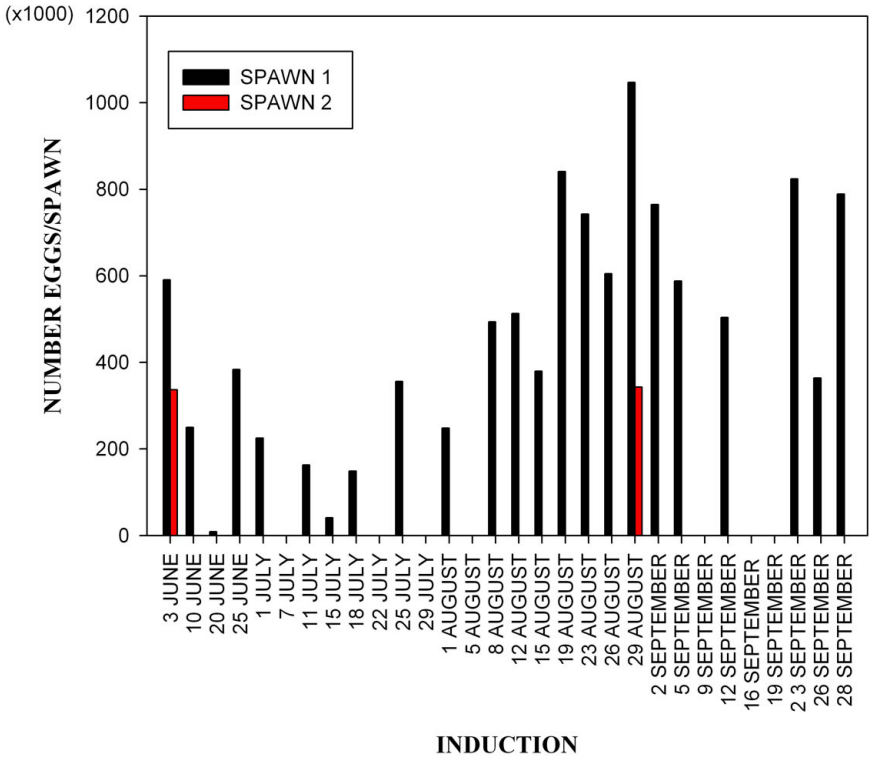

Figure 3.3.7 Trend of fecundity of females induced with multiple GnRHa injection.

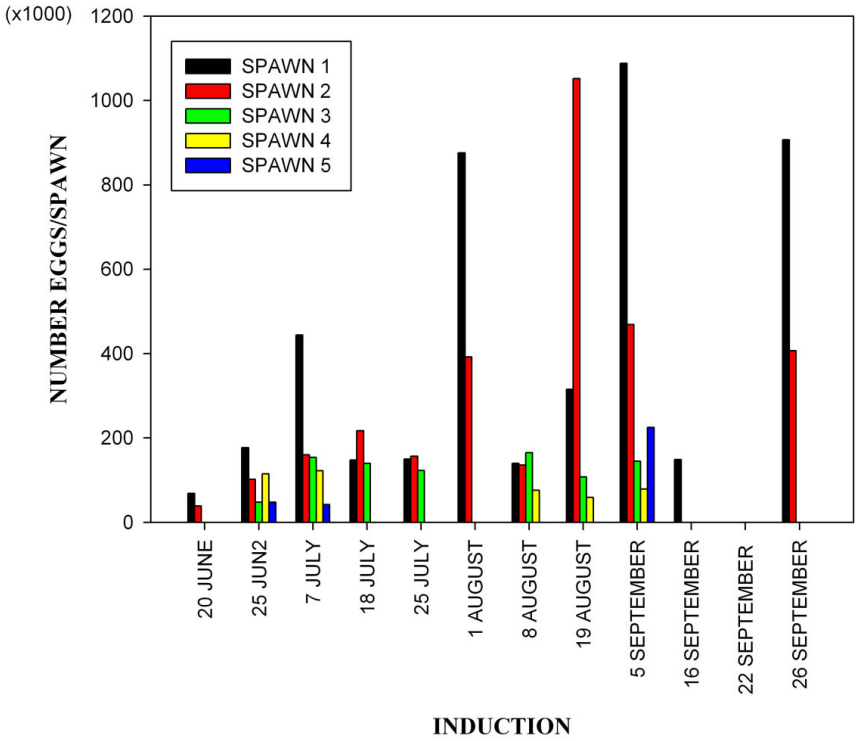

Figure 3.3.8 Trend of fecundity of females after each treatment with GnRHa implants.

The total number of eggs obtained (Figures 3.3.9 and 3.3.10) for each treatment was 21.880.600 in natural spawns, 10.763.600 after injection treatments, and 9.242.800 after implantations.

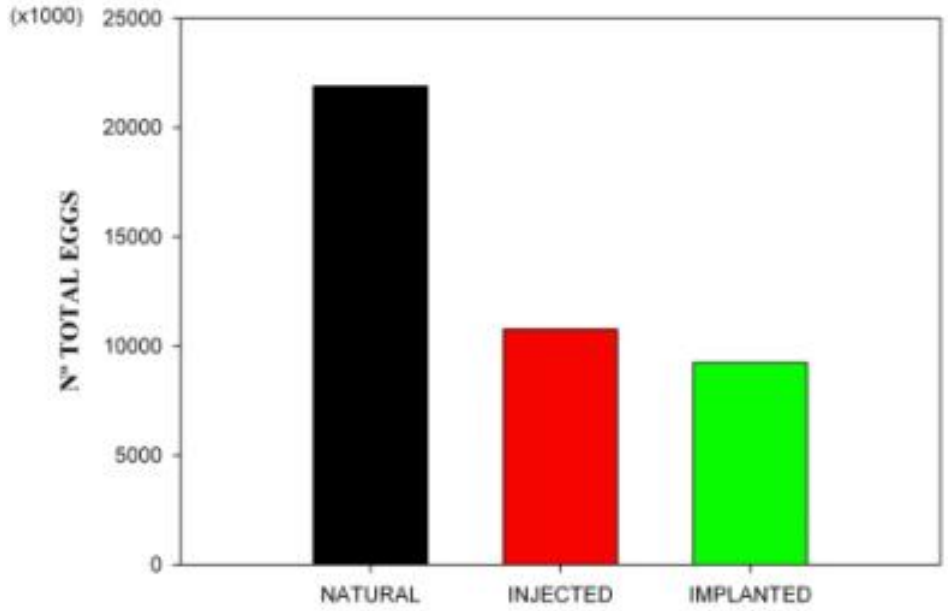

Figure 3.3.9 Total number of eggs obtained in each GnRHa treatment and non-induced control.

Figure 3.3.10 Eggs released from a natural spawn.

Spawning quality parameters for the three treatments are reported in **Table 3.3.2**. The best quality parameters have been achieved from natural spawns, excepts in % of alive larvae at 8 days, which was higher for implantation treatments. The spawns of injected broodstock and implanted showed differences in % fertilization.

Table 3.3.2 Spawning quality ratios from greater amberjack at P2. FCPCT.

Treatment	% Fertilization P < 0.01	% Viable 24 h P <0.01	% Hatching P < 0.01	% 4d Live P < 0.01	% 8d live P < 0.01
Natural	83±23[a]	94±8[a]	96±7[a]	70±17[a]	13±15[a]
Injected	56±28[b]	87±27[ab]	89±28[ab]	59±25[ab]	7±7[b]
Implanted	29±32[c]	76±35[b]	75±36[b]	46±27[b]	8±13[ab]

*Different superscripts in the same column indicate significant differences.

The diameter of eggs and lipid droplet, and the total length of larvae at day 0 and day 3 of life are shown in **Table 3.3.3**. Egg diameter and oil droplet diameter was significantly higher for natural spawned eggs. Total length of day 0 and day 3 larvae was significantly higher for natural and injected-induced spawns than in implanted spawns.

Table 3.3.3 Egg and larvae measurements (n = 450) from greater amberjack at P2. FCPCT.

Treatmet	Egg diameter (mm)	Oil droplet diameter (mm)	Total length of larvae at day 0 (mm)	Total length of larvae at day 3 (mm)
Natural	1.13±0.03[a]	0.30±80.02[a]	2.59±0.09[a]	3.85±0.13[a]
Injected	1.10±0.02[b]	0.27±0.02[b]	2,58±0.13[a]	3.82±0.13[a]
Implanted	1.10±0.02[b]	0.27±0.02[b]	2.45±0.13[b]	3.53±0.26[b]

*Different superscripts in the same column indicate significant differences (p< 0.01).

Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic (led by P8. IEO).

According to the decision taken at the kickoff meeting (Feb 2014), the main experimental work included in this task will be undertaken in 2015. Nevertheless, in order to obtain eggs and larvae to carry out other WP's, several activities related to reproduction have been carried out and the results may be of interest to the project.

In February 2014, a stock of greater amberjack broodstock born in captivity (F1 generation) in the Culture Unit of Canary Island Oceanographic Center (P8. IEO) was divided into three groups, which were placed in two raceway tanks of 500 m^3 and one circular tank of 50 m^3. Unfortunately, during this month, an infestation of *Zeuxapta seriolae* affected one of these groups that suffered significant losses, although the treatments administrated with hydrogen peroxide and copper were able to stop the mortality.

Therefore, two new broodstock groups were formed in March with the remaining fish. Individual identification code, body weight, and sex (obtained by means of gonad biopsy in previous year) of each fish are shown in **Table 3.4.1**. One group was stocked in a 500 m^3 raceway tank and the other in a 50 m^3 circular tank, both located outdoor and covered with shading nests.

Table 3.4.1 Identification code (ID), weight and sex of greater amberjack in each broodstock group at P8. IEO (March 2014).

Group Tank 500 m^3			Group Tank 50 m^3		
ID	Weight (kg)	Sex	ID	Weight (kg)	Sex
584663	11.3	Female	**562180**	9.3	Male
566201	13.6	Male	**899854**	9.4	Male
883043	7.6	Male	**584537**	10.0	Female
594669	15.8	Male	**588159**	12.8	Unsexed
558222	15.9	Unsexed	**897974**	11.5	Male
560004	24.5	Unsexed	**885828**	11.0	Female
569463	11.3	Unsexed	**908613**	11.8	Male
904365	10.9	Male	**559560**	17.0	Unsexed
883043	6.6	Male			
592680	21.4	Female			

Fish were feed to apparent satiation (three times a week) with frozen fish (Atlantic mackerel). An egg collector (mesh size 500 µm) was placed at the surface overflow of each tank in order to obtain the eggs released. For this purpose, the egg collectors were checked daily. Due to infestation previously suffered by the broodstock groups, the level of parasitism in each tank was monitored weekly using a device and method developed in our facilities. Its description and results are shown in WP 25 Fish health – greater amberjack.

The temperature of seawater increased approximately 1ºC, between July (22.2ºC) and August (23.0ºC), while food ingest/fish decreased continually since May (525 g/fish) to July (160 g/fish) and August (175 g/fish). Even though these parameters used as indicators of reproduction behaviour, were appropriate, the broodstock groups did not spawn spontaneously as in previous years.

Considering the need for eggs and larvae to develop the tasks scheduled in other WPs of the project, we took the decision to carry out hormonal induction in August. The group stocked in a 500 m^3 tank was treated with the induction method used by the enterprise Futuna España S.L. This method is subjected to confidentiality agreement between the enterprise and P8 .IEO. The group (males and females) stocked in a 50 m^3 was treated using intramuscular injection of GnRHa (Dose 30 µg/kg). Ovarian biopsies were not obtained from either group, in order to avoid handling stress, especially considering the previous pathology episode. However, this meant that the reproductive stage of the females at the time of the hormonal treatment was not known.

The broodstock group placed in a 500-m^3 tank released eggs from August 7 (2 days after to the induction treatment) until September 2. When spawning occurred, the eggs collector was checked at different times throughout the day. For each spawning, the date and number of eggs released were recorded, and the eggs were examined under a binocular microscope to check fertilization.

The spawning parameters obtained are shown in **Table 3.4.2**. Between the first (7/8/2014) and the last spawn (2/9/2014), six eggs batches were collected with a total of 1.4 x 10^6 eggs. The eggs collected, with a mean floating of 61%, showed a good appearance with clear spherical form and homogeneous diameter, but the fertilization rate was 0. Despite that, samples of these spawning were collected and preserved for biochemical (-80ºC) and genetic (Ethanol) analysis.

Table 3.4.2 Spawning events registered in the group of greater amberjack subject to induction treatment in 500 m^3 tank at P8. IEO.

Date	Time (hour)	N° eggs collected	Floating rate (%)
	9	501,384	65.11
	13	140,400	57.46
	18	101,268	46.05
07/08/2014		743,052	61.07
	9	157,140	57.08
	13	68,796	80.67
08/08/2014		225,936	64.27
	9	154,212	35.80
	18	74,376	74.62
09/08/2014		228,588	48.43
29/08/2014	9	158,100	88.25
01/09/2014	18	58,320	80.58
02/09/2014	9	44,160	5.43
Total-mean		1,458,156	61.63

The group of fish stocked in the 50-m^3 tank, subjected to intramuscular GnRHa injection did not spawn after the treatment.

In the Culture Unit of the P8. IEO at the Canary Islands, a group of greater amberjack broodstock captured from the wild and maintained under captivity for several years have spawned spontaneously for 8 consecutive years, producing eggs that have been successfully fertilized, but until now, these positive results have not been obtained with broodstock born in captivity (F1). Previous research conducted by P8. IEO in collaboration with P15. ULL that was focused on nutritional aspects of greater amberjack broodstock born in captivity as possible cause of reproductive failure, resulted in spontaneous releasing of eggs by the females (Rodriguez-Barreto et al., 2012, 2014, unpublished data). However, the eggs obtained were not fertilized by the males.

The results obtained now during 2014, together with our previous results show the difficulties to obtain fertilized eggs from broodstock born in captivity. We have achieved spontaneous and induced spawns from groups formed with F1females and males broodstock, but in all occasions, the eggs released were not fertilized. This is an important aspect to consider in the researching of F1 reproduction of this species in the future.

It is expected that the GnRHa implant treatment that will be carry out in 2015, achieve positive results. Adequate hormonal induction treatment together with improved nutritional and health status will contribute to solve the difficulties that have being seen for the first time in the reproduction of F1-generation greater amberjack.

Task 3.5 Spawning induction of greater amberjack and egg collection in cages (led by P1. HCMR).

Trials with spawning induction and egg collection were planed to be carried out in P1. HCMR and P23. ARGO. Following the agreement with GALAXIDI, another stock became available. Egg collection devices were mounted in cages of 40-m perimeter in the three rearing sites, Souda Bay, Crete (P1. HCMR), Salamina (P23. ARGO) and Galaxidi (GALAXIDI). The designed egg collector is a passive trapping device, which restricts the movements of floating eggs within the cage, on the water surface. Eggs of pelagic fish containing a lipid droplet rise to the water surface in calm weather. Egg collectors limit the movements of

eggs inside the cage because is mounted on the net of the cage, like a "curtain" and does not allow water movements.

The egg collector consists of two sections. The "lower" section is secured on the net of the cage throughout its perimeter through portholes in the tarpaulin every 30 cm (**Fig. 3.5.1**). This section starts at about 30 cm above the water line and goes down to about 3.5 m in depth. The "upper" section is hanging from the rails of the cage using ropes every 30 cm along the perimeter of the cage, and drapes down the cage over the lower section, overlapping with the top 1.5 m below the water surface (**Fig. 3.5.1**). The objective of this two-piece design is to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water, while the leeward side is push tightly against the net and the lower section, thus preventing any eggs from "jumping" over the cage and being lost (**Fig. 3.5.2**).

Figure 3.5.1 The "lower" section (left photo) together with part of the "upper" section (right photo) of the egg collector in Souda Bay, during installation. The lower section is attached to the cage net using cable ties, while the upper section is hanging from the rail using ropes.

Figure 3.5.2 The function of the two-piece design of the egg collector is to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water (left photo), while the leeward side (right photo) is pushed tightly against the net and the lower section, thus preventing any eggs from "jumping" over the cage and being lost.

The egg collectors (**Fig. 3.5.3**) were placed in each site after the initial sampling for reproductive evaluation (as described in Task 3.2), *i.e.* on 23/6/2014 in P1. HCMR, 25/6/2014 in P23. ARGO, 26/6/2014 in GALAXIDI. The evaluation of the reproductive stage of development, selection of broodstock for hormonal therapy, spawning induction experiments and egg collection data were described in detail above (Task 3.2).

Figure 3.5.3 Egg collection from GALAXIDI after the first spawning induction with GnRHa implants, and evaluation of fertilization success.

Spawning induction and egg collection of greater amberjack in cages was successful, but rather inefficient if we consider the number of eggs collected from the cages vs the tanks. Although both cage sites where were in areas protected from the wind and no strong wave action was observed, significant currents existed in both places. This probably resulted in eggs being moved outside the cage after spawning, before they had a chance to rise to the surface, where the egg collector would prevent them from exiting the cage. The lower section of the egg collector went down to 3.5 m in depth, while the cage was 8-m deep in GALAXIDI and 6-m deep in P1. HCMR. In next year's experiments, the bottom of the cage will be lifted higher (~5 m deep), to restrict the fish during spawning to the upper water layer, thus increasing the chances that rising eggs will be trapped by the egg collector before the current has a chance to move them outside the cage. Also, we expect that both because of their larger size/age next year, as well as because of a better acclimation of the broodstock, more eggs will be spawned next year, thus increasing the number that will be available for larval rearing experiments.

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Deviations from Annex I and their impact:

The main deviation from the work plan is due to the loss of the greater amberjack stock in P24. ITTICAL from a parasitic infestation. This stock was planned to be killed/sampled during 2015 for Task 3.1. During the Annual Coordination meeting in Nov 2014, it was decided to use wild-caught adult greater amberjack specimens already available in the facility of P23. ARGO. This Partner is involved in the spawning induction activities (Task 3.2 and 3.5) and had purchased another stock of fish from the same source, and have offered to make this population available to DIVERSIFY. The fish will be killed in the farm and the scientists from Italy responsible for this task (P13. UNIBA, P4. IOLR and P14, IFREMER) will travel for some of the sampling times, but P1. HCMR will be responsible to travel there from Crete to help them with the sampling.

This means that we will move a significant amount of the budget from P24. ITICAL to P23. ARGO, to cover the cost of these additional, as well as to HCMR and UNIBA for additional traveling and personnel cost. At the end, the task will be completed and delivered as proposed in the DOW (except that it will most likely be done with n=4 fish for each sampling as opposed to n=6), within the budget agreed (*i.e.*, no more money will be required, but with some reallocation of resources among partners and types of expenses).

As mentioned above, these fish will be killed/sampled in three different times of the reproductive cycle in 2015 according to the DOW. So, this deviation is not expected to have an impact on the deliverables of the Task.

Another small deviation relates to ***Deliverable 3.1 Establishment of quantitative PCR assays for target genes in greater amberjack (LHβ, FSHb, Leptin, Vg and VgR)*** will be delayed by 4 months. This is due to the late arrival of tissue samples derived from wild greater amberjack to P.7 IOLR (Eilat, Israel), which delayed the cloning of target genes (*i.e.*, pituitary gonadotropin beta subunits and liver leptin) still in progress. We expect to finalize this task and establish the respective quantitative real-time PCR assays, by March 2015. Therefore, Deliverable 3.1 will be delayed by 4 months, but this delay is not expected to have any impact on other Tasks or Deliverables from this WP.

WP No:	4	WP Lead beneficiary:	P1. HCMR
WP Title (from DOW):	Reproduction and Genetics – pikeperch		
Other beneficiaries (from DOW):	P1. HCMR	P9. UL	
Lead Scientist preparing the Report (WP leader):	Costas Tsigenopoulos		
Other Scientists participating:	Pascal Fontaine (P9)		

Objectives

1. Evaluate the genetic variability of captive broodstock in commercial RAS farms in Europe.
2. Compare this variability with the variability of wild individuals and define how a future genetic breeding program should be established for sustainable optimal performances through domestication of pikeperch.

Summary of progress towards objectives and details for each task:

Task 4.1 Evaluation of the genetic variation in available domesticated broodstocks of pikeperch has been completed and the associated deliverable was uploaded on the Participant's Portal. Two microsatellite multiplexes with seven and four loci were optimized and over 400 breeders sampled from 6 countries were genetically screened. Initial results indicate that some broodstocks have adequate genetic variation and few of them originate from few fish, and attention should be paid in the future to establish breeding programmes. In general, there is concordance with the stock origin and Finnish and Hungarian stocks from different companies are clustered together. Samples from wild stocks will provide new data in **Task 4.2 Evaluation of the genetic variation in non-domesticated broodstocks of pikeperch** and this data is expected to shed light into the origin of each captive broodstock and the caution to be taken into account when mixing pikeperch from different stocks in the future. Lastly it should be considered the sample size was small from a few broodstocks and further information on number of families is needed to define more precisely the needs to establish breeding program(s).

Task 4.1 Evaluation of the genetic variation in available domesticated broodstocks of pikeperch (led by UL, Pascal Fontaine)

Currently, there are no assessments of the genetic diversity in captive pikeperch stocks and because there are only a few commercial hatcheries that produce pikeperch (around 10 farms) in Europe, the genetic diversity is expected to be relatively lower compared to the genetic variability of natural populations (Säisä *et al.*, 2010). In principle, each pikeperch farm uses its own stock captured either from the wild or supplied by another farmer. Therefore, pikeperch populations differ from one farm to another depending upon the geographical origin of the captured wild populations, which are at the base of the captive stocks.

The primary objective in this first task (Task 4.1) was to develop a highly informative and efficient microsatellite multiplex for the species which ideally might consist of more than 10 markers to allow the adequate genotyping of all pikeperch populations sampled. This microsatellite multiplex is expected to be used for genotyping purposes on a capillary sequencer and evaluate the genetic variability of captive broodstock in commercial RAS farms around Europe and finally compare this variability with the variability of wild stocks/populations (Task 4.2). The outmost objective is to define how a future genetic breeding program should be established for sustainable optimal performance through domestication of pikeperch.

The microsatellite loci were chosen from other studies in pikeperch. Multiplex optimizations were performed for 22 loci (grouped into two multiplexes) that were reported in: Leclerc et al. (2000) for the yellow perch (*Perca flavescens* – Code: Pfla), Borer et al. (1999) and Wirth et al. (1999) in walleye *Stizostedion vitreum* – (Code: Zvi), and Dubut et al (2010) in the Rhone streber (*Zingel asper* – Code: Za) (**Table 4.1.1**). Those reported in pikeperch by Kohlmann & Kersten (2008) have shown low number of alleles (2-6 alleles in a population of 25 fish) and low expected heterozygosity (0.334 – 0.777) and have not been used previously in a multiplex to genotype populations. Microsatellite loci were first ordered by increasing size in base pairs (bp) and the size range (reported in the species described), and in each range one of the primers for each microsatellite locus the reverse (code: R) was fluorescently labelled with the dyes that conformed to the P1. HCMR's sequencing technology (ABI 3730).

Table 4.1.1 Characteristics of the 22 microsatellite loci used in pikeperch (*Sander lucioperca*). Loci were grouped into two multiplexes (upper and lower part of the table). For each locus, the accession number in NCBI is reported, the repeat motif (sequence), the size range, the number of alleles (Na) and the observed and expected heterozygosity (Ho and He, respectively) in the species first described (Pfla for *Perca flavescens*, Zvi for *Stizostedion vitreum* and Za for *Zingel asper*), the fluorescent dye used in the automatic sequencer for the reverse primer and the primers' sequences.

Locus	Acc.Number	Repeat Sequence	Size Range	Na	Ho	He	Dye	Forward sequence	Reverse sequence
Svi33	G36966	(AC)14	075–083	3	0.25	0.21	6-FAM	CAGGACTGCTGTGTATAGACTTG	GATATAGCTTTCTGCTGGGGTC
PflaL3	AF211828	(TG)18	101–119	8	0.34	0.29	6-FAM	GCCGAATGTGATTGAATG	CGCTAAAGCCAACTTAATG
SviL8	AF144741	(TG)22	107–145	8	0.34	0.20	Atto-565	GCTTATACGTCGTTCTTATG	ATGGAGAAGCAAGTTGAG
SviL11	AF144744	(TG)26G(TG)8	115–121	3	0.12	0.12	Atto-550	AGGGTATGGCATGATAAG	CTCTACATTTCATCAGACAG
Svi6	G36962	(AC)6	115–165	19	0.61	0.50	HEX	CATATTATGTAGAGTGCAGACCC	TGAGCTTCACCTCATATTCC
Svi18	G36964	(AC)18	132–182	10	0.67	0.59	6-FAM	GATCTGTAAACTCCAGCGTG	CTTAAGCTGCTCAGCATCCAGG
SviL9	AF144742	(CA)18AA(CA)3A(AC)4	161–223	10	0.17	0.17	Atto-550	TACTGTTCACTTATCTATCC	TGTATGTGTGTGTGTTCATGT
PflaL8	AF211833	(TG)39	167–203	16	0.72	0.51	Atto-565	GCCTTATTGTGTGACTTATCG	GGATCTTTCACTTTTTCTTTCAG
PflaL9	AF211834	(TG)24	182–214	4	0.65	0.52	HEX	GTTAGTGTGAAAGAAGCATCTGC	TGGGAAATGTGGTCAGCGGC
SviL7	AF144740	(TG)22	201–249	17	0.64	0.56	6-FAM	GATGTGCATACATTTACTCC	GCTTTAATCTGCTGAGAAC
PflaL2	AF211827	(CA)23	209–229	7	0.45	0.30	Atto-565	GTAAAGGAGAAAGCCTTAAC	TAGCATGACTGGCAAATG
Za121	HM622316	(CT)9	227-235	4	0.63	0.60	HEX	CAAAGTCATGAACGAGCTGC	AGCCAGGACCACTCTGTGAG
Za038	HM622298	(AC)11	107-130	6	0.80	0.77	6-FAM	TGAATCGCTGCTCTTTCTCA	TATGCAATTACATCGGAGCG
Svi4	G36961	(AC)16	120–166	15	0.70	0.65	Atto-550	ACAAATGCGGGCTGCTGTTC	GATCGCGGCACAGATGTATTG
Za024	HM622294	(AC)7	127–139	4	0.47	0.43	HEX	TGAACCTCCCTATCCCCTCT	TCTTTTCCACAGCAGGAAGC
Za138	HM622317	(AC)8	135-148	5	0.27	0.43	Atto-565	TTCTTTATACAAGAGGAATAGTTGCAG	TTTTTGTGATTGTGCTATTTTAAAGG
Za113	HM622314	(CA)11	169-229	9	0.70	0.85	HEX	ACCACGCACAATCACTCGTA	CCTGGCTTTACCCAGAAACA
Za237	HM622342	(CA)10	171-178	5	0.57	0.54	6-FAM	ATCTCAAGTCATGGGGCATC	GGTCCTCTGGTGCAGCTATAA
Za179	HM622329	(TCT)9	171-196	4	0.37	0.35	Atto-565	ATTTCCCATTGCGGGATTA	GGATTCTTGCATGCTTTGGT
Za144	HM622319	(AC)8	199-228	8	0.70	0.80	Atto-550	GCCCACAATAGCACCGTAAT	TTTGTGAATGTGAGTGAGAGTCAG
Za199	HM622334	(TCT)13	201-234	7	0.67	0.74	6-FAM	CCTTCCCCTCAAAAGCATGT	AGGAAATGGAAAGGGAATGC
Za207	HM622337	(GT)13	222-237	5	0.67	0.64	Atto-565	GGATTCCAGAAGCAAAAGAGG	TGGGACAAGGCTTTAACCAC

The Qiagen multiplex PCR kit was used to optimize PCR conditions and to determine the feasibility of working the two multiplexes (12-plex and 10-plex) and finally to determine the feasibility of developing one single multiplex as a powerful molecular tool for genotyping. Furthermore, the Qiagen multiplex PCR kit gives the advantage of maximal transferability of molecular protocols between labs. Unfortunately, the Qiagen multiplex PCR kit did not provide a single optimized multiplex that could be used for genotyping and the following two multiplexes were developed and optimized:

- **1st Multiplex: with loci** Svi18, PflaL3, Za138 and Za199.

- **2nd Multiplex: with loci** Za038, Svi4, Za024, Za237, Za144, Za207 and PflaL9

Polymerase chain reactions (PCR) were performed in 12.5 µl total volume, with the following cycling conditions. **1st Multiplex**: initial denaturation at 95°C for 5 min, 35cycles of 30 sec at 95 °C, 90 sec at 59°C, 90 sec at 72°C and a final extension for 30 min at 68°C. **2nd Multiplex:** initial denaturation at 95°C for 3 min, 30cycles of 15 sec at 95 °C, 30 sec at 58°C, 90 sec at 72°C and a final extension for 10 min at 72°C.

Raw allele sizes were scored using the STR and software (v. 2.4.59 http://www.vgl.ucdavis.edu/STRand). The number of alleles per locus, observed (H_O) and expected heterozygosity (H_E) and linkage disequilibrium (LD) were calculated in GENETIX v. 4.05 (Belkhir et al., 2004), FSTAT 2.9.3 (Goudet 1995) and GenAlEx 6.5 (Peakall and Smouse 2006, 2012) which offers a wide range of population genetic analysis options for the full spectrum of genetic markers within the Microsoft Excel environment on both PC and Macintosh computers. Deviations from Hardy-Weinberg equilibrium (HWE) across all samples were characterized by F_{IS}. In instances where the observed genotype frequencies deviated significantly from HWE, the Micro-Checker v.2.2.3 program (Van Oosterhout *et al*., 2004) was used to test for null alleles. The differentiation among locations was also quantified by F_{ST} (using the estimator θ of Weir & Cockerham, 1984).

Biological material

DNA extractions have been completed for all domesticated samples/populations following standard protocols (salt precipitation, Miller et al., 1988). PCR amplifications have been focused in the Aquaculture/domesticated samples in order to first fulfil Deliverable D4.1 (Month 12, *i.e.*, end of November). In total, DNA was extracted and analysed from 439 fish samples (**Table 4.1.2**); a wild population of 53 fish from Hungary was used as a reference for all population genetics parameters.

Table 4.1.2. List of domesticated pikeperch samples and number of fish per sample that were genotyped and analyzed; the first population marked in red is of wild origin.

A/a	Population	Sample size
1	*Gyori Elore, HTSZ, Hungary*	*53*
2	Szabolsi, Halaszati Kft, Hungary	50
3	VanMecklen, Holland, Aquapri A/S, Danemark	54
4	Czech Rep., Aquapri A/S, Danemark	38
5	Excellence fish, Hollande, Aquapri A/S, Danemark	14
6	Hungary, Aquapri A/S, Danemark	74
7	Mosso, Aquapri A/S, Danemark	19
8	IfB, Potsdam, Germany	48
9	FGFRI Kainuu fisheries research station, Finland	31
10	FGFRI Laukaa Fish Farm, Finland	20
11	ASIALOR, France	31
12	INAGRO, Belgium (German origin)	30
13	INAGRO, Belgium (Dutch Origin)	30

Results and Discussion

In domesticated stocks, the loss of genetic variability within the first generations of breeding practices limits the potential for future genetic improvement from selection practices. Considering a long term breeding program, ensuring sufficient genetic variation within populations is fundamental, because it determines the potential of adaptation to hostile changes in environmental /rearing conditions. Basic population genetics parameters (allelic richness, heterozygosity indices, inbreeding coefficients) were calculated for both wild and domesticated stocks

The number of alleles per locus ranged from 6-7 (PflaL3 and PflaL9, respectively) to 20 (Za138) (**Tables 4.1.3** to **4.1.5**). Therefore, microsatellite loci showed relatively high levels of polymorphism even though some samples were monomorphic (exhibited only one allele) for some loci like for Za199 and PflaL9 in the Excellence fish of Aquapri A/S (population 5), locus Za237 in Kainuu fisheries research station (population 9), and Za144 in Laukaa Fish Farm (population 10).

Table 4.1.3. Number of alleles per locus; populations numbers follow those in **Table 4.1.2**.

	Population													
Locus	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Total**
PflaL3	4	5	3	3	2	5	2	3	3	2	5	4	4	6
Svi18	6	12	3	4	4	13	4	4	4	3	6	7	5	18
Za199	5	5	2	3	1	6	3	4	4	3	5	9	7	14
Za138	9	14	3	3	2	13	2	7	4	2	6	9	6	20
PflaL9	3	4	2	3	1	3	4	5	3	5	3	5	4	7
Svi4	7	6	4	5	5	8	5	6	5	4	4	5	5	15
Za024	7	6	2	3	2	7	2	6	4	3	4	4	2	12
Za038	6	8	3	4	4	9	3	4	4	3	4	5	3	11
Za144	8	8	3	3	4	8	3	9	4	1	8	8	4	17
Za207	4	6	2	3	3	6	3	7	4	2	6	5	3	11
Za237	7	9	2	2	2	9	2	5	1	2	3	5	3	13

Table 4.1.4 Unbiased gene diversity per locus and population calculated using FSTAT 2.9.3 (Goudet 1995); populations numbers follow those in **Table 4.1.2**.

	Population												
Locus	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
PflaL3	0,6	0,66	0,63	0,64	0,07	0,67	0,49	0,50	0,49	0,50	0,66	0,74	0,64
Svi18	0,6	0,70	0,62	0,71	0,76	0,78	0,52	0,48	0,65	0,52	0,73	0,76	0,69
Za199	0,5	0,71	0,40	0,22	0	0,65	0,48	0,30	0,64	0,64	0,67	0,76	0,73
Za138	0,8	0,88	0,61	0,55	0,09	0,81	0,05	0,73	0,57	0,43	0,67	0,81	0,64
PflaL9	0,6	0,61	0,35	0,21	0	0,62	0,64	0,54	0,49	0,69	0,28	0,60	0,66
Svi4	0,7	0,72	0,56	0,68	0,61	0,78	0,59	0,78	0,69	0,65	0,58	0,71	0,70
Za024	0,6	0,58	0,49	0,19	0,2	0,54	0,05	0,53	0,62	0,49	0,62	0,48	0,33
Za038	0,6	0,69	0,62	0,53	0,54	0,73	0,52	0,52	0,62	0,65	0,71	0,77	0,59
Za144	0,7	0,81	0,61	0,51	0,60	0,82	0,66	0,61	0,57	0	0,70	0,85	0,59
Za207	0,6	0,71	0,5	0,56	0,58	0,66	0,64	0,58	0,52	0,29	0,69	0,65	0,66
Za237	0,7	0,82	0,01	0,47	0,15	0,84	0,10	0,52	0	0,42	0,60	0,66	0,61

Table 4.1.5. Allelic richness per locus and population calculated using FSTAT 2.9.3 (Goudet 1995); populations numbers follow those in **Table 4.1.2**.

	Population													
Locus	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Total**
PflaL3	3.327	4.074	2.994	2.994	1.714	3.93	2	2.213	2.868	2	3.862	3.989	3.541	4.664
Svi18	4.724	6.677	2.996	3.95	4	7.724	3.484	3.218	3.688	2.5	5.02	5.465	4.194	8.064
Za199	3.893	4.543	1.999	2.457	1	4.542	2.903	2.858	3.314	2.999	4.259	5.92	5.347	6.145
Za138	7.514	9.134	2.99	2.774	1.909	7.418	1.526	5.214	3.303	2	4.556	6.736	4.761	8.855
PflaL9	2.988	3.184	1.997	2.354	1	2.987	3.896	3.435	2.942	4.255	2.51	4.101	3.931	4.297
Svi4	5.536	4.737	3.161	3.883	4.354	5.554	3.835	5.707	4.33	3.554	3.171	4.044	4.534	7.622
Za024	4.429	3.368	2	2.311	2	3.515	1.526	4.233	3.314	2.756	3.529	3.534	1.997	5.028
Za038	4.783	4.472	2.994	2.526	3.429	5.242	2.526	2.742	3.833	2.999	3.921	4.917	2.992	6.051
Za144	6.147	5.905	2.978	2.263	3.64	5.998	2.999	4.665	3.089	1	5.626	6.757	3.827	7.979
Za207	3.221	5.072	2	2.804	3	4.924	2.997	4.02	2.645	1.996	4.939	4.194	2.999	6.202
Za237	5.272	6.606	1.185	2	1.978	6.731	1.782	3.351	1	2	2.991	4.089	2.981	5.663

For the thirteen populations analyzed, the least number of alleles was encountered in Aquapri's VanMecklen (2.64), Aquapri's Excellence fish and Laukaa Fish Farm (2.73) and the greatest in Hungarian Aquapri's (7.91) and Halaszati Kft (7.55) stocks which were greater than that in wild Hungarian stock (6.00). Likewise, expected heterozygosity (H_E) ranged from 0.3198 (in Aquapri's Excellence fish) to 0.7163 (in Aquapri's Hungarian fish) (**Table 4.1.6**)

Table 4.1.6 Basic population genetics parameters for all populations analyzed: mean number of alleles per locus, observed (H_O) and expected heterozygosity (H_E), and F_{IS} calculated in GENETIX v. 4.05 (Belkhir et al., 2004). Asterisks indicate significance at p=0.05.

A/a	Population	No Fish	Mean No Alleles	H_E	H_O	Fis
1	Gyori Elore, HTSZ, Hungary	53	6.00	0.6787	0.7325	-0.06881*
2	Szabolsi, Halaszati Kft, Hungary	50	7.55	0.7121	0.6712	0.06807*
3	VanMecklen, Holland, Aquapri A/S,	54	2.64	0.4921	0.7084	-0.43179*
4	Czech Rep., Aquapri A/S, Denmark	38	3.27	0.4781	0.5157	-0.0650*4
5	Excellence fish, Holland, Aquapri A/S,	14	2.73	0.3198	0.3922	-0.18608*
6	Hungary, Aquapri A/S, Denmark	74	7.91	0.7163	0.7193	0.00275
7	Mosso, Aquapri A/S, Denmark	19	3.00	0.4224	0.4149	0.04555
8	IfB, Potsdam, Germany	48	5.45	0.5516	0.5447	0.02399
9	FGFRI Kainuu fisheries research	31	3.64	0.5291	0.6003	-0.11825*
10	FGFRI Laukaa Fish Farm, Finland	20	2.73	0.4754	0.5801	-0.19545*
11	ASIALOR, France	31	4.91	0.6215	0.5909	0.06584*
12	INAGRO, Belgium (German origin)	30	6.00	0.7003	0.8057	-0.13374*
13	INAGRO, Belgium (Dutch Origin)	30	4.18	0.6152	0.6196	0.01019

A wide range of F_{IS} values were observed in the 13 populations analyzed (**Table 4.1.6** and **Table 4.1.7**). In principle, **positive** F_{IS} values indicate that individuals in a population are **more related** than you would expect under a model of random mating, whereas **negative** F_{IS} values indicate that individuals in a population are **less related** than you would expect under a model of random mating. The F_{IS} values are high and significant for Halaszati Kft (0.068), Aquapri's Mosso (0.0455) and ASIALOR (0.0658) samples. Such deviations from Hardy Weinberg equilibrium (HWE) may be due to i) the Wahlund effect, i.e. the reduction in the overall heterozygosity of a population as a result of subpopulation structures (that means if two or more subpopulations have independent allele frequencies then the overall heterozygosity is reduced, irrespective of whether those subpopulations are in Hardy-Weinberg equilibrium), ii) non-panmixia (inbreeding, groupings of relatives, selection against heterozygotes) or iii) to genotyping errors (null alleles and other scoring errors).

Table 4.1.7 F_{IS} values per locus and population; populations numbers follow those in Table 4.1.2. Asterisks indicate significance at p=0.05. The last row shows F_{IS} values per populations when locus PflaL3 is excluded due to null alleles.

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13
PflaL3	0,096	0,023	-0,26	-0,183	0	-0,108	0,036	-0,003	0,028	0,307	0,512	-0,3	0,537
Svil8	-0,409	0,199	-0,105	0,078	-0,305	-0,048	-0,003	-0,31	-0,091	-0,137	-0,366	0,254	-0,156
Za199	0,064	-0,202	-0,377	0,641	NA	-0,011	-0,094	0,002	-0,192	-0,238	0,136	-0,225	0,016
Za138	-0,032	0,33	-0,615	-0,091	0	-0,015	0	0,256	-0,211	-0,44	0,287	-0,025	-0,184
PflaL9	-0,242	0,027	-0,293	0,395	NA	0,03	0,344	0,219	0,15	-0,08	0,091	-0,27	0,043
Svi4	0,003	-0,047	-0,431	-0,142	-0,516	0,133	-0,668	0,138	-0,439	-0,53	-0,298	-0,267	-0,207
Za024	0,022	0,02	0,057	0,193	1	0,037	0	0,112	0,117	-0,109	-0,166	-0,029	-0,033
Za038	0,024	-0,06	-0,603	-0,341	-0,051	-0,05	0,402	0,012	-0,291	-0,302	0,252	0,265	-0,013
Za144	-0,116	0,069	-0,64	0,037	-0,3	0,007	0,465	-0,104	0,094	NA	0,013	-0,051	0,096
Za207	-0,161	0,039	-1	-0,289	-0,189	0,088	-0,138	-0,046	-0,23	-0,188	0,112	-0,483	0,052
Za237	0,01	0,244	0	-0,158	-0,048	-0,021	-0,029	-0,175	NA	-0,166	0,145	-0,456	-0,03
All	-0,069*	0,068*	-0,432*	-0,065*	-0,186*	0,003	0,046	0,024	-0,118*	-0,195*	0,066*	-0,134*	0,01
All-10L	-0.084*	0.072*	-0.454*	-0.049	-0.190*	0.013*	0.047	0.026	-0.132*	-0.248*	0.019	-0.116*	-0.045

Inbreeding seems an explanation in domesticated and non-random mating is also likely in our case, as deficits were homogeneous among loci (all significant and all non-significant F_{IS} values). Selection against heterozygotes cannot be demonstrated from our results; although microsatellite loci are typically recognized as neutral genetic markers, it is possible that one or more loci are linked to genes or gene groups under selection. The Walhund effect could also explain the deficit of heterozygotes due to the mixing of genetically variable populations to form a new domesticated stock which might be the case in some aquaculture companies' practices.

Moreover, one of the above microsatellite loci (PflaL3) showed significant probability ($P > 0.05$) of "large allele dropout" or "stuttering". However, when this locus is excluded from the analysis the F_{IS} values are slightly changed but remain significant in any case (see bottom of **Table 4.1.7**).

Finally, F_{ST} values are frequently used as a summary of genetic differentiation among groups. It depends on the allele frequencies at a locus, showing specific properties linked to genetic diversity: higher values for biallelic single-nucleotide polymorphisms (SNPs) than for multi-allelic microsatellites, low values among high-diversity populations viewed as substantially distinct, and low values for populations that differ primarily in rare alleles. Due to these reasons, several authors argued that F_{ST} measures may be poor measures of genetic differentiation when the level of diversity is high. Estimated population differentiation across samples using the F_{ST} estimate by Weir & Cockerham's (1984) showed that the smallest values were between Hungarian samples (wild-1 and domesticated-2) and also showed (as expected) a close relationship (F_{ST} <0.11) between the above two populations with the Aquapri's Hungarian one (population 6). There was also a close relationship of Aquapri's VanMecklen (population 6) with the Czech population from the same company and that from IfB Potsdam (F_{ST} <0.15). Lastly, a close relationship was found between ASIALOR and INAGRO's Belgian samples (F_{ST} =0.14) and Aquapri's Mosso sample with that from INAGRO's Dutch samples (F_{ST} =0.16).

Table 4.1.8 Pairwise Population Theta (θ) calculations [Weir & Cockerham's (1984) F_{ST}'s] between the thirteen populations; populations numbers follow those in **Tables 4.1.2** and **4.1.6**.

		2	3	4	5	6	7	8	9	10	11	12	13
1	Gyori Elore, HTSZ, Hungary	0	0,3	0,3	0,3	0,1	0,4	0,2	0,4	0,4	0,2	0,2	0,3
2	Szabolsi, Halaszati Kft, Hungary	--	0,3	0,3	0,3	0	0,3	0,2	0,3	0,3	0,2	0,2	0,2
3	VanMecklen, Holland, Aquapri A/S, Danemark	--	---	0,1	0,3	0,3	0,3	0,2	0,4	0,4	0,2	0,2	0,3
4	Czech Rep., Aquapri A/S, Denmark	--	---	---	0,2	0,3	0,3	0,1	0,4	0,5	0,2	0,2	0,3
5	Excellence fish, Hollande, Aquapri A/S, Denmark	--	---	---	---	0,3	0,4	0,2	0,4	0,5	0,3	0,3	0,3
6	Hungary, Aquapri A/S, Denmark	--	---	---	---	---	0,3	0,2	0,3	0,3	0,2	0,2	0,2
7	Mosso, Aquapri A/S, Denmark	--	---	---	---	---	---	0,3	0,2	0,4	0,2	0,2	0,2
8	IfB, Potsdam, Germany	--	---	---	---	---	---	---	0,3	0,4	0,1	0,2	0,3
9	FGFRI Kainuu fisheries research station, Finland	--	---	---	---	---	---	---	---	0,2	0,3	0,2	0,2
10	FGFRI Laukaa Fish Farm, Finland	--	---	---	---	---	---	---	---	---	0,3	0,3	0,3
11	ASIALOR, France	--	---	---	---	---	---	---	---	---	---	0,1	0,2
12	INAGRO, Belgium (German origin)	--	---	---	---	---	---	---	---	---	---	---	0,2
13	INAGRO, Belgium (Dutch Origin)	--	---	---	---	---	---	---	---	---	---	---	---

All results mentioned above based on F_{ST} values can also be visualized based on a Factorial Correspondence Analysis graph using the GENETIX v. 4.05 (Belkhir et al., 2004) software (**Fig. 4.1.1**).

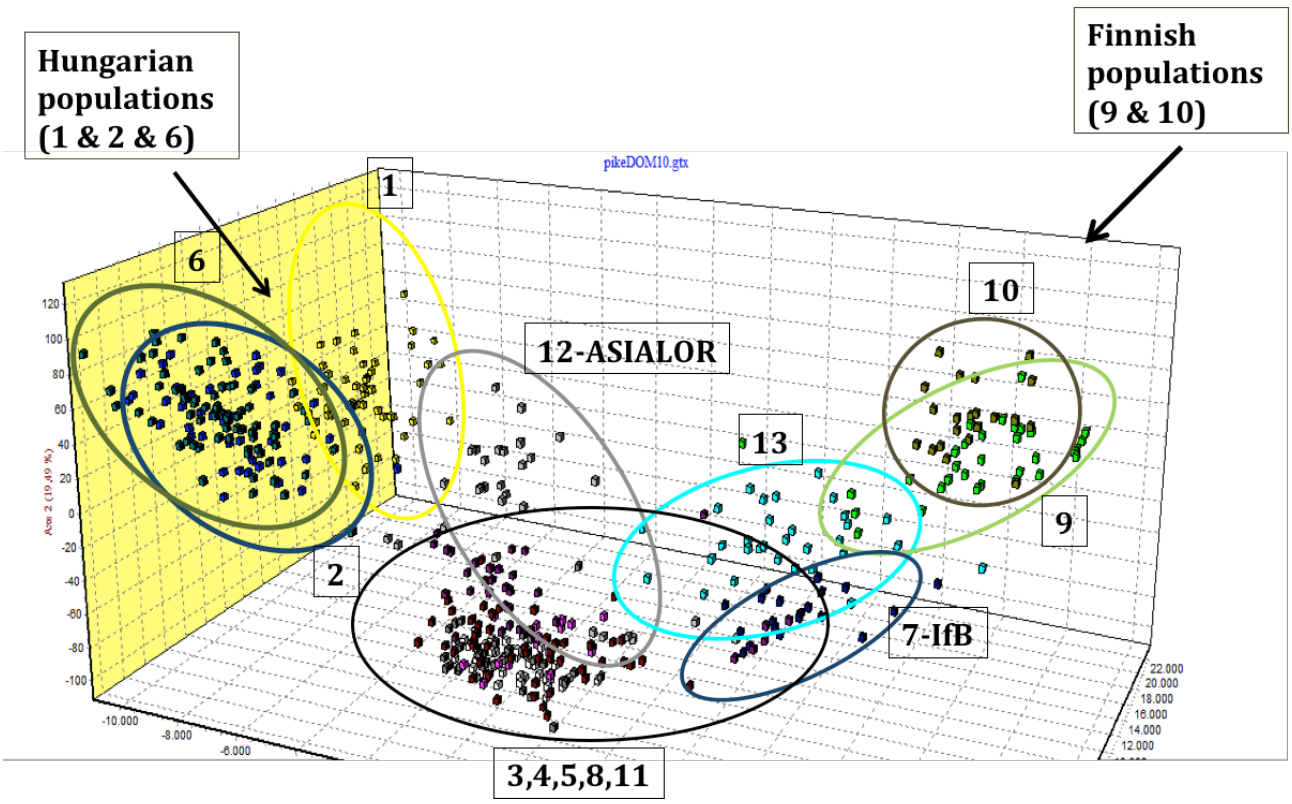

Figure 4.1.1 Factorial Correspondence Analysis (FCA) for all 13 populations and 10 loci using the GENETIX v. 4.05 (Belkhir et al., 2004) software; populations numbers follow those in **Tables 4.1.2** and **4.1.6**.

Task 4.2 Evaluation of the genetic variation in non-domesticated broodstocks of pikeperch (led by HCMR, Costas Tsigenopoulos).

Concerning the wild stocks, it is of particular importance to determine whether there is substantial genetic structure for management purposes, but also for the traceability of the species products. To date samples from seven wild populations have been received (**Table 4.2.1**) and others are expected from other colleagues. DNA extractions were finished for all samples that have been received and work is in progress on genotyping and population genetics analysis.

Table 4.2.1 List of wild pikeperch samples and number of fish per sample that were collected but are not yet genetically analysed.

a/a	Population of wild origin	Number of fish
14	URAFPA-DAC, Czech Rep.	70
15	Domaine de Lindre, France	51
16	Sarag Lake, Poland	14
17	Wymoj Lake, Poland	9
18	Lake Oulujärvi, Finland	32
19	Lake Hiidenvesi, Finland	31
20	INSTM, Tunisia	59

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Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP No:	5	**WP Lead beneficiary:**		P7. IMR
WP Title (from DOW):	Reproduction and Genetics – Atlantic halibut			
Other beneficiaries (from DOW):	P1. HCMR	P17. NIFES	P22. SWH	
Lead Scientist preparing the Report (WP leader):	Birgitta Norberg			
Other Scientists participating:	Constantinos Mylonas (P1), Borre Erstad (P22)			

Objectives

1. Improve fecundity and gamete quality in F1/F2 broodstock.

Summary of progress towards objectives and details for each task:

All tasks were initiated with good advances. **Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut** found that established wild caught broodstock had more regular ovulatory cycles and a higher fecundity than F1 broodstock. However, the F1 fish were first time spawners, which may have contributed to their apparently poor performance. **Task 5.2 GnRH implant therapy as a means to improve spawning performance** initiated with a pilot study of GnRH implantation in F1 breeders that showed that 50 µg GnRH per kg was sufficient to induce final maturation and ovulation. Most of the GnRH implanted fish ovulated earlier and gave more eggs than sham-implanted (control) females. However, due to a low number of individuals, results were not significant. This work will be repeated with more breeders. In **Task 5.3 Fecundity regulation**, samples have been taken for analysis of fecundity regulation.

Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut (led by IMR, Birgitta Norberg)

Spawning performance was documented through the spawning season from February to April 2014 in the wild-caught broodstock held at the P7. IMR facilities at Austevoll, Norway. Total number of egg batches, hours between ovulation, total volume of eggs spawned, volume of eggs per batch, fertilisation and hatching rates were recorded. Egg samples were taken of all batches for analysis of steroid content.

The F1 generation females were monitored for the same parameters and egg samples were collected. However, due to poor spawning performance more documentation will be needed for those fish. The F1 females were all first time spawners, and will be followed over one or more consecutive cycles in order to get systematic documentation on possible improvement in spawning as the fish get more experienced. Overall, established broodstock had more regulatory ovulatory cycles and a higher total and relative fecundity.

Due to a prolonged sick leave (August 2014 - December 2014) of the Principal Investigator of this WP (Dr Birgitta Norberg), planned analyses of data collections and egg samples from Task 1 have been put on hold. We expect to start the analyses in the first months of 2015 and there will be no delay in the deliverable for this task, which is due in month 30.

Task 5.2 GnRH implant therapy as a means to improve spawning performance (led by HCMR, Constantinos Mylonas)

The GnRHa implants were prepared by loading the agonist desGly^{10}, dAla^{6}, Pro^{9}-GnRH-NEthylamide (Alarelin, Bachem, Switzerland) into a matrix of poly [Ethylene-Vinyl Acetate] (EVAc, Dow Corning) according to the procedure of Mylonas et al. (2007). Briefly, 376 or 564 mg of GnRHa (for the 500 and 750 µg per implant, respectively) and 0.43 or 0.24 g of bovine serum albumin (BSA, Sigma, Germany) were dissolved in 7.5 ml dH_2O, and were mixed with 8 ml dH_2O containing 0.8 g of Inulin (Sigma, Germany). The mixture was frozen at –80°C and lyophilized for 48h (Alpha 1-2, Martin Christ, Germany). The dried powder was ground using a glass tissue-grinding rod connected to an overhead mixer (RZR 2020, Heidolph, Germany). Twenty ml of a 15% EVAc solution in $MeCl_2$ were then added to the GnRHa/BSA/Inulin powder, and the mixture was vortexed for 5 min and sonicated for 30s at 30 watts (UP 200S, dr. Hielscher GmbH, Germany). The produced emulsion was poured into a levelled aluminium cast (50 x 50 x 4 mm) placed on a block of dry ice. The solidified plate was then placed in a –20°C freezer for 3 days in order to evaporate the $MeCl_2$, followed by 48 h in a vacuum desiccator to remove any moisture. The implants were punched from the dried GnRHa/BSA/Inulin/EVAC plate using a 2 mm dermal punch (Keyes Punch 3mm, Miltex GmbH, Germany).

The *in vitro* release kinetics of the GnRHa implants were evaluated using the procedure of Mylonas et al. (2007) and Sarter et al. (2006). Briefly, the implants (n=4) were embedded in a 2% solution of low melting agarose (Sigma, Germany) in vitro buffer (3.36 g KH_2PO_4, 11.25 g $NaHPO_4$, 0.4 g sodium azide, 0.4 ml Tween 80 in 2 l of dd H_2O, adjusted to pH 7.0), at the bottom of 7 ml flat-bottom p[ethylene] vials. Once the agarose solidified, 5 ml of the in vitro buffer was added and the vials were placed on a rocking shaker in an incubator at 6°C (**Fig. 5.2.1**). At various times afterwards, a 500 µl sample of the in vitro buffer was taken from each replicate and stored at –80°C until analysis for GnRHa, using an ELISA developed earlier (Mylonas et al., 2007). The in vitro buffer was replaced with fresh one after each sampling.

The release from the implants is currently being determined using a specific ELISA developed for the GnRH agonist loaded in the impalnts. A representative release from a similar implant at 24°C is shown as n example of the *in vitro* release kinetics that can be expected. (**Fig. 5.2.1**).

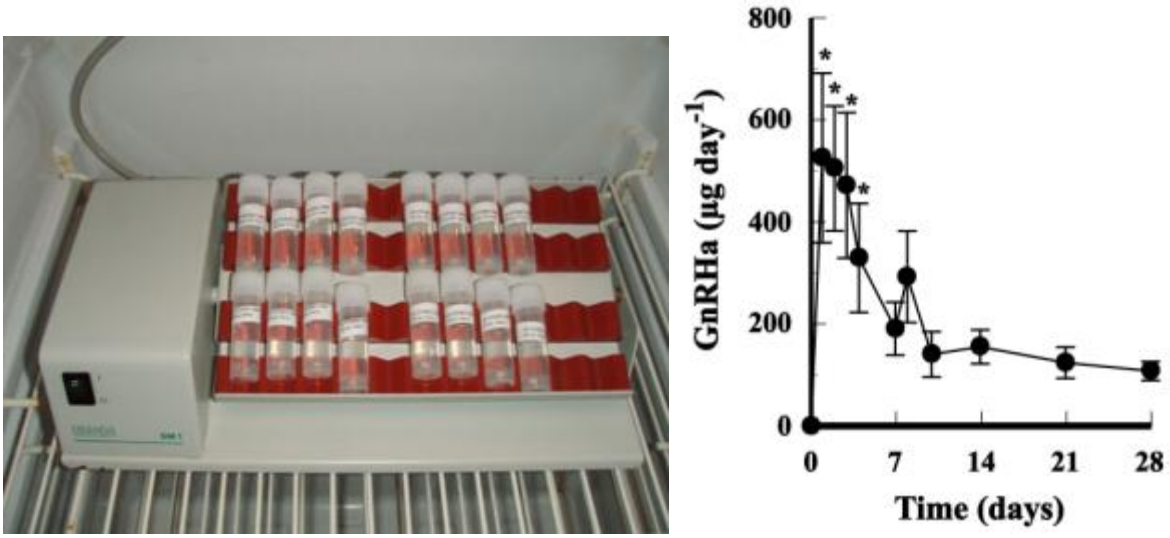

Figure 5.2.1 Set up for the *in vitro* release assay for the GnRHa implants (left). Mean (±SEM) GnRHa released from the GnRHa implants at 24°C (right).

In response to treatment with the GnRHa implants, Atlantic halibut started ovulating after 7 days (**Fig. 5.2.2**). There were no significant differences between fish receiving the two GnRHa doses, 500 or 750 µg per fish. Control fish ovulated a day later and ovulated fewer times over a more prolonged period.

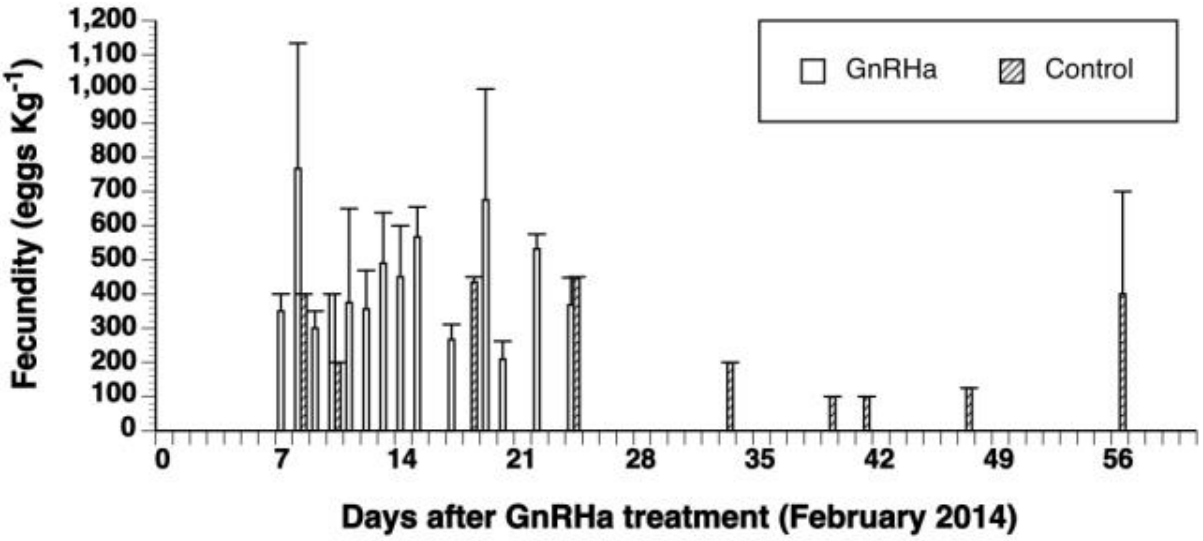

Figure 5.2.2 Mean (±SEM) daily egg production of Atlantic halibut treated with GnRHa implants (500 or 750 µg per fish) or left untreated as Controls.

The total egg production was higher but not statistically significantly higher in the GnRHa treated fish (**Fig. 5.2.3**), mainly because the fish spawned more times than control fish. It should be noted that in all groups, three individuals had only vitellogenic oocytes at the time of implantation, while one individual in each group was close to ovulation, as seen by presence of hyaline oocytes. Implantation did not appear to affect timing of ovulation in females with hyaline oocytes, as the control fish ovulated for the first time one day after the implanted females. Females implanted with vitellogenic ovaries all ovulated for the first time two to three weeks before controls at the same stage and had finished spawning by the time the first of comparable control fish ovulated.

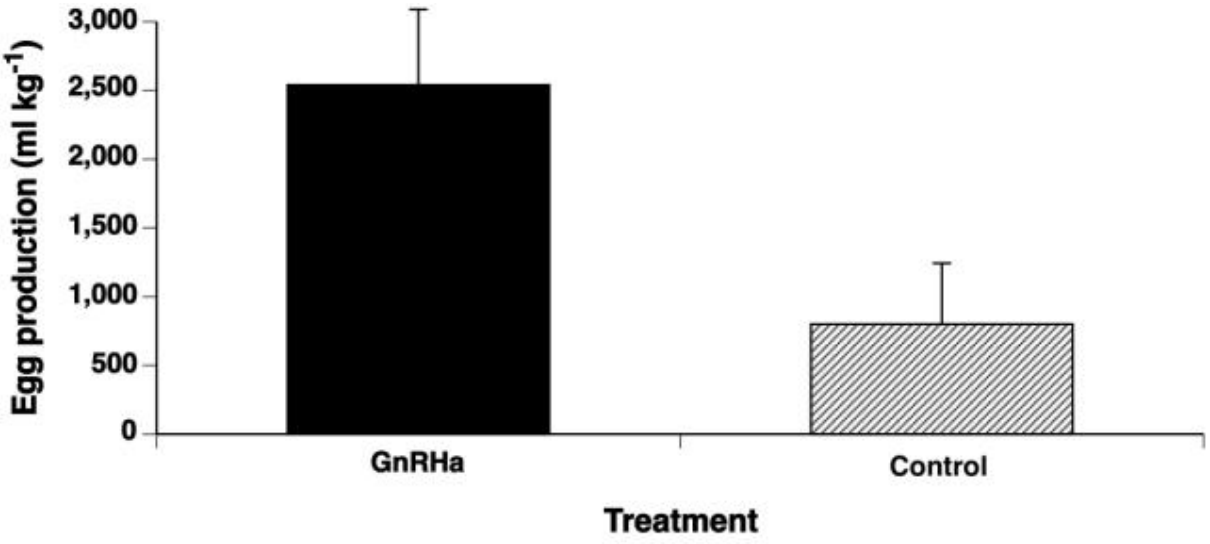

Figure 5.2.3 Total mean (±SEM) egg production of Atlantic halibut treated with GnRHa implants (500 or 750 µg per fish) or left untreated as Controls. There was no significant difference between the GnRHa treated and Control fish (ANOVA, P = 0.069).

Task 5.3 Fecundity regulation (led by IMR, Birgitta Norberg).

Ovarian biopsy samples were taken from F1 Atlantic halibut during vitellogenesis and spawning, frozen immediately on dry ice and stored at -80°C until processing. Total RNA was extracted from 50-100 mg of frozen tissue using the RiboPure kit, according to the manufacturers' instructions. A NanoDrop® NP-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) was used to measure the quantity and quality of the RNA samples. The quality of the RNA samples were checked using a 2100 Bioanalyzer (Agilent Technologies Inc. Santa Clara, USA), and no samples with RIN values below 8.5 were accepted for further analysis. Transcript levels of relevant genes will be analyzed in these samples using qPCR.

As mentioned earlier, due to the prolonged sick leave of (Dr Birgitta Norberg, planned analyses of data collections and egg samples from this Task have been also put on hold. Preliminary samples for histology and molecular biology were taken in 2014 and will be processed in the first half of 2015. The main sampling will start in the January 2015 reproductive season and will go on until 2016. The deliverable for this task is due in month 36 and should not be affected.

References

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Deviations from Annex I and their impact:

Due to a prolonged sick leave (August 2014 - December 2014) of the Principal Investigator of this WP (Dr Birgitta Norberg), planned analyses of data collections and egg samples from Task 1 have been put on hold. We expect to start the analyses in the first months of 2015 and there will be no delay in the deliverable for this task, which is due in month 30.

For the same reason, Task 3 was also delayed. Preliminary samples for histology and molecular biology were taken in 2014 and will be processed in the first half of 2015. The main sampling will start in the January 2015 reproductive season and will go on until 2016. The deliverable for this task is due in month 36 and should not be affected.

WP No:	6	**WP Lead beneficiary:**		P8. IEO
WP Title (from DOW):	Reproduction and Genetics - wreckfish			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P14. IFREMER	P15. ULL
P19. CMRM	P32. MC2			
Lead Scientist preparing the Report (WP leader):	Tito Peleteiro			
Other Scientists participating:	Constantinos Mylonas (P1), Christian Fauvel (P14), Fatima Linares, (Antonio Villar (P32),			

Objectives

1. Increase the availability of wreckfish broodstocks in captivity,
2. Describe the reproductive cycle in captivity at the level of the pituitary and gonad,
3. Develop spawning induction procedures for *in vitro* fertilization, as well as spontaneous tank spawning.
4. Develop a CASA for evaluation of wreckfish sperm and establish cryopreservation protocols for use in *in vitro* fertilization applications.

Summary of progress towards objectives and details for each task:

All tasks were initiated. Three juvenile potential breeders were collected and acclimatised to captivity in **Task 6.1. Collect wild fish to establish new broodstocks.** In **Task 6.2. Describe reproductive cycle,** two broodstocks were blood sampled and >60 wild fish were sampled. Initial results indicated that wreckfish held in captivity produced sufficient sperm over a long reproductive season and that females can complete vitellogenesis. However, two problems were encountered: many breeders sampled for blood did not mature and wild mature fish were prohibitively expensive. To increase the number of fish sampled in future years breeders from more broodstocks will be sampled and sampling of large mature wild fish will be coordinated with other WPs that also require large fish. **Task 6.3. Development of spawning induction procedures** has been initiated with work in three different broodstocks. Fish spawned spontaneously, were induced to spawn and eggs were stripped for *in vitro* fertilisation. However, few fertilised eggs were produced and no eggs were provided for larval culture studies. These preliminary results indicate how the work should progress to ensure fertilised eggs are obtained, with a focus on in vitro fertilisation. **Task 6.4. Evaluation of sperm characteristics and cryopreservation protocols** was initiated with the first characterisation of wreckfish sperm. The sperm had the highest reported velocity for a marine fish and duration of motility of 5 min. This work will be completed with more sperm samples. Altogether, the advances in all tasks in the work package represent a start that can be used to ensure the desired advances and deliverables in the coming years.

Task 6.1. Collect wild fish to establish new broodstocks (led by CMRM, Fatima Linares).

Regarding this task, 2 fish with a body weight of 2 kg each on 12/6/2014 and 1 fish weighing 1.5 kg on 26/08/2014, were captured using a hand net since these fish are usually found below floating objects. The fishing area was located to 5 miles West of Corrubedo Cape, La Coruña (**Fig. 6.1.1**). Fish were transported by sea on a ship in tanks with flow-through water, until the facilities from the "O Grove" Aquarium (Pontevedra), where the fish were maintained in quarantine until weaning to inert food. Morphometric measurements were performed and samples of the fin were taken for future genetic identification.

Fig. 6.1.1 Capture zone (Spain) (A), Hand net "salabre" used to capture juveniles (B), Quarantine tank (C).

Task 6.2. Describe reproductive cycle (led by IEO, Tito Peleteiro).

A stock of 5 wreckfish (captured from the wild as juveniles) has been maintained at P1.HCMR in two 15-m^3 tanks, under simulated natural photoperiod and constant temperature (16°C). The fish were fed 3 times a week with raw fish (mackerel). Unfortunately two of the fish stopped eating in the summer of 2013 and eventually died prior to the reproductive season in 2014. The remaining three fish (two males of 7.4 and 11.2 kg, and a single female of 11.8 kg) were followed during Year 1 of the project, beginning on 28 April 2014, collecting blood samples and gonadal biopsies. At this time, the female was undergoing vitellogenesis, having oocytes of 1325 µm in diameter (**Fig. 6.2.1**). The males were in full spermiation (Spermiation Index = 3, copious sperm released with very gentle abdominal pressure). On 12 May 2014, the female was examined again and at this time the ovaries contained not only vitellogenic oocytes (1250 µm), but also oocytes in oocyte maturation (1450 µm), while some eggs (25,000) were also released in the tank, but were not fertilized (**Fig. 6.2.1**).

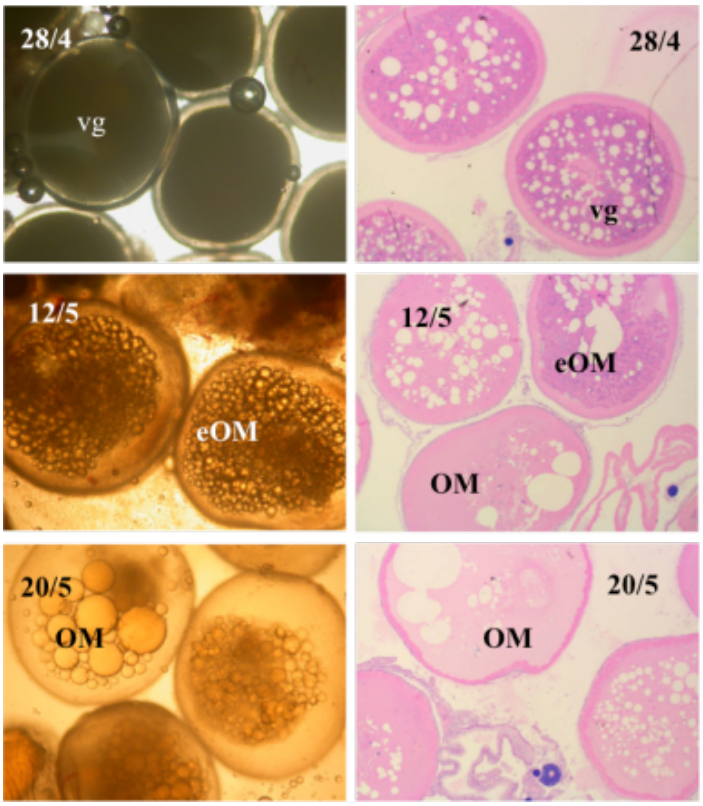

Fig. 6.2.1 Wet mount and histological sections of biopsies from wreckfish during the 2014 reproductive season (dates on each photo). eOM = early oocyte maturation, OM = oocyte maturation, Vg = vitellogenic

Sperm quality parameters that were evaluated included; sperm concentration (number of spermatozoa ml^{-1} of sperm), percentage of spermatozoa showing forward motility immediately after activation (initial sperm motility, %) and duration of forward sperm motility of at least 10% of the spermatozoa in the field of view (motility duration, min). Sperm concentration was estimated after a 21 to 4221-fold dilution (depending on spermatozoa density) with seawater using a Neubauer haemocytometer under 200X magnification (in duplicate) in a compound light microscope. Sperm motility (% spermatozoa showing forward motility) and motility duration (min) were evaluated on a microscope slide (400X magnification) after mixing 1 µl of sperm with a drop of about 50 µl of saltwater (in duplicate). Activated sperm samples were observed for the first time 10 sec after activation under the compound light microscope at a 400X magnification. Sperm motility was determined subjectively using increments of 10% and sperm was considered immotile when less than 5% of the spermatozoa were moving. Sperm was stored at 4°C for the following days, and was examined every other day for motility, until no forward motility was observed. The survival time (days) for each sample was considered as the day before the sample was found to have lost all its motility capacity.

Sperm quality was fairly high during the whole reproductive season and though some significant variations were observed in different parameters (ANOVA, DNMR, $P \leq 0.05$), there was no trend indicating either a reduction during the season or improvement in response to the GnRHa implantation (**Fig. 6.2.2**) that was given to the fish at the start of the spawning induction experiment (see Task 6.3 below).

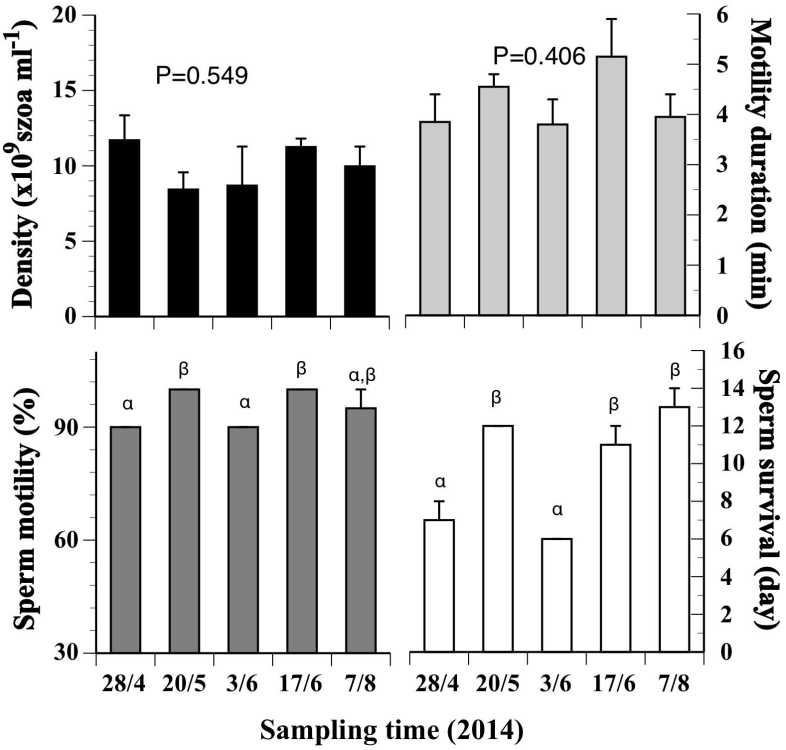

Fig. 6.2.2 Sperm quality parameters of wreckfish during the 2014 reproductive season. Different letter superscripts indicate the existence of significant differences (ANOVA, Duncan's New Multiple Range test, $P < 0.05$).

The results obtained so far demonstrated that males produce large volumes of good quality sperm for a very long period of time in captivity. Further sampling and analysis of the sex steroid hormones will provide more information as to the onset of the reproductive season, and the correlation with relevant hormones.

For the description of the reproductive cycle in captivity, blood samples were also obtained from the P19. CMRM stock of 12 wreckfish weighing between 9.94 and 18.28 kg, maintained in a 40-m^3 tank with natural

temperature (13-21ºC) and photoperiod (**Fig. 6.2.3**). Animals were fed dry broodstock pellets 3 times a week between January and June 2014. Blood was extracted from the gills using hypodermic needles. Samples were processed using the common protocol, centrifuged, and the plasma stored at – 80ºC until analysis. These blood samples were planned to be analysed at P1.HCMR for sex steroid hormones (Estradiol, Testosterone and 11 Ketotestosterone), together with the samples obtained from the other partners involved, in order to describe the annual reproductive cycle of male and female wreckfish. The fish that were blood sampled in P19. CMRM were expected to mature, which would have enabled the sampling of oocytes and sperm. However, these fish did not mature and could not be biopsied to obtain ovarian tissue or have sperm extracted. Therefore, the blood samples obtained cannot provide information of blood steroid parameters in association with known stages of gametogenesis. It is important that in coming years blood sampling is made in all broodstocks from breeders with a known stage of gametogenesis. This work must be made in all stocks as part of routine periodic sampling to assess maturity and to ensure sufficient samples are obtained during the years that remain to complete this work, 2015-2017. In this way the deliverable can be achieved whilst both increasing the number of fish sampled at a known stage of gametogenesis and reducing the stress to individual breeders or entire broodstocks.

Fig 6.2.3 Monitoring of sexual maturation, blood sampling and fin extraction samples.

Regarding the wild fish, a total of 60 fish were sampled between January and October of 2014 in the fish market in order to obtain information of this species (**Fig. 6.2.4**). For each animal sampled, total length (cm), total and eviscerated weight (kg), % of the peri-visceral fat, as well as the gonadosomatic ((gonad weight/body weight) x 100) and hepatosomatic index ((liver weight/body weight) x 100) was determined (**Table 6.2.1**). Samples from the stomach, liver, gonads, muscle and fins were taken for biochemical (P19. CMRM, P15. ULL) and histological studies. Total weights varied between 3.6 and 18 kg, and total length varied between 56 and 98 cm (**Fig. 6.2.5**). A relation between weight and length was established, both for males and females (**Fig. 6.2.6**).

Table 6.2.1 Biometric parameters and indexes from the 60 animals sampled from the wild in the Azores fisheries.

BIOMETRIC PARAMETER (60 WILD WRECKFISH)	MEAN	STD
TOTAL LENGHT	76,09	6,788
ST LENGHT	66,38	7,629
PERÍMETER	55,68	5,986
WEIGHT (Kg)	7,52	2,169
EVIS. WEIGHT (Kg)	6,99	1,967
GONAD WEIGHT (g)	17,10	20,831
LIVER WEIGHT (g)	95,70	71,671
FAT PERIVIS. WEIGHT (g)	76,25	72,233
STOMACH WEIGHT (g)	125,90	56,183
INTESTINE LENGHT (cm)	94,53	15,555
INTESTINE WEIGHT (g)	99,27	62,688
GSI FEMALES	0,30	0,184
GSI MALES	0,13	0,126
SHI	1,21	0,497
VSI	10,31	17,233

Fig. 6.2.4 Blood sampling from the gill (A), biometric sampling in the fish market (B), and gonad appearance inside the abdominal cavity (C and D).

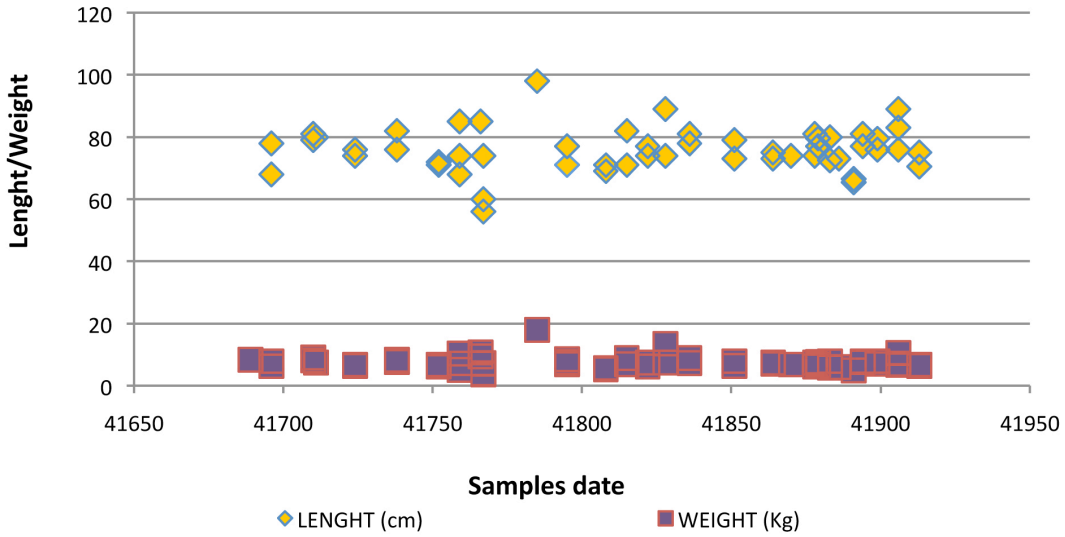

Fig. 6.2.5 Total length and weight from the 60 animals sampled from the wild in the Azores fisheries.

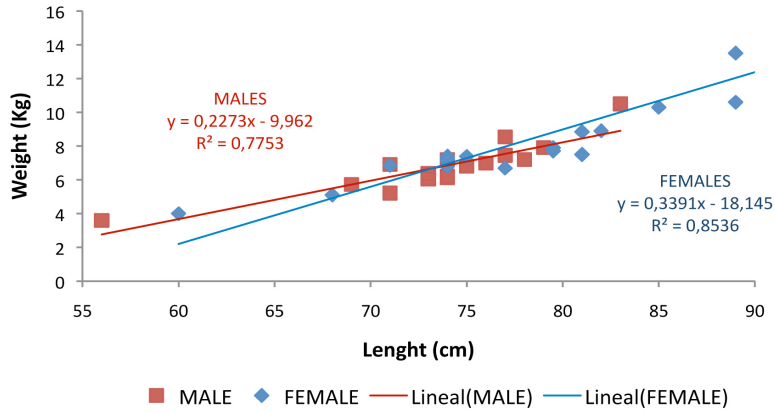

Fig. 6.2.6 Weight/length relationship from males and females captured from the wild in the Azores fisheries.

No relation was observed between weight, sex and perivisceral fat from the 60 fish sampled in the fish market (**Fig. 6.2.7**). No relation was observed between perivisceral fat % and date of capture from the 60 dead wild fish sampled (**Fig. 6.2.8**). This information would be important for nutrition studies and future elaboration of artificial feeds for this species (WP12 Nutrition - wreckfish).

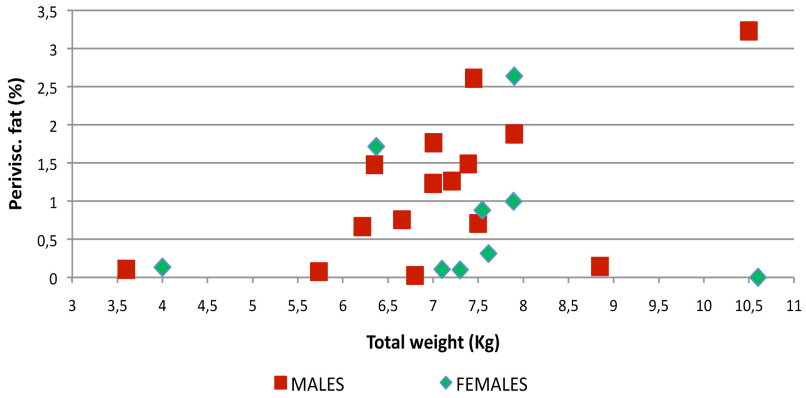

Fig. 6.2.7. Relation between weight, sex and perivisceral fat (%) from the 60 animals sampled from the wild in the Azores fisheries.

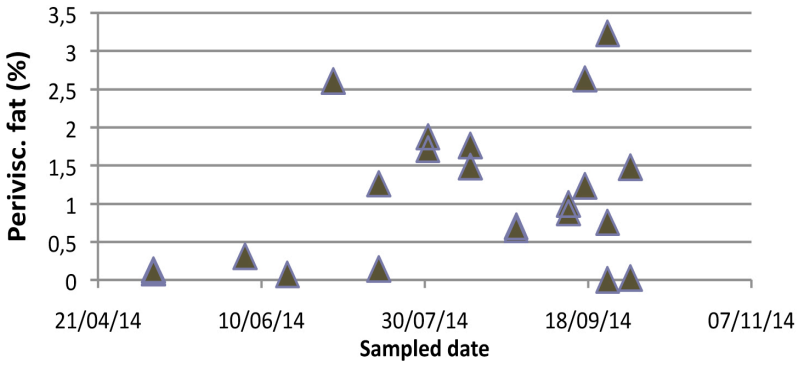

Fig 6.2.8. Relation between perivisceral fat % and time of capture from the 60 animals sampled from the wild in the Azores fisheries.

During the sampling all animals were visually identified as female and male based on gonad morphology. In addition histological analysis of the gonads was also performed in order to confirm sex and study the possibility of hermaphroditism in this species (**Fig. 6.2.9**).

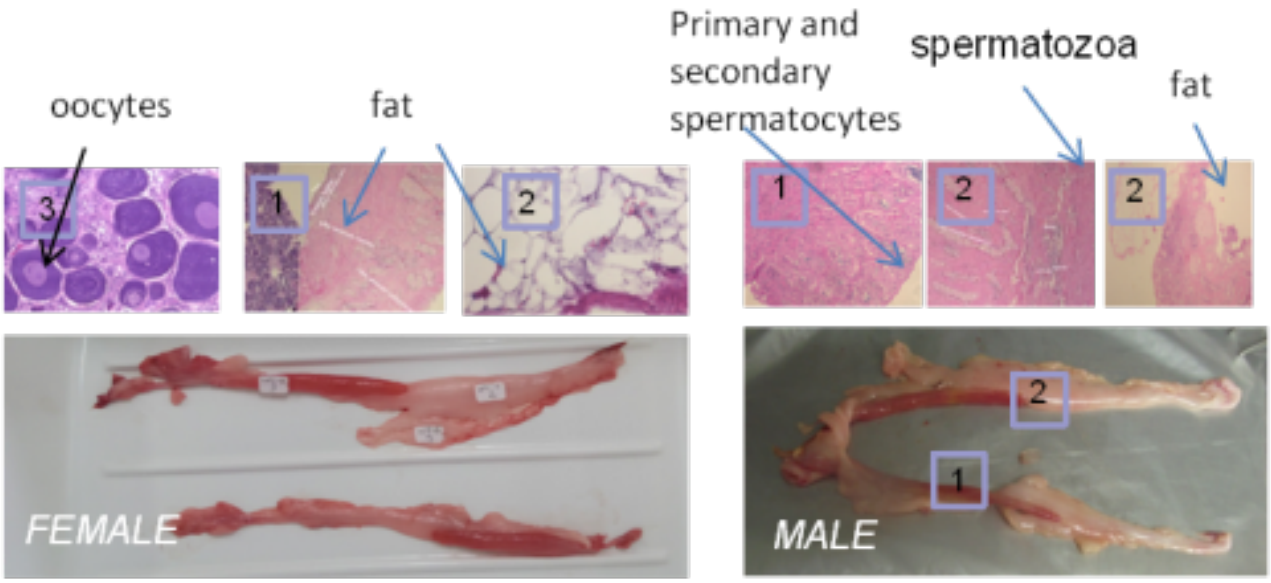

Fig. 6.2.9 Gonadal histology of females and males sampled from the wild in the Azores fisheries.

Task 6.3. Development of spawning induction procedures (led by IEO, Tito Peleteiro).

P1. HCMR Stock. When the female that was monitored for reproductive stage of development from Task 6.2 above was examined on 12 May 2014, the ovaries contained not only vitellogenic oocytes (1250 µm), but also some oocytes in oocyte maturation (1,450 µm), while some eggs (25,000) were also released in the tank, but were not fertilized (**Fig. 6.2.1**). The female was given a GnRHa implant (500 µg) and was placed together with one of the males (also given a 400 µg GnRHa implant), in a large tank (40 m^3) for spawning. The first spawn was obtained on 16 May (52,000 eggs) and another spawn was obtained on 19 May (58,000), but in both cases a very small number of eggs were fertilized <<1%. A day later (20 May) the fish were sampled for maturity (biopsied and sperm stripped) and the female contained post-ovulated eggs and many vitellogenic oocytes, some in atresia/apoptosis (**Fig. 6.2.1**), while the male was still in full spermiation. Both fish were implanted again with GnRHa and were returned to the tank for spawning. Another spawn was obtained on 23 May (3,000 eggs), but again no fertilization was observed.

A final effort to induce spawning was undertaken on 3 June, giving a higher dose of GnRHa (750 µg). At this time the female contained both post-ovulated eggs and vitellogenic oocytes, but with a high occurrence of atresia (**Fig. 6.3.1**). The fish did not spawn again, and all three fish were removed from the tank on 6 June and the experiment was concluded. Fish continued to be monitored for the following weeks, and the female maintained a large number of vitellogenic oocytes, while the males were in full spermiation condition until at least 8 July (**Fig. 6.3.1**).

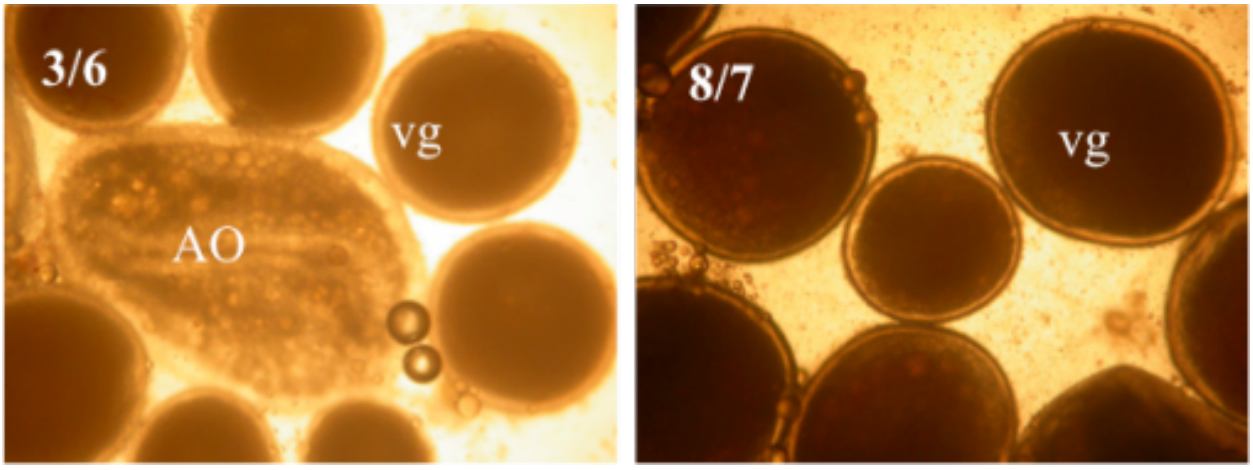

Fig. 6.3.1. Wet mount of biopsies from wreckfish during the 2014 reproductive season (dates on each photo). AO = apoptotic/atresic oocyte, Vg = vitellogenic.

This experiment demonstrated a few important aspects about wreckfish broodstocks in captivity. Firstly, as indicated in Task 6.2 above, males produce large volumes of good quality sperm for a very long period of time. Similarly, females do undergo vitellogenesis --and were also observed to undergo oocyte maturation spontaneously in captivity—and remain in fully vitellogenic stage for at least 3 months (under constant water temperature). Secondly, treatment of females with GnRHa implants can induce oocyte maturation and ovulation, but spontaneous spawning is somehow impaired and the resulting eggs were not fertilized in high percentages. It cannot be determined at this stage if this problem is because of the males were not releasing sperm at the time the female liberated eggs, or if the female does not release eggs soon after ovulation, thus undergoing over-ripening. In the following year, we will continue the work as planned in the DOW to try *in vitro* fertilization after the induction of maturation and ovulation with the GnRHa implants.

P8. IEO Stock. A stock of 9 wreckfish (4 females, 3 males and 2 undetermined), weighing between 9.5 and 18.9 kg, were maintained in a 130 m^3 tank with natural temperature (12-21ºC) and photoperiod. Animals were fed 3 times a week with semi-moist pellets elaborated at our research facility based on special fish paste normally used for parental diets. This stock was sampled twice a month during spawning season, in order to monitor sexual maturation (**Fig. 6.3.2**). No evidence of sexual maturation was observed on females. On the contrary, two males showed spermation, and were used in Task 6.4.

Fig 6.3.2. Periodic samplings from the P8. IEO stock to monitor stage of reproductive development.

P32. MC2 Stock. A stock of 27 wreckfish (11 females, 12 males and 4 unknown), weighing between 10.7 and 30.3 kg, were maintained in a 3500 m^3 exhibition tank (Nautilus) with natural temperature and photoperiod, and fed sliced fish and squid on a daily basis. When the first external evidence of reproductive maturation were detected (abdominal swelling), animals were transferred to a 50 m^3 tank for closer monitoring. Five females with evident abdominal dilatation were isolated, as well as three spermiating males.

These males were replaced in two occasions due to aggressiveness between them. During the breeding season, when the stock was sampled on a weekly basis to monitor the maturity stage of the females. Ovarian biopsies were taken from 9 females, to determine oocyte stage and diameter (**Table 6.3.1**)

Table 6.3.1 Evolution in oocyte diameter from 9 females from the P32. MC2 stock

Plastic tagg	Tagg	06/03/2014	20/03/2014	29/04/2014	13/05/2014
	Nº 8 00-0618-1E7D	973,5 ± 37,150 µ	1040 ± 50,7093 µ	1160 ± 109,5445µ	1126± 66,619 µ
	Nº 9 00-0618-16E6		1143,3 ± 104,9943 µ	1155± 114593102 µ	1563± 352,04 µ
	Nº 11 981023604036	660 ± 118,300 µ	733,3± 143,5104 µ	925± 125,13151 µ	976± 74,527 µ
	Nº 12 98102355554		980 ± 64,9175 µ	1010± 96,7906042 µ	1074± 62,549 µ
	Nº 19 98102357438		846,7 ± 107,6812 µ	1060 ± 68,0557 µ	995± 71,10 µ
Wide mouth	Nº 20 2356915	403,5 ± 176,000 µ	870 ± 99,6422 µ	965± 122,581874 µ	940 ± 82,115 µ
	Nº 21 00-0618-1779		976,66 ± 67,7882 µ	1095± 114,593102 µ	2138 ± 135,6044 µ
	00-0643-7B78			940± 114,248114 µ	1016± 65,5526 µ
	00-061D-5679				1066± 82,346 µ

From the 5 females in isolation, three were submitted to abdominal massage for oocytes extraction. Sperm was obtained from males. *In vitro* fertilization was performed, but spawn quality was poor, despite the oocytes were mature (2300 µm in diameter). The remaining two females spawned naturally in the tank, from May until August (**Fig. 6.3.3**). Eggs were collected and measured. Percentage fertilisation was determined. In almost all cases egg quality was poor, except the one from June 4th, with a percentage fertilisation was 70%. Nevertheless, this spawn was also of poor quality, since only 14% of the eggs hatched.

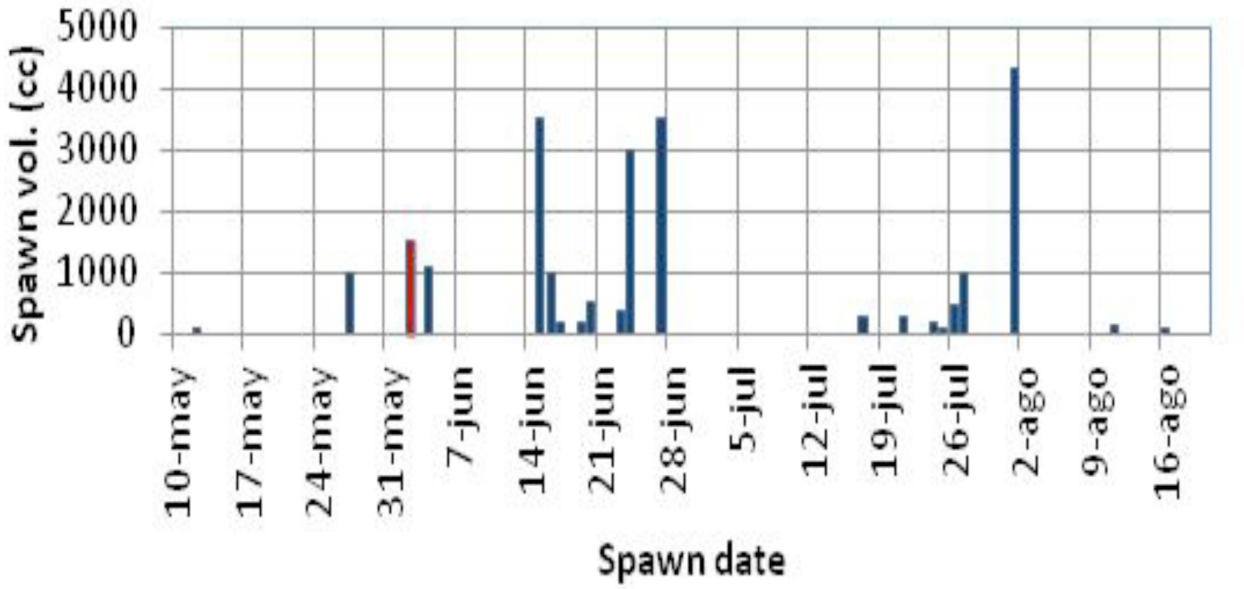

Fig. 6.3.3 Total volume of collected eggs in Acuarium Finisterrae (P32. MC2) in 2014.

Fig 6.3.4 Abdominal massage to obtain eggs and sperm to be used in artificial fertilization (P32. MC2).

Task 6.4. Evaluation of sperm characteristics and cryopreservation protocols (led by IFREMER, Christian Fauvel).

From April 8th to April 13th, sperm was collected from 6 males P32. MC2, 2 males of Luso Hispana de Aquicultura (LHA) and finally 2 males from the facilities of P8. IEO in of Vigo, in Galicia (Spain). To estimate sperm concentration, the collected semen were diluted to 1/1000 in distilled water and 2 aliquots were placed into Thoma counting plate. Pictures at X200 magnification were recorded using a digital camera. Then, the concentrations were assessed by image analysis using image J free software. The mean concentration of wreckfish sperm was 2.41 $x10^{10}$ (sd : 0.4 $x10^{10}$, n=9) spermatozoa per ml.

Only fresh sperm from Vigo could be correctly assessed for motility since gametes from the other sites were transported in suboptimal conditions of conservation before lab analyses (including temperature control and storage medium quality). At the P8. IEO laboratories, sperm activation and motility were recorded using a USB digital videocamera (imagingsource DMK 22BUCO3, Germany) at 30 frames s^{-1}. The videos were prepared through the Image J software and then analyzed using the plugin developed by Wilson-Leedy and Ingermann (2007). When the procedure was established and parameters adjusted, the method was recorded as a macro (**Fig. 6.4.3**). The two analyzed fresh sperm samples showed an initial velocity of more than 230µm per second which progressively decreased to 0 after 5 minutes (**Fig. 6.4.1**). The velocity of spermatozoa was one of the highest reported for marine fish and the trajectories vary from straight forward at activation to round as the speed decreased (**Fig. 6.4.2**).

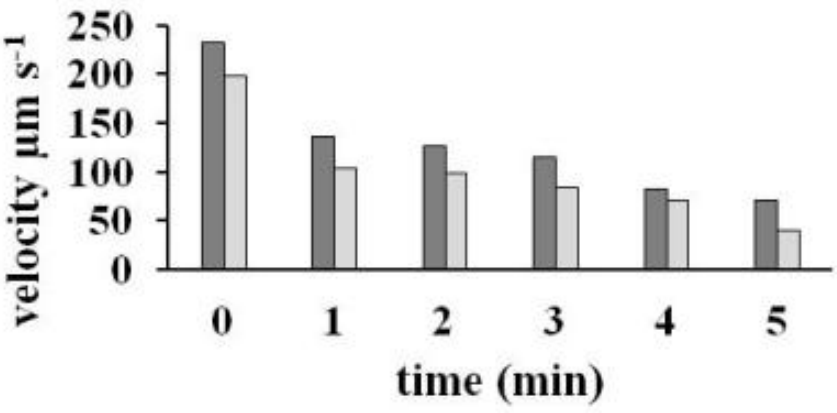

Fig. 6.4.1 Variations with time of Average Path Velocity i.e. along smoothed trajectory (dark grey) and Straight Line Velocity picturing the progressive movement (light grey).

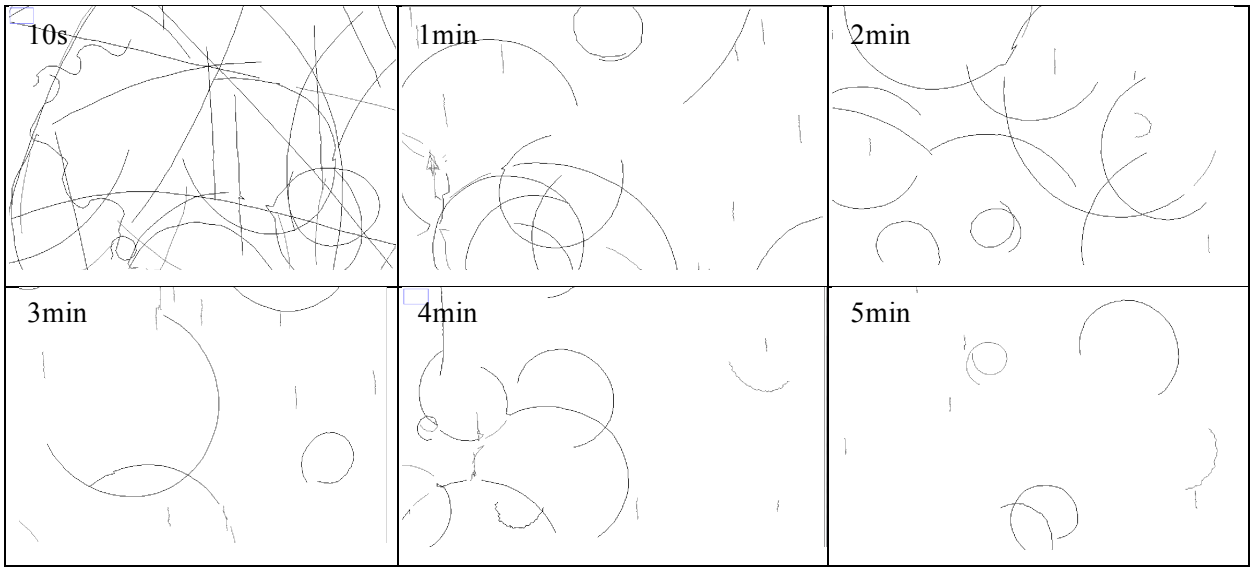

Fig. 6.4.2 Two second path tracks generated by computer assisted sperm analysis (CASA plugin of Image J) at 10 sec to 5minutes after sperm activation in wreckfish.

```
//run("Brightness/Contrast...");
setMinAndMax(160, 162);
run("Apply LUT", "stack");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(129, 255);
setOption("BlackBackground", false);
run("Convert to Mask", "method=Default background=Dark calculate");
run("CASA ", "a,=0 b,=1000 c,=5 d,=30 e,=20 f,=20.000000000 g,=20 h,=5.00000
i,=1   j,=25.000000000   k,=25.000000000   l,=35.000000000   m,=80.000000000
n,=80.000000000 o,=50.000000000 p,=60.000000000 q,=30 r,=460 s,=0 t,=1");
String.copyResults();
```

Fig. 6.4.3 Script of the macro established for automated CASA analysis of wreckfish sperm using Image J software and plug-in.

Sperm did not start moving at activation after 24h raw storage but exhibited motility if diluted at collection in storage media although lower speed and lower motility duration were recorded compared to fresh sperm. Conditions of sperm storage and transportation must be improved if male and female gamete collection occurred in separate locations or different times. For this, storage media and temperature of preservation would be adapted to this species.

As a conclusion, this first year experiment allowed establishing the assessment method for concentration and motility of sperm. Due to a problem of storage during transportation, the results are based on only two males so that they must be confirmed during the second year of the project. This type of initial problem was taken into account in the DOW so that this is not considered as a deviation.

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Deviations from Annex I and their impact:

There was no deviation to the work in this WP. However, a limited number and low quality of fertilised eggs were obtained under **Task 6.3 Development of spawning induction procedures**, which had a negative impact on WP18 Larval husbandry – wreckfish, as indicated by the poor results obtained in **Task 18.2 Development of feeding methodology**. In 2015, work will continue aimed to ensure more and better-quality fertilised eggs. In particular, the broodstock diets will be improved, and spawning trisals will be focused both on spontaneous spawning (P32. MC2) that has given tentative promising results, and *in vitro* fertilisation spawning (P1. HCMR, P8. IEO, P19. CMRM, P32. MC2) that when fully developed offers complete control to obtain of good quality gametes and eggs.

WP No:	7	**WP Lead beneficiary:**		P7. IOLR
WP Title (from DOW):	Reproduction and Genetics – grey mullet			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P13. UNIBA	P14. IFREMER
P15. ULL	P24. ITTICAL	P25. DOR		
Lead Scientist preparing the Report (WP leader):	Hanna Rosenfeld			
Other Scientists participating:	Constantinos Mylonas (P1), Neil Duncan (P3), Aldo Corriero (P13), Christian Fauvel (P14), Covadonga Rodriguez (P15), Fulvio Ceppolaro (P24), Gilad Safran (P25)			

Objectives

1. Evaluate the effectiveness of hormone-based treatments on synchronizing gonadal development and improving gamete (eggs and sperm) quality in mature grey mullet,
2. Develop hormone-based treatments for induced spawning of grey mullet,
3. Optimize a scaled-up breeding of grey mullet in captivity under natural and manipulated photo-thermal regimes,
4. Assess the effects of captivity on first sexual maturity and reproductive potential of captive-reared and hatchery-produced grey mullet broodstocks.

Summary of progress towards objectives and details for each task:

Lacking the natural spawning environment, captive grey mullet (*Mugil cephalus*) fail to reproduce spontaneously, largely due to a failure to undergo complete gametogenesis. Therefor, **Task 7.1 Evaluated the effectiveness of hormone-based treatments on synchronizing gonadal development**. A combined treatment consisting of follicle stimulating hormone (FSH) and dopamine antagonist (metoclopramide) on spermatogenesis in males and follicle growth and maturation in females was tested. The methylotrophic yeast (*Pichia pastoris*) expression system was used to produce large quantities of bioactive recombinant single-chain FSH, which was used in a series of *in vivo* assays. Unlike the controls, the hormonally treated groups (injected with rFSH and metoclopramide during the onset of the reproductive season) demonstrated synchronized gonadal development within and between sexes, with higher rates, over time, of spermiating males and post-vitellogenic females. In **Task 7.2 Development of hormone-based treatments for inducing spawning,** spawning induction trials that timed the administration of GnRH and metoclopramide with advanced stages of gamete maturation were relatively successful producing tens of millions of fertilized eggs. Nevertheless, our results highlight two major problems: (i) female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) episodic fertilization rate ranging between 0 to 98%, implicating the need to further fine tune and optimize the hormone-based breeding protocol for captive grey mullet.

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development (led by IOLR, Hanna Rosenfeld).

7.1.1 Recombinant gonadotropins production

Recombinant yoked FSH (i.e., both alpha and beta subunits are fused to a single chain) encompassing 6 histidine residues (6–His Tag) was produced using the *Pichia pastoris* yeast expression system under optimal

conditions. The His-Tag recombinant protein was purified by a Ni-NTA affinity column as described in Berkovitch *et al.* (2013) yielding a satisfying quantities (2-5 mg per liter of culture supernatant) allowing for *in vivo* experimentation (see Task 7.1.4 below).

In addition, the cDNAs encoding for the grey mullet gonadotropin hormone specific β-subunit (i.e., mLHβ and mFSHβ) were synthetically synthesised and introduced into pPIC9 expression vector. Work is in progress to produce the respective recombinant β-subunits in the yeast expression system. These recombinant proteins will be used as antigens for the immunization of rabbits, generation of polyclonal antibodies, with which the respective hormone-specific ELISAs will be established.

7.1.2 Broodstock source and conditioning

Grey mullet brooders consisted of P4. IOLR hatchery produced F1 fish (n= 186; age: 5-year old). The fish were tagged individually and maintained in 4-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). 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). S ex was predicted using a vitellogenin dotblot immunoassay as described in Aisen *et al*. (2005) and validated during gametogenesis when gonadal biopsies were performed.

7.1.3 Means to evaluate mullet sperm quality

In order to develop Computer Assisted Sperm Analysis (CASA) to characterize grey mullet sperm, a short term scientific mission (arranged through the AQUAGAMETE COST network) was completed between P4. IOLR where the broodstock are maintained and P14. IFREMER, which is the Partner responsible for the sperm quality quality assessment in all species. The purpose was to work together on the general methods for sperm quantification and qualification regardless of the species in order to pinpoint the critical steps in the methods required to establish a protocol of CASA to be implemented further in the assessment of the effects of hormonal treatments on mullet sperm.

Dr Iris Meiri Ashkenazi from P4. IOLR, travelled for one week to the experimental aquaculture station of P14. IFREMER, Palavas les flots. A complete cycle of sperm analysis from sperm collection to data treatment was performed using meagre sperm provided by a collaborating private company (Les Poissons Du Soleil, Balaruc, France). Moreover, in order to provide different situations, sperm was subject to contrasted storage conditions. Sperm samples of 8 males were studied. Concentration and motility were analysed using the free software Image J developed by NIH (National institute of Health, USA) and based on image analysis the concept of which was explained and detailed.

This training course was concluded by a short report available on internet (http://aquagamete.webs.upv.es/wp-content/uploads/2013/01/Iris-Meiri.pdf) showing the type of result to be expected from the presented methods. The latter protocol enabled to characterize, for the first time, motility parameters of activated grey mullet spermatozoa in high capturing speeds (>120 fps) facilitating enhanced accuracy, reliability and high throughput spermatozoa concentration measurements.

Finally, with a slight delay from the DOW, a public report entitled “Analysis of sperm motility: General protocol and propositions for mullet sperm quality assessment” will be released by the end of month 13 of Diversify project as the deliverable D7.1

7.1.4 Hormonal acceleration of gonadal development

An *in vivo* trial to enhance gametogenesis was carried out during the onset of the grey mullet natural reproductive season in the Mediterranean Sea (mid July 2014). Both mullet females and males were subjected to hormonal treatment consisting of recombinant FSH (7 mg per kg BW) combined with Metoclopramide (dopamine antagonist; Metoc) dissolved in ddH2O (15 mg/kg BW). 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 relative abundance of fully mature females exhibiting average oocyte diameter greater than 550 µm and spermiating males at early- and mid-spawning season (mid September and mid October, respectively) in control groups was lower than in hormonally treated fish (**Table 7.1.1**). The FSH-Metoc treatment also synchronised gonadal maturation in both grey mullet females and males, giving a high percentages of specimens adequate for the spawning induction trials as compared to numbers found in the control groups (see Task 7.2). The effect was more pronounced in females than in males.

Table 7.1.1 Relative abundance of post-vitellogenic grey mullet females and spermiating males at early- and mid-spawning season (mid September and mid October, respectively) 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

Task 7.2 Development of hormone-based treatments for inducing spawning (led by IOLR, Hanna Rosenfeld).

Ovarian development (oocyte diameter), was examined from September to October (natural spawning season) by obtaining ovarian biopsies. Females were considered post-vitellogenic when mean oocyte diameter was >550 µm (De Monbrison *et al*., 1997) and more than 50% of oocytes exhibited germinal vesicle migration. In parallel, males were checked for the presence of milt by applying gentle abdominal pressure. Once identified, a post-vitellogenic female was stocked with two or three spermiating males in a 1-m^3 tank supplied with seawater at 24-27°C. The selected fish were treated with GnRHa and Metoclopramide. Each treatment consisted of priming (GnRHa 10 µg/kg; Met 15mg/kg) and resolving injections (GnRHa 20 µg/kg; Met 15 mg/kg) given 22.5 h apart. Spawning trials were carried out from Mid-September throughout November 2014. The results (**Table 7.2.1**) highlight two major problems: (i) female's failure to ovulate (70% and 57% in control and hormonaly-treated groups, respectively) despite the spawning induction treatments and (ii) episodic fertilization rate ranging between 0 to 100%.

Table 7.2.1 Mean (±sem) spawning data from hormonally induced grey mullet in September-October 2014. Spawning ratio is the percentage females that ovulated after injection.

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		

Task 7.3 Optimization and scale-up of a breeding protocol for grey mullet in captivity (led by IOLR, Hanna Rosenfeld).

No work done during this period**.**

Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish (led by IOLR, Hanna Rosenfeld)

Following successful spawning induction trials (Task 7.2), the resultant high quality eggs and larvae (Task 19.1) gave rise to large numbers of robust juveniles (Task 13.1). These fish will be monitored until sexual maturity and compared to similarly reared wild caught juveniles.

Task 7.5 Establish a shipping protocol for grey mullet eggs (led by DOR, Gilad Safran)

In this task a short distance (440 km) mullet egg transport (10 h from egg collection to arrival in Eilat) in August 2014 was carried out between a hatchery in kibbutz Ma'agan Michael in the north of Israel to the IOLR facility in Eilat (these eggs were from a photoperiod brood stock and used for the taurine WP19 study). After arrival, egg hatching success was 91.1% and the survival of hatched larvae to 1 day post hatching was 91.4%. However, simulation studies of long distance egg transports were not carried out due to the large number of IOLR mullet eggs spawned in October, and the resultant juveniles, needed to perform Tasks 19.1, 13.1 and 23.2. These transport studies have been postponed to the IOLR autumn spawning in 2015 where egg stocking densities and temperatures will be tested in simulated egg transport up to 30 h.

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De Monbrison, D., Tzchori, I., Holland, M. C., Zohar, Y., Yaron, Z., Elizur, A., 1997. Acceleration of gonadal development and spawning induction in the Mediterranean grey mullet, *Mugil cephalus*: Preliminary studies. Isr. J. Aquac. Bamidgeh 49, 214-221.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

Group Work Packages

Nutrition

The importance of highly unsaturated fatty acids (HUFA) and the antioxidants vitamin E and vitamin C has not been investigated in meagre, despite the high oxidative risk that exist in this species with fast growing larvae. To improve current larval feeds, six weaning diets containing two levels of HUFA (0.4 and 3% dw), two of vitamin E (150 and 300 mg/100 g) and two of vitamin C (180 and 360 mg/100 g) were fed to 15 dph meagre larvae. Low HUFA/vitamin E/vitamin C diet reduced larval growth, lipid absorption and HUFA contents. Dietary HUFA levels of 3%, improved larval growth and lipid absorption and deposition. Among fish fed 3% HUFA, increased vitamin E and vitamin C improved body weight significantly, as well as lipid, 22:6n-3 and n-3 fatty acids contents in the larvae. Thus, the results demonstrated that weaning diets for meagre must be optimized by increasing HUFA levels up to 3% and vitamins E and C over 1500 and 1800 mg kg^{-1}, to spare these essential fatty acids from oxidation.

To improve larval enrichment products for greater amberjack, larvae were fed *Artemia* enriched with five levels of the essential docosahexaenoic acid (DHA). The lowest DHA content lead to poor survival, total length and body weight, whereas the increase in DHA levels in the *Artemia* up to 1-2% produced the highest survival, total length, body weight and fish welfare, but excess levels of DHA reduced growth. The results demonstrated the importance of adequate levels of DHA in *Artemia* (1-2% DHA) to prevent bone malformations and promote maximum growth and survival in greater amberjack. Also, rotifers were enriched according to the lipid composition of wild greater amberjack eggs, testing enrichment with different sources and levels of LC-PUFA rich lipids. The results indicated that rotifer enrichment with marine lecithin was the best for LC-PUFA enrichment based on the lipid composition of eggs from wild fish.

To increase the knowledge on the effect of nutrients essential for first feeding of pikeperch, fish were fed six different diets with increasing content of total phospholipids (TPL), as well as EPA and DHA. Survival rates were too low, due to the cannibalism and ranged between 1.9 and 12.3%. Samples were taken for growth, biochemical composition and enzyme activity, but the study will be repeated trying to avoid cannibalism.

Studies on wild fish composition, bibliography collection of feeding regimes in the wild and the feeding of wreckfish broodstock were conducted to allow the formulation of the broodstock diets. Thirty-four wild wreckfish were sampled from February to October 2104 to obtain samples of muscle, liver and gonads for biochemical analysis. Macroscopical and histological analysis showed that of 33 gonads examined, 15 were males and 18 females. Analysis showed that the level of proteins and lipids in muscle was 74-96% and 3-14%, respectively, whereas a high variability was observed in liver and gonads. Muscle polyunsaturated (PUFA), saturated (SFA) and monounsaturated (MUFA) fatty acids were 36-46%, 28-30% and 25-33%, respectively, whereas n-3 PUFA content reached 32-40%. Liver fatty acid profiles showed a broader variability with a lesser EPA, DHA and ARA content than muscle. Important differences in tissue composition were found between wild and cultured fish.

To improve enrichment products for live preys for grey mullet, the effect of dietary taurine on larval rearing at different stages of development was carried out. Each treatment was tested in replicates of 6 tanks: control (no taurine: T0-0), 400 mg taurine/l (T400-0) and 600 mg taurine/l of rotifer enrichment medium. Following the end of rotifer feeding at 14 dph, six rotifer-*Artemia* treatments (rotifer-*Artemia*) were tested to 19 dph in replicates of 3 tanks per treatment. These treatments were T0-0, T400-0, T0-400, T400-400, T600-0 and T600-600. The results showed a positive effect of taurine contents on rotifers on larval grow and survival at 44 dph, being stronger at the rotifer phase. Moreover, juveniles from the rotifer-only taurine treatments are being followed to see if the effect continues to give an advantage in older juveniles. To determine nutritional needs for improved weaning to a dry diet, samples were collected and will be studied in year 2015.

WP No:	8	WP Lead beneficiary:	P2. FCPCT
WP Title (from DOW):	Nutrition - meagre		
Other beneficiaries (from DOW):	P15. ULL	P20. SARC	P21. DTU
Lead Scientist preparing the Report (WP leader):	Lidia Robaina		
Other Scientists participating:	Covadonga Rodriguez (P15), Ramon Fontanillas (P20), Ivar Lund (P21)		

Objectives

1. Improve current larval weaning feeds for meagre,
2. Determine nutritional requirements to promote feed utilization, consistent growth rates and fish welfare to reduce size variation.

Summary of progress towards objectives and details for each task:

Task 8.1 Improvement of larval weaning feeds (led by FCPCT, Lidia Robaina).

A trial was conducted to determine the optimum level of essential fatty acids and related nutrients. Previous studies had pointed out the importance of highly unsaturated fatty acids (n-3 HUFA) for normal development of meagre larvae. However, as it has been shown in other species, dietary n-3 HUFA increase peroxidation risk and may lead to the production of free radicals that exert a great damage at a cellular and tissue level, markedly affecting fish health. Therefore, it is necessary to include optimum levels of antioxidants at a physiological level, such as vitamin E. In turn, vitamin C is required to re-cycle and protect vitamin E. Despite there are several studies regarding the importance of dietary n-3 HUFA and vitamin E in marine fish larvae, no information is available about its requirements in meagre larvae. Being a fast growing species with a fast metabolism, a high n-3 HUFA and antioxidant vitamins requirements could be expected in this species. Thus, the aim of the present study was to investigate the requirements of larvae for n-3 HUFA, and its nutritional interrelation with vitamin E and vitamin C.

Experimental conditions

The study examined the combined effect of several dietary contents of vitamin E and highly polyunsaturated fatty acids (HUFA) with the inclusion of two levels of vitamin C on culture performance, morphometric parameters, gut occupancy, larval organ and skeleton development, and biochemical composition. To determine this combined effect, a feeding experiment was conducted in larvae from 14 day after hatching (dah) to 28 dah in which two levels of n-3 HUFA (0.5% and 3.5% were tested). For each n-3 HUFA dietary inclusion, three combined levels of vitamin E+C were tested: 150 vitE+180 vitC, 300 vit E+180 vitC and 300vit E+360 vitC (**Table 8.1.1**). Diets had the same proximate composition (**Table 8.1.1**) and different levels of dietary n-3 HUFA/ vit E / vit C inclusion (**Table 8.1.2**).

Table 8.1.1 Main ingredients and proximate composition (g 100 g^{-1} dw) of the early weaning diets containing several n-3 HUFA, vit E and vit C levels used to fed larval meagre from 14 to 28 dah.

	Diet					
	0.5/150/180	**0.5/300/180**	**0.5/300/360**	**3.5/150/180**	**3.5/300/180**	**3.5/300/360**
Main ingredients						
Fish oil	0.00	0.00	0.00	10.00	10.00	10.00
Oleic acid	10.00	10.00	10.00	0.00	0.00	0.00
Vitamin E *	150.00	300.00	300.00	150.00	300.00	300.00
Vitamin C*	180.00	180.00	360.00	180.00	180.00	360.00
Proximate composition						
Lipid	16.01	17.09	17.06	17.52	17.34	17.44
Protein	65.14	64.72	64.97	65.43	65.45	64.88
Moisture	10.32	10.59	9.38	9.67	9.39	9.35
Ash	5.47	5.55	5.70	5.88	5.73	5.81

*mg $100g^{-1}$, Vitamin E: α-tocopheryl acetate (Sigma-Aldrich (Madrid, Spain), Vitamin C: Ascorbyl monophosphate ROVIMIX Stay-C-35 (Roche, Paris, France).

Culture performance and morphometric parameters

At the end of the feeding trial (28 dah), larval survival was not significantly different among the different groups of larvae, being in average 13.45±3.08 % (mean±SD)(**Table 8.1.3**). However, after only 10 days of feeding (24 dah), growth in terms of total length and dry body weight was significantly lowest in larvae fed diet 0.5/150/180 containing the lowest HUFA, vit E and vit C levels (**Table 8.1.3**). The highest growth was obtained in meagre fed diets 3.5/150/180, 3.5/300/180 and 3.5/300/360 (**Table 8.1.3**). Among fish fed 0.5% HUFA, elevation of dietary vitamin E from 150 to 300 mg/100 g significantly improved total length in 24 dah larvae ($P<0.01$) (**Table 8.1.3**). Among fish fed 3.5% HUFA, increase in both vit E and vit C significantly improved body weight ($P<0.05$) (**Table 8.1.3**) and a significant positive linear correlation was found between dry body weight and dietary vit E+vit C levels ($y=9E-05x+0.18$ $R^2= 0.995$).

The same trends were observed at the end of the feeding trial (28 dah). Thus, the two-way ANOVA analysis comparing the effect of dietary HUFA and vit E showed an improvement in growth, particularly body weight, when dietary HUFA levels were raised from 0.5 to 3.5%, whereas the effects of vit E or the interaction between both nutrients were not significant (**Table 8.1.4**). Similarly, the two-way ANOVA analysis comparing the effect of dietary HUFA and vit C showed a significant positive effect of dietary HUFA on fish weight, whereas the effects of vit C or the interaction between both nutrients were not significant (**Table 8.1.5**).

Table 8.1.2 Main fatty acid composition of the early weaning diets containing several n-3 HUFA, vit E and vit C levels used to fed larval meagre from 14 to 28 dah (% dry weight).

	Diet					
	0.5/150/180	**0.5/300/180**	**0.5/300/360**	**3.5/150/180**	**3.5/300/180**	**3.5/300/360**
14:0	0.09	0.09	0.08	0.60	0.63	0.63
14:1n-5	0.00	0.00	0.00	0.01	0.01	0.01
15:0	0.01	0.01	0.01	0.08	0.09	0.08
15:1n-5	0.00	0.00	0.00	0.00	0.00	0.01
16:0	2.41	2.17	2.00	3.29	3.84	3.26
16:1n-7	0.03	0.03	0.03	0.69	0.64	0.76
16:1n-5	0.00	0.00	0.00	0.03	0.03	0.00
16:2n-4	0.00	0.00	0.00	0.05	0.05	0.06
17:0	0.00	0.00	0.00	0.04	0.04	0.05
16:3n-4	0.01	0.01	0.01	0.05	0.05	0.05
16:3n-3	0.00	0.00	0.00	0.02	0.02	0.03
16:3n-1	0.01	0.01	0.01	0.01	0.01	0.02
16:4n-3	0.00	0.00	0.00	0.06	0.05	0.06
18:0	0.28	0.58	0.52	0.80	0.94	0.79
18:1n-9	9.85	10.08	10.12	3.43	3.16	3.29
18:1n-7	0.09	0.15	0.15	0.43	0.42	0.47
18:1n-5	0.00	0.00	0.00	0.02	0.02	0.02
18:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
18:2n-6	2.51	3.16	3.28	2.60	2.30	2.70
18:2n-4	0.00	0.00	0.00	0.02	0.02	0.03
18:3n-6	0.00	0.00	0.00	0.05	0.05	0.05
18:3n-4	0.00	0.00	0.00	0.02	0.02	0.02
18:3n-3	0.14	0.18	0.20	0.41	0.37	0.43
18:4n-3	0.00	0.00	0.00	0.15	0.14	0.16
18:4n-1	0.00	0.00	0.00	0.01	0.01	0.02
20:0	0.04	0.03	0.03	0.05	0.06	0.05
20:1n-9	0.02	0.01	0.00	0.06	0.05	0.06
20:1n-7	0.12	0.13	0.13	0.48	0.67	0.53
20:1n-5	0.00	0.00	0.01	0.03	0.04	0.04
20:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
20:2n-6	0.00	0.01	0.01	0.06	0.05	0.06
20:3n-9+n-6	0.00	0.00	0.00	0.01	0.00	0.01
20:3n-6	0.01	0.00	0.00	0.02	0.02	0.02
20:4n-6	0.01	0.02	0.02	0.13	0.12	0.15
20: 3n-3	0.03	0.01	0.01	0.04	0.03	0.04
20:4n-3	0.00	0.00	0.00	0.08	0.07	0.08
20:5n-3	0.09	0.10	0.11	0.95	0.86	0.99
22:1n-11	0.03	0.01	0.01	0.34	0.56	0.39
22:1n-9	0.03	0.01	0.02	0.07	0.09	0.07
22:4n-6	0.00	0.02	0.00	0.02	0.02	0.02
22:5n-6	0.00	0.01	0.01	0.06	0.05	0.06
22:5n-3	0.00	0.01	0.00	0.18	0.16	0.19
22:6n-3	0.17	0.22	0.27	1.64	1.52	1.67

Table 8.1.3 Culture performance and morphometric parameters of larval meagre (initial total length 4.07±0.26 mm and dry weight 0.058±0.01 mg) fed early weaning diets containing several n-3 HUFA, vit E and vit C levels from 14 dah to 28 dah.

	Diet					
	0.5/150/180	**0.5/300/180**	**0.5/300/360**	**3.5/150/180**	**3.5/300/180**	**3.5/300/360**
Total length (24dah)	4.754±0.44^{b}	4.999±0.39^{a}	4.906±0.40^{ab}	4.955±0.45^{a}	4.964±0.48^{a}	5.055±0.38^{a}
Total length (28dah)	5.155±0.46^{ab}	5.198±0.43^{ab}	5.139±0.51^{ab}	5.290±0.44^{a}	4.969±0.31^{b}	5.340±0.59^{a}
Dry weight (24dah)	0.192±0.04^{c}	0.208±0.02^{bc}	0.202±0.03^{bc}	0.207±0.02^{bc}	0.223±0.02^{ab}	0.238±0.03^{a}
Dry weight (28dah)	0.233±0.02	0.214±0.04	0.207±0.03	0.267±0.05	0.234±0.05	0.244±0.04
Survival (%)	12.09±4.96	8.04±5.20	15.12±4.14	14.16±8.29	16.68±3.45	15.16±7.67

*Values (mean ± standard deviation) with the same letters are not significantly different; ANOVA, $P_{Length} < 0.01$; $P_{Weight} < 0.05$.

Table 8.1.4 Culture performance in total length (mm) and dry weight (mg) (mean ± S.E.) of meagre larvae fed two fatty acids and two vit E dietary levels.

			24 dah	28 dah
Total length (mm)	AG	0.5	4.854±0.052	5.180±0.054
		3.5	4.959±0.052	5.238±0.054
	Vit E	150	4.855±0.060	5.222±0.062
		300	4.958±0.042	5.196±0.044
	Interaction		NS	NS
Dry weight (mg)	AG	0.5	0.198±0.008	0.222±0.010^{b}
		3.5	0.216±0.008	0.254±0.010^{a}
	Vit E	150	0.200±0.010	0.250±0.11
		300	0.215±0.007	0.226±0.008
	Interaction		NS	NS

Values (mean ± standard deviation) with the same superscripts are not significantly different. Two-way ANOVA P< 0.05. NS: not significant.

Table 8.1.5 Culture performance in total length (mm) and dry weight (mg) (mean ± S.E.) of meagre larvae fed two fatty acids and two vit C dietary levels.

			24 dah	28 dah
Total length (mm)	AG	0.5	4.892±0.055	5.195±0.051
		3.5	4.984±0.055	5.250±0.051
	Vit C	150	4.895±0.045	5.168±0.041
		300	4.981±0.063	5.277±0.058
	Interaction		NS	NS
Dry weight (mg)	AG	0.5	0.201±0.008	0.215±0.011^{b}
		3.5	0.224±0.008	0.248±0.011^{a}
	Vit C	150	0.205±0.007	0.238±0.009
		300	0.220±0.009	0.226±0.012
	Interaction		NS	NS

Values (mean ± standard deviation) with the same superscripts are not significantly different.
Two-way ANOVA P< 0.05. NS: not significant.

Gut occupancy

Studies on 15 samples collected from each tank after 2 h of feeding showed guts of all samples with a high percentage of occupation. Studies of image analysis showed no significant differences among the different treatments, denoting the good feed acceptance of all the weaning diets (**Fig. 8.1.1**).

Biochemical composition

The diets without fish oil and containing oleic acid (0.5/150/180, 0.5/300/180 and 0.5/300/360) were characterized by a high level of monoenoic and n-9 fatty acids, particularly oleic acid (18:1n-9), as well as n-6 fatty acids such as linoleic acid (18:2n-6) (**Table 8.1.2**). On the contrary, diets containing fish oil (3.5/150/180, 3.5/300/180 and 3.5/300/360) were high on saturated fatty acids and n-3 fatty acids (Table 8.1.2.). Elevation of dietary HUFA and vit E+vit C tend to increase larval lipid contents (Table 8.1.6). Larval contents of n-3 polyunsaturated fatty acids clearly reflected dietary levels, being significantly higher in larvae fed fish oil. In larvae fed 3%HUFA inclusion of vit E increased n-3, n-3 HUFA and n-6 fatty acids (**Table 8.1.6**), regardless similar levels were found in the respective diets (**Table 8.1.2**). Besides, in larvae fed either 0.4 or 3% HUFA diets, the combined increase of vit E and vit C, tend to increase larval lipid contents by increasing 14:0, 15:0, 16:0iso, 16:0, 16:1n-7, 16:1n-5, 18:0, 18:1n-7, 20:0, 20:1n-7 and 22:1n-11, end-products of non-essential fatty acid synthesis in marine fish, as well as the levels of 20:2n-6, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3, suggesting the antioxidant protection of these vitamins (**Table 8.1.7**).

Table 8.1.6 Lipid (% dw) and moisture composition of larval meagre before (14 dah) and after (28 dah) feeding with early weaning diets containing several n-3 HUFA, vit E and vit C levels.

	14 dah	28 dah					
		0.5/150/180	**0.5/300/180**	**0.5/300/360**	**3.5/150/180**	**3.5/300/180**	**3.5/300/360**
Lipid	19.49± 2.81	17.54±2.12^{b}	17.63±3.79^{b}	21.11±1.12^{ab}	18.72±1.71^{b}	19.59±0.13^{ab}	23.61±0.86^{a}
Moisture	92.15±0.57	90.65±0.52	88.43±1.71	88.23±0.84	88.01±0.71	88.77±2.42	89.48±2.52

Values (mean ± standard deviation) with the same superscripts are not significantly different (P<0.05).

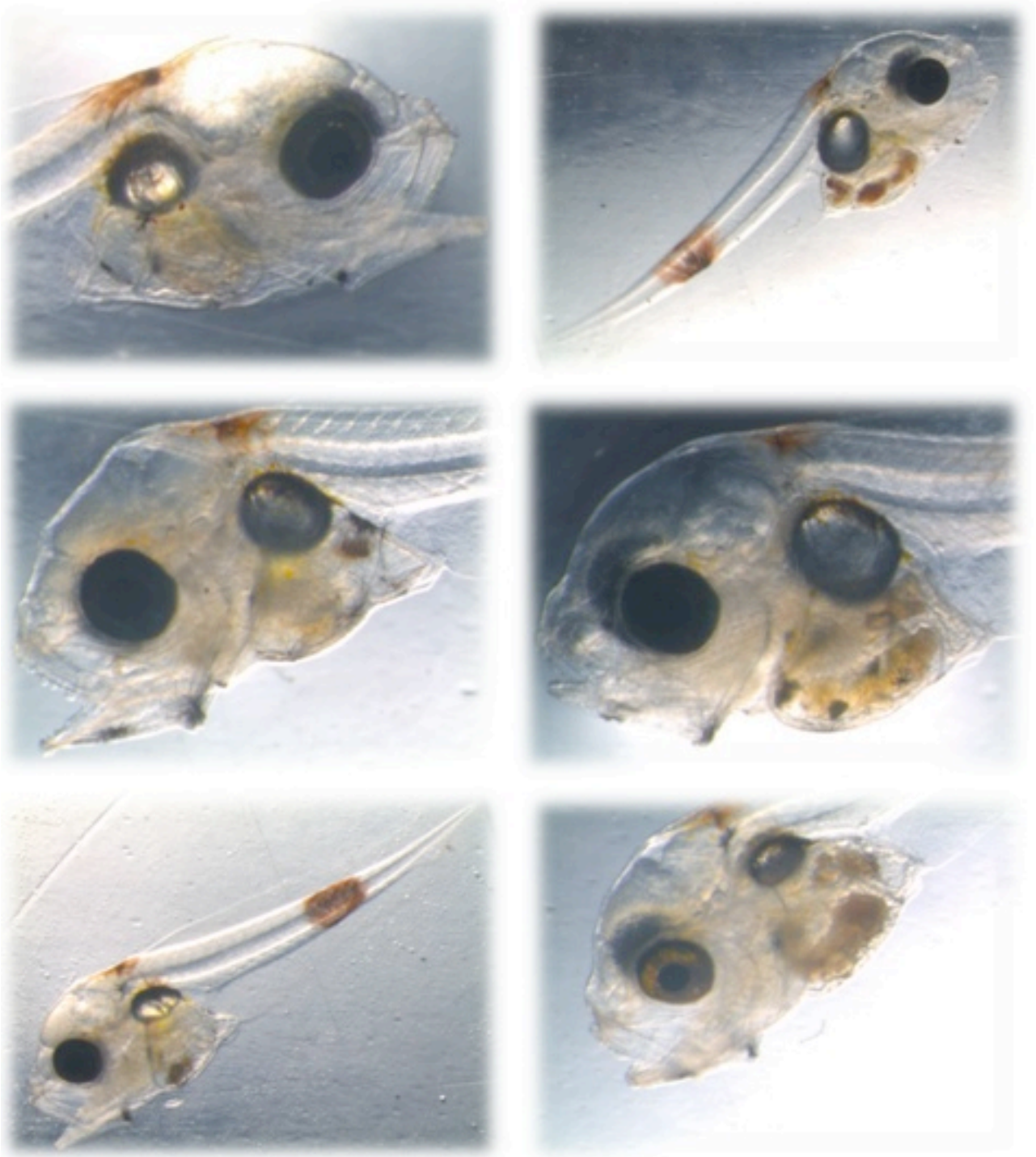

Figure 8.1.1 Images of gut occupancy by the early weaning diet in 16 dah larval meagre fed early weaning diets containing several n-3 HUFA, vit E and vit C levels.

Table 8.1.7. Fatty acid composition of larval meagre before (14 dah) and after (28 dah) feeding with early weaning diets containing several n-3 HUFA, vit E and vit C levels (% total fatty acids).

	14 dah	28 dah					
		0.5/150/180	0.5/300/180	0.5/300/360	3.5/150/180	3.5/300/180	3.5/300/360
14:0	0.17	0.08	0.07	0.11	0.14	0.12	0.14
14:1n-5	0.05	0.01	0.01	0.02	0.01	0.01	0.01
14:1n-7	0.00	0.00	0.00	0.01	0.01	0.00	0.00
15:0	0.10	0.05	0.05	0.06	0.08	0.07	0.08
15:1n-5	0.01	0.00	0.00	0.00	0.00	0.00	0.01
16:0iso	0.20	0.14	0.14	0.16	0.02	0.15	0.18
16:0	4.86	3.82	3.88	4.42	4.50	4.21	4.67
16:1 n-7	1.10	0.14	0.13	0.17	0.24	0.29	0.34
16:1n-5	0.12	0.04	0.04	0.05	0.05	0.05	0.06
16:2n-6	0.01	0.00	0.00	0.00	0.00	0.00	0.00
16:2n-4	0.20	0.10	0.09	0.11	0.17	0.17	0.20
17:0	0.03	0.02	0.02	0.02	0.02	0.02	0.02
16:3n-4	0.11	0.03	0.03	0.04	0.05	0.05	0.07
16:3n-3	0.03	0.01	0.01	0.01	0.02	0.02	0.02
16:3n-1	0.50	0.32	0.34	0.38	0.35	0.34	0.43
16:4n-3	0.09	0.14	0.14	0.35	0.07	0.09	0.11
16:4 n-1	0.00	0.03	0.03	0.05	0.03	0.03	0.01
18:0	1.81	2.43	2.36	2.80	2.95	2.46	2.81
18:1 n-9	3.85	4.87	5.00	5.45	3.01	2.58	3.12
18:1 n-7	0.72	0.38	0.37	0.43	0.54	0.49	0.59
18:1 n-5	0.04	0.02	0.01	0.02	0.03	0.02	0.04
18:2n-9	0.17	0.03	0.03	0.03	0.01	0.02	0.03
18:2 n-6	1.46	2.45	2.55	2.83	1.70	1.83	2.31
18:2n-4	0.01	0.00	0.00	0.00	0.01	0.02	0.02
18: 3n-6	0.06	0.05	0.05	0.06	0.07	0.06	0.07
18:3n-4	0.01	0.01	0.01	0.03	0.01	0.01	0.02
18:3 n-3	0.19	0.10	0.07	0.34	0.42	0.12	0.17
18:4 n-3	0.02	0.02	0.01	0.24	0.02	0.01	0.02
18:4 n-1	0.01	0.02	0.02	0.02	0.01	0.02	0.02
20:0	0.12	0.14	0.13	0.17	0.14	0.12	0.14
20:1 n-9	0.04	0.01	0.01	0.01	0.02	0.02	0.03
20: 1n-7	0.36	0.40	0.41	0.49	0.34	0.33	0.42
20: 1n-5	0.08	0.03	0.03	0.04	0.04	0.04	0.05
20: 2n-9	0.05	0.00	0.01	0.02	0.01	0.02	0.02
20:2 n-6	0.13	0.11	0.11	0.34	0.13	0.12	0.16
20:3 n-6	0.08	0.04	0.04	0.04	0.04	0.05	0.06
20:4 n-6	0.51	0.28	0.29	0.30	0.41	0.55	0.70
20: 3n-3	0.04	0.01	0.01	0.03	0.03	0.03	0.04
20:4 n-3	0.05	0.03	0.01	0.22	0.05	0.04	0.06
20:5 n-3	0.27	0.14	0.14	0.15	0.36	0.65	0.85
22:1 n-	0.03	0.01	0.01	0.04	0.09	0.07	0.10
22:1 n-9	0.09	0.24	0.22	0.22	0.29	0.28	0.24
22:4 n-6	0.04	0.02	0.02	0.02	0.03	0.04	0.06
22:5 n-6	0.09	0.04	0.04	0.04	0.09	0.14	0.18
22:5 n-3	0.13	0.06	0.06	0.08	0.17	0.31	0.43
22:6 n-3	1.44	0.67	0.66	0.69	1.94	3.51	4.50

Organ development

Study of larval foregut histological characteristics by haematoxilin and eosin staining showed that larvae fed 0.4% HUFA presented very pigmented enterocytes with centered nucleous and very little lipid vacuoles (**Fig. 8.1.2, A, B and C**). However, larvae fed higher levels of dietary HUFA, such as in 3.5/150/180 (**Fig. 8.1.2,**

D), showed larger and more developed enterocytes containing lipid vacuoles around the nucleous, reflecting the higher lipid absorption activity. Similar features were observed in larvae fed diets 3/300/180 and 3/300/360 (**Fig. 8.1.2, E and F**), with lipid droplets also found in the basal part of the enterocyte. Regarding the liver, larvae fed low HUFA diets showed very condensed hepatocytes with centered nucleous and marked cytoplasm staining, suggesting a scarce deposition of energy reserves (**Fig. 8.1.2, A, B and C**). On the contrary, larvae fed higher HUFA levels showed hepatocytes with a higher vacuolization, denoting the accumulation of energy reserves. Generally speaking, increase in dietary vit C seemed to produce more condensed hepatocytes (**Fig. 8.1.2**, C in comparison to A and B, and F in comparison to D and E).

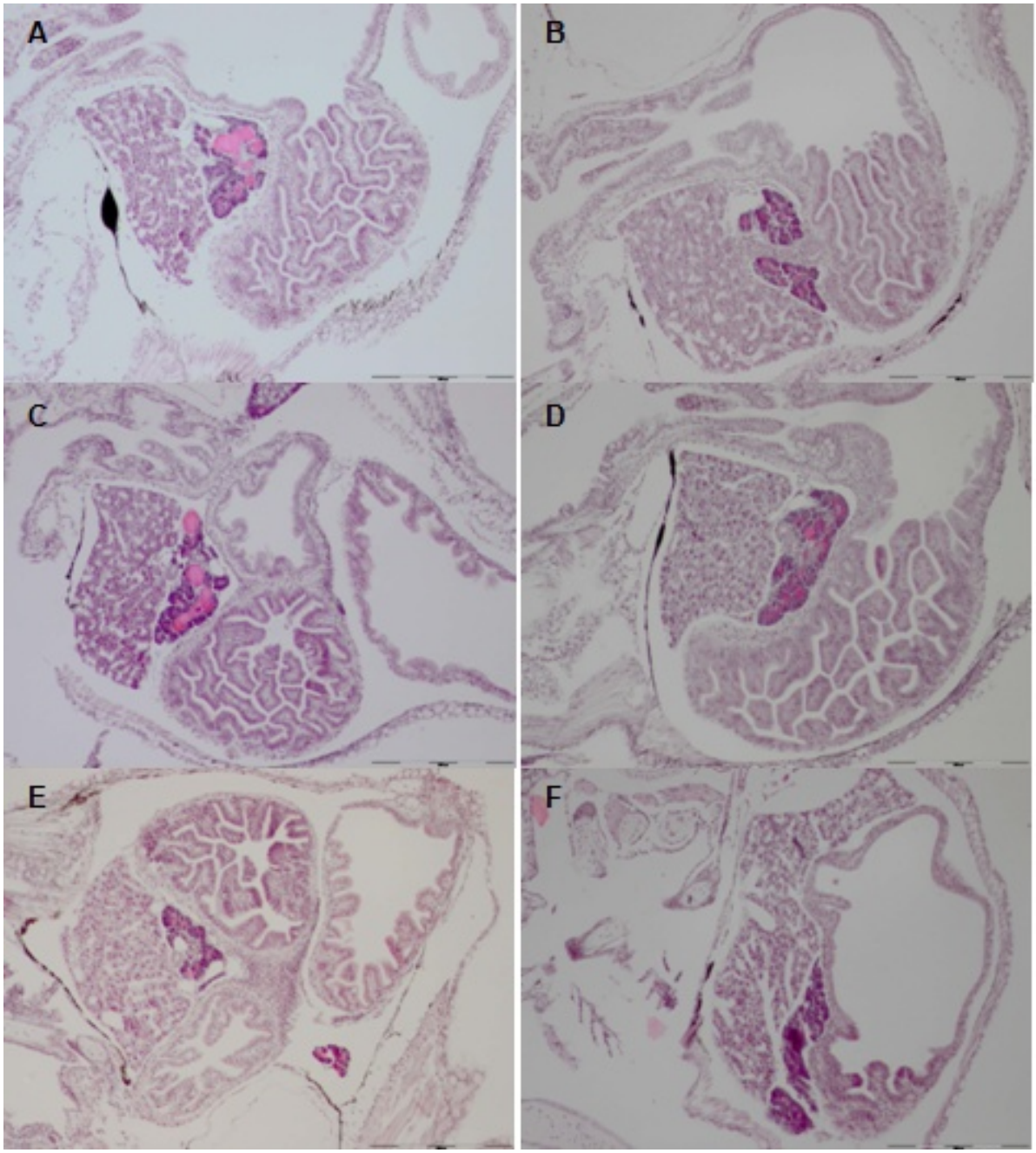

Figure 8.1.2. Images obtained from sections of 28 dah meagre fed early weaning diets containing several n-3 HUFA, vit E and vit C levels, showing intestine, pancreas and liver. Increase in n-3 HUFA increased lipid absorption in the gut and lipid deposition in liver. A: 0.5/150/180; B: 0.5/300/180; C: 0.5/300/360; D: 3.5/150/180; E: 3.5/300/180; F: 3.5/300/360.

Conclusion

In summary, the work done at the facilities of P2. FCPCT (**Fig. 8.1.3**) demonstrate the high requirement of this species for HUFA to promote growth and Vit E and Vit C to prevent fatty acid oxidation during larval stages, and denote that weaning diets for larval meager must be supplemented with increased n-3 HUFA, Vit E and Vit C in order to be improved.

Based on the stress response, diets 0.5/150/180, 3.5/150/180, 0.5/300/350 and 3.5/300/350 were selected to conduct the studies on resistance to handling stress, stress bio-markers such as gene expression of HSPs (P1. FCPCT), specific fish behaviour, evaluation of metabolic cost after sub-lethal stress, video analysis of activity, escape responses and sensory acuity (P21. DTU) and digestive enzyme (protease, amylase and lipase) and gut ATPase activities (P15. ULL).

Figure 8.1.3. Experimental tanks used for the trial with larval meagre fed early weaning diets containing several n-3 HUFA, vit E and vit C levels.

Task 8.2 Determination of nutritional requirements to promote feed utilization, consistent growth rates and fish welfare (Led by FCPCT, Lidia Robaina)

To achieve objective 2, the essential fatty acid requirements will be examined in grow out diets (P20. SARC) for meagre by feeding six levels of docosahexaenoic, eicospentaenoic and araquidonic acids (P2. FCPCT). During the last three months of this reporting period, information on the nutritional requirements of meagre and related species have been collected and a basal diet formulation has been defined as part of Milestone MS19.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP No:	9	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Nutrition – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P8. IEO	P15. ULL	P.20 SARC
P.28 CANEXMAR				
Lead Scientist preparing the Report (WP leader):	Marisol Izquierdo			
Other Scientists participating:	Yannis Kotzamanis (P1), Salvador Jerez (P8), Virginia Martín (P8), Covadonga Rodriquez (P15), Jose Pérez (P15), Ramon Fontanillas (P20), Rafael Guirao (P28)			

Objectives

1. Improve of larval enrichment products for live-preys to enhance production of larvae and juvenile,
2. Develop diets for grow-out in order to maximize growth potential,
3. Development of an appropriate broodstock diet to improve unreliable reproduction in amberjack.

Summary of progress towards objectives and details for each task:

Task 9.1 Improve larval enrichment products to enhance production of larvae and juveniles (led by FCPCT).

Sub-task 9.1.1(FCPCT, Marisol Izquierdo). Optimum essential fatty acids levels in enrichment products for live preys for greater amberjack.

To achieve this objective, greater amberjack larvae obtained from the FCPCT broodstock (WP3 Reproduction & genetics – greater amberjack) were fed *Artemia* enriched with five levels of Docosahexaenoic acid (DHA), to determine the requirements for this essential fatty acid during early larval development. Larval performance in terms of survival, growth and welfare (survival to handling stress test) was studied. Proximate and fatty acid composition of enrichment products, live preys and larvae were analysed. Morphogenesis of digestive system and bone, as well as morphogenetic biomarkers (IGF, BMP, ALP) and related parameters (bone mineralization and deformities) will be studied by P2. FCPCT.

Experimental conditions

In this trial, 50,000 greater amberjack larvae produced under a semi-intensive system until 19 dah, were distributed into 15 tanks of 200 L at a density of 5 larvae/L. Five different groups of *Artemia* metanauplii were fed enrichment products with 5 different levels of DHA. After the enrichment the content of *Artemia* in DHA ranged from 0% to 2.5% DHA d.w. (**Fig. 9.1.1.1**). Triplicate groups of larvae were fed with one of this enriched *Artemia* groups until 35 dah. Survival was determined by manually counting all the larvae at the beginning and at the end of the trial. At the beginning and end of the feeding trial 25 fish per tank were sampled for growth determination, including total length and body weight. At the end of the feeding trial two welfare tests were conducted by handling the fish during 30" or 60". Proximate and fatty acid composition of *Artemia* and 35 dah larvae was conducted. Bone morphogenesis was also studied.

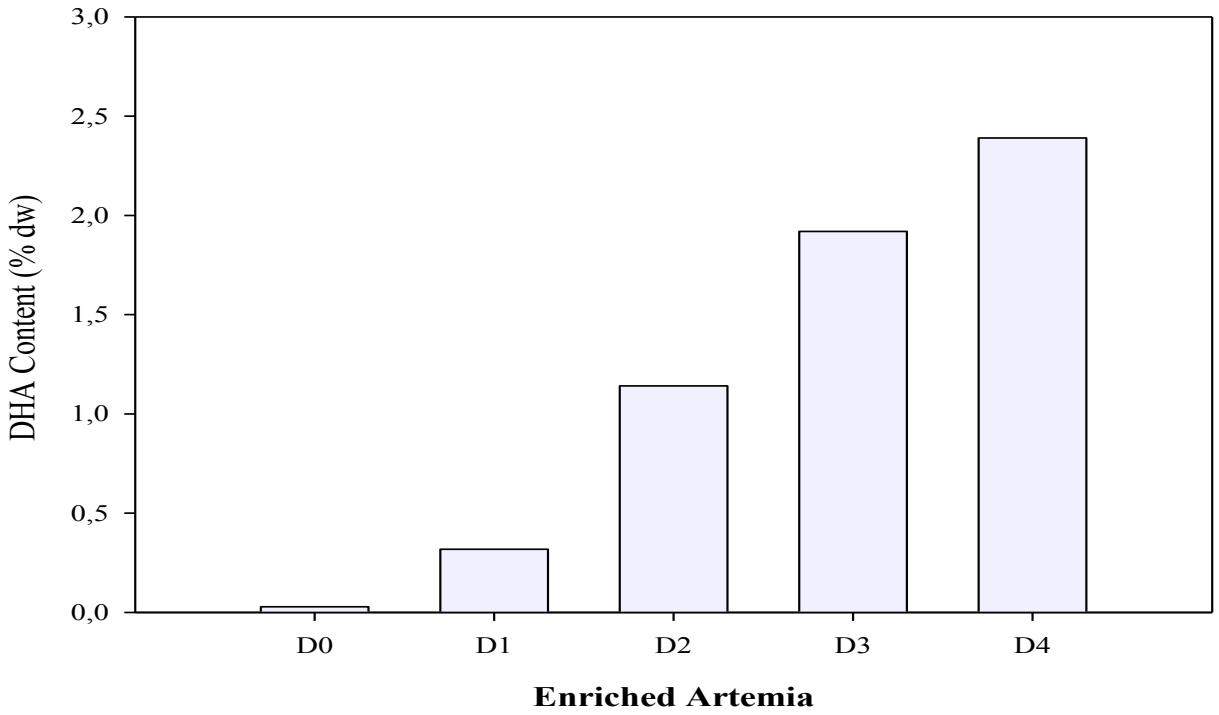

Fig 9.1.1.1. Fatty acid composition, in terms of DHA content (% d.w) in *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for greater amberjack.

Larval performance

The results showed that feeding greater amberjack larvae with *Artemia* enriched with the lowest DHA level lead to significantly lower larval survival (**Fig. 9.1.1.2**). However, increase in DHA levels in the *Artemia* up to 1-2% (D2 and D3) improved larval performance in terms of survival (Fig 9.1.1.2.). Similar results were also found in larval growth, with larvae fed the lower DHA content in *Artemia* (D0 and D1) showing a significantly lower final total length (Fig 9.1.1.3) and body weight (Fig 9.1.1.4). Nevertheless, growth tent to be also reduced when larvae were fed *Artemia* with the highest DHA levels (**Fig. 9.1.1.3** and **Fig. 9.1.1.4**). Thus, the highest growth was found in larvae fed D2 and D3 *Artemia*. Regarding larval welfare, all the larvae fed D0 *Artemia* died after the handling stress, whereas survival of those fed D2 was six and three times higher than that of larvae fed D1 and D4 *Artemia*, respectively (Fig 9.1.1.5.). Thus, the highest survival after activity test was obtained in fish fed D2 and D3 *Artemia* (1-2 % DHA) (**Fig. 9.1.1.5**).

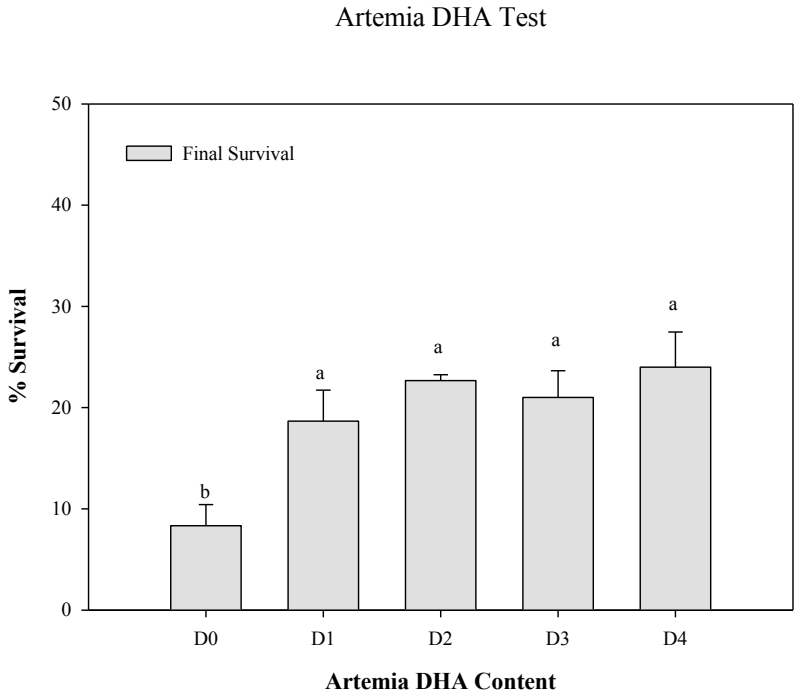

Figure 9.1.1.2. Survival of greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P<0.05, n=3

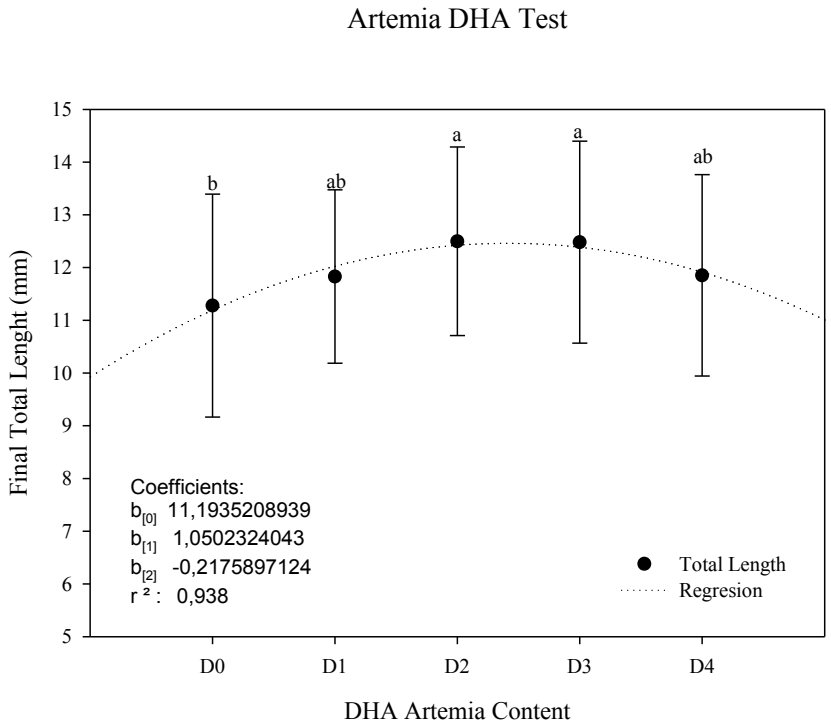

Figure 9.1.1.3. Growth in terms of final total length (mm) of greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P<0.05, n=75).

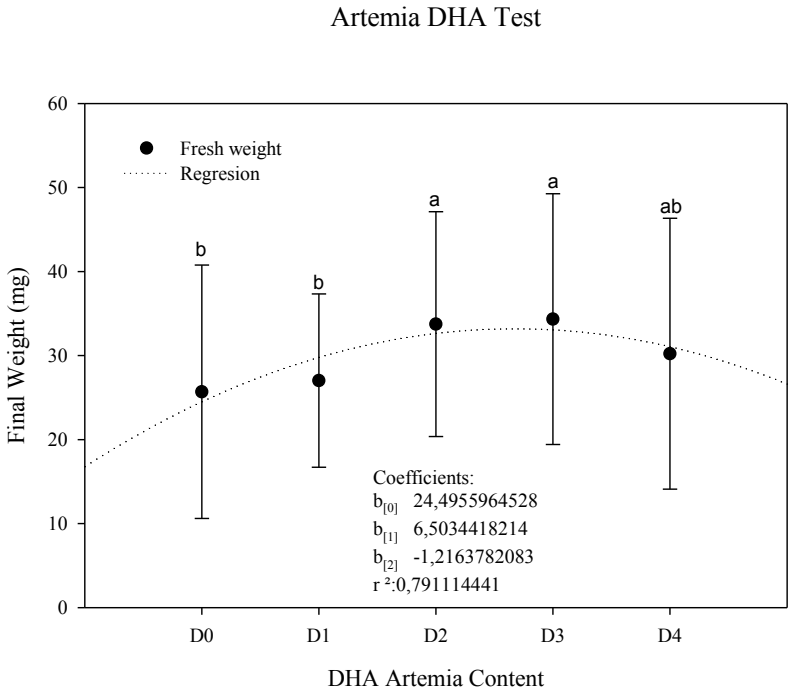

Figure 9.1.1.4. Growth in terms of final body weight (mg) of greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P<0.05, n=75).

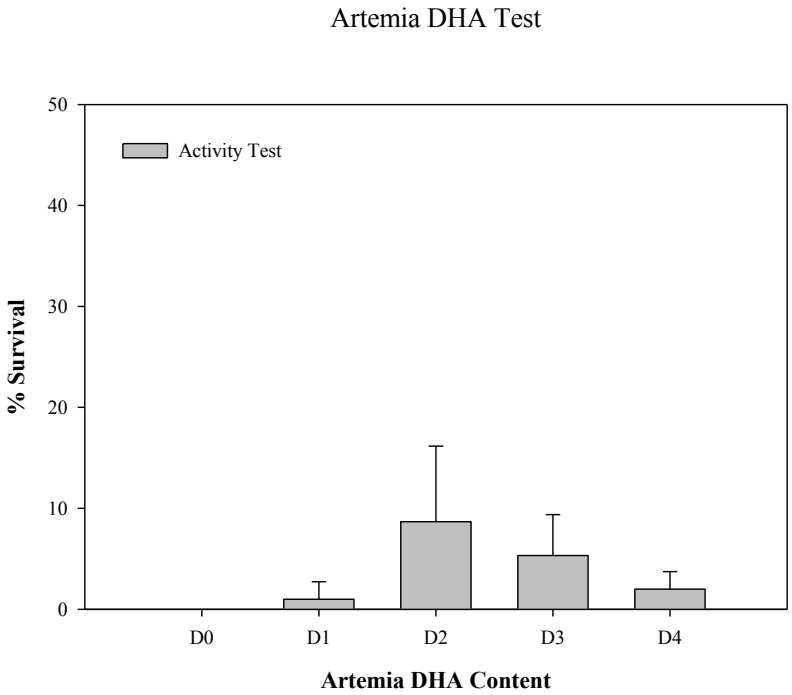

Figure 9.1.1.5. Welfare conditions in terms of survival to handling stress of greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P>0.05, n=15)

Proximate and fatty acid composition

No significant differences were obtained in the proximate composition of *Artemia* enriched with different DHA contents. However, fatty acid composition of enrichment products and live prey showed that DHA contents in *Artemia* increased proportionally to the elevation of DHA levels in the enrichment products (**Fig. 9.1.1.1**). Study of the proximate composition of the greater amberjack larvae fed *Artemia* with different DHA contents showed no differences, except for a slightly lower lipid content in larvae fed the lowest DHA content in *Artemia* (**Fig. 9.1.1.6**). Fatty acid composition of larvae showed an increase in the DHA content when DHA was increased in *Artemia* (**Fig. 9.1.1.7**). However, no differences were found in the Arachidonic (ARA) or Eicosapentaenoic (EPA) acids contents in the larvae (**Fig. 9.1.1.7**).

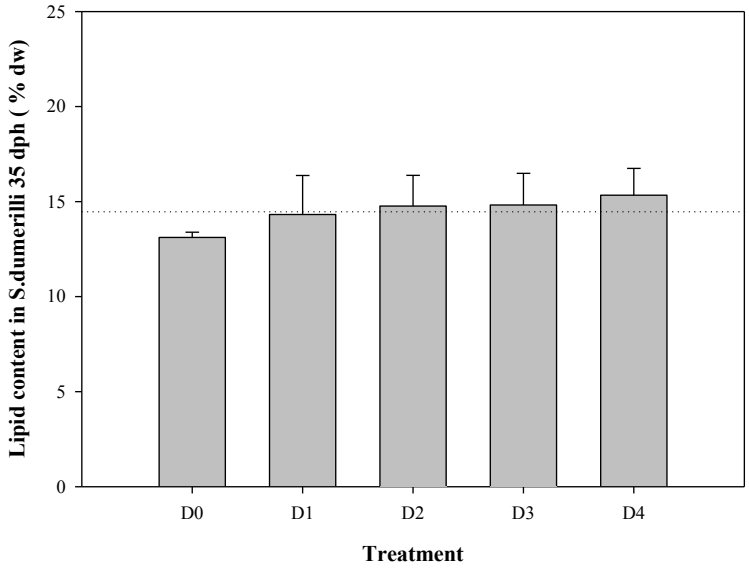

Figure 9.1.1.6. Proximate composition in terms of body lipid content of greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P>0.05, n=3).

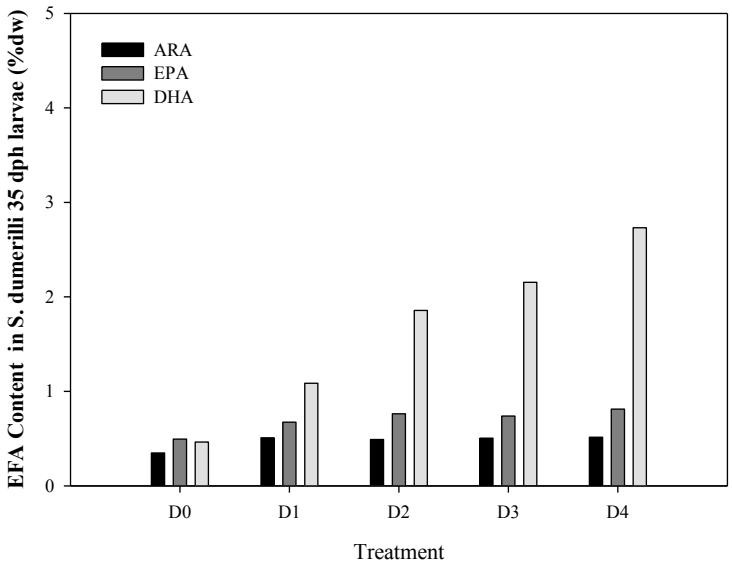

Figure 9.1.1.7. Fatty acid composition in terms of essential fatty acid contents in greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine

optimum essential fatty acids levels in enrichment products for live preys for this species. (Different letters indicate significant differences, P>0.05, n=3).

Morphogenesis of bone and anomalies incidence

The bone morphogenesis study denoted similar bone development among larvae fed the different DHA containing *Artemia* (**Fig. 9.1.1.8**). However, differences were found in the incidence of bone deformities, depending on the type of deformity. Thus, the increases in DHA content in *Artemia* tend to reduce vertebral anomalies (**Fig. 9.1.1.9**), whereas increased DHA content over 2% dw in *Artemia* tends to increase cranial anomalies (**Fig. 9.1.1.9**).

Figure 9.1.1.8. Study of bone morphogenesis by alizarine red staining of a 35 dah greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys.

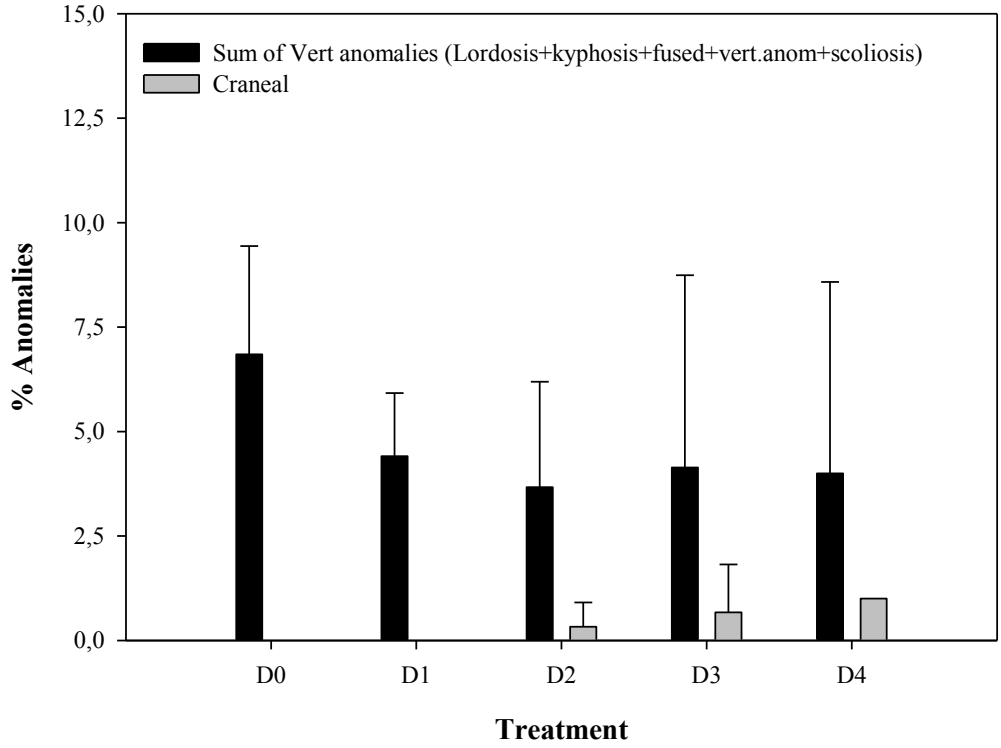

Figure 9.1.1.9. Deformities incidence in terms of cranial and vertebral anomalies in 35 dah greater amberjack larvae fed *Artemia* metanauplii enriched with 5 different emulsions containing different DHA levels to determine optimum essential fatty acids levels in enrichment products for live preys.

Conclusion

In summary, these results indicate that a DHA level between 1-2% is required in *Artemia* for abetter larval performance of greater amberjack larvae in terms of survival, growth and welfare.

Sub-task 9.1.2 (IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez) will examine the combined effect of PUFA-rich lipids and carotenoids. Triplicate trials will be carry out with amberjack larvae fed live preys enriched using three different levels of PUFA-rich lipids combined with two carotenoids levels. Larval performance (survival and growth parameters) and indicators of welfare (survival to handling stress test and cortisol, glucose, osmolality and total protein in whole final larvae body homogenates) will be studied (P8. IEO). Biochemical composition including lipid classes, fatty acid and carotenoid profiles of enrichment products, live preys and larvae will be analyzed (P15. ULL). This Sub-task will contribute to deliverable D9.1 Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids in greater amberjack enrichment products.

To achieve the objectives proposed in this task, a first trial of rotifer enrichment was designed (P15. ULL/P8. IEO) and performed at P8. IEO, in order to establish a good protocol for LC-PUFA enrichment according to the lipid composition of wild greater amberjack eggs. A similar new trial is intended, by using several levels of carotenoids (esterified astaxanthin), in order to establish the best final experimental enrichment products (LC-PUFA/carotenoids) to be administered to amberjack larvae through live prey (rotifers).

Experimental conditions.

Four lipid enrichment treatments were tested in triplicate. Three experimental treatments (8% lipids) (E1, E2 and E3) and a commercial enrichment product as control treatment (C) were assayed.

These preliminary assays combined different times of enrichment with different sources and levels of LC-PUFA rich lipids. The combinations of lipids were made in order to supply high polar lipids, and high LC-PUFA levels, but remained DHA/EPA/ARA ratios similar to those present in amberjack wild eggs, by using a range of lipid sources mainly rich in polar lipids (PL) (E1), triacylglycerols (TAG) (E3) or a mixture of them (E2).

The assays of rotifer culture were carried out at P8. IEO during 24 h, with the following conditions:

- Tank volume of 10 l, continuous light and aeration, maintained at room temperature (20ºC) at an initial density of 300 rot/ml.
- Sampling at 0, 3, 6, 10 and 24 hours for register: survival (%), ovigerous females (%), temperature (ºC), oxygen (% saturation). In order to obtain enough sample for biochemical analyses (approx. 1 g of fresh weight, approx. 0.5 x 10^6 rot.) a volume of 2 litters of each tank were filtered at each sampling point and tank. A total of 51samples of rotifers were collected and immediately frozen (-80ºC) until analysis of lipids (total lipid contents, and lipid classes and fatty acid profiles) at P15. ULL.

Culture performance

The results on rotifers culture parameters are shown in **Table 9.1.2.1**. The survival rates of treatments E1 and E3 were similar to that of the commercial treatment (C), whereas treatment E2 produced lower survival despite the oxygen saturation remained unchanged among treatments. The lowest oxygen saturation was registered under control conditions after 24 h of enrichment. Overall, the experimental treatment E1 showed the best results in terms of survival and ovigerous females in the rotifer population.

Lipid composition

Preliminary lipid analyses (**Table 9.1.2.2**) demonstrate that longer enrichment protocols and higher total lipid (TL) levels in rotifers, increased the proportions of TAG. The highest levels of TL, TAG and n-3 HUFA, specifically DHA in TL were obtained with E2, coinciding with the worst results in terms of rotifer survival. In addition, the richest emulsion in PL (E1) resulted in highest proportion of PL and n-3 HUFA in rotifer PL after 3h of enrichment, together with best results in rotifer population.

Table 9.1.2.1. Survival (%), ovigerous female (%), temperature (ºC) and oxygen saturation (%) from the different experimental treatments (E1, E2 and E3) vs. control treatment (C) used to live prey (rotifers) enrichment, for 24 hours.

Treat.	Time (h)	Survival (%)			Ovigerous (%)			Temp. (ºC)	Oxygen (%)		
C	0	100. ± 0.0	a		14.2 ± 0.0			22.2 ± 0.1	95.1 ± 0.7	a	
	3	72.0 ± 8.0	bc	12	10.9 ± 2.4		12	22.6 ± 0.0	81.5 ± 0.7	ab	
	6	74.4 ± 3.2	b	1	9.8 ± 2.3		1	22.8 ± 0.0	81.5 ± 2.1	ab	
	10	60.1 ± 2.9	c	2	8.5 ± 1.9		1	22.9 ± 0.1	71.0 ± 8.7	b	
	24	66.8 ± 5.3	bc	1	9.8 ± 3.6		1	22.9 ± 0.2	73.2 ± 13.1	b	2
E1	0	100. ± 0.0	a		14.2 ± 0.0			22.1 ± 0.0	95.3 ± 1.0	a	
	3	79.3 ± 3.0	b	12	10.3 ± 2.1		12	22.8 ± 0.0	84.9 ± 3.6	b	
	6	76.6 ± 6.4	b	1	10.2 ± 2.5		1	22.9 ± 0.1	82.7 ± 2.9	b	
	10	68.7 ± 3.4	b	12	6.4 ± 1.9		1	23.0 ± 0.1	80.5 ± 3.1	b	
	24	78.4 ± 14.4	b	1	9.0 ± 4.2		1	23.0 ± 0.1	94.2 ± 2.1	a	1
E2	0	100. ± 0.0	a		14.2 ± 0.0			22.1 ± 0.0	95.0 ± 3.7	a	
	3	70.8 ± 1.5	b	2	9.7 ± 2.4	a	2	22.7 ± 0.1	83.8 ± 0.7	b	
	6	18.6 ± 4.9	c	2	1.7 ± 1.5	b	2	22.9 ± 0.1	83.0 ± 1.9	b	
	10	12.7 ± 5.2	c	3	0.0 ± 0.0	b	2	23.0 ± 0.1	76.4 ± 0.8	c	
	24	18.4 ± 1.4	c	2	8.5 ± 1.3	a	1	22.9 ± 0.0	94.6 ± 1.5	a	1
E3	0	100. ± 0.0	a		14.2 ± 0.0			22.1 ± 0.1	95.2 ± 1.1	a	
	3	80.1 ± 4.7	ab	1	15.6 ± 5.2	a	1	22.7 ± 0.1	84.5 ± 1.7	b	
	6	72.6 ± 5.2	ab	1	8.5 ± 1.5	ab	12	22.9 ± 0.1	81.3 ± 0.9	b	
	10	70.6 ± 15.7	ab	1	5.5 ± 4.1	b	12	22.9 ± 0.0	78.1 ± 3.4	b	
	24	50.7 ± 18.9	b	1	3.4 ± 1.3	b	2	22.8 ± 0.1	93.7 ± 4.5	a	1

C, commercial enrichment product; E1: 100% marine lecithin; E2: 30% marine lecithin + 50% DHA-rich TG oil + 20% cod liver oil; E3: 60% DHA-rich TG oil + 40% cod liver oil. Values are means ± SD (n=3). Different letters within a column denote significant differences among hours for a dietary treatment; different numbers within a column denote significant differences among dietary treatments for an enrichment period ($P<0.05$).

Table 9.1.2.2. Total lipid content (% DM), polar lipid and tryicylglycerols (% TL) and main PUFA fatty acid composition of rotifers enriched with the control or one of three experimental emulsions at two different enrichment periods.

	C		E1		E2		E3	
	3 h	6 h	3 h	6h	3 h	6h	3 h	6 h
TL (% DW)	12.73 ± 1.07 b	17.59 ± 0.08 B*	19.95 ± 1.38 a	15.10 ± 0.23 B*	19.95 ± 1.38 a	22.92 ± 0.83 A	12.68 ± 1.62 b	13.95 ± 1.44 B
20:4n-6	1.22 ± 0.07 d	1.62 ± 0.04 D*	7.35 ± 0.02 a	8.36 ± 0.21 A*	4.79 ± 0.42 b	4.66 ± 0.15 B*	3.83 ± 0.30 c	3.79 ± 0.41 C*
20:5n-3	2.27 ± 0.16 c	3.21 ± 0.22 D*	8.01 ± 0.19 a	9.28 ±0.09 A*	8.55 ± 0.33 a	8.44 ± 0.31 B	4.79 ± 0.28 b	5.76 ± 0.11 C*
22:6n-3	13.54 ± 0.46 c	19.10 ± 0.73 B*	17.41 ± 0.48 b	20.35 ± 0.08 B*	31.87 ± 0.45 a	32.10 ± 1.25 A	16.11 ± 1.95 bc	19.08 ± 1.32 B*
Total n-3 HUFA	16.76 ± 0.63 d	23.51 ± 0.99 D*	25.92 ± 0.60 b	30.45 ± 0.15 B*	42.91 ± 0.77 a	42.71 ± 1.60 A	21.86 ±2.34 c	26.91 ± 1.27 C*
PL (% TL)	26.12 ± 1.86 bc	25.98 ± 2.17 AB	31.47 ± 2.87 c	31.11 ± 0.80 A	13.56 ± 0.45 a	17.46 ± 2.74 C	24.89 ± 0.16 b	24.05 ± 1.85 B
20:4n-6	0.78 ± 0.10 b	1.61 ± 0.04 C*	4.03 ± 0.01 a	4.55 ± 0.50 A	3.74 ± 0.26 a	4.38 ± 0.27 A*	3.72 ± 0.16 a	3.60 ± 0.02 B
20:5n-3	1.13 ± 0.11 d	3.32 ± 0.16 B*	6.36 ± 0.15 a	6.51 ± 0.72 A	5.01 ± 0.48 b	6.35 ± 0.64 A*	3.06 ± 0.29 c	4.06 ± 0.58 B
22:6n-3	2.36 ± 0.16 c	11.35 ± 0.44 B*	16.26 ± 0.45 a	16.48 ± 2.79 A	15.31 ± 1.06 a	18.08 ± 2.77 A	7.36 ± 0.10 b	9.54 ± 0.14 B*
Total n-3 HUFA	4.32 ± 0.29 c	16.00 ± 0.66 B*	23.19 ± 0.51 a	23.72 ± 2.40 A	21.02 ± 1.68 a	25.61 ± 2.23 A*	11.16 ± 0.39 b	14.93 ± 0.51 B*
TAG (% TL)	33.53 ± 0.70 b	39.84 ± 1.78 A *	24.49± 0.87 c	33.47 ± 0.33 B *	43.40 ±0.82 a	42.17 ± 2.39 A	35.93 ± 2.45 b	41.59 ± 2.38 A *

C, commercial enrichment product; E1: 100% marine lecithin; E2: 30% marine lecithin + 50% DHA-rich TG oil + 20% cod liver oil; E3: 60% DHA-rich TG oil + 40% cod liver oil. Values are means ± SD (n=3). Different letters within a row denote significant differences among treatments for an enrichment period; * denote significant differences between hours for a treatment ($P<0.05$).

Conclusion

In summary, these results indicate that rotifer enrichment treatment E1 (100% marine lecithin) is the best protocol for LC-PUFA enrichment according to the lipid composition of wild seriola viable eggs. A second phase of enrichment protocols is necessary in order to adjust the n-3 HUFA and lipid levels in the rotifer culture and the enrichment period for the treatment (E1) assayed. These are being designed and will be performed this year. With this information and that obtained from carotenoid enrichment protocols, the best combinations of LC-PUFA and carotenoids to be assayed on amberjack larval rearing will be established. Therefore these last trials on amberjack larvae will be developed during 2015.

Task 9.2 Development of diets for grow-out of amberjack to maximize growth (led by HCMR).

Sub-task 9.2.1 (HCMR, Yannis Kotzamanis)
This Sub-task does not start until month 15.

Sub-task 9.2.2 (CANEXMAR, Rafel Guirao)
This Sub-task does not start until month 36.

Task 9.3 Design adequate feeding regimes for broodstock to optimize reproduction (led by IEO).
Sub-task 9.3.1 (FCPCT, Hipólito Fernández-Palacios)
To achieve objective 3, published information on the nutritional requirements of greater amberjack and related species have been collected, and a basal diet formulation will be defined at the beginning of next reporting period. Besides, published information has been collected regarding the spawning quality parameters more sensitive to feeding regimes for greater amberjack. Moreover, based on the spawning quality results from Task 3.3, the spawning quality parameters were defined to be part of milestone MS21. The literature collected to define broodstock requirements and spawning quality parameters are listed below:

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Sub-task 9.3.2 (IEO, ULL, Salvador Jérez). With the aim of approach the lipid and carotenoids eggs profile released by cultured females to their wild counterparts, experimental diets with optimized EFA and carotenoid contents are being designed. Fecundity, egg quality and haematological and biochemical indicators of fish health will be studied (IEO).

In a previous work conducted by our research group (Rodríguez-Barreto et al., 2014), an experimental diet (Diet-E) was formulated and tested. When greater amberjack broodstock born in captivity were fed on this Diet-E, the lipid and fatty acid composition of the muscle, liver and ovary of these specimens approached that of the wild fish, although some imbalances in certain fatty acids were still observed.

Taking into account previous results, two new experimental diets (**Table 9.3.2.1**) have been formulated to be tested. Both diets will have a total content of proteins (52 %), crude fat (20 %) and carbohydrates (6 %) equal to that of Diet-E, but the new formulas will differ in the proportions of certain essential fatty acids. These two preliminary experimental diets could be improved with the results obtained in further studies, including the analysis of the composition of fertilized eggs and several tissues of greater amberjack broodstock fed with mackerel, a diet that has allow obtaining fertilized eggs from broodstock in captivity for several consecutive years. Experimental diets will be supplemented also with Vitamin A (10.000 U.I./kg), Vitamin D3 (1.500 U.I./kg), Vitamin E (150 mg/kg) and Astaxanthin (25 ppm). In contrast, a diet based on mackerel (*Scomber scombrus*) will be used as reference due to the optimal results obtained in feeding wild broodstocks.

Table 9.3.2.1 Diets composition (fatty acid content expressed as % of total fatty acids)

Diet tested in previous work		Experimental diets to be tested	
Diet-E		**Diet-A**	**Diet-B**
0.5	**20:4n-6**	0.5	1.0
4.2	**20:5n-3**	2.0	2.0
10.3	**22:6n-3**	10.0	15.0
34.4	**18:1n-9**	40.0	40.0
12.4	**18:2n-6**	7.0	7.0
2.5	**DHA/EPA**	5.0	7.5
9.1	**EPA/AA**	4.0	2.0
2.8	**18:1n-9/18:2n-6**	5.7	5.7

References

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Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP No:	10	WP Lead beneficiary:	P21. DTU
WP Title (from DOW):	Nutrition – pikeperch		
Other beneficiaries (from DOW):	P2. FCPCT	P15. ULL	P16. FUNDP	P.29 ASIALOR
Lead Scientist preparing the Report (WP leader):	Ivar Lund (P21)		
Other Scientists participating:	Marisol Izquierdo (P2), Covadonga Rodriquez (P15), Patrick Kestemont (P16), Robert Mandiki (P16), Kevin Debes (P29), Erik Høglund (P21)		

Summary of progress towards objectives and details for each task:

Objectives

1. Increase knowledge on the effect of nutrients essential for first feeding of pikeperch.
2. Develop specific enrichment products and formulated diets to improve pikeperch larval performance.

During the first year, the Partners have exchanged ideas regarding design and protocols of experimental studies. This included a special "pikeperch" meeting held in Namur, Belgium on March 2, 2014 with the presence of most partners involved this and other WPs related to pikeperch. During the first year, one study was planned in Task 10.1. This study is currently carried out (started 1. October 2014).

Task 10.1 Effect of selected dietary nutrients on pikeperch larval development and performance (led by DTU). A first experimental study is carried out according to the scheduled plan for this task (see below). The survival was much lower than expected (due to cannibalism), and the study will be repeated during December 2014.

Effect of phospholipid levels and HUFA levels on ontogenetic development and performance of pikeperch larvae. (Experimental study (Oct. 2014 - Nov. 2014).

Objectives of the study: To increase the knowledge of nutrients essential for first feeding pikeperch. The study investigates the effects of increasing inclusion of phospholipids and the additional effect of single highly unsaturated fatty acids (HUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on pikeperch larval performance (growth and stress sensitivity) and larval development (*i.e.*, organ development, tissue/liver morphology, digestive tract development). Further effects on digestive enzymatic activity, liver proteomics, candidate gene expression and skeleton morphogenesis will also be examined. This study will be the first in a series towards Deliverable 10.1 Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch.

Six different diets with increasing content of PL and DHA are tested. Cold extruded feed was prepared with a pellet size of 0.4 mm and 0.8 mm by SPAROS, Portugal (**Table 1**).

Table 1. Dietary ingredients and composition of the 6 experimental diets.

Ingredients	D1	D2	D3	D4	D5	D6
	%	%	%	%	%	%
MicroNorse	45,000	45,000	45,000	45,000	45,000	45,000
CPSP 90	7,000	7,000	7,000	7,000	7,000	7,000
Squid meal	13,000	13,000	13,000	13,000	13,000	13,000
Fish gelatin	1,000	1,000	1,000	1,000	1,000	1,000
Wheat Gluten	4,400	4,400	4,400	4,400	4,400	4,400
Wheat meal	6,100	5,900	5,600	6,100	5,900	5,600
Algatrium DHA70	0,000	0,000	0,000	0,550	2,000	3,400
Olive oil	18,900	12,100	3,400	18,350	10,100	0,000
Vit & Min Premix PV01	1,000	1,000	1,000	1,000	1,000	1,000
Soy lecithin – Powder	3,000	10,000	19,000	3,000	10,000	19,000
Binder (guar gum)	0,200	0,200	0,200	0,200	0,200	0,200
Antioxidant powder (Paramega)	0,200	0,200	0,200	0,200	0,200	0,200
Antioxidant liquid (Naturox)	0,200	0,200	0,200	0,200	0,200	0,200
Total	**100,000**	**100,000**	**100,000**	**100,000**	**100,000**	**100,000**

As fed basis	D1	D2	D3	D4	D5	D6
Crude protein	52,74	52,72	52,69	52,74	52,72	52,69
Crude fat	27,01	26,99	27,01	27,01	26,99	27,01
Fiber	0,14	0,14	0,13	0,14	0,14	0,13
Starch	4,02	3,90	3,72	4,02	3,90	3,72
Ash	8,12	8,12	8,11	8,12	8,12	8,11
Gross Energy	24,02	23,34	22,48	24,02	23,34	22,48
Lys	4,22	4,22	4,22	4,22	4,22	4,22
Methionine & cysteine	1,91	1,91	1,91	1,91	1,91	1,91
Taurine	0,52	0,52	0,52	0,52	0,52	0,52
Available P	1,30	1,30	1,30	1,30	1,30	1,30
Ca	1,84	1,84	1,84	1,84	1,84	1,84
EPA (eicosapentaenoic acid)	0,41	0,41	0,41	0,47	0,61	0,75
DHA (docosahexaenoic acid)	0,66	0,66	0,66	1,04	2,06	3,04
PC (phosphatidylcholine)	1,51	2,88	4,64	1,51	2,88	4,64
PE (phosphatidylethanolamine)	0,62	1,58	2,81	0,62	1,58	2,81
PI (phosphatidylinositol)	0,69	2,10	3,90	0,69	2,10	3,90
TPL (total phospholipids)	3,16	7,45	12,96	3,16	7,45	12,96

Eggs were obtained by AquaPri SA in October and hatched in an incubation system (0.5 m^3 tank) at DTU Aqua at ambient temp of app. 18°C and increased gradually to 19-20°C. From 3-11 dph, larvae were fed with newly hatched unenriched AF *Artemia* nauplii, kept alive in a suspension and fed continuously for 10-12 h by an automatic feed dispenser.

At 11 dph, larvae were moved to a temperature-controlled, flow through larval system consisting of 30 conical tanks of 50 l each. The experiment was carried out in 18 of these tanks in a triplicate set up with 3 tanks per feed type (6x3). Each tank was stocked initially with 13 larvae/l. Approximately 600 larvae/tank were used and kept at 22-23°C in freshwater.
From 12 to 35 dph, larvae in each triplicate group were fed one of six experimental diets and from 12 to 17 dph larvae are co- fed with newly hatched EG *Artemia* nauplii. During the first week, 0.4 mm pellets was grinded further to obtain sizes of 200-400 µm and this was gradually exchanged with 0.4 and 0.8 mm, which was used for the last week of the experiment. Feed was given by automatic feeders for a period of 1-2 min every 30 min. (daily from 0800-1800). Larvae were fed dry feed in surplus approximately 25% of expected larval wet weight biomass in the first week, decreasing to 10 % during the fourth week. Tanks were cleaned with a siphon once daily, removing dead larvae (which were counted daily) and feed remainings.

Samplings & procedure:
Growth & fatty acid content. At 1 and 11 dph, larvae were sampled (2 x 30) for measurement of dry weight (DW), lipid, FA content and were stored frozen at -80°C. Similarly, for each tank 30 larvae at 20 dph and 30 dph were sampled. At 35 dph, 50 larvae per replicate were sampled for examination of the rate of malformations (lordosis, scoliosis and jaw malformations).

Digestive enzyme activities and proteomics (FUNDP). For digestive enzymatic activity 200 larvae were sampled at 0 dph, and 90 larvae at 11 dph . At 20 dph (40 larvae replicate^{-1} were sampled and at 30 dph (30 larvae replicate^{-1}). 20* larvae replicate^{-1} were sampled for proteomic analyses at dph 35 and 40, respectively. Since livers are used for the proteomic analyses, samplings were done only at this late -larval developmental stage. (-*i.e. at 35 dph, survival was so low in some replicates that that not enough larvae were left for samplings at 35 and 40 dph, **Table 2**).

For both enzymatic and proteomic analyses, larvae from each tank were pooled, and stored at -80°C. Larvae are analysed for organ development and tissue morphology of digestive tract, liver, digestive enzyme activity in stomach, pancreas, intestine, and liver proteomics involving use of *in situ* hybridization techniques.

Radiographic studies and gene expression. At 35 dph 50* larvae per replicate was anesthetized with clove oil and frozen for radiographic studies (P2. FCPCT). (- *i.e. at 35 dph, survival was so low in some replicates (**Table 2**) that that not enough larvae were left for these samplings and it was decided to redo the experiment to obtain the necessary samples). The larvae sampled for specific staining of bone and cartilage will be kept in buffered formalin in flat glass flasks to keep the fish unbent (P2. FCPCT). This study will be completed by molecular approaches such as real time PCR, in order to quantify expression of some relevant genes involved in the skeletal system (P2. FCPCT) for this further 10 larvae per replicate will be stored in RNAlater at 35 dph.

Table 2. Overall survival at 35 dph for the 6 diets tested (excl. larvae sampled at 21 dph).

Diet	D1	D2	D3	D4	D5	D6
Survival (dph 35)	1.9±1.8	3.8±2.4	6.0±5.2	12.3±9.2	2.8±2.2	6.9±5.8

Milestone MS23 (M12): Definition of parameters for skeleton studies in pikeperch

Minerals, vitamins, lipids and fatty acids all have essential biological influence on the larval skeleton development. The understanding of the action of some of these components at the molecular level at critical phases will allow the improvement of the quality of hatchery reared larvae. Thus, studies are performed (2015) using various diet compositions to test the influence of HUFAs, phospholipids, Ca/P, dietary vitamin A, C, D levels on malformations (lordosis, scoliosis (vertebral curvatures), bone deformities (central fusion and compression of vertebra) operculum and distortion of gill filament cartilage and influence on skeleton related gene expression.

Task 10.2 Effects of pikeperch early fatty acid nutrition on long-term stress sensitivity (led by DTU).

No work was planned during this period in this task.

Deviations from Annex I and their impact:

There were no deviations from Annex 1, but the experimental study was 1 month delayed due to late spawning of pikeperch breeders, the experimental study will be redone in December, where no activities are planned as so the expected work carried out during year 1 is within the expected time frame.

WP No:	11	**WP Lead beneficiary:**		P17. NIFES
WP Title (from DOW):	Nutrition – Atlantic halibut			
Other beneficiaries (from DOW):	P7. IMR	P15. ULL	P20. SARC	
Lead Scientist preparing the Report (WP leader):	Kristin Hamre			
Other Scientists participating:	Torstein Harboe (P7)			

Objectives

1. Develop a protocol for early weaning,
2. Develop a production strategy for on-grown *Artemia,*
3. Improve growth in late larval stages, and juvenile quality, through feeding with on-grown *Artemia,*
4. Better understand the effects of RAS vs FTS on Atlantic halibut larval nutrient utilization,
5. Investigate how dietary phospholipids after weaning affects growth and lipid metabolism.

Summary of progress towards objectives and details for each task:

Task 11.1 Early Weaning of Atlantic halibut (led by IMR, Torstein Harboe)

The work has been planned and the practical experiments will be performed during 2015 and 2016.

Task 11.2 Development of a production strategy for on-grown *Artemia* (led by IMR, Torstein Harboe).

A strategy for production of on-grown *Artemia* has been made. The strategy is a modified production method used at IMR in 2005. *Artemia* cysts will be hydrated and hatched according to standard procedures. The nauplii will be transferred to on-growing tanks (250 l). These tanks will have continuous water supply. The off let sieve is made of plankton net. Water temperature is 20 to 22°C and pH is adjusted with NaOH. Initial number of *Artemia* will be 100 ind. /ml. We will try two different feeds, micronized fish meal and ORI-culture from Skretting, as on-growing feed. MultiGain (Danafeed) or ORI-Gold (Skretting) will be used for short time enrichment just before *Artemia* are given to the larvae. Expected growth during the 4 day culture is from ~2.1 to ~12 µg/*Artemia*.

Practical preparation of the facilities and purchase of feed have been peformed and the experiments will be run in week 50-51. Samples will be analyzed in the beginning of 2015.

Task 11.3 Nutrient retention and digestive physiology of Atlantic halibut juveniles fed *Artemia* nauplii or on-grown *Artemia* (led by NIFES, Kristin Hamre).

Planning of the work has started and the experiment will be done in spring 2015.

Task 11.4 Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS (led by NIFES, Kristin Hamre).

The experiment will be run in spring 2016.

Task 11.5 Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles (led by NIFES, Kristin Hamre)

The experiment will be run during spring/summer 2017

Deviations from Annex I and their impact:

Although according to the DOW work should start from the beginning of the project period, there are no results yet. This has to do with the relatively few activities in this WP and the need to organize the work in a practical way. Furthermore, the spawning season of the halibut in late winter/spring determines when the activities can be executed. However, we do not expect any delays in the deliverables

WP No:	12	WP Lead beneficiary:	P19. CMRM
WP Title (from DOW):	Nutrition – wreckfish		
Other beneficiaries (from DOW):	P2. FCPCT	P8. IEO	
Lead Scientist preparing the Report (WP leader):	Fatima Linares (P19)		
Other Scientists participating:	Marisol Izquierdo (P2), Tito Peleteiro (P8), J.L Rodriguez (P19), Blanca Alvarez Blázquez (P8) and Rosa Cal (P8)		

Objectives

1. Test the effectiveness of live prey and influence of enrichment on wreckfish larvae,
2. Determine the influence of broodstock feeds on fecundity and spawning quality.

Task 12.1 Live preys and enrichments for wreckfish larvae (led by CMRM, Fatima Linares).
No work done during this period

Task 12.2. Influence of broodstock feeding regimes for fecundity and spawn quality
The activities initiated in this task are related to the composition of wild fish and the feeding of wreckfish broodstock. Sampling of wild wreckfish from February to October were carried out on a fornightly basis in two places: O Grove (Pontevedra) and Vigo, although these fish came from the same fishing area, the Azores. The number of fish examined were 34 from O Grove (mean fresh weight of 7.4±1.2 Kg and size 76±4 cm) and 26 from Vigo (mean fresh weight of 7.7±2.9 Kg and size of 76±8.9 cm (**Fig. 12.2.1**).

Figure 12.2.1. Wild wreckfish and visceras from the sampled speciments.

Data of weight, size (total and standard), perimeter, and weight of liver, stomach, intestine and gonads were obtained and used to estimate the GSI ((gonad weight / whole body weight) x100), HSI ((liver weight/whole body weight) x100) and VSI ((viscera weight / whole body weight) x 100) as part of WP.6. Reproduction and genetics – wreckfish (data shown there). The stage of the reproductive development of each individual was checked. Fish dissection and sample collection of muscle, liver and gonads were carried out for

biochemical analysis to know the nutritional status of wild fish. The stomach content was also observed. Additionally samples of fin of each fish were taken out for genetic analysis.

Samples of gonads were stored in formol 4% adjusted to pH 7.2 for histological analysis. Additionally, samples of muscle, liver and gonad from one female wreckfish cultured in the P8. IEO and oocytes from different spawns from the Acuario A Coruña (P32. MC2) broodstock were analysed.

Samples were processed by P19. CMRM, stored at -80ºC and freeze dried before the biochemical analysis. Analysis of proteins, total lipids and fatty acids were carried out.

Macroscopical and histological analysis showed that of 33 gonads examined, 15 were males and 18 females, and no evidence of hermaphroditism was obtained.

The number of samples of different wreckfish tissues analysed were 10 samples of muscle, 20 of liver and 39 of gonad. All the analyses were carried out in triplicate and values are reported as mean ± std.

Concerning biochemical composition, the first results (%DW) showed that on wild wreckfish the level of proteins and lipids in muscle (**Fig. 12.2.2**) varied between 74-96% and 3-14% respectively. In liver and gonad (**Figs. 12.2.3 and 12.2.4**) a high variability was observed: 19-69% in proteins of liver and 10-80% in gonad. Lipid content varied among 15-73% and 9-90% in liver and gonad respectively. When the samples of gonad were analysed with peri-gonadal fat, the mean lipid level was 51±25.5% and decreased when the analyses were carried out without the perigonadal fat (25±7.4%).

The fatty acid profile (% of total fatty acids, mean ±std) in wild wreckfish is shown in **Table 12.2.1**. Muscle PUFA (polyunsaturated fatty acids), SFA (saturated fatty acids) and MUFA (mono-unsaturated fatty acids) fractions are among 36-46% (mean 38.5±5.1), 28-30% (29±1.4) and 25-33% (32.5±5.9) respectively, n-3 PUFA content reached 32-40% (33.8±4.3); the EPA (eicosapentaenoic acid) value was 4-6% (4.3±0.7), DHA (docosahexaenoic acid) 24-31% (26.2±3.8) and ARA (arachidonic acid) 2-4% (3±0.7).

The liver fatty acid profile showed a high variability as it was said above for total lipids: PUFA levels were 4-33% (mean 17.3±9.4), SFAs 21-41% (26.3±4.5) and MUFA 37-69% (57.3±10.4). n-3 PUFA, n-6 PUFA, EPA, DHA and ARA represent a lesser percentage of total fatty acids that it was found in muscle, therefore some fatty acids like DHA are relatively retained in muscle.

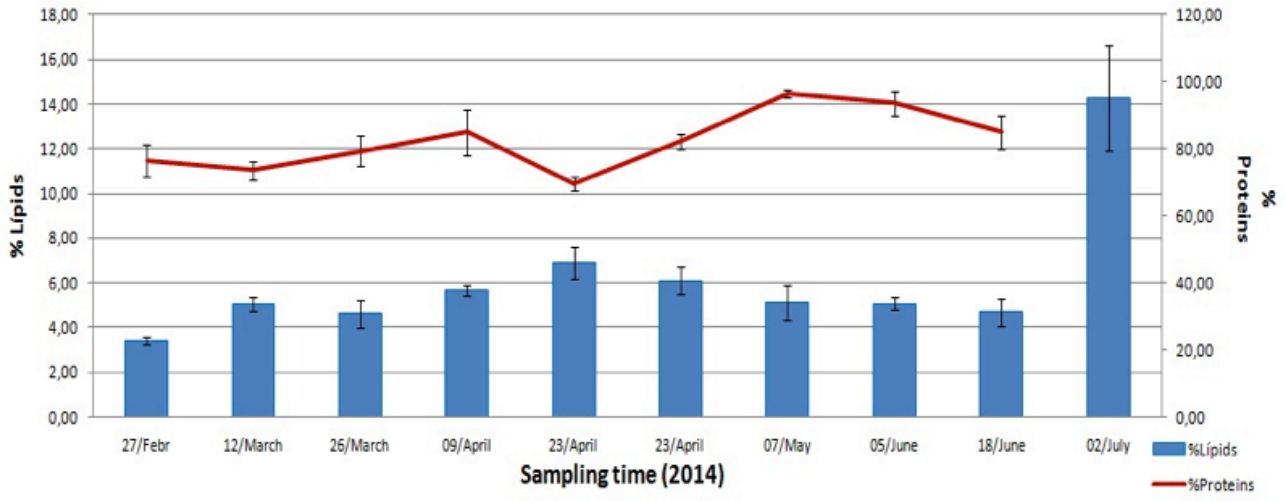


Figure 12.2.2. Proteins and lipids (% dry weight±std) of wild wreckfish muscle.

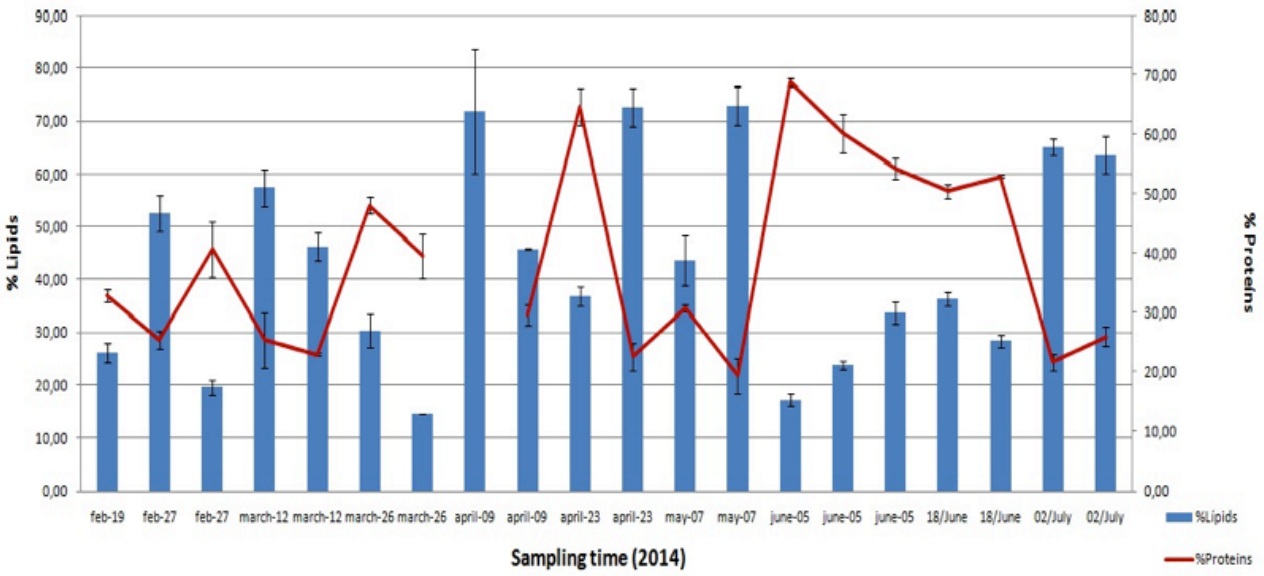


Figure 12.2.3. Proteins and lipids (% dry weight±std) of wild wreckfish liver.

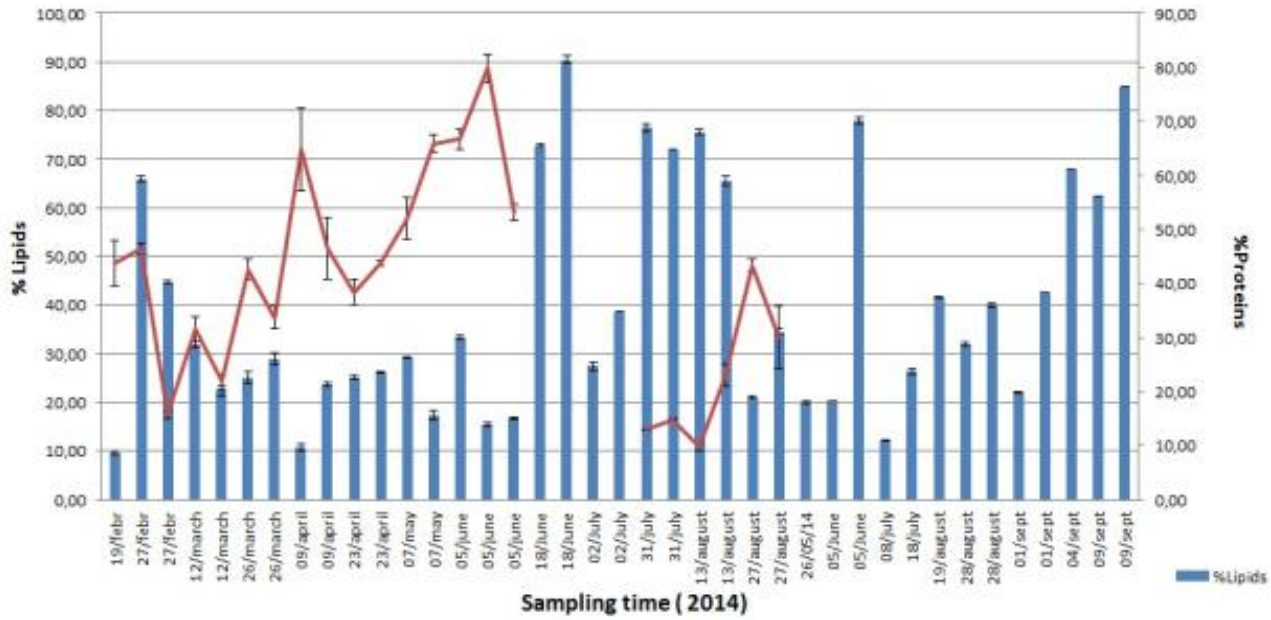


Figure 12.2.4. Proteins and lipids (% dry weight±std) of wild wreckfish gonad.

The gonad fatty acid profile shows a PUFA content among 23-37% (29±4.2), SFAs 27-31% (28.1±1.5) and MUFA 36-50% (43±4.6). n-3 PUFA represented 20-29 % (24.6±2.8) of total fatty acids, n-6 PUFA the 3-7% (4±1.7), EPA the 3-6% (4.5±0.7), DHA the 12-19 % (16.3±2.3) and ARA 1-6% (2.9±1.8).

The first results about biochemical composition of wild wreckfish indicated that they have a high amount of proteins in muscle (82%DW) and a low lipid content (6% DW). Also some fatty acids as DHA, present a very high content (26% of total fatty acids) in the muscle of these fish.

Table 12.2.1. Biochemical composition, proteins, lipids and fatty acids (mean±std) of muscle, liver and gonad of wild wreckfish.

	Muscle	Liver	Gonads
Proteins (%DW)	82.09±9.33	39.00±16.45	40.05±19.20
Lipids (%DW)	6.03±2.86	42.87±19.15	39.78±23.52
Fatty acids (%)			
14:0	1.95±0.34	1.91±0.59	2.84±0.55
15:0	0.42±0.06	0.33±0.17	0.54±0.08
16:0	19.67±0.97	17.56±3.64	17.80±0.96
17:0	1.03±0.21	1.12±0.38	1.23±0.23
18:0	5.90±0.58	5.43±1.09	5.66±0.90
ΣSFAs	28.96±1.43	26.33±4.51	28.07±1.51
16:1n-9	0.44±0.06	0.98±0.29	0.56±0.08
16:1n-7	5.33±1.50	8.54±3.41	6.48±1.01
18:1n-9	16.97±3.75	30.77±7.14	22.19±2.55
18:1n-7	4.88±0.91	9.77±1.57	6.26±0.56
20:1n-9	1.69±0.24	2.82±0.72	2.78±0.99
22:1n-9	0.32±0.09	0.27±0.12	0.54±0.21
22:1n-7	0.07±0.04	0.05±0.05	0.09±0.04
ΣMUFAs	32.50±5.94	56.33±10.37	42.96±4.56
18:2n-6	0.95±0.11	0.97±0.43	1.06±0.19
18:3n-3	0.29±0.05	0.34±0.14	0.40±0.10
18:4n-3	0.20±0.05	0.18±0.08	0.33±0.13
20:4n-3	0.54±0.08	0.54±0.45	0.74±0.17
ARA	3.04±0.70	1.45±0.92	2.91±1.76
EPA	4.32±0.69	2.95±1.52	4.46±0.75
DPA	2.22±0.31	1.44±0.77	2.32±0.30
DHA	26.20±3.77	9.27±5.75	16.33±2.31
ΣPUFAs	38.53±5.06	17.34±9.44	28.97±4.18
Σn-3	33.76±4.33	14.71±8.19	24.57±2.77
Σn-6	3.99±0.71	2.42±1.29	3.97±1.75
n-3/n-6	8.59±0.95	6.01±1.58	7.08±2.34
DHA/EPA	6.10±0.74	3.06±0.96	3.71±0.51
EPA/ARA	1.50±0.41	2.21±0.64	2.04±1.07

With respect to oocytes composition values of proteins and lipids are shown in **Fig. 12.2.5**. Samples are obtained from 3 different spawnings and the results showed there were no significant differences among them.

With respect to the influence of the broodstock food composition on the reproductive development, some samples of semi-moist diet (two lots) were collected at different times of freezing to perform the biochemical analysis (**Table 12.2.2**). This diet is supplied to the P8. IEO broodstock and was a mixture of 14.8% white fish,14.8% oily fish 14.8%, 18% mussels, 17.6% squid and 24.8% fishmeal. The first results of its lipid composition showed that lipid level was 14-16%. The PUFA level reached 35-39%, SFAs 29-32% and MUFA 32-34%; EPA represented 9-10%, DHA 14-16% and ARA 1-2%. No differences were found between samples taken at different times and with different freezing times.

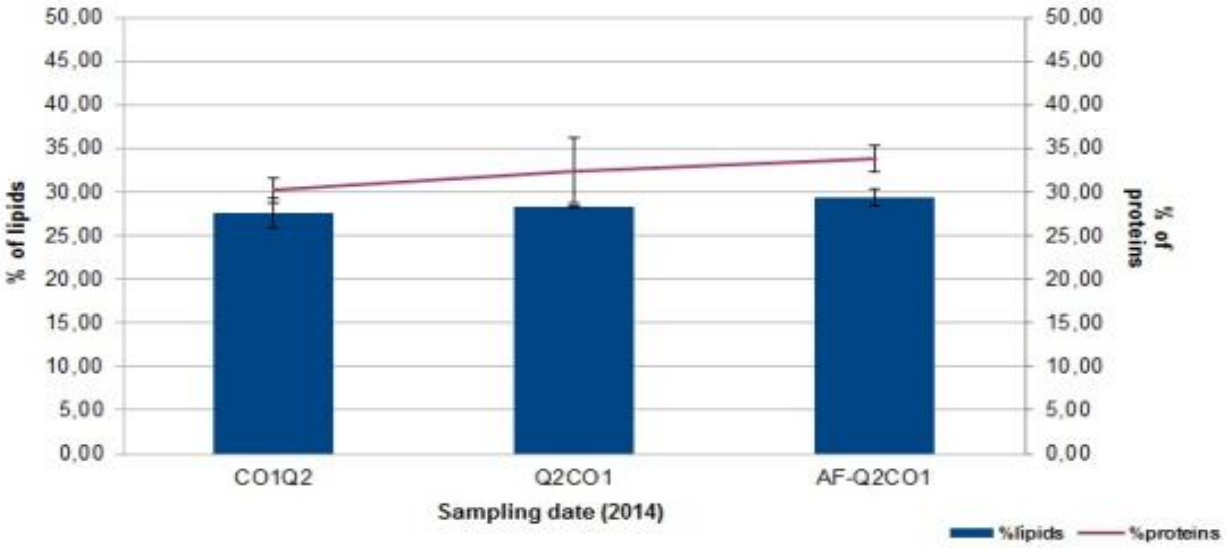


Figure 12.2.5. Oocytes composition from different spawnings in the Acuario A Coruña (P32. MC2).

Table 12.2.3. Lipid and fatty acid composition (mean±std) of semi-moist diet supplied to IEO broodstock.

	Lot 1			Lot 2				
Freezingdays	**0**	**6**	**9**	**0**	**1**	**4**	**9**	**11**
Lipids (%DW)	13.86±1.15	13.96±1.98	13.83±2.33	15.14±66	15.85±0.86	16.44±0.43	16.26±1.32	15.85±1.90
Fatty acids (%)								
PUFA´s	39,15±0.99	37,38±0.43	37,61±0.81	36,39±0.85	35,51±0.71	36,04±0.50	37,10±0.29	35,65±2.63
SFA´s	29,12±0.48	30,96±0.24	30,23±0.40	31,11±1.18	32,05±1.07	31,14±0.32	30,56±0.14	30,71±0.46
MUFA´s	31,73±0.54	31,66±0.19	32,17±0.69	32,49±1.80	32,45±1.77	32,82±0.18	32,34±0.16	33,64±2.35
Σn-3	29,39±0.89	27,53±0.76	28,08±0.22	27,57±0.93	26,68±0.70	27,50±0.47	27,81±0.52	27,61±1.30
Σn-6	8,67±0.52	8,83±0.17	8,22±0.51	7,63±0.20	7,77±0.46	7,29±0.33	8,02±0.38	6,79±1.50
n-3/n-6	3,40±0.21	3,12±0.14	3,42±0.19	3,61±0.07	3,44±0.20	3,78±0.20	3,48±0.21	4,19±0.86
EPA	9,69±0.89	9,17±0.04	9,11±0.09	9,00±0.19	8,83±0.53	8,96±0.04	8,89±0.06	8,96±0.06
DHA	16,04±1.13	13,96±0.69	14,90±0.31	14,40±0.46	13,81±0.39	14,50±0.05	14,93±0.24	14,94±0.35
ARA	0,96±0.10	0,90±0.05	0,94±0.04	0,89±0.04	0,93±0.03	0,92±0.02	0,93±0.01	1,99±1.78
DHA/EPA	1,66±0.04	1,52±0.08	1,63±0.03	1,60±0.02	1,57±0.13	1,62±0.00	1,68±0.04	1,67±0.04
EPA/ARA	10.08±0.44	10.17±0.53	9.75±0.44	10.06±0.23	9.46±0.28	9.74±0.14	9,61±0.12	6.98±4.14

The effect on the reproductive development of three feeding regimes will be compared in the next years: Commercial feed (Vitalis Repro and Vitalis Cal) will be supplied to the CMRM broodstock and semi-moist diet and a new dry food with a new specific formulation for wreckfish, which will be supplied to the P8. IEO broodstock 1 and 2, respectively. The studies will focus particularly on protein/energy ratios and essential fatty acids.

Deviations from Annex I and their impact:

Task 12.2 was scheduled to begin in December of 2014, but in order to have information about the biochemical composition of the wild wreckfish and connected with task 6.2 (describe reproductive cycle), samplings of wild wreckfish were performed to know some parameters as weight, size, the stomach content, perivisceral fat content, GSI, HSI, VSI etc. Furthermore, samples of some tissues (muscle, liver and gonad) were analysed to know the nutritional status of wild fish. The bibliography about feeding habits of this specie is scarce, therefore the information obtained will be very useful for the development of a specific formulation for wreckfish broodstock feeding.

WP No:	13	WP Lead beneficiary:	P19. IOLR
WP Title (from DOW):	Nutrition –grey mullet		
Other beneficiaries (from DOW):	P2. FCPCT	P3. IRTA	P13. UNIBA	P18. CTAQUA
Lead Scientist preparing the Report (WP leader):	Bill Koven		
Other Scientists participating:	Marisol Izquierdo(P2), Alicia Estevez (P3), Aldo Corriero (P13), Rocio Robles (P18)		

Objectives

1. Improve enrichment products, weaning, grow out and broodstock diets,
2. Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.

Summary of progress towards objectives and details for each task.

Task 13.1 Improvement of larval performance (led by IOLR, Bill Koven).

Sub-task 13.1.1

This was planned to first test the effect of different DHA/EPA/ArA ratios on larval rearing and then selecting the best treatment from these studies to investigate the effect of dietary taurine to further improve the larval rearing protocol. However, due to the unexpected availability of eggs from photoperiod grey mullet brood stock at kibbutz Ma'agan Michael in the north of Israel, we decided to run the taurine experiment first, which fulfils Sub-task 13.1.2 and postpone Task 13.1.1 to next year's breeding season.

Sub-task 13.1.2

The effect of dietary taurine on grey mullet larval rearing at different stages of development was carried out in early September. The experimental system consisted of eighteen 400 l v-tanks (**Fig. 13.1.2.1**) with computer salinity and temperature control, which allowed the testing, in replicates of 6 tanks per treatment, of the control (no taurine: T0-0), 400 mg taurine/l (T400-0) and 600 mg taurine/l of rotifer enrichment medium. Following the end of rotifer feeding at 14 dph, six rotifer- *Artemia* treatments (Trotifer-*Artemia*) were tested to 19 dph in replicates of 3 tanks per treatment. These treatments were T0-0, T400-0, T0-400, T400-400, T600-0 and T600-600. The treatment T0-600 could not be tested due to the lack of available tanks. Sufficient sampling of larvae, rotifers and *Artemia* for taurine and fatty acid composition were taken as well as larval samples for the expression of key genes and these samples will be processed during 2014-2015. This study is also reported in Task 19.5 as the results contributed markedly to improving the larval rearing protocol for grey mullet, which will be tested later on in the project at the SME P25. DOR.

In summary, the analyses to date shows a significant ($P<0.05$) effect of rotifer taurine on larval growthat (a) 12 dph (end of rotifer feeding), (b) 19 dph (end of *Artemia* feeding) and (c) 44 dph (fed weaning and starter diets) (**Fig. 13.1.2.2**) as well as in survivalat 44 dph (**Fig. 13.1.2.3**). Therotifer taurine effect appears stronger and significantly ($P<0.05$) effecting much later stages of development than *Artemia* taurine

enrichment (data not shown). Moreover, juveniles from the rotifer-only taurine treatments have been presently stocked (ca 100mg wet wt) in nine 80 l cages floating in 1000 l flat bottom tanks (**Fig. 13.1.2.4**) that are situated outside in the IOLR nursery where they will be grown to 500 mg. This study is being conducted to see if the rotifer taurine effect continues to give an advantage in older juveniles. In addition, samples for the gene expression of the proteins CSD (rate limiting enzyme in taurine synthesis) and CYP7a1(rate limiting enzyme in bile salt synthesis) will be taken.

Figure 13.1.2.1 Experimental 400 l system used to test the dietary taurine effect during rotifer and/or *Artemia* feeding.

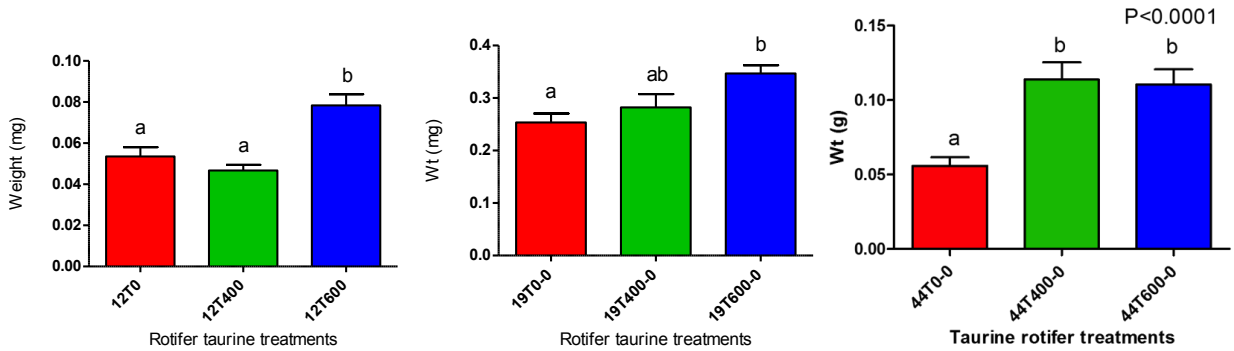


Fig. 13.1.2.2. Mean (±SEM) weight values (mg) of (a) 12, (b) 19 and (c) 44 dph juvenile grey mullet that ingested rotifers from 2-14 dph containing no taurine (T0-0) or enriched with either 400 or 800 mg taurine/l of enrichment medium (T400-0, T600-0, respectively). All treatment fish were fed on non taurine -enriched *Artemia* nauplii from 14 to 19 dph followed by weaning and starter diets until 44 dph. The one-way ANOVA (wt vs taurine treatment) indicated the existence of a significant interaction ($P<0.0001$), showing the early importance of dietary taurine and its far-reaching effects into much later development in grey mullet. Wt values having different letters were significantly ($P<0.05$) different.

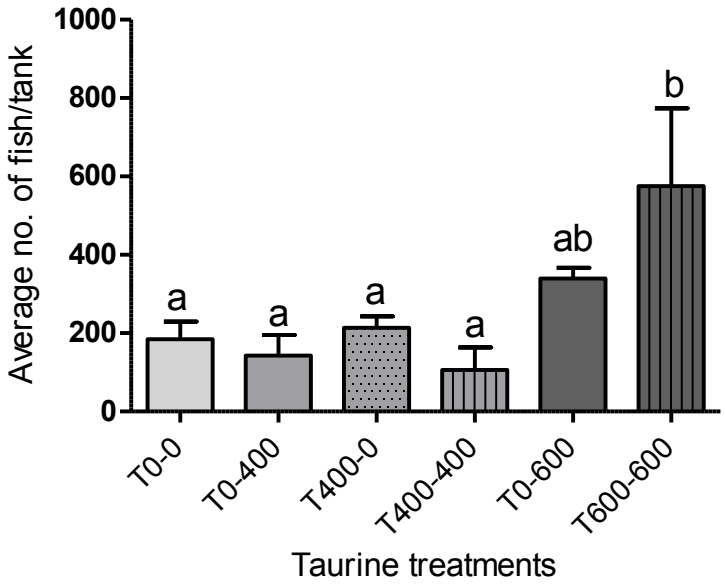


Figure 13.1.2.2. Mean (±SEM) survival values (mean no. of fish remaining/tank) of 44 dph juvenile grey mullet that ingested rotifers and/or Artemia (rotifer-Artemia) containing no taurine (T0-0) or enriched with either 400 or 800 mg taurine/l of enrichment medium. The one-way ANOVA (mean surviving individuals/tank vs taurine treatment) indicated the existence of a significant interaction ($P<0.05$) showing that the highest fish survival occurred when both rotifers and Artemia were enriched with 600 mg taurine/l. Survival values having different letters were significantly ($P<0.05$) different.

Figure 13.1.2.4. Nursery rearing system testing the long term effect of feeding taurine to 2-14 dph larvae on the performance of 100-500 mg juvenile grey mullet. The experimental system consisted of three 1000 l flat bottom tanks where larvae from the treatments (T0-0, T400-0, T600-0) were tested in triplicate 80 l floating cages. In each tank there was one replicate cage from each of the taurine treatments.

Task 13.2 Determining mullet nutritional needs for improved weaning to a dry diet (led by IOLR, Bill Koven).
This task, and its constituent Sub-tasks, is scheduled to start in month 21 (2015) and its design depends on results from WP19 Larval rearing – grey mullet, which is taking place now (December 2014). This WP19 study will determine the ratio of the brush border enzyme alkaline phosphatase (AP) over the cytosolic intestinal enzyme leucine alanine peptidase (leu-ala). When the ratio peaks, this is a signal that maturation of the digestive tract has occurred and weaning can commence. On the other hand, when this ratio decreases, the fish changes from carnivorous to omnivorous feeding, which means the weaning diet should include more amylolytic energetic compounds such as starch

Sub-task 13.2.1
Samples from the larval and juvenile taurine experiments (Sub-task 13.1.2), which will determine the age dependent gene expression of the rate limiting enzyme for taurine synthesis; cysteine sulfinate decarboxylase (CSD), will be analyzed in 2015.

Sub-task 13.2.2
Samples from the larval and juvenile taurine experiments (Sub-task 13.1.2), which will determine the age dependent gene expression of the rate limiting enzyme for bile salt synthesis, cholesterol7α-hydroxylase (CYP7A1) will be analyzed in 2015.

Task 13.3 Determining grey mullet nutritional needs for a more cost-effective production (led by IOLR, Bill Koven).
The Sub-tasks 13.3.1, 13.3.2, 13.3.3, 13.3.4, 13.3.5 will be performed in 2015 on juveniles from the larvae that were reared in 2014.

Task 13.4 Design adequate feeding regimes for brood stock to optimize reproduction success (led by UNIBA, Aldo Corriero).
Sub-tasks 13.4.1 and 13.4.2 are not scheduled to begin until month 39 in 2017 according to the DOW. Nevertheless, in years 2 and 3 biological samples will be collected from grey mullet broodstock fed different diets in collaboration with P4. IORL.

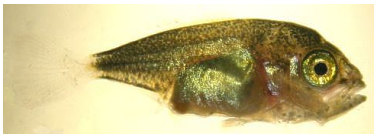
Deviations from Annex I and their impact:
There were no deviations from Anex I during this reporting period.

Group Work Packages

Larval husbandry

Progress was hampered by limited, and poor quality spawns and larvae, resulting in high mortality and the necessity to repeat some experiments. Moreover, the natural spawning of some of the species occurs late in the year (*e.g.*, gry mullet), which means key studies are still on-going at the time of preparing this report. On the other hand lessons have been learned and modifications to future studies such as transport of eggs from other sources, improved spawning conditions and quality of implants, as well as modifying tank management to increase larval feeding on weaning diets will be implemented so it is expected that most of the deliverables will still be on time.

Nevertheless, there were encouraging results and achievements during the first reporting period. Although clear results advancing the age of weaning in meagre were limited by a large larval size distribution leading cannibalism and poor survival, there were no significant differences, in terms of length and weight, between the control larvae weaned at 19 dph and those fed a full *Artemia* ration, and the larvae weaned at 15 dph and fed a half *Artemia* ration. Moreover, larval performance in this treatment was significantly greater than larvae fed only half the *Artemia* ration (not weaned early) or weaned at 12 dph. This suggests the possibility of successful early weaning and reduced dependence on feeding *Artemia* nauplii.



Work in to determine the most promising rotifer enrichment protocol, in terms of biochemical composition, for larval greater amberjack is near completion and will be a major tool in the 2015 studies to determine optimal prey density and feeding frequency in the larviculture of this species. Larval rearing methodologies for semi-intensive mesocosm (aeration, algae and rotifer addition, feeding and photophase) and intensive (tank management, algae and rotifer addition, egg stocking density) rearing systems for rearing amberjack larvae were established. These management protocols will be critical for the growth studies scheduled in 2015.

The studies on pikeperch to test the effect of selected environmental factors did not succeed, due to a poorly functioning biofilter resulting in high ammonia and nitrite levels and massive larval mortality. A new trial is planned at the beginning of 2015, which will insure a properly aged and functioning biofilter. Similarly, studies on wreckfish failed due to a small number and poor quality spawns and larvae, resulting in high mortality. Measures to prevent a repeat of these limitations will be implemented in a new study, which will begin in 2015.

A study is presently running to compare the efficacy of a recirculation aquaculture system (RAS) and flow through (FT) for the rearing of Atlantic halibut. The RAS showed higher larvae mortality during the first week after hatching and an increased proportion of jaw deformities at the end of the yolk-sack larva stage.

An experiment on grey mullet larvae is also currently underway and on schedule testing the effect of algal type and concentration used to "green" the rearing tanks on fish performance, biochemical profile and ontogeny of digestive tract enzymes. This study is proceeding normally and the modified grey mullet larval rearing protocol has so far demonstrated very good survival, as well as little or no cannibalism.

WP14 Larval husbandry - meagre

WP No:	14	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Larval husbandry - meagre			
Other beneficiaries (from DOW):	P15. ULL			
Lead Scientist preparing the Report (WP leader):	Estevez, Alicia			
Other Scientists participating:	Gisbert, Enric (IRTA), Rodriguez, Covadonga (ULL), Perez, Jose (ULL)			

Objectives

1. To reduce costs by early weaning in meagre larvae and improve growth, survival and larval quality

Larval rearing of meagre is relatively easy with a protocol based generally on rotifer and *Artemia* nauplii feeding methodologies implemented in European sea bass and gilthead sea bream. However, meagre larvae are quite sensitive to high light intensity (>500 lux) or a long photoperiod (Roo et al., 2010; Vallés & Estévez, 2013). Meagre producers do not consider larval rearing to be a major bottleneck for meagre culture, although cannibalism and variable size distribution in larvae and juveniles are a main concern, as they increase production costs and limit yields. Advancing the early weaning of larvae from its dependence on *Artemia* to a dry feed is a priority, and the major focus and objective of the larval work planned for meagre.

Summary of progress towards objectives and details for each task:

Task 14.1 Determining the earliest and most cost effective weaning period (led by IRTA, Estevez, Alicia). Larvae were cultured intensively (4 x 3 design using an IRTAMar recirculation unit) following a standard technique: rotifers from 2 days post-hatch (dph), *Artemia* from 12 dph until 30 dph at 100 larvae/l density and 12hL:12hD photoperiod with a light intensity of 500 lux from May 29th until June 20th 2014. Live prey were enriched using a commercial diet (Red Pepper, Bernaqua, Belgium) as shown in **Table 1**.

Larvae were obtained from two spawnings (tanks V6 and V8) after hormonal induction (see WP2) on May 27th. In the experimental groups, live prey were replaced at 12 (group D) and 15 (group C) dph using a commercially available weaning diet (Gemma Micro, Skretting, Norway) and half (groups B, C and D) of the usual concentration of *Artemia* metanuplii. Control group was fed using the standard regime. Larvae were sampled at 1, 8, 15 and 24 dph to measure larval growth in length, and weight and size distribution, and samples for quality (typology and incidence of skeletal deformations), maturation of the digestive system in terms of activity of pancreatic and intestinal enzymes, as well as survival and larval biochemical composition were also taken periodically (**Fig. 1, 2 and 3**).

Table 14.1.1 Feeding protocol and quantities of prey for meagre weaning. Darker colours indicate start of weaning with artificial diet (Gemma Micro, Skretting, Norway)

							Control A			Group B			Group C			Group D		
Date	Dph	rot/ml	Rot/tq	Total Rotifero	Enrichment (L)	Enrichment (gr)	NA/ml	NA/tq	Total NA	NA/ml	NA/tq	Total NA	NA/ml	NA/tq	Total NA	NA/ml	NA/tq	Total NA
29-maig	2	10	1000000	12000000	24	2,4												
30-maig	3	10	1000000	12000000	24	2,4												
31-maig	4	10	1000000	12000000	24	2,4												
1-juny	5	10	1000000	12000000	24	2,4												
2-juny	6	10	1000000	12000000	24	2,4												
3-juny	7	10	1000000	12000000	24	2,4												
4-juny	8	10	1000000	12000000	24	2,4	0,5	50000	150000	0,25	25000	75000	0,25	25000	75000	0,25	25000	75000
5-juny	9	10	1000000	12000000	24	2,4	0,5	50000	150000	0,25	25000	75000	0,25	25000	75000	0,25	25000	75000
6-juny	10	10	1000000	12000000	24	2,4	1	100000	300000	0,5	50000	150000	0,5	50000	150000	0,5	50000	150000
7-juny	11	10	1000000	12000000	24	2,4	1	100000	300000	0,5	50000	150000	0,5	50000	150000	0,5	50000	150000
8-juny	12	10	1000000	12000000	24	2,4	1	100000	300000	0,5	50000	150000	0,5	50000	150000	0,75	75000	225000
9-juny	13	10	1000000	12000000	24	2,4	1,5	150000	450000	0,75	75000	225000	0,75	75000	225000	0,75	75000	225000
10-juny	14	10	1000000	12000000	24	2,4	1,5	150000	450000	0,75	75000	225000	0,75	75000	225000	1,5	150000	450000
11-juny	15						1,5	150000	450000	0,75	75000	225000	0,75	75000	225000	1,5	150000	450000
12-juny	16						3	300000	900000	1,5	150000	450000	1,5	150000	450000	1,5	150000	450000
13-juny	17						3	300000	900000	1,5	150000	450000	1,5	150000	450000	1,5	150000	450000
14-juny	18						3	300000	900000	1,5	150000	450000	1,5	150000	450000	1,5	150000	450000
15-juny	19						6	600000	1800000	3	300000	900000	1,5	150000	450000	0,75	75000	225000
16-juny	20						6	600000	1800000	3	300000	900000	1,5	150000	450000	0,75	75000	225000
17-juny	21						3	300000	900000	1,5	150000	450000	0,75	75000	225000	0,75	75000	225000
18-juny	22						3	300000	900000	1,5	150000	450000	0,75	75000	225000	0,75	75000	225000
19-juny	23						3	300000	900000	1,5	150000	450000	0,75	75000	225000	0,75	75000	225000
20-juny	24						3	300000	900000	1,5	150000	450000	0,75	75000	225000			
21-juny	25						3	300000	900000	1,5	150000	450000	0,75	75000	225000			
22-juny	26						1,5	150000	450000	0,75	75000	225000						
23-juny	27						1,5	150000	450000	0,75	75000	225000						
24-juny	28						1,5	150000	450000	0,75	75000	225000						
25-juny	29						1,5	150000	450000	0,75	75000	225000						
26-juny	30						1,5	150000	450000	0,75	75000	225000						
Enrichment for rotifers = 0.1 gr/l and 500 rot/ml																		
Enrichment for Artemia 1,5 gr/l and 500 NA/ml																		

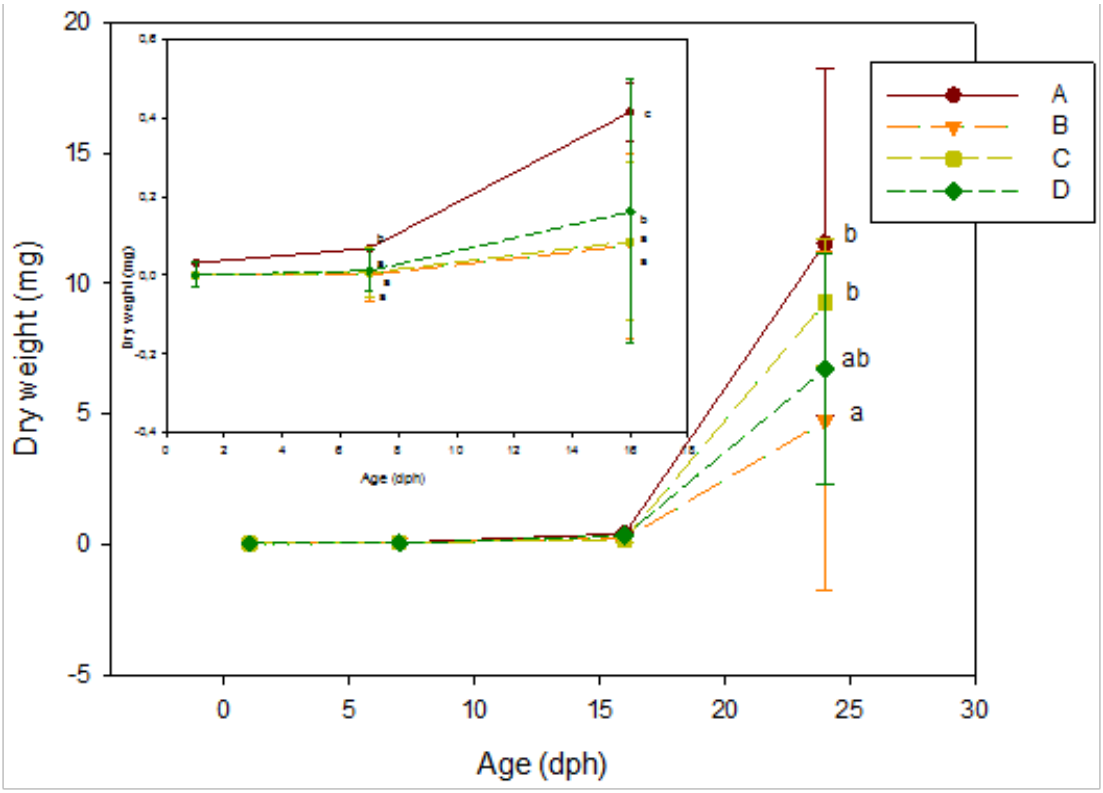


Figure 14.1.1. Growth in dry weight (mg, Mean ±SD) of the larvae from the different groups. Different letters show significant differences (ANOVA, P<0.05).

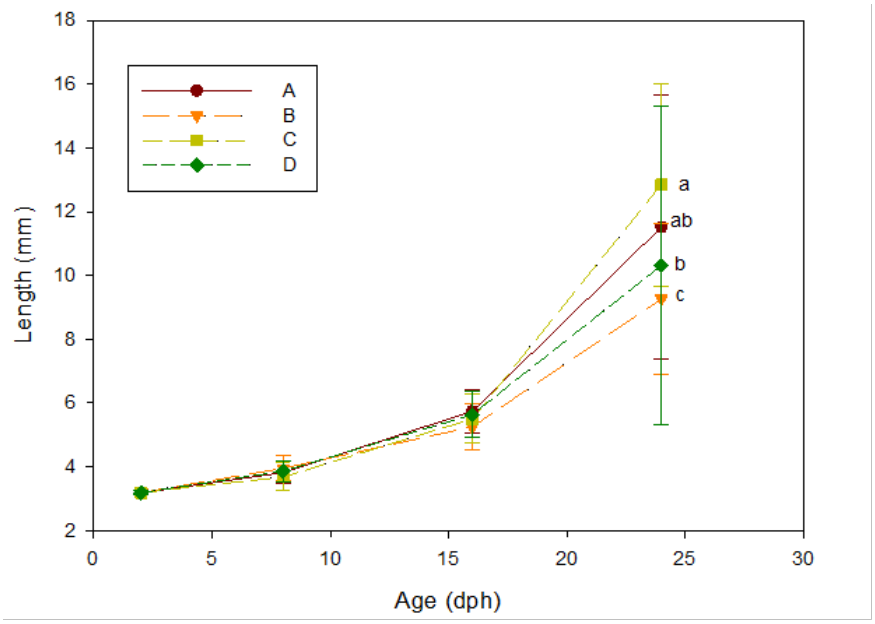


Figure 14.1.2. Growth in length (mm, Mean ±SD) of the larvae from the different groups. Different letters show significant differences (ANOVA, P<0.001)

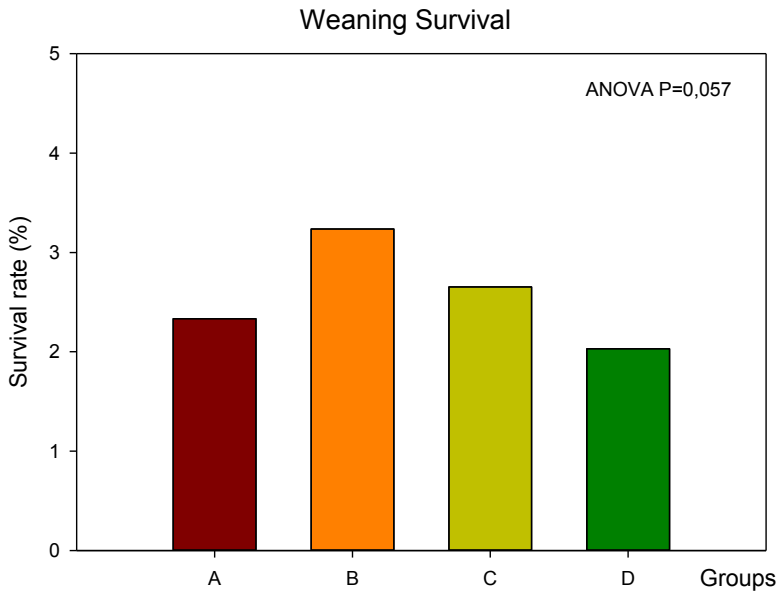
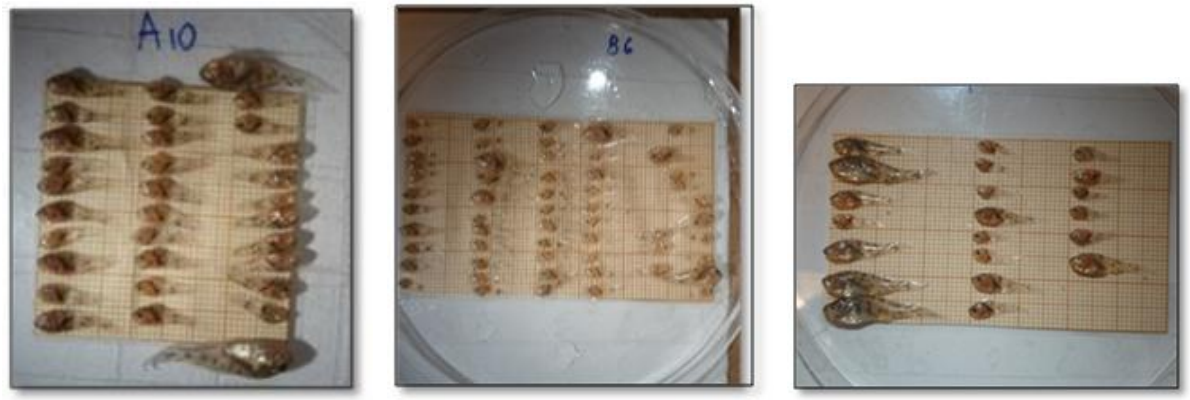


Figure 14.1.3. Survival rate (%, Mean ±SD) of the 4 groups. No significant differences could be found (ANOVA= 0.057) although groups B and C showed a tendency to have better survival than groups A and D.

Group C, weaned at 15 dph and fed half the amount of *Artemia* nauplii showed similar final weight to controla (group A), but higher length than all the groups, although in this group the incidence of cannibalism, and subsequently the growth in length of some individuals, was higher than the rest of the groups --with the exception of group D that showed the highest mortality rate (**Fig. 14.1.3**). On the other hand, group B showed a significantly lower growth in weight and length and the highest survival rate with lower incidence of cannibalism (**Fig. 14.1.4**) and a similar distribution of size among the larvae. Although no significant differences were obtained in survival rate, overall this parameter was very low (2 – 3.3%, **Fig. 14.1.3**). At the end of the experiment high differences in growth of the larvae were detected in each tank (**Fig. 14.1.4**), due to the high incidence of cannibalism.



A. **B.** **C.**

Figure 14.1.4. Photographs showing the differences in the size of the larvae at the end of the experiment (24 dph) in groups A, B and C, due to the high incidence of cannibalism.

Fatty acid composition of the larvae at the end of the assay is presented in **Table 2**. Total monounsaturated fatty acids (utilized as an energy reserve) were significantly (P<0.05) higher in groups A and B compared to C and D, while total n-6 PUFA was significantly (P<0.05) higher in groups A, C and D compared to B. The DHA content was markedly (P<0.05) higher in groups C and D compared to A and B, possibly as a consequence of increased feeding on the enriched live prey/weaning diet or sibling larvae.

Table 2. Fatty acid composition (%TFA, mean and SD) of the larvae at the end of the weaning period, letters indicate significant differences.

	A		B		C		D		
14:0	0,38	0,15	0,26	0,05	0,17	0,04	0,23	0,02	
15:0	0,16	0,02	0,08	0,03	0,09	0,05	0,06	0,05	
16:0	15,10	0,74	14,67	1,19	15,31	0,22	15,84	0,32	
18:0	11,63	0,35	11,73	0,69	12,13	0,56	12,53	0,58	
Total saturated	27,26	0,92	26,74	1,94	27,70	0,51	28,66	0,79	
16:1	0,38	0,12	0,30	0,11	0,34	0,04	0,28	0,03	
18:1n-9	17,25	0,16	18,24	1,69	16,44	2,54	15,12	0,49	
18:1n-7	4,29	0,28	2,62	2,28	2,70	2,36	3,71	0,29	
20:1	0,63	0,15	0,55	0,09	0,69	0,04	0,70	0,05	
Total monounsaturated	22,55	0,21a	21,71	0,73a	20,17	0,21b	19,81	0,34b	P<0.001
18:2n-6	5,84	0,22	5,39	0,13	4,56	0,37	4,26	0,48	
18:3n-6	0,74	0,05	0,59	0,05	0,55	0,08	0,43	0,11	
20:3n-6	0,30	0,06	0,18	0,02	0,20	0,05	0,15	0,01	
20:4n-6	4,68	0,26	4,53	0,25	5,99	0,62	6,39	0,30	
22:4n-6	3,81	0,15	3,96	0,18	4,31	0,06	4,23	0,12	
22:5n-6	0,07	0,00	0,00	0,00	0,16	0,08	0,06	0,10	
Total n-6 PUFA	15,43	0,11b	14,65	0,20a	15,77	0,43b	15,52	0,28b	P=0.006
18:3n-3	13,70	1,14	13,12	1,86	7,77	1,77	7,71	1,72	
18:4n-3	1,42	0,16	1,37	0,03	0,95	0,16	0,97	0,16	
20:4n-3	0,87	0,17	0,85	0,15	0,55	0,08	0,74	0,26	
20:5n-3	5,49	0,44	5,38	0,55	4,25	0,16	4,25	0,09	
22:5n-3	0,67	0,03	0,65	0,09	1,30	0,24	1,32	0,16	
22:6n-3	11,33	0,50a	13,60	0,65a	19,19	2,10b	17,39	1,56b	P<0.001
Total n-3 PUFA	33,48	1,28	34,97	2,90	34,02	1,01	32,39	0,31	
Total PUFA	48,92	1,23	49,63	2,87	49,79	0,58	47,91	0,59	P=0,355
Total Fatty acids (mg)	518,48	76,70	616,13	82,98	700,56	25,41	588,19	64,61	P=0.056

The analysis of the digestive enzymes to detect differences in digestive capabilities among the larvae due to the different feeding regimes and the estimation of skeletal deformities are in progress at the time of writing this report and we expect to have them finished at the end of 2014 (mo 13).

Deviations from Annex I and their impact:

Due to the high cannibalism observed in all the groups, particularly the groups fed the microdiet from day 12 onwards, we are planning to repeat the experiment in 2015 in order to increase the survival rate and dietary performance, and obtain more data to address the objective of the WP. We do not expect any delays in the submission of the planned deliverable and milestone.

WP No:	15	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Larval husbandry –greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P8. IEO	P15. ULL	P27. FORKYS
Lead Scientist preparing the Report (WP leader):	Hernandez Cruz, Carmen Maria (P2)			
Other Scientists participating:	Papandroulakis, Nikos (HCMR), Jerez, Salvador (IEO), Rodriguez, Covadonga (ULL), Diakogeorgakis, Ioannis (FORKYS)			

Objectives

1. Effects of different feeding strategies on larval performance in intensive systems.
2. Development of feeding protocols in mesocosm and semi-intensive systems.
3. Development of industrial protocol for larval rearing.

Summary of progress towards objectives and details for each:

Task 15.1 Effect of feeding regime and probiotics (led by IEO, Jerez, Salvador).

The effect of different live prey protocols (concentration and supply frequency) together with probiotics and immunostimulants as health promoting/nutritional supplements will be evaluated. To achieve the objectives proposed in this task, a first trial of rotifer enrichment was performed. Different proportions of *Echium* oil (four experimental treatments by triplicate) were used to enrich the rotifers considering 4 different enrichment periods (3, 6, 10 and 24 hours). The *Echium* oil was combined with either a commercial emulsion or a marine lecithin rich in LC-PUFA. Biochemical analysis of enriched rotifers is currently being carried out at P15. ULL to establish the best combination (time and concentration) to feed the larvae. Once the best results are obtained, prey density and the frequency supplying enriched rotifers will be assayed in trials on greater amberjack larvae, which will occur during 2015.

Task 15.2 Comparison of semi-intensive and intensive rearing (led by HCMR, Papandroulakis, Nikos).

Sub-task 15.2.1 (by HCMR, Papandroulakis, Nikos).

The objective of this particular sub-task was the comparison between intensive (in RAS of 500 l tanks) and semi-intensive (Mesocosm of 40,000 l tanks) larval rearing. The evaluation will be based on a comparative study of the:

(i) Ontogeny of the visual system of the larvae, (influenced by feeding) through histological procedures.

(ii) Larval oxidative stress through the activity of specific enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase and the concentration of glutathione).

(iii) Investigating the larval somatotropic axis consisting of the growth hormone-releasing hormone (GHRH), growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factors (IGF-I and II), associated carrier proteins (IGFBPs) and receptors that represents the endocrine and autocrine regulators for skeletal-muscle growth.

During the reporting period preliminary trials were performed in order to establish the larval rearing methodologies in the two rearing systems. In the following paragraphs the methodology applied and the

results obtained are presented. The implementation of this work was achieved with the participation of the following HCMR personnel: N. Papandroulakis, N. Mitrizakis, S. Stefanakis, P. Anastasiadis, M. Vassilakis, Y. Strakantounas E. Sfakaki, Y. Tsoukali, N. Kopidakis.

Egg origin

Eggs from induced spawning of breeders kept in the Souda cage farm of P1. HCMR were used for the rearing. After collection, eggs were transported to the hatchery facilities in polystyrene boxes (~ 4 hours) and then incubated. Approximately 40 g of eggs were incubated in a Mesocosm tank, and ~35 g of eggs/ tank in two 500 l tanks for intensive RAS rearing.

Semi-intensive Mesocosm larval rearing

The **Mesocosm methodology** is an intermediate approach between the intensive and extensive methods of larval rearing and can thus be considered as a semi-intensive technique of mass production. The most important characteristic of the infrastructure required by this method is the size of the Mesocosm larval tank, which at P1. HCMR is 40 m^3 (**Fig. 15.2.1**.)

Figure 15.2.1 Mesocosm tanks at P1. HCMR.

Mesocosm tanks were filled with filtered and UV-treated seawater (salinity 40 psu) that was also the water for subsequent renewal. Temperature was at 24°C and pH fluctuated from 7.1 to 8.1. The rate of water renewal was increased progressively during the rearing. Aeration was provided in the tanks by means of five pipes (without any wooden or stone diffuser) distributed in the perimeter and the centre of the tank. A surface skimmer was operational during the appropriate period to keep the surface free from lipids, a requisite for good swim bladder inflation.

The photophase was 24L: 00D from mouth opening until 25 dph, then turned to 18L: 06D for the remaining period. Light intensity varied between 20-150 µmole m^2 s^{-1} during the day while during the night, when prolonged photophase was applied, light intensity was about 25 µmol m^{-2} s^{-1}. The phytoplankton organism used was *Chlorella sp*. The rotifers, *Brachionus plicatilis,* were mass-produced at P1. HCMR. Prior to distribution to the larvae, rotifers were enriched with commercial products and were maintained during their use with *Chlorella sp*. Instar II *Artemia* nauplii were also enriched with commercial products according to the specifications of the provider.

Feeding was based on daily administration of enriched rotifers (3 to 18 dph), Instar II *Artemia* nauplii (from 15 to 30 dph), and occasionally eggs and pre-larvae from 16 dph. Phytoplankton was added daily until 20 dph. The concentration of rotifers in the tank was maintained at 1.5 rotifers/ml, while *Artemia* concentration was 0.1 nauplii/ml after measurements of the concentration twice daily. Mesocosm tanks presented also an inert productivity of zooplankton (copepods) that could potentially contribute to larval feeding. Artificial diet delivery was initiated on 22 dph and was distributed with an automatic feeder. The growth of the individuals in terms of total length during the first 30 days of rearing was exponential with a rate of 0.062 daily (R^2=0.95) and reached 23.3 ± 2.5 mm (**Fig. 15.2.2**). On day 30, 950 individuals were transferred for pre-growing.

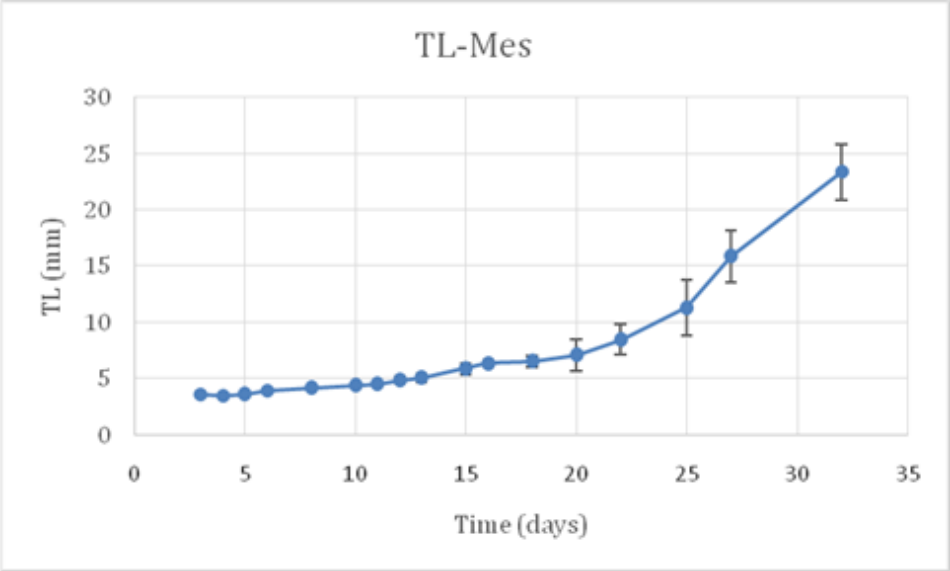


Figure 15.2.2 Progression of total length during Mesocosm rearing (± SEM, n=10).

Intensive rearing in RAS

The second methodology applied was the **intensive rearing,** which is characterized by controlled conditions of water quality, light intensity, photophase and feeding. The variant of the methodology applied is the so-called "pseudo-green" water that is based on the frequent addition of phytoplankton and zooplankton in the larval rearing tanks. It integrates principles of both "clear water" and "green water" methods and minimizes biological problems and many of their technical, human and environmental constraints. The main difference from the classical "green water" technique is that phytoplankton is not produced or bloomed in the rearing tank, but its concentration remains constant by daily addition.

The tanks used at P1. HCMR were 500-l cylindro-conical and were organized in pairs in a closed water system with a biological filter (**Fig. 15.2.3**). The tanks were filled with borehole water (35 psu). Temperature was kept at 22°C and pH fluctuated from 7.9 to 8.2. Water circulation was achieved in two ways according to the stage of rearing. During embryogenesis, egg hatching and the autotrophic larval stage, water circulated in the tanks through a biological filter. Aeration was also provided in the tanks (150–250 ml min^{-1}). After first feeding, water circulation was autonomous for each tank by means of an airlift pump. The water in the biological filter was used for renewal in larval rearing tanks. A small renewal of water (3%) was realized daily. The temperature was controlled. A skimmer was installed at the appropriate period to keep the surface free from lipids. The light intensity was controlled and varied according to the species and stage of rearing.

Feeding was based on daily administration of enriched rotifers (3-21 dph), Instar II *Artemia* nauplii (12 dph onwards), and artificial diet (21 dph onwards). The concentration of rotifers in the tank was maintained at 3.0 rotifers/ml, while of *Artemia* it was 0.1 nauplii/ml, with the concentration being measured twice daily. Phytoplankton was added daily until 22 dph. The administration of the zooplankton was implemented with the use of an automated feeding system allowing the administration of food several times daily and also during the night. The growth of the individuals in terms of total length during the first 30 days of rearing was exponential with a rate of 0.047 daily (R^2=0.93) and reached about 20 mm (**Fig. 15.2.4**). On day 30, about 2500 individuals were transferred for pre-growing. With the results obtained, the methodology for larval rearing is established and the actual trial will be implemented during the second year of the project.

Figure 15.2.3 Larval RAS rearing tanks in P1. HCMR.

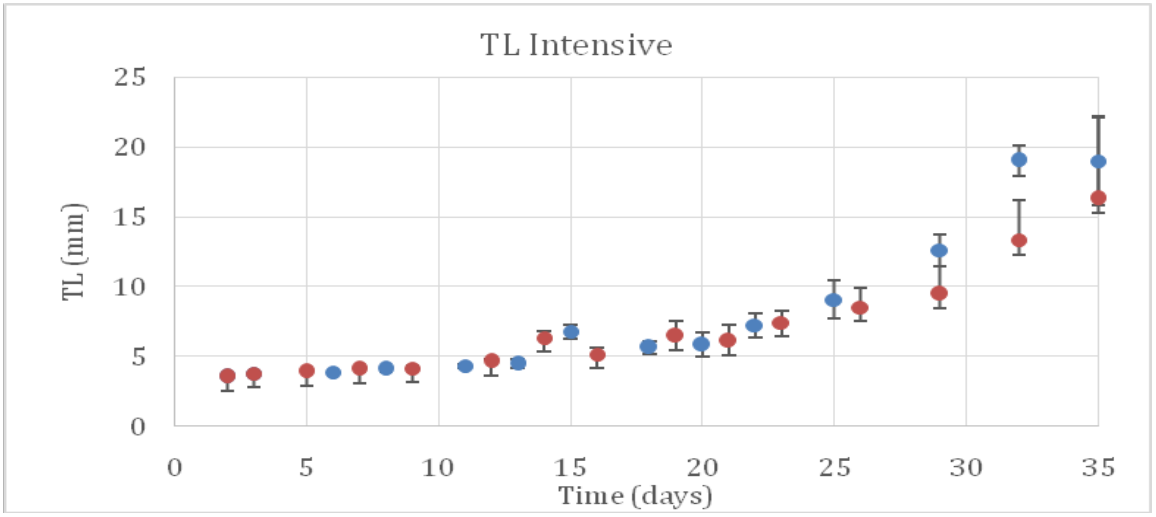


Figure 15.2.4 Progression of TL during intensive rearing (± SEM, n=10).

Sub-task 15.2.2 (by FCPCT, Hernández-Cruz, Carmen Maria).

The effect of stocking density on larval performance in terms of growth, survival, skeletal deformities and gene expression of stress and skeleton genes was studied. Three different larval rearing densities were evaluated: 25, 50 and 75 eggs/l in triplicate tanks for a period of 30 days. The implementation of this work was achieved with the participation of the following personnel: Antonio La Barbera, Samira Sarih, Hipólito Fernández-Palacios Barber, Carmen María Hernández Cruz, Antonio Mesa, and Javier Roo.

The eggs that were used in these experiments come from natural spawning of broodstock maintained in the facilities of P2. FCPCT. Greater amberjack eggs were stocked at densities of 25, 50 and 75 eggs/l in nine tanks of 2,000 l allowing the testing of each treatment in triplicate replicates (**Fig. 15.2.5**). Tanks were

supplied with filtered and UV-treated seawater. The inlet water entered at the bottom and exited at the above outlet, which was outfitted with a mesh filter of 500 µm. All tanks were equipped with a surface skimmer for removing any lipid residue from the water surface while in the center there was an aeration tube.

Figure 15.2.5 Larval rearing tanks at P2. FCPCT.

Larval rearing was according to the established protocol of P2. FCPCT, which required a natural photoperiod as well as recommended salinity (37 psu) and temperature conditions (27±0.2 ºC). Water renewal flow was increased progressively during the rearing, as described in the **Table 15.2.1**. The feed was based on rotifer (*Brachionus plicatilis*) and brine shrimp (*Artemia*) enriched with commercial products as well as providing an artificial diet that progressively increased in size with larval age. The feeding and larval rearing protocol and sampling schedule are shown in **Table 15.2.1** and **15.2.2**, respectively.

To date, three experiments were done: The first experiment started on 12/8/2014 with the aim of producing fry for future experiments. In this trial 900,000 eggs were stocked, and after 20 dph about 3,000 individuals were transferred to the pre-growing tank facility. In **Fig. 15.2.6** the growth in terms of larval total length is shown. No significant (P>0.05) differences were found among the density treatments.

The second experiment started on 1/9/2014. The aim of this experiment was also to determine the effect of stocking density of 25, 50 and 75 eggs/l. In this experiment larval growth was significantly higher from 15 dph as shown in **Fig. 15.2.7**.

The third experiment started on 03/10/2014 and the results will be available by the end of the year. In all experiments, severe cannibalism and variable total length were observed from 15-20 dph (**Fig. 15.2.6** and **Fig. 15.2.7**). Larval samples (15 dph) were given to ULL for enzyme analysis. With the results obtained, the methodology for larval rearing is established and the actual trial will be implemented during the second year of the project.

Table 15.2.1 Larval rearing protocol

<table>
<tr><th rowspan="2">Dph</th><th rowspan="2">Photop.</th><th rowspan="2">Renewal</th><th rowspan="2">Phyrop.</th><th colspan="2">Rotifer</th><th colspan="2">Artemia</th></tr>
<tr><th>8:00</th><th>15:00</th><th>8:00</th><th>11:00</th></tr>
<tr><td>Stocking</td><td rowspan="30">Natural</td><td rowspan="3">25%/h</td><td rowspan="3">Clear water</td><td rowspan="3"></td><td rowspan="3"></td><td rowspan="20"></td><td rowspan="13"></td></tr>
<tr><td>Hatching</td></tr>
<tr><td>1</td></tr>
<tr><td>2</td><td rowspan="4">15%/h</td><td rowspan="2">40 l</td><td rowspan="25">10 rot/ml</td><td rowspan="17">10 rot/ml</td></tr>
<tr><td>3</td></tr>
<tr><td>4</td><td rowspan="9">20 l</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td rowspan="4">25%/h</td></tr>
<tr><td>7</td></tr>
<tr><td>8</td></tr>
<tr><td>9</td></tr>
<tr><td>10</td><td rowspan="2">50%/h</td></tr>
<tr><td>11</td></tr>
<tr><td>12</td><td rowspan="4">75%/h</td><td rowspan="11">30 l</td><td rowspan="2">0,5 art/ml</td></tr>
<tr><td>13</td></tr>
<tr><td>14</td><td rowspan="19">0,5 art/ml</td></tr>
<tr><td>15</td></tr>
<tr><td>16</td><td rowspan="2">100%/h</td></tr>
<tr><td>17</td></tr>
<tr><td>18</td><td rowspan="2">150%/h</td></tr>
<tr><td>19</td></tr>
<tr><td>20</td><td rowspan="11">200%/h</td><td rowspan="6">15 l</td><td rowspan="9"></td></tr>
<tr><td>21</td></tr>
<tr><td>22</td></tr>
<tr><td>23</td></tr>
<tr><td>24</td></tr>
<tr><td>25</td><td rowspan="6">Clear water</td><td rowspan="9"></td></tr>
<tr><td>26</td></tr>
<tr><td>27</td></tr>
<tr><td>28</td></tr>
<tr><td>29</td></tr>
<tr><td>30</td></tr>
</table>

Table 15.2.2 Larval samplings for different actions.

DPH	Height and weight	Histology	Osteology	Genetic	Activity test	Total larvae/Tank
0	30 larvae	20 larvae	-	-	-	50
5	30 larvae/T	20 larvae/T	-	-	-	50
10	30 larvae/T	20 larvae/T	-	-	-	50
15	30 larvae/T	20 larvae/T	-	-	-	50
20	30 larvae/T	20 larvae/T	-	-	-	50
30	30 larvae/T	20 larvae/T	50 larvae/T	10 larvae/T	15 larvae/T	125

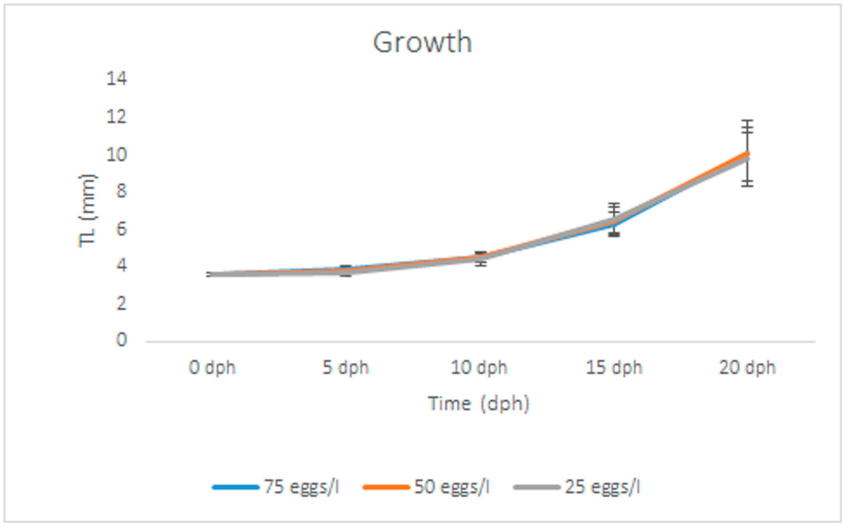


Figure 15.2.6 Growth expressed total length during the 20 days of larvae of experiment one.

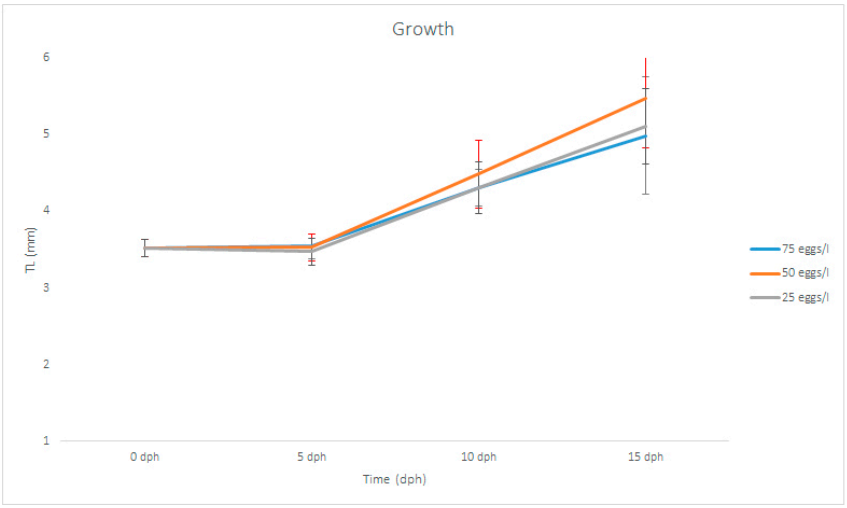


Figure 15.2.7 Growth expressed as total length during the 15 days of experiment two.

Sub-task 15.2.3 Ontogeny of digestive system of greater amberjack larvae focusing on proteases, lipases, amylases and ATPase (by ULL).

No work was done during this period.

Task 15.3 Effect of environmental parameters during rearing (led by FCPCT Hernández-Cruz, Carmen Maria).

Sub-task 15.3.1 (by FCPCT) The effect of tank hydrodynamics will be studied in different tanks types.

No work was done during this period.

Sub-task 15.3.2 (by HCMR) Effect of light (intensity and duration) on larval rearing.

No work was done during this period.

Task 15.4 Development of industrial protocol (led by IEO, Jerez, Salvador).

Sub-task 15.4.1 (by IEO) Development of an industrial protocol for larval rearing based on the results of the previous tasks.

No work was done during this period.

Sub-task 15.4.2 (by FCPCT) Ossification pattern and incidence of skeletal deformities for amberjack larvae evaluated under different levels of intensification.

No work was done during this period.

Sub-task 15.4.3 (by FORKYS) Validation of the developed protocol initially at FCPTC and over two successive years in an SME hatchery

No work was done during this period.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP No:	16	WP Lead beneficiary:	P9. UL
WP Title (from DOW):	Larval husbandry – pikeperch		
Other beneficiaries (from DOW):	P3. IRTA	P21. DTU	P29. ASIALOR
Lead Scientist preparing the Report (WP leader):	Fontaine, Pascal		
Other Scientists participating:	Gisbert, Enric (IRTA), Lund, Ivar (DTU), Debes, Keves (ASIALOR)		

Objectives

1. Improvement of pikeperch larval rearing protocols by using a multifactorial approach,
2. Reduction of cannibalism rate to increase survival,
3. Development of industrial protocol to improve larval performance during rearing.

Summary of progress towards objectives and details for each task:

Task 16.1 Optimal combinations of factors to improve larval rearing (led by UL, Pascal Fontaine).

Using a pilot scale larval rearing system (RAS, 10 tanks of 700L each, see **Fig. 16.1.1**), a multifactorial experimental design was developed to study the effect of four environmental factors and their interaction on pikeperch larval rearing using a factorial design. To inoculate and accelerate the development of the nitrifying bacteria in the biofilter, volumes of Aquaclean were added before the introduction of pikeperch larvae. This was due to the fact that our larval system was not used for one year (moving of our facilities), implying that the biofilter was inactive.

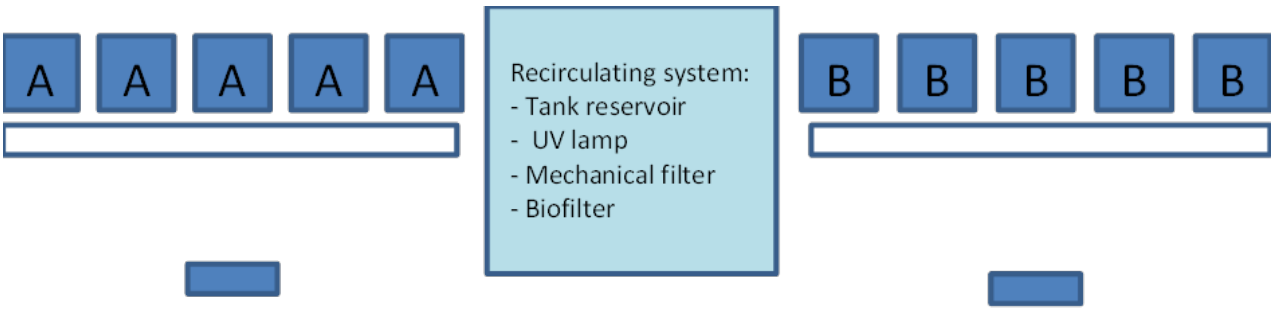


Figure 16.1.1 Recirculated system used for pikeperch larval rearing trials.

After discussing with the SME partner P29. Asialor and reviewing the scientific literature, we have decided to test the effect of light intensity (5 *vs* 50 lx; Summerfelt, 1996 ; Hamza *et al*, 2008 ; Lund and Steenfeldt, 2011), water renewal rate (50 vs 100% per hour; Szkudlarek and Zakes, 2007 ; Lund and Steenfeldt, 2011; Lund *et al*, 2012 ; Ott *et al*, 2012), direction of the water flow (up-stream *vs* down-stream; Summerfelt, 1996) and stress related to tank cleaning timing (cleaning the tank during morning just after the light switch-

on and feeding or at the end of the afternoon before the departure of the technical personal), as depicted in **Table 16.1.1**.

Table 16.1.1 Experimental matrix developed to study the effect of four environmental factors and their interactions on pikeperch larval rearing (8 tanks were used).

Tank number	Combination	Light intensity (lx)	Water renewal rate (% per hour)	Water stream	Time of tank cleaning
A4	4	5	100	Down-stream	afternoon
A3	6	5	50	Down-stream	afternoon
A2	8	5	100	Up-stream	morning
A1	2	5	50	Up-stream	morning
B1	3	50	100	Up-stream	morning
B2	1	50	50	Up-stream	afternoon
B3	5	50	50	Down-stream	morning
B4	7	50	100	Down-stream	afternoon

Considering firstly husbandry and economic constraints related to the SME management and secondly our bibliographical review, other parameters were consistent with SME farm protocols and conditions or according to available data:

1. Photoperiod (L:D 12:12, switch on at 08:00 and switch off at 20:00, Hamza *et al*., 2007),
2. Temperature (19-20°C, Hamza *et al*, 2007; Kestemont et al, 2007; Szkudlarek and Zakes, 2007),
3. Feeding protocol (a meal every 1.5 hour during the lighting period, Hamza *et al*, 2007, 2008, 2010, 2012 ; Kestemont et al, 2007) (see table 16.1.2),
4. Dissolved oxygen level above 6 $mg.L^{-1}$,
5. Larval density (90 $larvae.L^{-1}$).

In our experiment, the initial water temperature of 14-15°C was identical to that of hatching (as observed in the Domaine de Lindre, Moselle, France). The temperature was increased up to 20°C in 5 days (+ 1°C/ day) and the analyses of water quality parameters (T°C, pH, TAN, $N\text{-}NO_2^-$) were performed 3 times/week (Monday, Wednesday, Friday). A theoretical growth curve and a feeding protocol (**Table 16.1.2**) was developed to determine feeding ratio and regime.

In the framework of a collaboration with P3. IRTA, a sampling strategy was carried out to study the effect of the four factors on survival, growth and development of pikeperch larvae:

1. Samples of 30 larvae/tank on days 0, 7, 14, 21, 28 35, 42, 49, 56 and 60 for morpho-metric analyses (larva size, eye diameter, length of yolk sac).
2. Samples of 150 larvae/tank on days 0, 7, 14, 21, 28 35, 42, 49, 56 and 60 for allometric analyses.
3. Samples of 180 larva/tank on day 60 for the evaluation of the deformity rate.
4. Samples of 150 larvae/tank on days 0, 7, 14, 21, 28 and 35 for histological study of the digestive tract development.
5. Samples of 150 larvae/tank on days 0, 7, 14, 21 and 42 histological study of the eyes and retinal development.
6. Samples of 150 larvae/tank on day 60 for histological study of jaw muscle development.

7. Samples of 600 mg of larvae/tank on days 0, 7, 14, 21, 28 and 35 for study of digestive enzymes.
8. Samples of 300 mg of larvae/tank on days 0, 7, 14, 21, 28 35, 42, 49, 56 and 60 for gene expression.

Table 16.1.2 Feeding protocol (DPH : days post-hatching).

Type of food	Product	Supplier	Period (DPH)
Artemia naupli	High HUFA Premium Artemia cysts	Catvis	4-6
Artemia nauplii	Salt Lake Aquafeed Premium Artemia cysts	Catvis	4-18
Formulated food	Prostart 100	BioMar	8-15
Formulated food	Prowean 100	BioMar	16-19
Formulated food	Prowean 300	BioMar	18-25
Formulated food	Prowean 500	BioMar	24-29
Formulated food	Prowean 700	BioMar	28-32
Formulated food	Inicio plus 0,8 mm	BioMar	31-64
Formulated food	Inicio plus 1,1 mm	BioMar	56-64

The experiment started on April 29th with the arrival of 500,000 larvae from the Domaine de Lindre (Moselle, France) and was stopped 5 five days later due to very high levels of ammonia and nitrite in the water of the RAS and a total mortality of larvae. Consequently Deliverable D16.1 (Determine the effect of environmental factors on pikeperch larval rearing) could not be delivered as planned (Mo12) and will be delayed for 8 months so that the experiment will be repeated.

Task 16.2 Development of an industrial protocol (led by ASIALOR, Debes Kevin).

No work was done this year.

References

Hamza, N., Mhetli, M., Kestemont, P., 2007. Effects of weaning age and diets on ontogeny of digestive activities and structures of pikeperch (*Sander lucioperca*) larvae. Fish Physiology Biochemistry 33, 121–133.

Hamza, N., Mhetli, M., Ben Khemis, I., Cahu, C., Kestemont, P., 2008. Effect of dietary phospholipid levels on performance, enzyme activities and fatty acid composition of pikeperch (*Sander lucioperca*) larvae. Aquaculture 275, 274–282.

Hamza, N., Silvestre, F., Mhetli, M., Ben Khemis, I.B., Dieu, M., Raes, M., Cahu, C., Kestemont, P., 2010. Differential protein expression profile in the liver of pikeperch (*Sander lucioperca*) larvae fed with increasing levels of phospholipids. Comparative Biochemistry and Physiology 5, 130–137.

Hamza, N., Kestemont, P., Ben Khemis, I.B., Mhetli, M., Cahu, C., 2012. Effect of different sources and levels of dietary phospholipids on performances and fatty acid composition of pikeperch (*Sander lucioperca*) larvae. Aquaculture Nutrition 18, 249–257.

Kestemont, P., Xueliang, X., Hamza, N., Maboudou, J., Imorou T. I., 2007. Effect of weaning age and diet on pikeperch larviculture. Aquaculture 264, 197–204.

Lund, I., Steenfeldt, S.J., 2011. The effects of dietary long-chain essential fatty acids on growth and stress tolerance in pikeperch larvae (*Sander lucioperca L.*). Aquaculture nutrition 17, 191–199.

Lund, I., Skov, P. V., Hansen, B. W., 2012. Dietary supplementation of essential fatty acids in larval pikeperch (*Sander lucioperca*), short and long term effects on stress tolerance and metabolic physiology. Comparative Biochemistry and Physiology 162, 340–348.

Ott, A., Löffler, J., Ahnelt, H., Keckeis, H., 2012. Early development of the postcranial skeleton of the pikeperch *Sander lucioperca* (Teleostei: Percidae) relating to developmental stages and growth. Journal of Morphology 273, 894–908.

Summerfelt, R.C., 1996. Walleye culture manual. (1rst Ed.). NCRAC Culture Series 101. 416 pp.

Szkudlarek, M., Zakes, Z., 2007. Effect of stocking density on survival and growth performance of pikeperch, *Sander lucioperca* (L.), larvae under controlled conditions. Aquaculture International 15, 67–81.

Deviations from Annex I and their impact:

Deliverable 16.1 - Determine the effect of environmental factors on pikeperch larval rearing, will be delayed by 8 months. The experiment pf Task 16.1 planned for April-June 2014 has been postponed for December 2014-February 2015. Partner P9. UL has initially scheduled the experiment first in spring 2014, because this period corresponds to the natural pikeperch spawning season in France and contacts have been previously established with the fish farm "Domaine de Lindre" to supply pikeperch larvae. As planned, 500,000 larvae were purchased from the company. To do this experiment our laboratory was going to use the brand-new and modern experimental platform (with many ARS, at a cost of €3 million. The facility was completed and delivered in late April 2014 (3 months later than scheduled, after a long period of technical tests by companies (to test cooling, ventilation and warming systems). That meant that regardless of our efforts, such as introduction of nitrifying bacteria in commercial solution (AQUACLEAN N), the ARS did not operate with mature biofilters. Then as the biofilter was not balanced, very high levels of total ammonia nitrogen and nitrite (more than 10 mg L^{-1} for each each substance) were measured and larvae died five days after introduction. From that moment, we have continued to manage our larval rearing station in order to obtain a well-balanced biofilter; that was achieved in July 2014 only. We have contact several pikeperch farms (Aquapri in Denmark, Excellence Fish Farm in the Netherlands) to buy a new batch of larvae, but they had no larvae available at that period. Consequently, we have decided to postpone this experiment to late 2014 because P29. ASIALOR will be able to supply us with larvae produced out-of-season spawning.

WP No:	17	WP Lead beneficiary:	P7. IMR
WP Title (from DOW):	Larval husbandry – Atlantic halibut		
Other beneficiaries (from DOW):	P17 NIFES		
Lead Scientist preparing the Report (WP leader):	Norberg, Birgitta		
Other Scientists participating:	Harboe, Torstein (IMR), Bergh, Øivind (IMR), Sandlund, Nina (IMR), Hamre, Kristin (NIFES)		

Objectives

1. Improve larval survival and quality during early development of Atlantic halibut.

Summary of progress towards objectives and details for each task:

Task17.1 Determine if RAS is a more effective protocol than FT for Atlantic halibut larvae.

Introduction:

The commercial production of halibut fry is currently carried out in large incubators, silos, and with flow through water systems (FT) (Harboe *et al*., 1994), while there is a growing consensus that a recirculation system (RAS) would offer more stable environmental and chemical water parameters that would lead to improved larval performance. The yolk sac stage for halibut lasts for 43 days at 6 C° and a small change in temperature or salinity will influence the location of the larvae in the water column. In turn this can lead to higher larvae mortality. There is also an ongoing discussion on when the intestine of the larvae is colonized with bacteria. Atlantic halibut larvae start drinking activity approximately 6 days after hatching and could be colonized during the yolk sac stage. However, some findings indicate that the bacterial flora in the gut is determined by what they are eating, rather than the water itself. The aim of this study was to reveal possible differences in larvae performance (yolk utilisation, survival and development) and bacterial flora in both larval gut and rearing water, during the yolk sac stage.

Materials and methods:

A RAS system for halibut yolk sac incubators was constructed by connecting two identical silos (**Fig. 17.1.1**): one silo for larvae and one as a bio-filter (C). Silo A had normal water flow through (FT) with seawater taken from 160 metres depth, filtered through 15um filter and temperature regulated (heat pump). Between the two silos a water cooling system was mounted (D). Water from the silo incubated with halibut larvae was passively led from the outlet (top of silo) into the silo functioning as a bio-filter and thereafter pumped further back to the bottom of the silo incubated with larvae.

The RAS (C) silo was filled with seawater from the same water source as the FT silo and run for two weeks prior to incubation of halibut eggs. Atlantic halibut eggs were administered to silo A and B, three days prior to hatching. From incubation until hatching was completed, both silos were run as flow through incubators. This is due to addition of freshwater during this period, in order to create a density gradient in the upper part of the silos (prevents the newly hatched larvae from contact with the outlet sieve). After completion of hatching, the RAS (B) silo was connected to the bio-filter (C) for the entire yolk sac period (43 days).

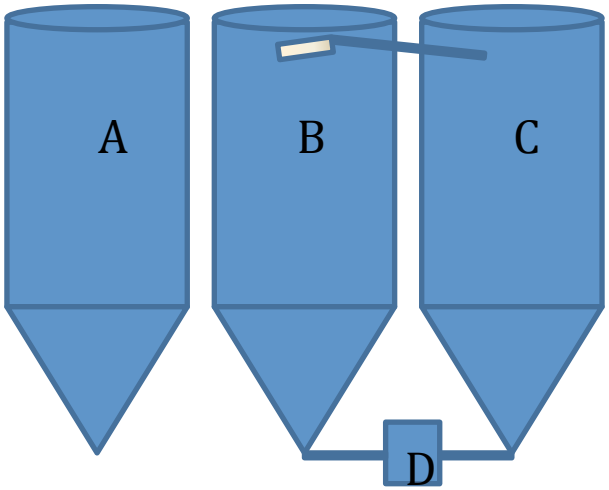


Figure 17.1.1 Flow through (A) and RAS (B and C) silos with water cooling system (D).

To monitor the bacterial microflora during the yolk sac period both water and larval samples were collected. The first water samples were collected from each silo prior to larval transfer and from then on every week throughput the whole yolk sac period. Samples of egg and larvae were also collected. We also dissected larvae for microbial analyses of skin and intestine. These samples will be used for DGGE analysis (Polymerase chain reaction denaturing gradient gel electrophoresis) of bacterial DNA (Brunvold *et al*., 2007; Meeren *et al.*, 2011). Samples were also taken from the bio-filter for isolation of probiotic bacteria candidates to be used in Task 17.2 The effect of probiotics on larval microbiota and survival. This activity will mainly be carried out in the next three years of the project.

Results:

Recently, (after the 6 month report) we have examined larvae survival in the two systems (**Fig. 17.1.2**).

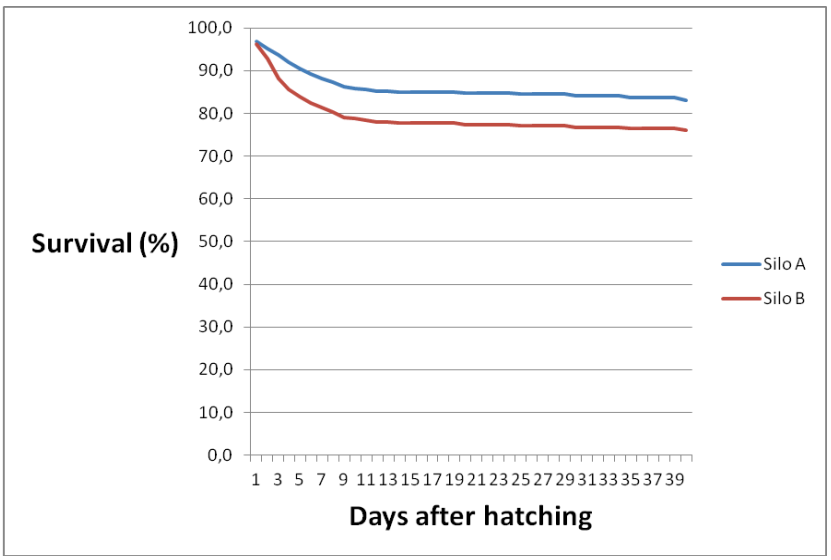


Figure 17.1.2 Larval survival in Flow through (FT silo A) and Resirculation (RAS silo B).

Larval mortality was higher in the RAS system the first week after hatching. Thereafter there were no differences in mortality. No differences in larval size at the end of yolk sac stage were found. There was

however, a higher proportion of jaw deformed larvae in the RAS system, 23 compared to 12%. Previous work with halibut yolk sac larvae in silos has strongly indicated that jaw deformities are more frequent when the larvae are exposed to water movement the first days after hatching. Apparently, there was a difference between the two siloes in such water movement, and we suggest that this, rather than water quality itself has caused the observed differences in larval development.

Denaturing Gradient Gel Electrophoresis analysis of bacterial DNA was delayed due to methodological difficulties (see Deviations, below). The analyses are expected to start early in 2015 and no delay in the deliverable is foreseen.

Task 17.2 The effect of probiotics on larval microbiota and survival

A major challenge experiment with Atlantic halibut larvae was carried out in November-December 2014. The aim of the study was to test 35 differerent *Vibrio* spp. strains for virulence towards halibut larvae. Some of the strains are known to be associated with bacteriophages, making phage therapy possible. Other possible strains are to be used as model strains in challenge experiments with probiotics following the model by D`Alvise *et al*. (2012).

Bacteria from frozen stock cultures (−80°C) were streaked on half-strength Marine Agar (½MA; 27.6 g Difco 212185 Marine Agar, 15 g Instant Ocean Sea Salts, 7.5 g Agar, 1 l deionized water). *Vibrio anguillarum* was counted on Tryptone-Soy Agar (TSA; Oxoid CM0131) containing 6 mg/l chloramphenicol. The cells were harvested at 5,000 x *g*, washed twice, and used as inoculum for algae and rotifer experiments. Bacteria were diluted and washed in 80% autoclaved oxygenated seawater (ASW; 2% Sigma Sea Salts). Axenicity of algae and rotifer cultures was controlled by plating 100 µl on ½MA and incubating for 7 days at 25°C.

For the challenge trials, *V. anguillarum* HI610 was grown in tryptone-soy broth with additional 0.5% NaCl at 20°C with shaking at 60 rpm to an OD_{600} of about 0.5. The *Phaeobacter gallaeciensis* strains were grown in MB without shaking at 20°C until stationary phase was reached. All strains were harvested by centrifugation (1,825 x *g*), washed twice, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations in these suspensions were determined using a counting chamber for *V. anguillarum*, and for the *P. gallaeciensis* strains by measuring OD_{600} after centrifugation and dissolving in 0.1M NaOH.

The protocol was adapted from Sandlund and Bergh 2008 and Sandlund et al. 2010. Atlantic halibut embryos were obtained from the commercial hatchery Norsk Kveite, in Askøy municipality, Western Norway. Transport of the embryos in polystyrene containers at around 6-7°C took 1,5 hr. in total by car. Upon arrival, the embryos were randomly picked and distributed to the wells of 6-well dishes (Nunc, Roskilde, Denmark) filled with 10 ml 80% autoclaved, aerated seawater, placing one embryo in each well. In each trial twelve dishes for each treatment (72 embryos) were prepared and inoculated immediately. All inocula were prepared in a volume of 100 µl, initial bacterial concentrations were 1×10^6 cfu/ml and 1×10^4 cfu/ml for *V. anguillarum* HI610 in the two positive control groups (high and low concentration, respectively. The plates were incubated in the dark at 7°C. The day when 50% of the larvae had hatched was defined as day 0, which was 3 days after the start of the experiment. Dead larvae were registered every second day for 30 days.

A total of 35 different *Vibrio* strains were tested for pathogenicity towards the halibut larvae. The results are at present not ready.

Task 17.3 Production protocol of on-grown *Artemia*.

A strategy for production of on-grown *Artemia* has been made. The strategy is a modified production method used at IMR in 2005. *Artemia* cysts are hydrated and hatched according to standard procedures. The nauplii are transferred to on-grown tanks (250 litres). These tanks have continuous water supply. The off let sieve is made of plankton net. Water temperature is 20 to 22°C and pH is adjusted with NaOH. Initial number of *Artemia* is 100 *Artemia* pr. ml. Two different feeds, micronized fish meal and ORI-culture from Skretting, are tried as on-growing feed. MultiGain (Danafeed) or ORI-Gold (Skretting) is used for short time

enrichment just before given to the larvae. Expected growth during the 4 day culture is from ~2,1 to ~12 µg/ *Artemia*. The work is ongoing at the time of this report, and will be reported in full in the second periodic report.

Task 17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on larval performance.

No work done during this period.

References:

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Deviations from Annex I and their impact:

Development of the Denaturing Gradient Gel Electrophoresis (DGGE) method took longer than expected. However, the method is now up and running in the laboratory but results are delayed as a consequence of the methodological problems. This should not affect the Deliverable for Task 17.1, which is due month 36.

A mistake was made when preparing the Gannt Chart for Task 17.3 Development of a production protocol for on-grown *Artemia*. This task should have been set to start in the last quarter of year 1 (November-December 2014), due to overlap in personnel and facilities with task 17.1. The work is now well underway and the deliverable is expected to be on time.

WP No:	18	**WP Lead beneficiary:**		P8. IEO
WP Title (from DOW):	Larval husbandry- wreckfish			
Other beneficiaries (from DOW):	P1. HCMR	P19. CMRM	P32. MC2	
Lead Scientist preparing the Report (WP leader):	Peleteiro, Tito			
Other Scientists participating:	Papandroulakis, Nikos (HCMR), Linares, Fatima (CMRM), Vilar, Antonio (MC2)			

Objectives

1. Development of larval rearing protocol based on the most effective prey density, succession of prey type, light regime (intensity and duration), temperature and culture system,
2. Description of ontogeny of digestive system, vision, taste and smell organs in response to larval rearing methods.

Summary of progress towards objectives and details for each task:

Task 18.1 Development of feeding methodology (lead by HCMR, Papandroulakis, Nikos). This task was not accomplished, due to insufficient number of eggs/larvae available.

Task 18.2 Defining optimum conditions for larval rearing (lead by IEO, Peleteiro, Tito).

Sub-task 18.2.1. Egg were obtained after spontaneous spawning of the broodstock from P32. MC2 (**Fig. 18.2.1**). Nevertheless, hatching success and larval quality was very poor compared to the previous year, possibly due to extremely variable and changing weather conditions, which was the only changing variable. From all the spawns, only one had acceptable quality to attempt further larval culture. Information regarding this spawn is the:

- Viable Spawn date: 04/06/2014
- Volume: 1000 ml
- Fertilization : 70%
- Number of eggs: 270,000
- Egg diameter: 2,405±32 µm
- Number of eggs/ml: 270±12
- Incubation tank volume: 100 l
- Incubation period: 7 days at 14.6 ± 0.5ºC

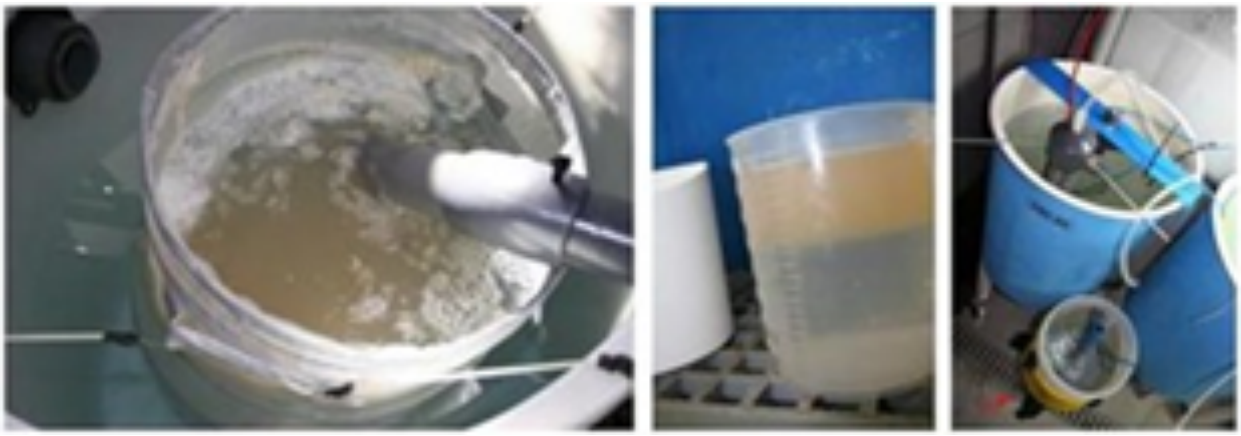


Fig. 18.2.1. Eggs inside a collector tank, separation of viable eggs and incubation, at the MC2 (La Coruña) facilities (P32).

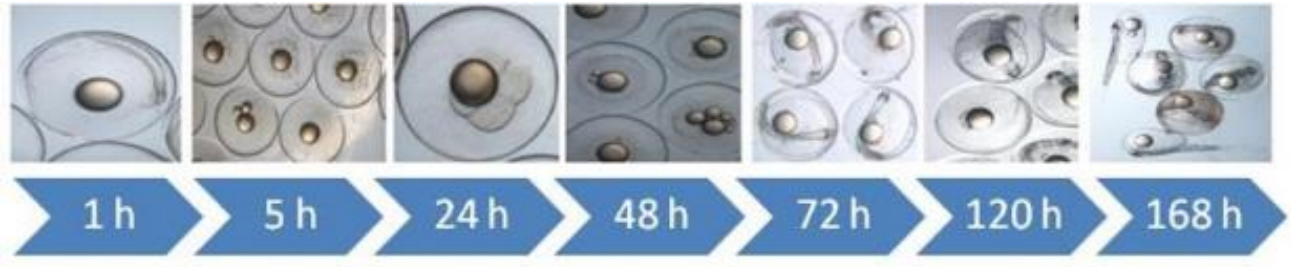


Fig 18.2.2. Incubation of wreckfish eggs at 14 ± 0.6ºC

Once egg incubation was concluded (hatching percentage: 14%), the resulting 11,340 larvae (3.8 ± 0.3 mm) with 100% yolk sac were transferred to a 85-l culture tank (**Fig. 18.2.2**). The yolk sac was consumed in 6 days post hatching (dph) (**Fig. 18.2.3**); mortality was 100% at 20 dph. Rotifers (*Brachionus plicatilis*), at a concentration of 8 rot/ml and phytoplankton were used as diet, but at no time ingestion of live feed was observed. Larvae at 20 dah had a functional digestive system, but all had empty stomachs.

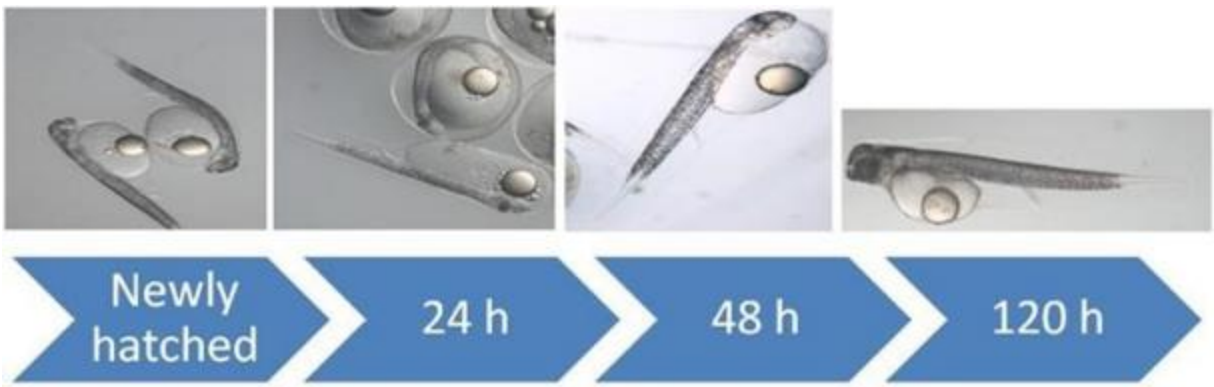


Fig. 18.2.3. Yolk consumption from 1 to 120 hours after hatching.

Sub-task 18.2.2. This task was not accomplished, since eggs were not available, due to the poor larval quality from the P32. MC2 spawns (WP6 Reproduction & genetics – wreckfish).

Deviations from Annex I and their impact:

Task 18.1 Development of feeding methodology (lead by HCMR, Nikos Papandroulakis).

It was not possible to perform this task, since as explained before, probably due to change in breeding season that occurred this year, the egg quality was very poor, compared to the previous year. Also, the female that spawned at P1. HCMR in response to hormonal therapy (WP6 Reproduction & genetics – wreckfish), produced very low fertilization percentages (<11%). This did not allow us to perform this task. Although this task began during this reporting period, the deliverable for this task is not until month 36, consequently we believe that this task deliverable will still be on time.

Task 18.2 Defining optimum conditions for larval rearing (lead by IEO, Tito Peleteiro).

It was not possible to perform this task, due to low availability and poor quality of the larvae. In this case, only larval development was completed, and mortality was 100% at 20 days after hatch. Improvement of breeders' parental diets and hormonal induction planned for 2015 provides make us optimistic regarding the availability of good larval quality for next year. Although this task began during this reporting period, the deliverable for this task is not until month 36, consequently we believe that this task deliverable will still be on time.

WP No:	19	WP Lead beneficiary:	P4. IOLR
WP Title (from DOW):	Larval husbandry – grey mullet		
Other beneficiaries (from DOW):	P2. IRTA	P25. DOR	
Lead Scientist preparing the Report (WP leader):	Koven, Bill		
Other Scientists participating:	Estevez, Alicia (IRTA), Gisbert, Enric (IRTA), Shafran, Gilad (DOR)		

Objectives

1. Investigating environmental and nutritional factors that affect larval rearing.
2. Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production.
3. Determine when to wean larvae and to feed weaning diet type according DT maturation and the shift from carnivorous to omnivorous feeding.

Summary of progress towards objectives and details for each task

Task 19.1 Effect of algal type and concentration on larval performance (led by IOLR, Bill Koven).

Sub-task 19.1.1 (IOLR) Determine the effect of algal type and concentration in rearing tanks on larval performance

As the mullet spawning season is in October, this task began on Oct 31st 2014 and is presently being carried out. At the writing of this report the fish were 23 dph (2 days before the end of the algal treatments) and survival was very good. The experimental system consists of twenty 1500 l tanks in a flow through system where filtered (10 µm) and UV treated sea water temperature and salinity is computer controlled. The system allows for the testing of 5 treatments in replicates of 4 tanks/treatment which are: (1) no algae control, (2) *Nannochloropsis* sp.-low (0.2 x 10^6 cells/ml, (3) *Isochrysis galbana* – low (0.0144x106 cells/ml), (4) *Nannochloropsis* sp. – high (0.5x10^6 cells/ml), (4) *Isochrysis galbana*-high (0.228x106 cells/ml)-high and are shown in **Table 19.1.1**.

Each pair of treatments (2 and 3, 4 and 5) differ in concentration (algal cell size is different in each species) but are identical in their level of tank turbidity. In addition, treatments 4 and 5 provide twice the turbidity as 2 and 3. The NTU units in the control, the low turbidity (2 and 3) and high turbidity (4 and 5) were 0.2-0.4, 0.55-0.8, 1.1-1.4, respectively. Eggs from IOLR brood stock were stocked in the experimental system (100 eggs/l) where the hatchability a few days later was 83.2%. After yolk sac absorption, eye pigmentation and the opening of the mouth and anus (2 dph), the larvae were reared according to the IOLR mullet rearing protocol, which included the addition of algae treatments from 2-25 dph while feeding rotifers and *Artemia* nauplii according to enrichment and feeding protocols but adding 600 mg taurine/l of enrichment medium.

Figure 19.1.1 shows one of the 1.5 m^3 tanks in the *Isochrysis-low* (isoA) treatment which is similar to other tank replicates in that it shows good survival and the start of "silvering" in certain tank individuals in the centre of the tank. The sampling schedule is shown in **Table 19.1.2.** Note larval samples at 18, 25 and 35 dph will be sent to P3. IRTA for analysis of digestive tract enzymes.

Table 19.1.1 Algal treatments testing the effect of turbidity

No.	Treatment	Concentration	turbidity
1	Control	without algae	Clear
2	*Nannochloropsis* spp. low	$0.2x10^6$ cells/ml	Identical turbidity
3	*Isochrysis galbana* low	$0.0144x10^6$ cells/ml	0.55-0.8 NTU
4	*Nannochloropsis* spp. high	$0.5x10^6$ cells/ml	Identical turbidity
5	*Isochrysis galbana* high	$0.0288x10^6$ cells/ml	1.1-1.4 NTU

Figure 19.1.1 Treatment isochrysis low (A) tank with 23 dph mullet larvae. Note that silvering has started to occur in individual fish swimming in the centre of the picture.

Table 19.1.2 The sampling schedule for the first 35 days after hatching. Samples of digestive tract enzymes will be analysed by P3. IRTA.

DPH	Mastax no.	FA/AA	Wt	Length	Digestive tr.	Comments
Eggs	-	√	-		-	
0	-	√	√	√	-	
1-5	√	-	-	-	-	
5	-	-		√	-	
10	-	√	√	√	-	Unenriched Artemia
14	-			√	-	Enriched Artemia
18	-	√	√	√	√	Weaning feed + Artemia
25	-	√	√	√	√	Starter feed only
35	-	√	√	√	√	Transfer to nursery

Sub-task 19.1.2 (IOLR) Determine if the benefit of algal addition to rearing tanks due to background lighting or other factors that contribute to larval performance

This task will be based on the results of 19.1 and will be performed in 2015.

Task 19.2 Comparing the selected microalgae type and protocol (Task 19.1) with lyophilized substitute (led by IRTA, Alicia Estevez/Enric Gisbert)

This task is also based on the results of 19.1. and 19.2 and will be performed in 2016 (see Gant table).

Task 19.3 Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production (led by IOLR, Bill Koven).

The Israeli start-up company Zoopt that was going to be sub-contracted to produce ciliates for these studies has closed. We are presently trying to arrange for a Norwegian company that produces copepods to be subcontracted to provide these zooplankters for our co-feeding experiments.

Task 19.4 Determine when to wean larvae and to feed weaning diet type according to DT maturation and the shift from carnivorous to omnivorous feeding (led by IOLR, Bill Koven).

This study is not scheduled to start until 2016.

Task 19.5 Testing the improved grey mullet larval rearing protocol in a commercial hatchery (led by DOR, Gilad Shafran)

This task is not scheduled to start until the third quarter of the 3rd year of the project. However, the clear results of a taurine experiment that was carried out recently at the IOLR have been now implemented in the IOLR mullet larval rearing protocol and is described in Task 13.1.

Deviations from Annex I and their impact:

In general, there are no deviations from the work plan as grey mullet is a fall spawner. Consequently, the mullet experiments are only now underway and at the writing of this report, the larvae were 23 dph and the studies are proceeding as planned including the use of resources and person months. The only deviation was the closing of the company Zoopt. However, the deliverable of this task is not due until month 36, therefore there is time to resolve this issue and find a suitable substitute. The budget for subcontracting has not been used. There are no deliverables or milestones due in this reporting period.

Group Work Packages

Grow out husbandry

Regarding size variability of meagre juveniles, the first trial showed that (a) high cannibalism at the early stages may result in significant size variability of juveniles and (b) different size classes performed similarly following grading. Regarding optimal rearing conditions; results so far show that depth of the cage net during on-growing affects the behavior of the fish, but it is not yet clear, whether it has any effect on growth. There is also a significant difference of fish behavior during day and night.

For greater amberjack, during the first reporting period, the preparatory activities resulted in the availability of the required fish groups for the implementation of the trials planned to start during the second year of the project.

The work for pikeperch targeted the definition of the physiological and immune response of pikeperch when exposed to stressors since there is limited information for the species. Results showed a high sensitivity of pikeperch to captive environment. Application of emersion stressor (single or repeated) increased the cortisol, as well as the glucose levels in plasma. Furthermore, long-term application of stress significantly decreased growth performance of the fish. Diets containing L-tryptophan (TRP) induced a significant decrease in cortisolemia, in a dose related manner, indicating that TRP may interfere with the primary stress response in pikeperch. On the contrary TRP supplementation suppressed growth and feed intake.

Work performed on grey mullet during the period was preparatory for the actual trials. Hence, the collection of the required wild juveniles was implemented in Greece and Spain. Also for the multifactorial comparison of different stocking densities and rearing systems with individuals of different origin (wild vs F1) planned for the second year of the project the formulation of an improved diet was required. Preliminary nutritional trials have been performed and a formula is now available.

WP20 Grow out husbandry - meagre

WP No:	20	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Grow out husbandry - meagre			
Other beneficiaries (from DOW):	P1. HCMR	P23. ARGO	P30. CULMAREX	
Lead Scientist preparing the Report (WP leader):	Neil Duncan			
Other Scientists participating:	Nikos Papandroulakis (P1), Tassos Raftopoulos (P23), Marilo Lopez (P30),			

Objectives

1. Adaptations in the existing methodology for grow out in cages related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed,
2. Development of an appropriate feeding method that respects the species specificities.

Summary of progress towards objectives and details for each task:

The task related to meagre was targeted to adaptations of the existing methodology for grow out in cages. In particular the conditions related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed will be studied. Also the development of an appropriate feeding method that respects the species specificities will be addressed.

During the first reporting period experimental trials have been performed and some results are already available. Regarding size variability of meagre juveniles the first trial showed that (a) high cannibalism at the early stages may result in significant size variability of juveniles and (b) different size classes performed similarly following grading. For the definition of the optimum rearing environment during on growing, there is an on-going trial and another one in preparation. The results obtained until now show that depth of the cage net during on-growing affect the behaviour of the fish, but it is not yet clear whether it has any effect on growth. There is also a significant difference of fish behaviour during day and night.

In general terms the WP is progressing as indicated in the DOW and the timing of deliverables remain as programmed.

Objectives

Task 20.1 Size variability at juveniles (led by IRTA, Neil Duncan).

Size variability in juvenile pre-grow out makes regular grading essential to avoid cannibalism and grades of smaller fish may be related to poor performance when transferred to sea cages. Experiments will be carried out with meagre juveniles of a mixture of 5-6 known families, to simulate the commercial hatchery situation and in order to study differences in growth rate. Juvenile fish will be stocked in triplicate tanks at the same initial density and fed the same commercial diet (P3. IRTA). At the end of the experiment, fish will be characterised genetically for parentage assignment (P1. HCMR, Task 2.4 from WP2 Reproduction and genetics - meagre) to establish if differences in growth rate are a consequence of genetic origin. Fish with low growth rates will be used for compensatory growth studies to determine growth potential of small juveniles and estimate the economic cost of using these fish for production, compared to discarding and using only larger juveniles. This task will result in deliverable D20.1 Methodology to avoid size variability in meagre juveniles.

A preliminary trail was conducted to obtain base data to define the size variability problem and focus the development of the methodology to manage size variability during the juvenile stage. Six different

spawnings obtained from hormonal induction of paired fish (see WP2 Reproduction and genetics - meagre) were used for larval rearing, 2 groups (V8-1 and C2 spawning on April 24th) hatched on April 28th and the other 4 (V8-1 (2), C1, V6 and V8-2, spawning on May 1st) on May 5th (**Table 20.1.1**). Three spawns were from half-sib families (families 1-3) and three families that were unrelated (families 3-4). Two cultured females were used and all other fish were from wild origin. Analysis in WP2 will determine the relationship between the cultured females.

Table 20.1.1 Parents that contributed to each family or half-sib family and spawning date. The female and male number refers to the breeders unique ID and wild or cultured indicates the origin of the breeder.

Family	Related half-sib family	Spawning Date (Tank)	Female	Male
1	2 and 3	24/04/2014 (V8-1)	5-wild	19-wild
2	1	01/05/2014 (V8-1)	5-wild	20-wild
3	1	01/05/2014 (V8-2)	1-wild	19-wild
4	-	24/04/2014 (C2)	16-cultured	21-wild
5	-	01/05/2014 (C1)	2-wild	22-wild
6	-	01/05/2014 (V6)	13-cultured	17-wild

Larvae were reared following the standard procedure, that is rotifers from 2 to 14 days post hatching (dph), *Artemia* metanauplii from 8 to 30 dph (both feed items enriched with Red Pepper), 22ºC, 16L:08D photoperiod and 500 lux light intensity. The larvae were weaned on dry diets from 20 dph onwards. Growth in dry weight was recorded every week and growth was variable between families / tanks (Fig 20.1.1).

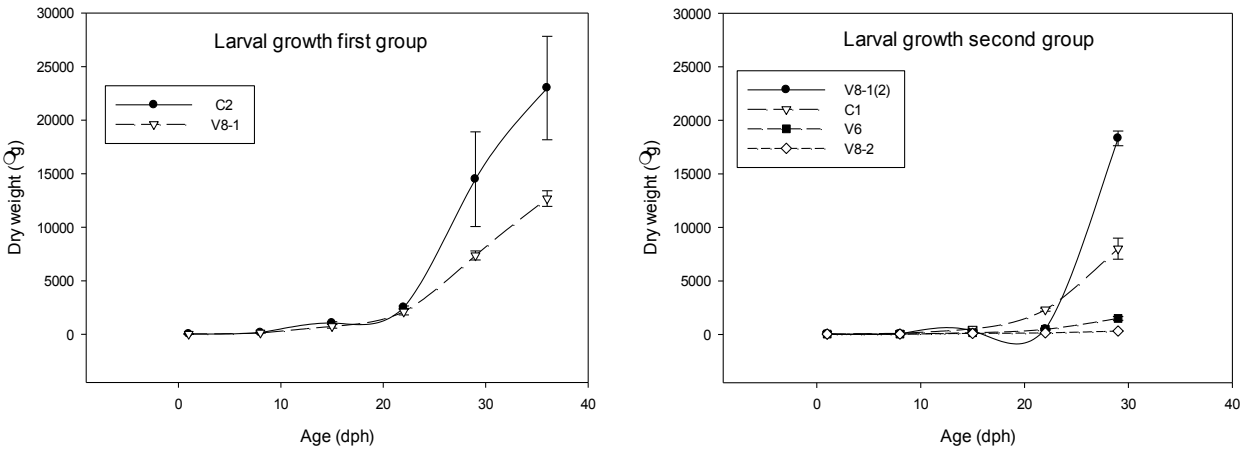


Figure 20.1.1 Growth (DW, g) of the six groups of larvae during the first month

On June 19th all the fish were graded and separated in big and medium juveniles and transferred to the nursery. The results of the number of fish graded are shown in **Table 20.1.2** and the distribution of sizes of the different tanks in **Fig 20.1.2**.

Table 20.1.2 Big and small juveniles transferred to the nursery on June 19th 2014.

Tank	V8-1	C2	V8-1 (2)	C1	V6	V8-2
Big	5	8	5	14		
Medium	219	141	176		36	89
Total	224	181	181	14	36	89
% Survival	0.64	0.43	0.52	0.04	0.10	0.25

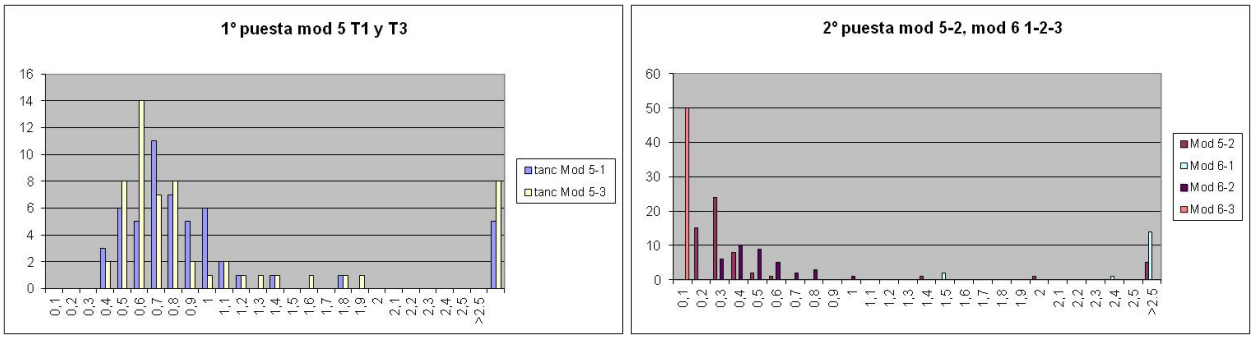


Figure 20.1.2 Weight (WW, g) distribution of fish from first and second week spawnings.

The number and weights of the fish transferred to the nursery (1500-l tanks) is shown in **Table 20.1.3**. The fish were stocked into tanks of 1500 l with >400% flow through water supply daily. Water temperature and photoperiod was natural. The fish were fed a commercial meagre diet to satiation, by both hand feeding and automatic feeders.

Table 20.1.3 Distribution of fish in Nursery on June 19th 2014.

	Week 1 Big	Week 1 Medium	Week 2 Big	Week 2 Medium
Nº	13	360	19	212
Avg Weight (g)	8.83	0.64	2.82	0.35
SD	2.24	0.30	0.8	0.16
Biomass (g)	114.73	230.4	53.66	73.78

A new classification of fish was carried out on July 21st to separate big, medium and small fish, the results are shown in Table 20.1.4

Table 20.1.4 Results of the second grading on July 21st 2014.

	Week 1			Week 2		
	Big	**Medium**	**Small**	**Big**	**Medium**	**Small**
Nº	12	46	168	19	49	122
Av Weight	114.54	14.14	9.22	59.59	16.60	11.01
SD	8.71	3.73	1.48	5.84	3.58	3.80
Min	50.60	11.00	5.20	23.26	12.97	5.65
Max	78.50	30.3	11.80	41.23	23.70	22.70

During the first two months in the nursery, fish exhibited good growth, the largest group from the week 1 spawning grew from 8.8±2.2 g to 101.8±22.3 g and the largest fish from week 2 spawn grew from 2.8±0.8 g to 56.7±13.6 g (**Fig. 20.1.3**). The small and medium grades of fish grew from 0.6±0.3 g to 21.8±5.0 g (small grade) and 35.2±4.3 g (medium grade) for the first spawning and from 0.3±0.2 g to 26.0±4.1 g (small grade) and 50.7±12.7 g (medium grade) for the second spawning. Specific growth rate (SGR) was calculated as $(\ln W2 - \ln W1)/\Delta T$ (where ΔT is the number of days between times T1 and T2 and W1 and W2 are wet weight on respective days). The large fish exhibited SGR of 5.6 and 6.9% day^{-1} for the first period (June to July) and 1.8 and 2.1% day^{-1} for the second period (July to August). The medium and small grades exhibited SGR of 8.0 and 10.1% day^{-1} for the first period (June to July) and a range from 3.1 to 4.0% day^{-1} for the second period (July to August). The proportion or percentage of the population attaining larger weights was low indicating a few fish grow faster than the majority of the population. The large grades of fish represented 3 and 8% of the population, the medium grades 21 and 26% and the small grades 76 and 65% of the population (**Fig. 20.1.3**)

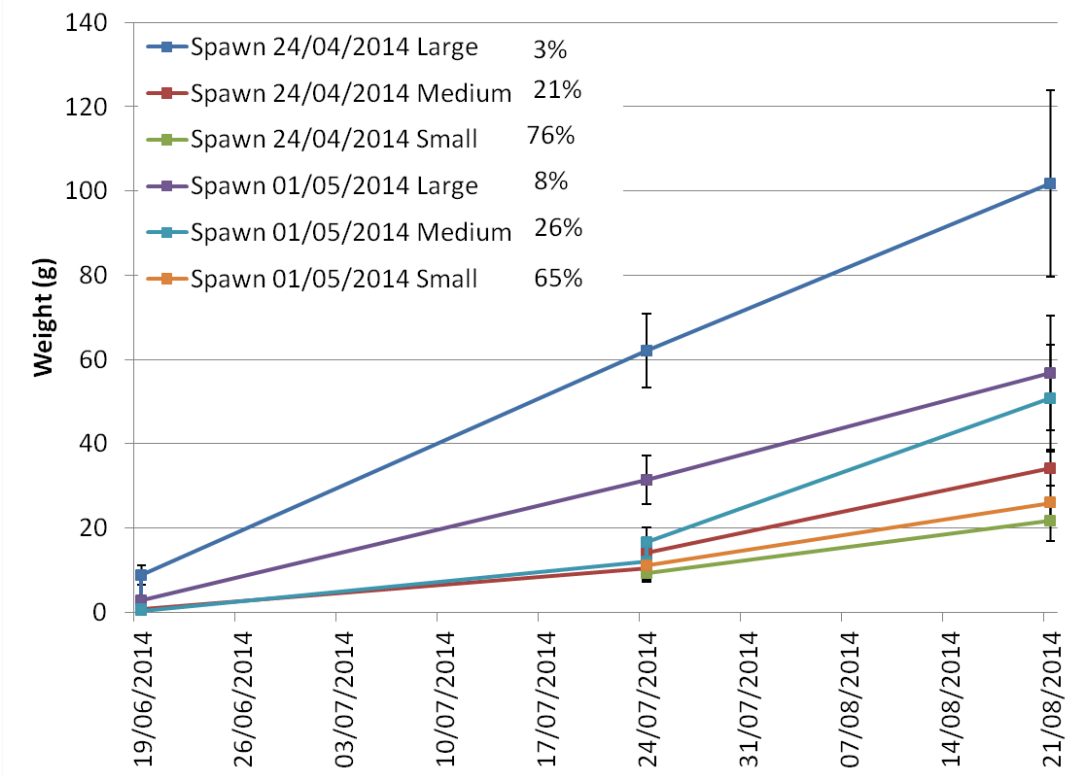


Figure 20.1.3 Growth, mean wet weight (g) with 1 standard deviation of the juveniles from different spawning dates and graded by weight. Initially the population was divided into large and medium fish and then the medium fish were divided into medium and small. The percentages in the legend refer to the percentage of the population in each grade.

On the 21st August 2014 all fish were weighed, measured (length), photographed and fin clips were taken for genetic analysis and in addition to fin clips 16 fish were sacrificed and samples of liver and muscle stored in RNA-Later for transcriptome analysis (see WP2, Task 2.5). The distribution of all the size grades across the different tanks / grades was compared and 70% of the population was observed in the size range from 15 to 30 g (**Fig. 20.1.4**). This 70% of the population was graded into three grades 73 large (25-30 g) fish, 89 medium (20-25 g) fish and 86 small (15-20 g) fish and growth was monitored.

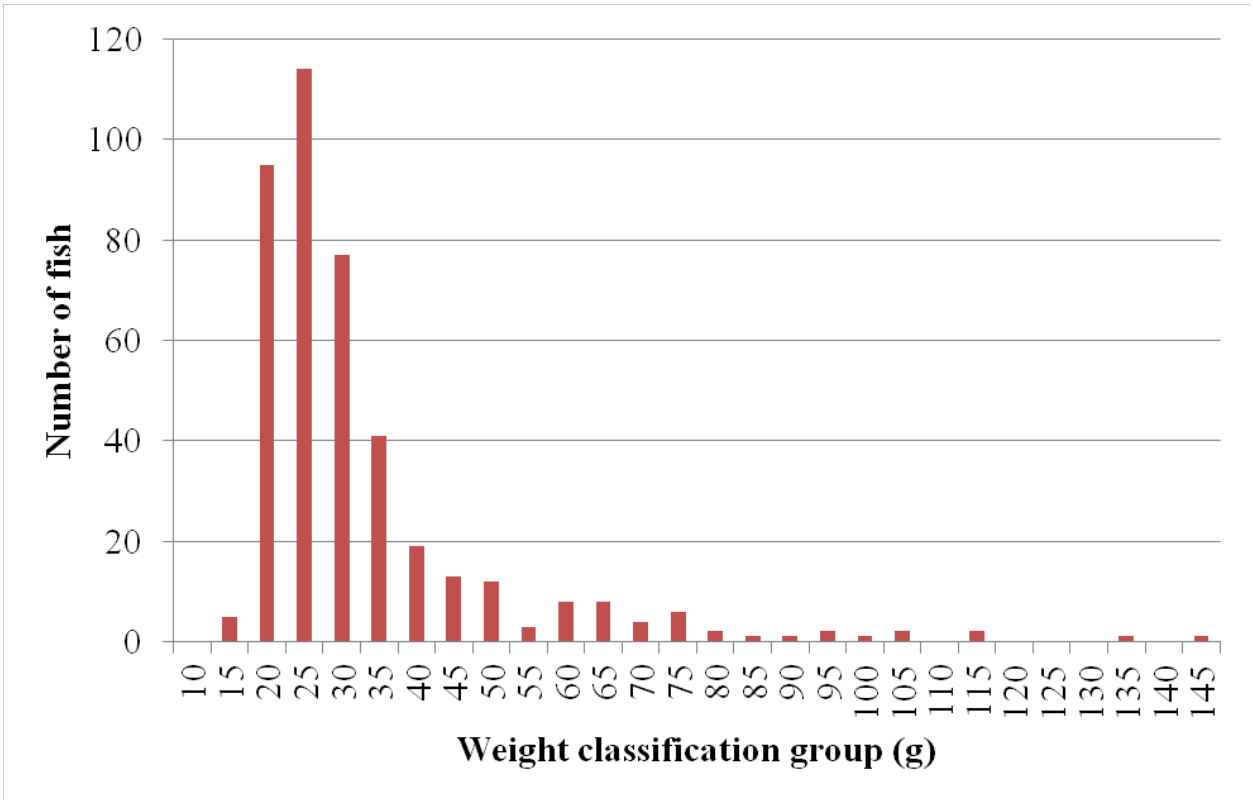


Figure 20.1.4 Frequency distribution of number of fish in each 10g size classification. The weight shown is the upper value of the classification, for example classification 15 g contains fish from 10.1 g to 15 g.

The fish were stocked into tanks of 1500 l with >400% flow through water supply daily. Water temperature and photoperiod was natural. The fish were fed to satiation with a commercial meagre diet, by both hand feeding and automatic feeders. A random sample of 50 fish from each group has been weighed and measured (length) on the 18th Sept, 8th Oct, 29th Oct, 19th Nov 2014. The growth of these three groups has been similar and the SGR in the first period (21st August to 18th Sept.) was 2% day^{-1} in all groups, 1.6-1.8% day^{-1} across all groups in the second period (18th Sept. to 8th Oct.), 1.4-1.7% day^{-1} in the third period (8th Oct. to 29th Oct.) and 0.8-0.9% day^{-1} in the fourth period (29th Oct. to 19th Nov.),. The large fish have grown from 27.2±1.5 g to 113±21.0 g, medium fish have grown from 22.7±12.2 g to 94.2±19.8 g and small fish have grown from 17.9±1.8 g to 71.6±31.31 g (**Fig. 20.1.5**). On all sample dates there have been significant differences ($P<0.05$) between the grades and the fish in each group have grown significantly ($P<0.05$). The different size grades appear to have very similar growth potential. This trial will be continued until 11th Dec. 2014.

Problems observed during rearing, weaning and ongrowing

Larval growth was very different among the six families, thus the larvae from the first week grew faster and bigger than those of the second week except for the group V8-1 (2), siblings of V8-1 larvae. Groups C1 and V6 grew very slowly, larvae were always very small and the weaning became very difficult because the larvae were too small to eat the microdiet.

During the weaning phase a high incidence of cannibalism was detected causing very high mortality rates (some days, especially in groups V6 and V8-2 more than 1000 dead larvae were siphoned in the bottom of the tanks) and very high dispersion of sizes was detected. As a consequence on June 19th 2014 when the first grading was carried out, the survival was very low and a clear high dispersion of weights was detected, with fish of 8 grams and of 0.4 g together in the same tank.

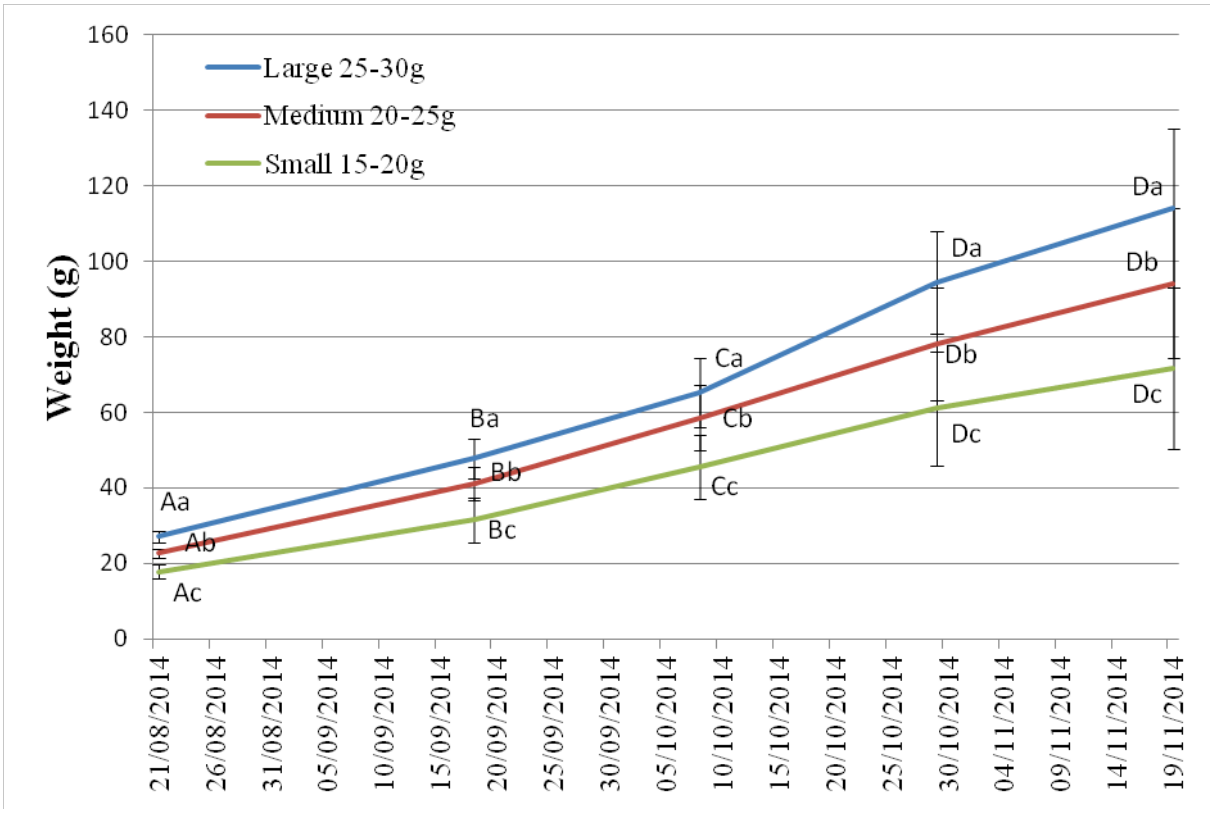


Figure 20.1.5 Growth, mean wet weight (g) with 1 standard deviation of the juveniles classified to three grades large (initially 25-30 g), medium (initially 20-25 g) and small (initially 15-20 g). These fish represented 70% of the population from five spawns on two different dates. Capital letters represent significant differences ($P<0.05$) between sample dates for the same size grade. Lower case letters represent significant differences ($P<0.05$) between size grades on the same sample date.

Task 20.2 Effect of rearing environment (led by HCMR, Nikos Papandroulakis)

The technologies and practices used currently for meagre grow out are the same as those used for gilthead sea bream and European sea bass, although this fish exhibits significant differences in growth rates, feeding and spatial behavior in the cage. Meagre exhibits a distinct feeding behavior and has a tendency to stay in the bottom of the cage, feed low in the water column and take time to rise towards the surface to feed. As fish are not very visible to the farmer, feeding may often not be adequate for maximum growth, resulting in large size dispersions. The objective of this Task is the modification of existing methodologies for cage culture related to volume and light conditions, in order to maximize the performance.

Sub-task 20.2.1 Effect of cage depth (HCMR, Nikos Papandroulakis).

The specific objective of the trial was to test the performance of meagre in cages of different depth. The trial started in May 2014 using cages of 180 (6x6x5) and 290 (6x6x8) m^3 at the P1. HCMR pilot farm in duplicates indicated as Shallow and Deep.

Fish origin was the hatchery of P1. HCMR. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery of the institute. Juveniles of 2 g were transferred at the cage facility and they were reared under similar conditions until the beginning g of the trial. Four groups were created, two of ~5,150 for the 180 m^3 cages and two of ~8,240 for the 290 m^3 cages. The wet weight at the beginning of the trial was 200 ± 20 g. The duration of the trial was planned to be 8 months and will be finished as expected at the end of 2014. During the initial period, growth performance was estimated with monthly samples (**Fig. 20.2.1**).

Every second month, blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid, free fatty acids), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. The samples are currently being analyzed. Furthermore, the vertical distribution in cages has been monitored using an echo integrator. Although a technical problem has not allowed the monitor during the first month of the trial, an upgraded system (CageEye 1.3, Lindem Data Acquisition AS, Norway) was installed in June and the trial has been implemented as planned without further alterations.

Although the analysis of the data is not completed an interesting observation has been already made. The vertical distribution of meagre was mostly in the lower half of the cage for a period of approximately 12 hours, while the rest of the period the meagre were distributed almost homogeneous in the whole available volume of the cage (**Fig. 20.2.2**). This observation is independent of the cage depth and it is correlated with the light and dark period of the day. The pattern was repeated during the implementation period. To our knowledge this is the first time that such a behavior has been observed. The results will be analyzed after the termination of the trial in order to provide a better understanding on the meagre behavior in cages.

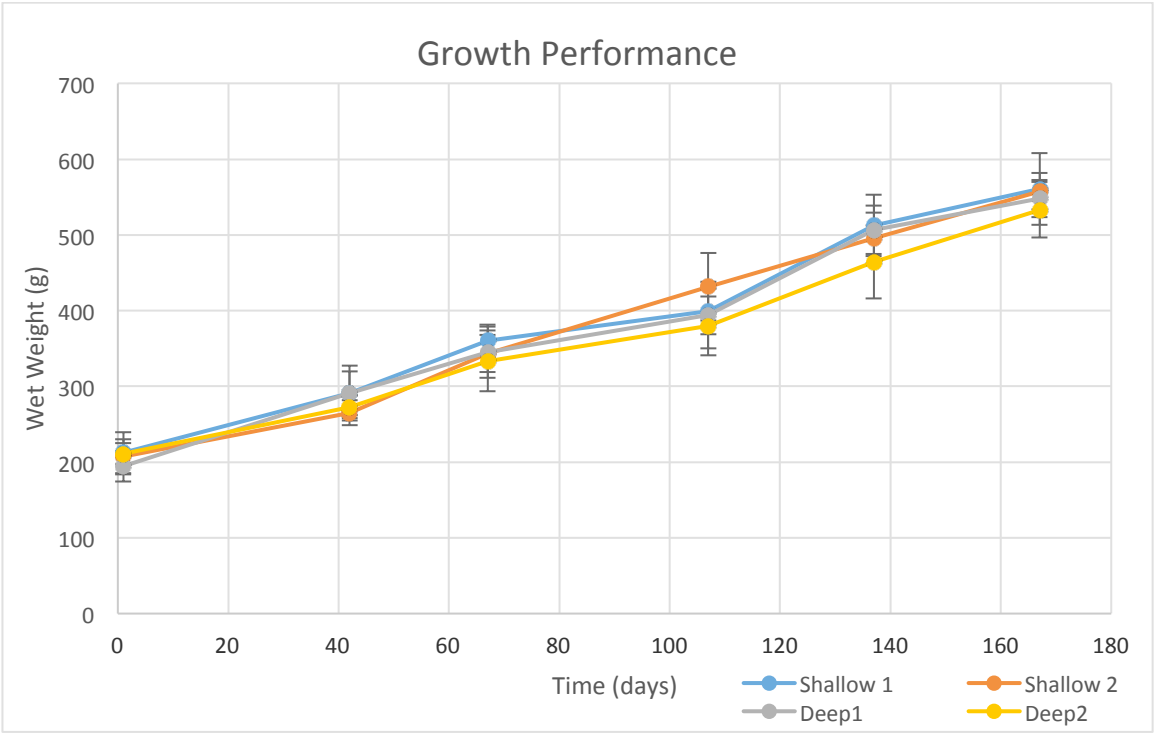


Figure 20.2.1 Growth performance, mean weight, of meagre. Error bars are the standard deviation (n=10).

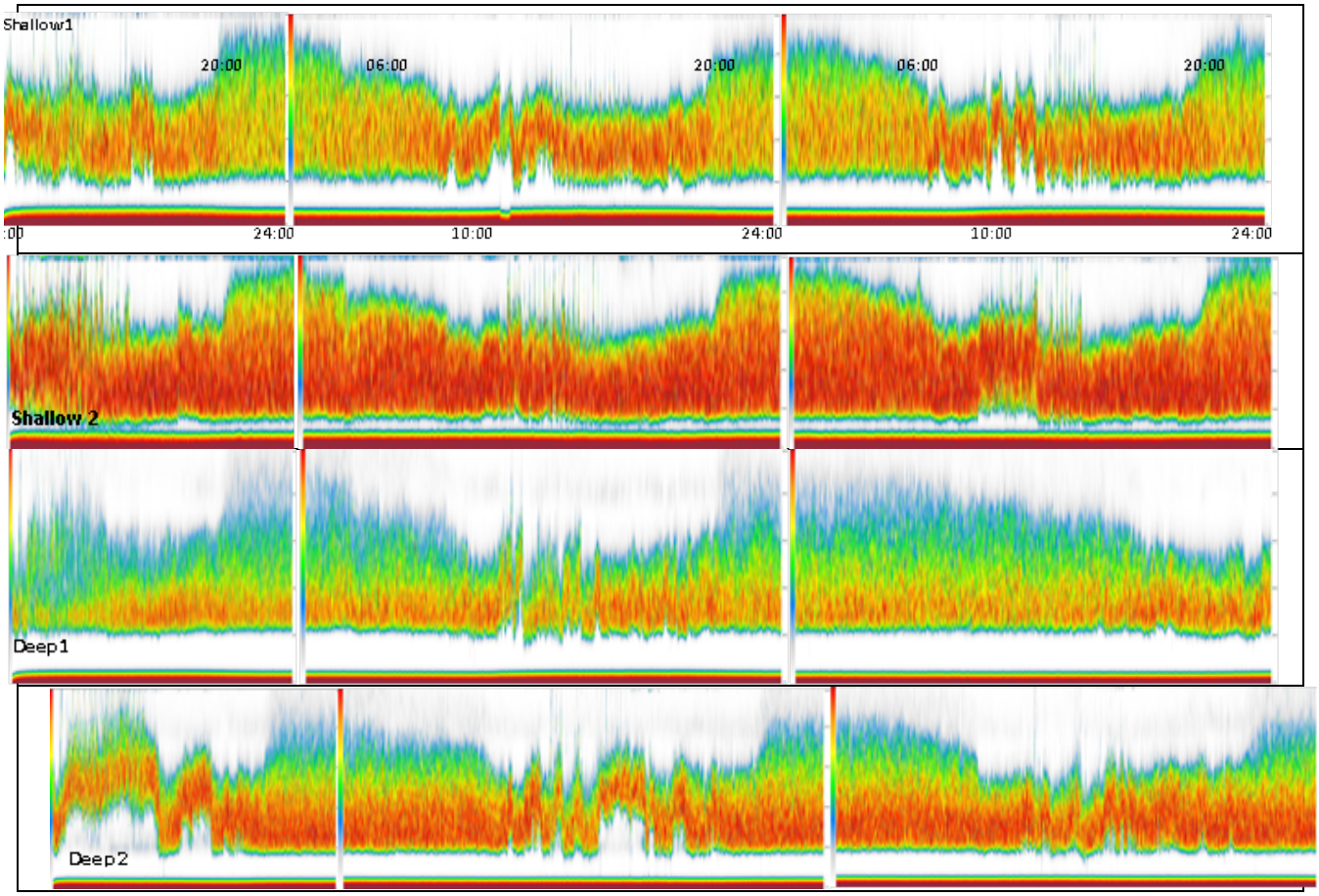


Figure 20.2.2 Vertical distribution of meagre in the experimental cages for a period of 3 days.

Sub-task 20.2.2 Effect of light intensity in the cage (ARGO, Tasos Raftopoulos; HCMR, Nikos Papandroulaksi)

The trial is under preparation and has started as initially scheduled in November 2014. The objective is to test cage rearing with and without shading at the installations of P23. ARGO applying standard commercial procedures for 2 rearing periods. Two cages will be used for the trial with groups of different sizes (200-600 g) and (0.8 - 1.5 kg). The cages to be used are rectangular of 10x10x10. The meagre population to be used is already in the farm with an average weight of ~100 g at the beginning of October 2014. During the trial, one of the cages will be covered by net of 90-95% shading. It is planned that feeding will be provided daily by hand, while every 45 days weight samples will be taken to monitor the growth performance. For the behavior of the fish, transducers are already available and have been tested. They will be in place for monitoring the vertical distribution in cages using an echo integrator.

Task 20.3 Development of feeding methodology (led by HCMR, Nikos Papandroulakis).

Sub-task 20.3.1 Test of different feeding stimuli (HCMR, Yiannis Papadakis).

The objective of the task is to test different feeding stimuli (mechanical, optical, etc) in different age groups of meagre (50-100 and 700-900 g) and at different tank sizes (500 and 5,000 l respectively) for a period of 4 months (each group).

The trial is planned to start in December 2014, starting with the younger individuals (now at ~40 g). The stimuli to be used include aeration at the feeding area before feeding (mechanical) and also a light of different intensity and duration at the feeding area before feeding. Monitoring with video recordings will

allow the definition of the fish behavior, the optimal feeding stimuli and the most appropriate for industrial application.

Sub-task 20.3.2 Test of different feeding methods (HCMR, Yiannis Papadakis).

This task has not started yet and it will be planned after the implementation of Sub-tasks 20.3.1.

Sub-task 20.3.3 Test in cages of 2 feed distribution methods (HCMR, Nikos Papandroulakis).

This task has not started yet and it will be planned after the implementation of Sub-tasks 20.3.1 and 20.3.2.

Sub-task 20.3.4 Comparison of hand and industrial demand type feeding in cages. (CULMAREX, Marilo Lopez).

This task has not started and will be started in 2015. The seasonality of juvenile meagre production, poor performance of existing stocks in CULMAREX and the company's production plan have delayed the start of the task. Poor performance of an existing meagre stock indicated that the planned stocking should be made when the poor meagre performance was recovered or explained. It was considered that an early stocking in spring-summer may give better results than the autumn stocking programmed in the DOW. To stock earlier than planned in the DOW in spring 2014 was not feasibly possible considering the logistics needed and the company's production plan. The stocking of meagre for the first trial will be made in spring-summer 2015 and this will not delay the deliverable.

Two meagre batches stocked in July and August 2013 in two different Culmarex sites in Spain performed very differently (**Fig. 20.3.4**). The batch stocked in July performed normally and has been termed Standard performance batch, whilst the as batch stocked in August performed poorly and has been termed poor performance batch. These meagre performances can be compared to those of generation 2013 batches of sea bass and sea bream, which gave standard performance and were harvested after 17 and 20 months.

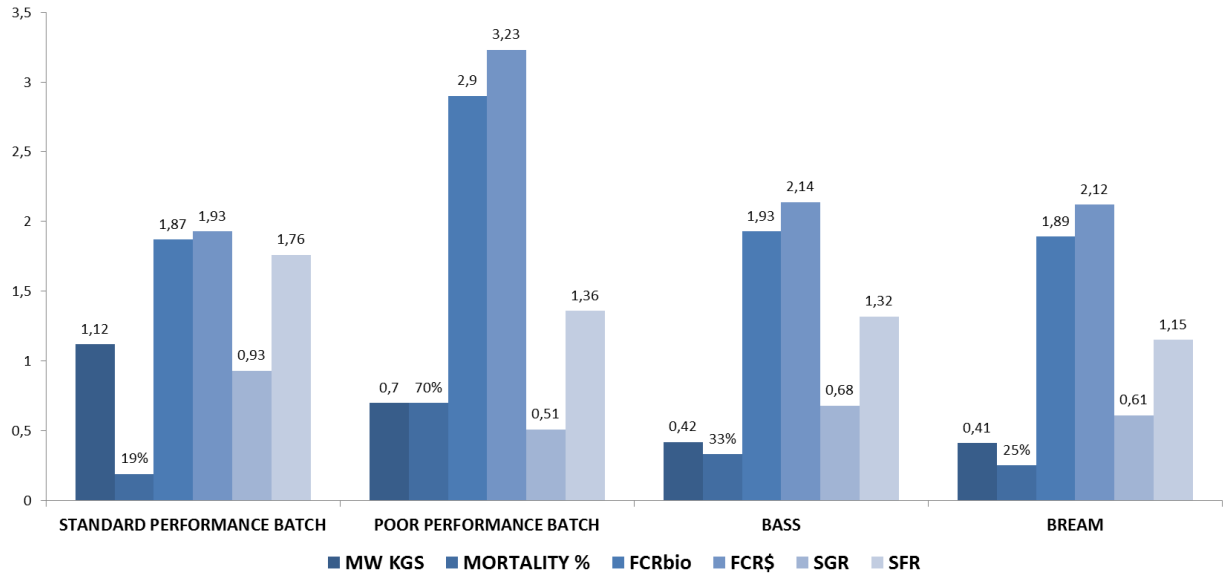


Figure 20.3.4 Meagre 2013 batches performance compared to those of European sea bass and gilthead sea bream already harvested and reared during the same period of time. "Standard" performance batch was meagre stocked in July 2013 and "Poor "performance batch was meagre stocked in August. MW is mean weight (kg) of meagre Oct. 2014 and harvest weight of bass and bream. FCRbio is feed conversion ratio excluding mortality. FCR$ is feed conversion ratio including mortality. SGR is the specific growth rate. SFR is the specific feed rate.

The two meagre batches have performed very differently after 15-16 months. The one stocked in July displays production ratios much better than those of bream and bass at the same weight and after approximately the same rearing period. On the other hand the meagre batch stocked one month later shows very poor performance, even compared to those of sea bass and bream.

European sea bass and gilthead sea bream with survivals of 75-80% at 410 g exhibited an FCR of around 2.1 (1.9 biological). Whereas meagre's FCR (biological) are 1.87 – 2.9 for 1,120 g and 700 g fish. Growth rate in the July meagre batch is much higher than those in bream and bass but the August batch of meagre exhibited a poor growth performance of 0.51 SGR% day^{-1}, which is well below European sea bass and gilthead sea bream. In terms of survival there is a big difference between the two meagre batches 81% and 30%. It is not clear the reason for the high mortality occurred in the batch stocked in August 2013.

Sub-task 20.3.5 Development of feeding system for industrial application (HCMR, Nikos Papandroulakis).

This task has not started yet.

Deviations from Annex I and their impact:

Task 20.3.4 has not started and will be started in 2015. Poor performance of existing meagre stocks at P30. CULMAREX indicated that the planned autumn stocking in the DOW should be avoided until the cause of the poor performance is understood. Therefore, a spring-summer stocking period would be preferred. This deviation will not delay the deliverable.

WP21 Grow out husbandry – greater amberjack

WP No:	21	**WP Lead beneficiary:**		P1. HCMR
WP Title (from DOW):	Grow out husbandry –greater amberjack			
Other beneficiaries (from DOW):	P2. FCPCT	P8. IEO	P15. ULL	P27. FORKYS
P28. CANEXMAR				
Lead Scientist preparing the Report (WP leader):	Nikos Papandroulakis			
Other Scientists participating:	Lidia Robaina(P2), Salvador Jerez, Eduardo Almansa (P8), José Pérez (P15), Ioannis Diakogeorgakis (P27)			

Objectives

1. Development of appropriate rearing methods for cages including rearing volume and type of cage,
2. Development of feeding methods for fry and juveniles by identifying daily rhythms and feeding frequency.

Summary of progress towards objectives and details for each task:

For on growing of greater amberjack, the rearing methodologies for the cage rearing of the greater amberjack will be studied with emphasis on (a) the cage technology (depth and type), (b) the feeding method and (c) the husbandry practice (temperature, stocking density).

During the first reporting period, preparatory activities took place for the implementation of the various trials that will start later during the project.

Task 21.1 Development of rearing method in cages (led by FCPCT, Lidia Robaina**)**

Sub-task 21.1.1 Effect of rearing volume (depth) on performance (FORKYS, HCMR).

This task has not started yet. It is anticipated to start by the end of 2015, provided that a sufficient number of juveniles will be available.

Sub-task 21.1.2 Effect of cage type on performance. (CANEXMAR, FCPCT)

This task has not started yet. Two commercial cages types, one submerged and one surface are being prepared by P28. CANEXMAR according to the DOW. A protocol for fish management in both cages will be prepared by P28. CANEXMAR and P2. FCPCT teams in order to establish fish management practices for the trial, which will start in middle 2015.

Task 21.2 Development of feeding methods (led by IEO, Salvador Jerez).

Sub-task 21.2.1 Definition of feeding pattern for 5 g fish reared in 500 l-tanks for 4 months (led by FCPCT, Lidia Robaina).

This task has not started yet. It is planned to start in 2015, according to the DOW.

Sub-task 21.2.2 Definition of feeding pattern for 200 g reared in 500 l-tanks for 4 months (led by IEO, Eduardo Almansa).

The lack of juveniles of greater amberjack due to the absence of spawning at P8. IEO during 2014 (See WP 3) has not allowed IEO to carry out this activity planned for 2014. However, IEO has acquired the necessary juveniles and thus is expected to implement the task in early 2015. Juveniles were obtained in November 2014, and are currently being acclimated in the facilities. A first experimental trial to define the optimum feeding methods for greater amberjack grow out will be conducted in early 2015, when the initial weight of the fish will be 200 g.

Task 21.3 Development of appropriate husbandry practise (led by HCMR, Nikos Papandroulakis)

Sub-task 21.3.1 Determination of minimum-maximum temperature ranges (led by HCMR, Nikos Papandroulakis).

This task is planned to be performed with three size classes (5 g, 200 g and 500 g). The trials with the first and second size classes have not started yet and they are planned to start during 2015. The trial with the size class of 500 g fish that will be performed by FCPCT has already been initiated. The required juveniles are available and are expected to reach the appropriate size for the trial.

Sub-task 21.3.2 Definition of optimal stocking density (led by IEO, Salvador Jerez).

As described before, IEO faced difficulties with the availability of juveniles to implement the tasks planned for 2014. However, juveniles will be acquired from a commercial hatchery, arriving to the culture facilities between December 2014 and January 2015, and it is expected that the first trial for the definition of the optimal stocking density will be initiated immediately after fish arrive. It is expected that the task can be completed in 2015.

Deviations from Annex I and their impact:

The main deviation from the work plan is the short delay in the implementation of Sub-tasks 21.2.2 and 21.3.2, due to the lack of greater amberjack juveniles at P8. IEO, as already explained. Partner 8. IEO, however, has already acquired some juveniles and is going to acquire more, so that a sufficient number of juveniles from a commercial hatchery (Futuna España SL) will be used for the planned work. These juveniles born in captivity will be acquired in sufficient number and adequate size to allow conducting trials related to stocking density and feeding strategies. Thus, although the implementation of few tasks is slightly behind schedule, they will be initiated at the end of 2014 and completed during 2015 without any significant impact in the implementation of the project or the deliverables.

WP22 Grow out husbandry - pikeperch

WP No:	22	**WP Lead beneficiary:**		P16. FUNDP
WP Title (from DOW):	Grow out husbandry – pikeperch			
Other beneficiaries (from DOW):	P9. UL	P21. DTU	P29. ASIALOR	
Lead Scientist preparing the Report (WP leader):	Patrick Kestemont			
Other Scientists participating:	Robert Mandiki (P16), Jessica Douxfils (P16), Pascal Fontaine (P9), Ivar Lund (P21), Kevin Debes (P29)			

Objectives

1. Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status,
2. Characterization of pikeperch growth, immune and physiological status in farm conditions,
3. Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity.

Summary of progress towards objectives and details for each task:

The WP is targeted on the study of the husbandry requirements during on-growing, with emphasis on the effect on growth, immune and physiological status (a) of the environmental parameters, (b) of the farm conditions and (c) of the domestication level and geographical origin. During the reporting period, a preliminary experiment was conducted to better define the methodological requirements of a multifactorial stress screening, which was initially planned to start between the months 8 to 12 of the project, and will effectively start on month 17.

Task 22.1 Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status (led by FUNDP, Patrick Kestemont).

In order to identify the most stressful factors, which hamper the growth and welfare of pikeperch juveniles, a multifactorial experiment including 8 factors in two modalities was planned to start between the months 8-12 of the project in close collaboration with P9. UL partners (Nancy) that have suitable RAS for such type of experiments. To adapt the rearing conditions of the facilities to the multifactorial protocol requirements, it was planned that the multifactorial experiment will start by early June 2015 since the deliverables are expected on month 24.

Before the actual start of that multifactorial experiment, two preliminary experiments were necessary in order:

(a) To standardize some methodological aspects, especially concerning the physiological and immune analyses since there is limited information on stress response for pikeperch.
(b) To define the lethal concentration (LC50) of *Aeromonas salmonicida* that will be used for the disease tests after stress experiments.

1. First preliminary experiment:

The objectives of the first preliminary stress experiment were: (i) to determine the sensitivity of pikeperch to a single or repeated emersion stress, (ii) to estimate the time amplitude for stress response of pikeperch juveniles and (iii) to test whether dietary tryptophan may mitigate the primary stress response, and mitigate the overall effects of stress emersion.

Methodology: The fish used were juveniles of ±10-12 g BW from Excellence Fish farm, Netherlands that were transferred to Namur on May 28, 2014. They were acclimatized to RAS and to the experimental feeds for about 3 weeks before transferred to the experimental tanks (in triplicate), each stocked with 42 fish.

The experimental protocol included (a) stress with complete removal of tank water for +30 s-emersion (mimicking grading) once per week and (b) two experimental diets containing L-tryptophan (TRP).

Thus, four experimental variants were created:

- CT: control groups without any stress and feed additive
- CTs: control groups submitted to emersion stress but without any feed additive
- 3TRPs: groups receiving 3-time TRP diet and submitted to emersion stress
- 6TRPs: groups receiving 6-time TRP diet and submitted to emersion stress

Six fish per tank were sampled on D0 (start of TRP feeding); D7 (after the 1st emersion stress); D37 and D91 (after 5 or 15 emersion stress). For stressed fish, samplings were done one hour after the emersion manipulations.

Sampled organs: plasma, spleen, liver, brain and head kidney

Results

(a) Physiological response:
Cortisol response: Control fish presented high cortisol level in plasma (CT=88-122 ng/ml) (**Fig. 22.1.1**) compared to salmonids such as rainbow trout (<25 ng/ml, Jentoft *et al*., 2005) confirming a higher sensitivity of pikeperch to captive environmental conditions. Emersion stress induced a significant increase in plasma cortisol (**Fig. 22.1.1**) both after a single stress ($F=23.36$, $p=1.51*10^{-10}$) or repeated stress ($F=14.09$, $p=4.60*10^{-7}$). Dietary TRP supplementation induced a significant decrease in cortisolemia in a dose related manner, indicating that TRP may interfere with the primary stress response in pikeperch as already demonstrated for some salmonids (Basic *et al*., 2013).

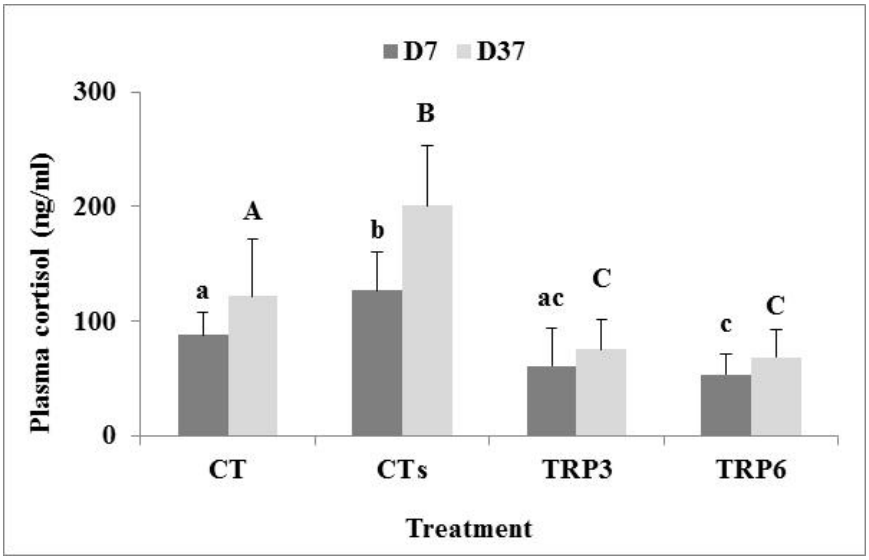


Figure 22.1.1 Mean (±SD) plasma cortisol of pikeperch juveniles submitted once a week to emersion stress, D7: single stress, D37: repeated stress. CT or CTs = fish receiving the control diet or CT diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times of the amount of TRP in the control diet.

Glucose response: Emersion stress induced a significant increase in plasma glucose (**Fig. 22.1.2**) both after a single stress ($F=15.96$, $p=1.50*10^{-7}$) or repeated stress ($F=7.71$, $p=0.00019$). Dietary TRP showed a trend of decrease after a single emersion on D7 of TRP supplementation but long-term dietary treatment did not seem effective in reducing stress-inducing glycaemia.

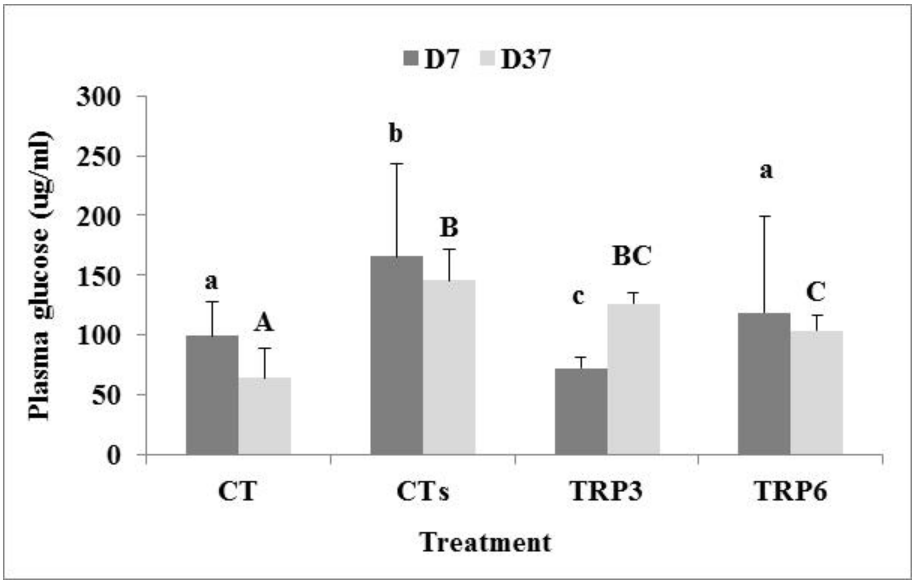


Figure 22.1.2 Mean (±SD) plasma glucose in pikeperch juveniles submitted to emersion stress once a week; D7: single stress, D37: repeated stress. CT or CTs = fish receiving the control diet or CT diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times of the amount of TRP in the control diet.

(b) Growth response to emersion stress:

Emersion stress once a week did no show marked effect on the growth rate (**Fig. 22.1.3**) and feed intake (**Fig. 22.1.4a**) or feed conversion ratio (FCR, Figure 22.1.4b) at D37. However, after a long-term application of stress a significant decrease in growth was observed at D91 ($F=84.46$, $p=2.13*10^{-6}$) associated with a low food utilization ($F=56.91$, $p=8.45*10^{-5}$). TRP dietary supplementation induced a significant decrease in growth whatever the dose, more specifically after a long-term treatment at D91. The depression in growth rate by the long-term TRP supplementation was associated to a decrease in daily feed intake and to negative feed utilisation for both doses.

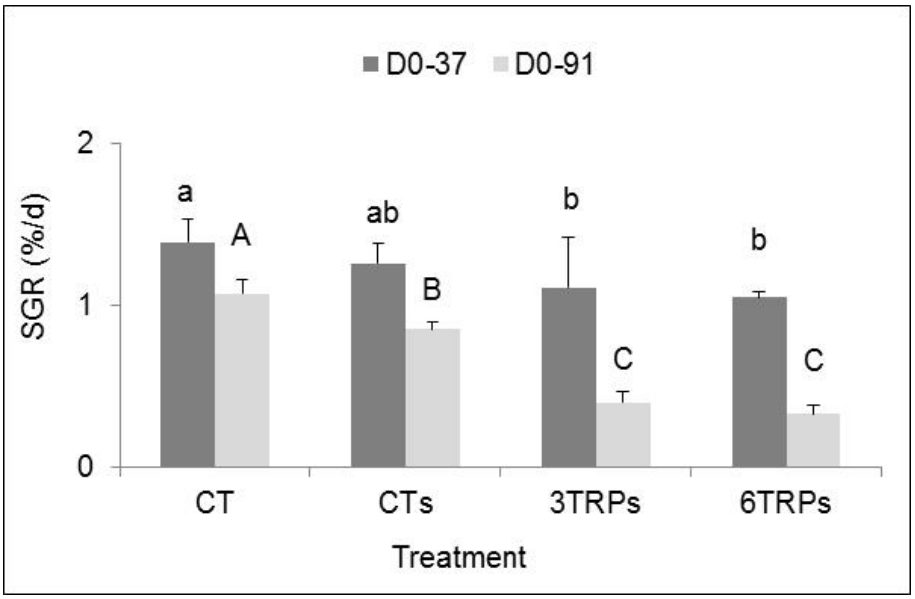
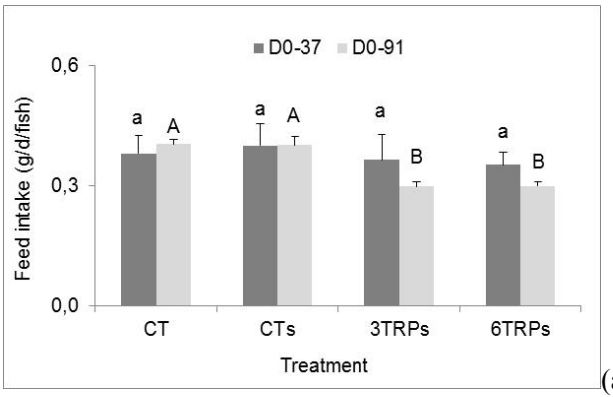
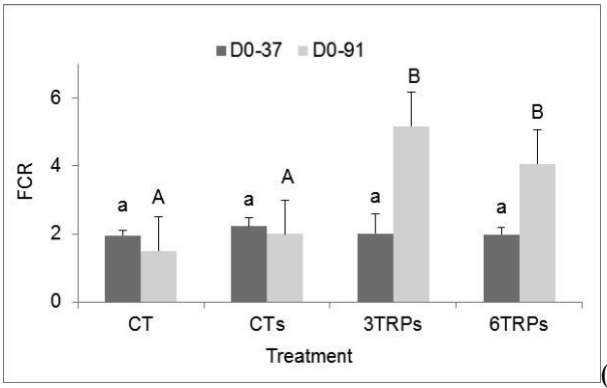


Figure 22.1.3 Mean (±SD) specific growth rate (SGR) of pikeperch juveniles submitted to emersion stress once a week during three months. CT or CTs = fish receiving the control diet or CT diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times of the amount of TRP in the control diet.



(a)



(b)

Figure 22.1.4 Daily feed intake (g/d/fish) (a) and feed conversion ratio (FCR) (b) of pikeperch juveniles submitted to emersion stress once a week during three months. CT or CTs = fish receiving the control diet or CT diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times of the amount of TRP in the control diet.

c) Immune response:
Analyses of immune markers are ongoing.

(d) Resilience response to emersion stress:
After the samplings at D91, the remaining fish were divided in three groups, which will be used to determine the amplitude of emersion stress response, as well as the TRP resilience on the stress response. Three experimental groups will be compared:

- CT: control groups without any stress and feed additive
- CTs: control groups submitted to emersion stress but without any feed additive
- TRPs: groups receiving TRP diet and submitted to emersion stress

Serial samplings will be done at 0, 0.5, 1, 3, 6, 24 h

The test is planned to start in January 2015, and the results are expected in mid-March 2015.

2. Second preliminary experiment:
The objective of the 2nd preliminary trial is to define the lethal dose of *Aeromonas salmonicida* that will be used for the disease tests after stress experiments. Part of the remaining fish from the 1st preliminary experiment will be used

Methodology
Three doses will be tested: $5x10^6$, $1x10^7$, $1x10^8$ CFU/fish based on a previous study on Polish pikeperch (*Stizostedion lucioperca*) juveniles (Siwicki *et al*., 2012) for which a concentration of 10^7 CFU/fish was used. It will be a duplicated trial with 15 fish/tank. It is planned to start in mid-December 2014, and the results are expected at the end of January 2015.

3. Screening of the major stress factors for pikeperch:

The *objectives* are *(a)* to identify the most stressful husbandry and environmental factors, which affect the growth and welfare of pikeperch juveniles*; (b)* to determine the optimal conditions for increasing the production and quality of pikeperch juveniles.

3.1. Multifactorial screening (P9. UL Nancy – P16. FUNDP):
Eight factors combining husbandry and environmental conditions will be tested using an orthogonal design: (1) Size grading, (2) Rearing density, (3) Hypoxia, (4) Ammonia level, (5) Light intensity, (6) Temperature, (7) Feed type and (8) Light type.

Two modalities will be tested for each factor; so it means for the multifactorial design that each factor-modality is repeated 8 times in combination with the other experimental conditions.

- Size grading (one grading/15 days vs no grading). Grading stress is processed by harvesting the fish with net from each tank, anesthetizing them and select them according to size as applied currently in pikeperch farms (*e.g.*, Asialor).
- Rearing density: low (10kg/m^3) vs high (50kg/m^3) stocking density.
- Hypoxia, low O2 (4 mg/L) vs high (8 mg/L).
- Ammonia: low (0.15-0.25 mg TAN/L) vs high (0.80-1.2 mg TAN/L)
- Light intensity: low level (5-10 lx) vs high level (80-100 lx) of white light as measured at the water surface.
- Temperature: 20-21 vs 26-27 °C
- Feed type: sinking vs floating feed
- Light type*:* white light vs red light

RAS availability and planning:
Large experimental facilities will be available in P9. UL from early June 2015. The experiment is planned from June to August 2015, including three periods of fish samplings: D0, D30 and D60.

Fish availability:
Juveniles of ±100 g BW will be used. Approximately 8,000 young juveniles are now available at P29. ASIALOR farm, and it is expected that they will have the right body weight at the onset of the experiment.

Output variables:
The analyses and some stress indicator data will be done between August-September 2015, and immune markers from end of August to October 2015

3.2. Impact of directive stressors on disease resistance (P16. FUNDP):
Some directive stress factors will be re-evaluated in terms of immune-competence and mortality after experimental bacterial infection in the AL2 facilities of P16. FUNDP. The planning of this experiment includes:

- In-vivo stress experiment and bacterial challenge: September – November 2015
- Immune analyses: October – December 2015

Task 22.2 Characterization of pikeperch growth, immune and physiological status in farm conditions (led by ASIALOR, Kevin Debes).

This task has not started yet. It is expected to start in the second part of 2015.

Task 22.3 Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity (led by FUNDP, Patrick Kestemont).

This task has not started yet.

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Jentoft S, Aastveit AH, Torjesen PA, Andersen O, 2005. Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology, Part A 141 : 353-358.

Siwicki AK, Lepal A, Zakęś Z, Kowalska A, Kazuń B, Kazuń K and Głąbski E, 2012. Influence of Dietary Administration of β-hydroxy-β methylbutyrate (HMB) on the Innate Immunity and Resistance against Bacterial Infections in Pikeperch (*Sander lucioperca*). Journal of Agricultural Science and Technology B2: 965-970.

Deviations from Annex I and their impact:

The Milestone 48 that was for month 18 will be delayed for month 22. Indeed, the multifactorial study was planned to start between month 8 and 12, but it appeared necessary to adapt the rearing conditions of the UL facilities to the protocol requirements of the multifactorial design for a better implementation concerning the identification of the major stress factors for pikeperch juveniles. This adaptation took more time than expected. Due to limited information on stress responsiveness for pikeperch, it was also necessary to standardize some methodological aspects, especially concerning the physiological and immune analyses as well as the bacterial LC50 doses for the evaluation of the disease resistance of stressed fish. As a consequence, the multifactorial trial is postponed and is planned to start by early June 2015, but should be completed by November 2015. Therefore, Task 22.1 will be completed in time through preliminary methodological refinement, without any major delay on the Deliverables associated with this task (except some data on immune responses to bacterial challenge tests) since the only Deliverable is expected on Mo24.

WP23 Grow out husbandry – grey mullet

WP No:	23	**WP Lead beneficiary:**		P4. IOLR
WP Title (from DOW):	Grow out husbandry – grey mullet			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P18. CTAQUA	P25. DOR
P26. GEI	P31. IRIDA			
Lead Scientist preparing the Report (WP leader):	Bill Koven			
Other Scientists participating:	Yannis Kotzamanis (P1), Alicia Estevez, Enric Gisbert (P3), Rocio Robles (P18), Gilad Shafran (P25), Evangelos Geitonas (P26), Nikos Papaioannou (P31)			

Objectives

1. Evaluating the geographic range for grow-out of grey mullet in the Mediterranean basin,
2. Determine the cost-benefit of different weaning diets on the performance and health status of juvenile grey mullet.

Summary of progress towards objectives and details for each task:

The objectives of WP 23 is the study of some parameters during the grow-out of grey mullet. The first study is related to the definition of an optimal weaning diet. The second study is a multifactorial comparison of different stocking densities and rearing systems with individuals of different origin (wild VS F1) fed an improved diet. During the period preliminary actions took place related to the collection of the required wild juveniles but also the definition of the optimal diet that will be tested.

Task 23.1. Determine the cost-benefit of different weaning diets on the performance and health status of wild juveniles (led by IRTA, Enric Gisbert).

The collection of wild grey mullet post-larvae (about 300 mg) began in September 2014, in order to carry out this task, which is presently underway until late December. It is impossible to capture smaller fish as this is the size at which they move into coastal lagoons in our area. It is expected that the deliverable D23.1 will be reported on time in month 18 according to the original planning.

Task 23.2 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of F1 juveniles stocked at two different densities in cement and earthen ponds (led by IOLR, Bill Koven).

This task will begin in the spring of 2015. The P31. IRIDA (a Greek feed company) will produce the feed based on an improved P4. IOLR mullet grow-out diet formula. Presently P4. IOLR and P31. IRIDA are in the final stages of signing a non-disclosure agreement. Shortly after this, the formula will be transferred to IRIDA and the extruded feed produced to fulfil the requirements to carry out Tasks in 23.2, 23.3 and 23.4. In task 23.2 the F1 juveniles will come from P4. Partner 4. IOLR produced larvae that were used in experiments in WP13 and WP19. At the time of writing this report about 80,000 juveniles were transferred from the hatchery to the nursery and it is expected that another 200,000 will be transferred in the next two weeks (end of December 2015).

Task 23.3 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild caught juveniles stocked at two different densities in cement ponds in Greece (led by HCMR, Yannis Kotzamanis).

The collection of wild juveniles started in September 2014 and was completed in October 2014, with 5,000 fish collected. The fish were <1 g in weight at the time of collection and have been placed in two concrete ponds at P26. GEI. The fish are currently fed with an eel feed until the development of the grey mullet feed, and the start of the experiment in the Spring 2015.

Task 23.4 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild juveniles at two different densities in ponds in Spain (led by CTAQUA, Rocio Robles).

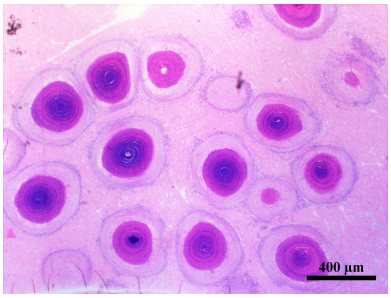
This task has not started yet. Juvenile collection is planned for beginning of the year. Feeding trial will be performed according to the original planning, starting Spring 2015.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period. As the trial is foreseen to start in Spring 2015, it is not expected to have any delay in the starting of the trial as the juveniles will be available before that date.

Group Work Packages

Fish Health



Good progress has been made in all three WPs relating. The first diet trials to attempt to ameliorate the effects of systemic granulomatosis in meagre have been performed using three levels of vitamin D supplementation. All the samplings were performed successfully and the analysis is on-going. Diets for a second trial looking at the impact of vitamin E, C and carotenoids have been prepared and supplied to the relevant Partners. Pathology assessment of systemic granulomatosis-affected fish has already started, and a scoring system has being developed.

Attempts have been made to isolate pathogens from cultured meagre and greater amberjack. In meagre bacteria belonging to the Vibrionacae family (*Vibrio* sp. and *Photobacterium damselae* subsp. *damselae*) have been discovered, as well as *Nocardia* spp. The type strain of *Nocardia seriolae* 44129 has been obtained from DSMZ and NCIMB for comparative analysis. Mass mortality occurred in one culture tank where no bacteria could be isolated and virus analysis is in progress. Greater amberjack bacteria isolated from skin ulcers have been identified as belonging to *Vibrios* of the *harveyi* clade and *Staphylococcus epidermidis* (FCPCT). Gill parasites were identified as the monogenean *Zeuxapta seriolae*, with cysts attributed to eggs of digenean parasites of the *Paradeontacylix* genus. A skin monogenean was also identified as *Neobenedenia melleni*, that belongs to the family *Benedeniiae* family. In addition, attempts to isolate the aetiological agent of Epitheliocystis in amberjack have been made during larval rearing trials.

As a means to control infection of greater amberjack by monogenean parasites, a collector device has been designed and tested as a method to detect and quantify the level of infestation of monogenean parasites in fish without the need to manipulation the fish. This simple device has proved effective in controlling the level of infestation of several species of monogenean parasites (including *Benedenia seriolae, Neobenedenia melleni* and *Zeuxapta seriolae*). The adult fish can support a certain level of infestation without showing signs of disease over a long period of time. This demonstrates the importance of carrying out a regular monitoring of the parasite populations in the fish tanks, in order to improve the effectiveness of any therapeutic treatment.

A first experiment has been undertaken to characterise the ontogeny of the immune response in meagre with samples collected at various times after hatching. Duplicate sets of samples were collected at each time point; one set was fixed in formalin for histological analysis, and a second set was collected in RNAlater for extraction of RNA to be used in immune gene expression analysis. As fish became more developed and organ tissues were easily recognized, individual tissue samples were collected in formalin and RNAlater. Primers to relevant meagre and amberjack immune genes have been designed and are currently being tested to confirm they can amplify the genes of interest (IRTA, FCPCT, ABDN). First attempts to develop a challenge model have also been performed with *Photobacterium damselae* subsp. *piscicida* in meagre and amberjack.

Lastly, production of Viral Neural Necrosis (VNN) capsid protein has been progressing well, and successful expression in *E. coli*, tobacco plants and *Leishmania* has been achieved. The methods are currently being optimised prior to larger scale production for vaccination of Atlantic halibut against this viral disease.

WP No:	24	WP Lead beneficiary:	P1. HCMR
WP Title (from DOW):	Fish Health - Meagre		
Other beneficiaries (from DOW):	P2. FCPCT	P3. IRTA	P5. UNIABDN	P20. SARC
Lead Scientist preparing the Report (WP leader):	Pantelis Katharios		
Other Scientists participating:	George Rigos (P1), Daniel Montero (P2), Ana Roque, Karl Andree (P3), Chris Secombes (P5), Ramon Fontanillas (P20)		

Objectives

1. Identify the causes of systemic granulomatosis (SG), and chronic ulcerative dermatopathy,
2. Investigate anti-parasite treatments in juvenile meagre,
3. Undertake preliminary characterisation of immune genes and study specific immune responses post-vaccination,
4. Evaluate the occurrence of *Nocardia* infections in meagre and develop an autogenous vaccine,
5. Develop diagnostic-prevention-treatment protocols for diseases in meagre.

Summary of progress towards objectives and details for each task:

Task 24.1. Systemic Granulomatosis (led by HCMR, Pantelis Katharios).

Meagre is a new promising candidate for the diversification of the European aquaculture, due to its attractive and competitive attributes. The most important bottleneck of meagre production is systemic granulomatosis (SG), a pathological condition affecting the majority of cultured populations. Systemic granulomatosis is characterized by multiple granulomas in all soft tissues, which become progressively calcified and necrotic. The aetiology of the disease is unknown. However, in addition to problems with various pathogenic microorganisms, nutritional issues including vitamin deficiencies have been reported.

Sub-task 24.1.1. Feeding trials (HCMR, Eleni Fountoulaki)

Trial 1. *The effect of Vitamin D in the development of systemic granulomatosis* (HCMR)

The aim of this study is to investigate the effect of 3 levels of vitamin D3 in SG. The best known action of vitamin D is in the endocrine system, where it has a role in calcium and phosphate homeostasis, however it also plays an important role in other biological processes, such as muscle function, autoimmune diseases and cardiovascular physiology, cell proliferation and differentiation.

Experimental diets

The basal diet was formulated (**Table 24.1.1**) to contain ~53% crude protein and 13% crude lipid. Vitamin D3 was supplemented separately to the basal diet of the mixture to obtain concentrations of 4550 (D0), 7000 (D1), 10000 (D2) and 20000 (D3) IU/kg respectively.

Table 24.1.1. Formulation of the experimental diets for meagre.

Ingredient	D0	D1	D2	D3
Soybean meal	10	10	10	10
Fish meal	50	50	50	50
Wheat	17,4	16,9	16,3	14,3
Corn gluten	14,6	14,6	14,6	14,6
Fish oil	7,5	7,5	7,5	7,5
Premix	0,5	0,5	0,5	0,5
Vitamin D		0,49	1,1	3,1
Vitamin D (IU/Kg)	4550	7000	10000	20000

Experimental fish and feeding trial

For the feeding trial, 3 month old meagre were used, produced in May 2014 in the facilities of the Institute of Aquaculture, Hellenic Centre for Marine Research, Crete, Greece. In total, 600 fish of an average weight of 4.72 ± 0.21 g were weighed and allocated into 12 500-l cylindrical tanks at a density of 50 fish per tank (0.47 kg m^{-3}). Three replicates were allocated to each diet. Prior to the commencement of the experiment, the fish had been acclimated for 1 week by being fed on the D0 diet. The feeding trial lasted for 93 days (July 2014-October 2014).

Tanks were supplied with borehole water and all had similar light conditions and temperature (20^{o}C). Airstones in each tank provided aeration and nets were placed over the tanks in order to prevent the fish from jumping out of the tanks. The fish were hand-fed to visual satiety twice a day for 7 days a week.

Samplings

Until now, 3 samplings have been performed:

- ✓ Initial sampling (prior to the commencement of the experimentation)
  - 8 fish were anaesthetized with 2-phenoxyethanol and samples of heart, liver, intestine and kidney were fixed in 4 parts formaldehyde to 1 part glutaraldehyde (4F:1G) for histology and evaluation of granulomas.
  - 50 fish were anaesthetized and stored at -80^{o}C for whole body composition analysis of CYP27A1, vitamin D3, Ca, P and antioxidant enzymes (SOA, CAT, GSH).
- ✓ 1st month (August 2014)
  - 10 fish/treatment were weighed and anaesthetized and samples of heart, liver, intestine and kidney were evaluated stereoscopically for granulomas. Also a small part of those tissues fixed in 4F:1G for histology.
- ✓ 2nd month (September 2014)
  - 10 fish/treatment were weighed and anaesthetized and samples of heart, liver, intestine and kidney were evaluated stereoscopically for granulomas. Also a small part of those tissues fixed in 4F:1G for histology.
  - 39 fish/treatment were weighed and stored at -80oC for whole body composition analysis of CYP27A1, vitamin D3, Ca, P and antioxidant enzymes (SOA, CAT, GSH).
- Final sampling was performed in October 2014
  - In this sampling all fish will be weighed for the estimation of growth rate and feed performance. Samples of heart, liver, intestine and kidney will be evaluated stereoscopically for granulomas. Also a small part of those tissues will be fixed in 4F:1G for histology.

***Trial 2**. The effect of Ca/P ratio in the diets in the development of granulomatosis* (HCMR)

This trial is not scheduled for this reporting period.

***Trial 3**.The effect of high plant protein diets in the development of granulomatosis* (HCMR)

This trial is not scheduled for this reporting period.

***Trial 4.**The combined effect of Vitamin E, C and carotenoids in the development of granulomatosis* (FCPCT)

During this first annual period, mass production of meagre juveniles to be used for feeding trials (Sub-task 24.1.1.) has been conducted. The production of more than 10,000 juveniles was done and animals were stocked in 1-m^3 tanks, at a density of 5 kg fish per m^3. Animals are being fed three times per day, for 6 days a week, and will be used in the first feeding trial (trial 4) that started in November 2014, as scheduled in the DOW. The diets for feeding trial 4 (Sub-task 24.1.1) were received at P2. FCPCT in November 2014, when the experiment started. In this feeding trial we will study the combined effect of vitamins E, C and carotenoids in SG prevention and the diets were produced by P20. SARC. The effect of these nutrients on survival, growth and metabolic indicators (oxidative enzymes and health in terms of SG and other parameters will be monitored.

***Trial 5**.The effect of Se, Mn and Fe in the development of granulomatosis* (FCPCT)

This trial is not scheduled for this reporting period.

Sub-task 24.1.2.Health and pathological assessment (HCMR, Pantelis Katharios).

Pathology assessment of the SG-affected fish has already started and it is a task that will run throughout the lifespan of WP24. Pathological assessment is performed in the following levels:

Level 1. Assessment of fresh samples. Sampled fish from the feeding trials are visually inspected and tissue samples from various organs are dissected and examined under a stereoscope for the presence of granulomas. Further analysis is conducted for the presence of crystal-like material in the soft tissues using a microscope with polarised filter. For the overall assessment of the health status of the fish we have developed a scoring system which is described in Table **24.1.2.1**.

Table 24.1.2.1. Severity scale for systemic granulomatosis (SG) assessment.

Condition	Score
No granulomas present	0
Granulomas visible only with the aid of microscope	1
Granulomas visible with naked eye	2
Calcification of the tissue	5

Using this scoring system we assess samples from the heart, kidney, liver, gonad, intestine, spleen, peritoneum, and swim bladder from all fish participating in the feeding trials. The results generated are in an ordinal scale and can be analysed statistically using appropriate methodologies such as a chi-square test.

Level 2. Histology. Excised samples are fixed in 4F:1G and are processed for routine histology using a Technovit 7100 resin embedding kit, and following sectioning they are stained with either polychromatic stain (methylene blue/Azzure II/basic fuchsine) or special stains such as PAS and alizarin red.

Level 3. Electron Microscopy (SEM and TEM) coupled with X-Ray microanalysis. Selected samples are fixed in cacodylate buffered glutaraldehyde and are processed for microscopy depending on the technique used at the Electron Microscopy facility of the University of Crete.

Level 4. Blood biochemical analysis. Blood is being collected from the fish of the feeding trials, centrifuged and serum stored at -20°C until analysed. Analysis of the samples has not started yet.

To date we have sampled the fish of the 1st feeding trial (Vitamin D) and the samples have been partly processed. In addition we have included several fish with various degrees of granulomatosis severity from different sources (fish farms, previous samples etc) in order to create a reference library for the pathology assessment. In the following figures we present some of the basic characteristics of the disease at its extreme cases (**Fig. 24.1.2.1** and **24.1.2.2**).

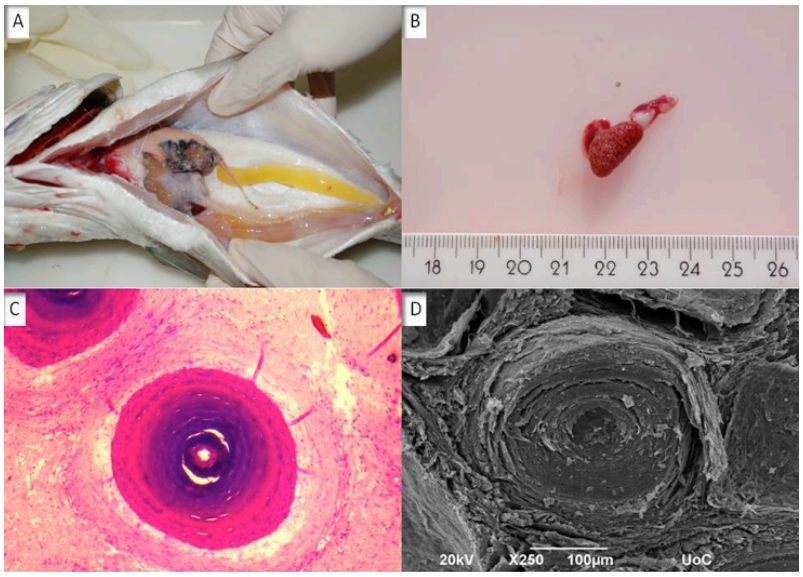


Figure 24.1.2.1 (A) Extensive calcification of the liver of a cage-cultured meagre. (B) Excised heart showing numerous visible granulomas. (C) Histological section of a heart showing the typical appearance of a "mature" granuloma with concentric lamellation and central necrosis. (D) SEM picture of a heart granuloma showing the typical lamellation that give this onion-like appearance in the granulomas.

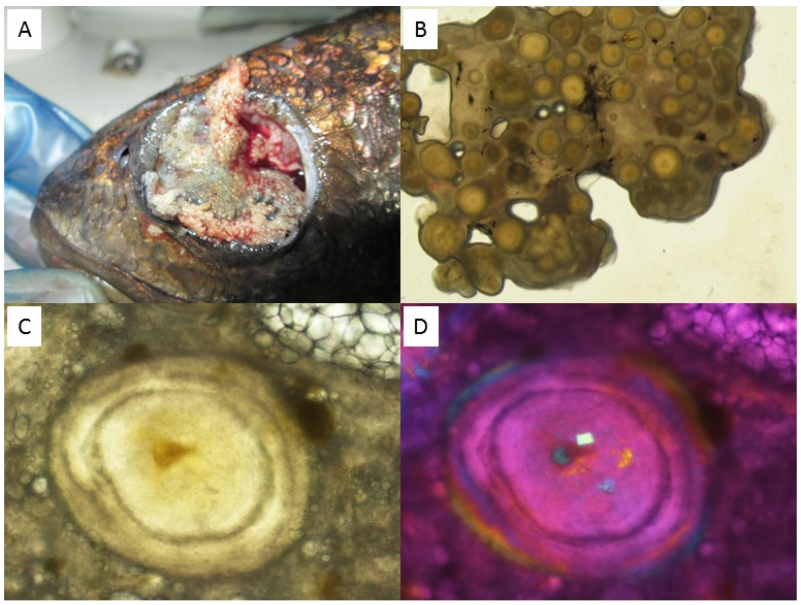


Figure 24.1.2.2 (A) Extreme case of granulomatosis in a cage-cultured meagre where a mass of granulomas has mechanically pushed out the eye of the fish. (B) Fresh preparation of liver tissue showing multiple granulomas. (C) granuloma in the liver as shown in light microscope and (D) the same granuloma using a polarised filter showing crystal structures in the centre.

Task 24.2. Chronic Ulcerative Dermatopathy (led by HCMR, Pantelis Katharios).

The task is not scheduled for this reporting period.

Task 24.3. Anti-parasitic treatments (led by IRTA, Ana Roque,).

Although the task is not scheduled for this reporting period, initial preliminary trials are currently being performed on the acceptance of medicated feed by juveniles.

Task 24.4.*Nocardia* infection in meagre (led by HCMR, Pantelis Katharios).

Sub-task 24.4.1 Isolation and characterization of the pathogen (HCMR, Pantelis Katharios).

Monitoring of cage cultured meagre for Nocardia is in progress. To date we have made several samplings from various geographic locations for the isolation of the pathogen. The samplings are made on a seasonal basis. We have received fish from Crete, Greece from the cage-farm facilities of HCMR and from collaborating fish farms from West and Central Greece. For the isolation of the pathogen several media are being used including TSA with 0.5% or 2% NaCl, LB, Brain Heart Infusion Agar (BHIA) and Marine Agar. All isolates are separated and recultured in new agar plates. When purity is obtained strains are partially characterized and identified, at least to genus level, using basic bacteriological techniques and the BIOLOG GEN III identification system.

So far, only few bacterial strains have been obtained, mostly belonging to the Vibrionacae family (*Vibrio* sp. and *Photobacterium damselae* subsp. *damselae*), which are considered environmental isolates since apart from SG the fish did not exhibit clinical signs of bacterial infection. We have also bought the type strain of *Nocardia seriolae* 44129 from the DSMZ collection for comparative analysis.

Sub-task 24.4.2.Preparation of an autogenous vaccine (HCMR, Pantelis Katharios).

The task will start after the isolation of Nocardia from cultured meagre.

Task 24.5.First characterisation of the immune system (led by UNIABDN, Chris Secombes).

Meagre being grown for WP20 were sampled at IRTA for collecting data on specific growth rate and to collect chronological samples for the immune ontogeny study. Duplicate sets of samples were collected at each time point; one set was fixed in formalin for histological analysis, and a second set was collected in RNAlater for extraction of RNA to be used in gene expression analysis. As fish became more developed and organ tissues were easily recognized individual tissue samples were collected in formalin and RNAlater. Tissues collected were spleen, head kidney, gills, and intestine. Samples for immune gene expression analysis are being stored at -80ºC.

This sampling was finished on Oct. 13th 2014. The original plan was to collect animals that were of a medium size, as well as animals from the larger end of the growth spectrum to see how differential growth may lead to premature immune maturation. We abandoned this idea due to a reduction in the overall size of the population. The original population was greatly diminished due to cannibalism during the grow-out period.

During the grow-out of fish being reared for WP20, samples have been collected for documentation of SG and to collect chronological samples for the immune ontogeny study (**Table 24.5.1**). During the early rapid growth phase fish were sampled every week, but weight data was only collected once per week. Later, during the juvenile stage fish were only sampled once per week.

Table 24.5.1 Samples collected for documentation of SG and to collect chronological samples for the immune ontogeny study.

Species	Nº sample	dph	Date		Size (mm)	W Wt. (mg)	n
Corvina	**L0**	1	28-apr	√		0,20	30 - 50
Corvina	**L1**	3	30-apr	√			
Corvina	**L2**	8	05-may	√		0,65	
Corvina	**L3**	12	09-may	√			
Corvina	**L4**	15	12-may	√		4,73	
Corvina	**L5**	19	16-may	√			
Corvina	**L6**	22	19-may	√		10,75	
Corvina	**L7**	26	23-may	√			
Corvina	**L8**	29	26-may	√		61,22	
Corvina	**L9**	33	30-may	√			
Corvina	**L10**	36	02-jun	√		99,41	
Corvina	**L11**	40	06-jun	√			
Corvina	**L12**	43	09-jun	√			
Corvina	**L13**	47	13-jun	√			
Corvina	**L14**	50	16-jun	√			
Corvina	**L15**	54	20-jun	√		1590,00	
Corvina	**L16**	59	25-jun	√			
Corvina	**Wm1**	64	30-jun	√			2
Corvina	**Wm2**	71	07-jul	√	6400	2693,5	2
Corvina	**Wm3**	78	14-jul	√	7050	3940	2
Corvina	**Wm4**	82	18-jul	√	7900	8600	2
Corvina	**Wm5**	92	28-jul	√	8500	9560	2
Corvina	**Wm6**	99	04-aug	√	9200	12104	2
Corvina	**Wm7**	107	12-aug	√	9350	15080	2
Corvina	**Wm8**	113	19-aug	√	17255	14185	2
Corvina	**Mm1**	127	02-sep	√	11200	20915	
Corvina	**Mm2**	141	16-sep	√	12600	30035	2
Corvina	**Mm3**	155	29-sep	√	15500	54470	2
Corvina	**Mm4**	162	06-oct				
Corvina	**Mm5**	169	13-oct				

A search of the online database GenBank was performed to identify and collect existing sequences for genes of interest from extant marine teleost species for the study of the immune system (**Table 24.5.2**). The sequences collected were used for the preparation of alignments for designing degenerate/consensus primers for amplification from cDNA of meagre tissues.

Samples for the preparation of RNA and subsequent synthesis of cDNA for preparation of these gene expression assays has already been done during the grow out period of fish being used in WP20. All of this process for isolation of gene sequences and development of the specific gene expression assays will be initiated in the next quarter at IRTA. Samples from IRTA have been sent to Aberdeen where the first PCR results look promising for IgM and IgT amplification (**Fig. 24.5.1**)

Table 24.5.2 Genes targeted for characterization of the immune system of meagre. The unknown gene sequences should provide amplicon sizes approximating those shown, if there exists a high degree of conservation between species. These estimates are based upon data from existing sequences found in GenBank.

	Target Gene	Degenerate/ ConsensusPrimers	Ampliconsize
EndogeneousControls	EF1 (Elongation Factor)	X	230
	GAPDH (GlyceraldehydePhosphateDehydrogenase)	X	239
	18S	X	-
InnateImmunity	Piscidin1 ("Defensin")	X	110
	Piscidin2 ("Defensin")	-	-
	Piscidin3 ("Defensin")	-	-
	Lysozyme	X	220
	Metallothionein	X	80
	MX protein	X	570
	NOD2 (Toll Like Receptor - TLR)	X	1390
Adaptive Response	RAG1 (RecombinationActivating Gene)		
	IgM		
	IgT		
	TcR (T-cell Receptor)		
	C3 (complement)	X	1202
	TNFa (Tumor Necrosis Factor)	X	250
	IFN alpha (interferon)		
	IFN gamma		
	IL-1beta (Interleukin)		
	IL-2		
	IL-4		
	IL-10		
	IL-17		
	IL-22		
Inflammatory Response	COX2 (cyclooxygenase 2)	X	1500
	MyD88 (myeloiddifferentiating factor)	X	130

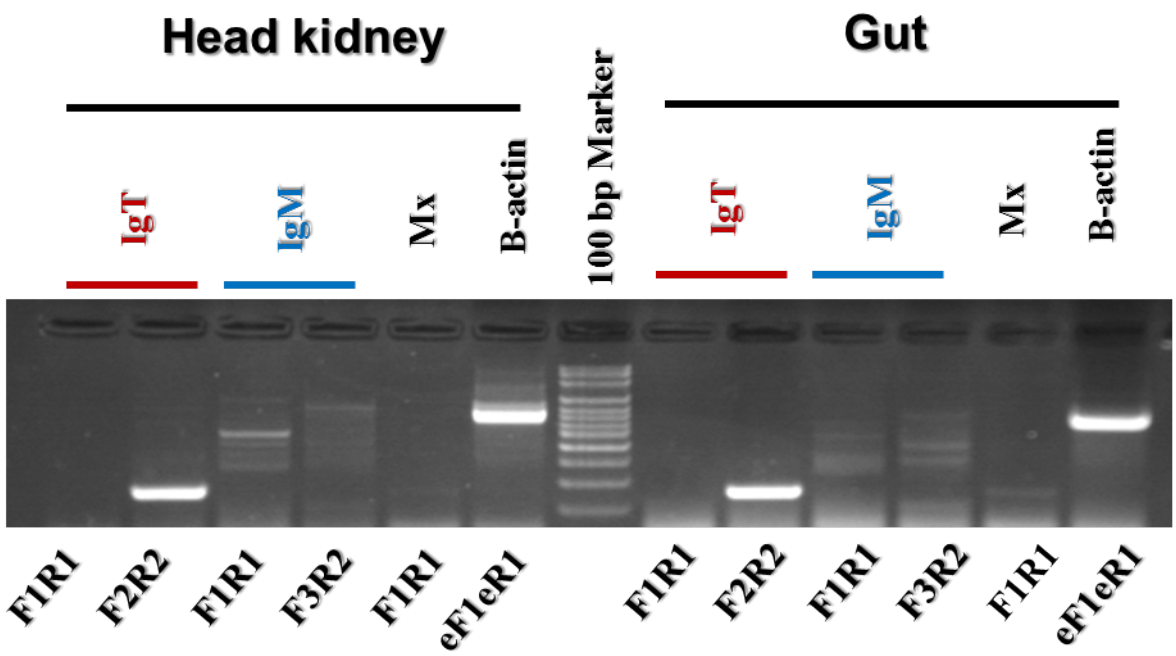


Figure 24.5.1 PCR was performed using primers designed to amplify meagre IgT (two pairs used), IgM (two pairs used), Mx and B-actin (housekeeping gene). Promising bands of the correct size were seen for IgT and IgM in both kidney and gut samples, in addition to B-actin amplification.

Task 24.6. Monitor specific immune responses (led by UNIABDN, Chris Secombes).
The task is not scheduled for this reporting period.

Task 24.7. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in meagre (led by FCPCT, Daniel Montero).
Some analysis has been conducted to monitor meagre to establish the seasonality of the potential diseases throughout the project. External morphology, necropsy and samples of liver, spleen, kidney, gut and nervous system are being studied for bacterial content.

The samples obtained were cultured in (BHIB) supplemented with 1.5% NaCl at 25ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl, and the bacteria grown in the culture media were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16SRNA.

Routine Samples obtained during the first months:

July 2014

Clinical signs: None. Routine sampling. Juvenile fish.

Microbiology: Some different strains of bacteria were isolated (**Fig. 24.5.2**). However, when the *Nocardia* spp. were sent for typing, the rest of the bacteria were discarded because they are frequently found in culture conditions and are considered to be normal microbiota.

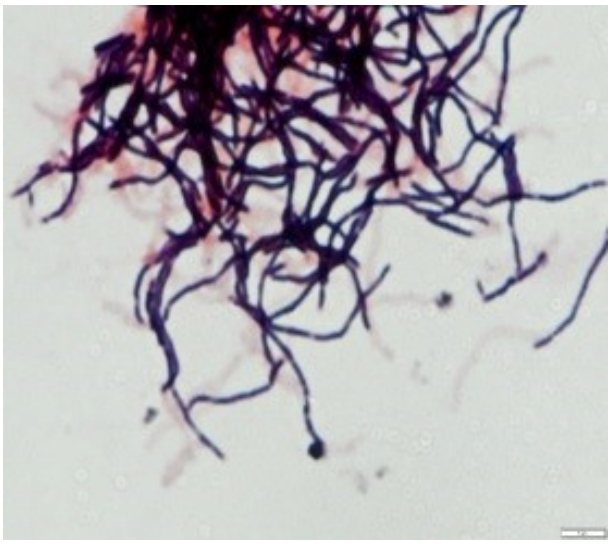


Figure 24.5.2 Nocardia spp. isolated from meagre juveniles.

August 2014 Meagre fry, 3.6 g

Clinical Signs: Ulceration, erosion of the tail

Microbiology: Bacterioscopy and culture - Different strains of bacteria were isolated. Species determination is in progress.

Probable cause of ulceration: cannibalism

September 2014. Meagre adults

Clinical Signs: Mass mortality in one culture tank.

Microbiology: Bacterioscopy and culture – No pathogenic bacteria isolated . Virus analysis is in progress.

Challenge test: Sub-lethal dose of opportunistic bacteria.

Sampled fish Meagre fry (n=30)

Bacteria/Dose: *Photobacterium damselae* subsp. *piscicida* - 10^3cfu/fish by intraperitoneal injection.

Mortality/Microbiology

No fish died but bacteria were recovered from 3 fish post-challenge.

Sub-lethal dose did not produce losses and seems to be useful for immunological studies in this species.

Task 24.8 Diagnostic-recommendation manual for meager health (led by HCMR, Pantelis Katharios).

This task is not scheduled for this reporting period.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP25 Fish health – greater amberjack

WP No:	25	**WP Lead beneficiary:**		P5. UNIABDN
WP Title (from DOW):	Fish health – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P8. IEO	P15. ULL
Lead Scientist preparing the Report (WP leader):	Chris Secombes			
Other Scientists participating:	Pantelis Katharios, George Rigos (P1), Daniel Montero (P2), Juana R. Cejas (P8), Covadonga Rodriguez (P15)			

Objectives

1. Provide early diagnosis tools for Epitheliocystis,
2. Develop "antiparasite diets" to be used prior to sea cage culture,
3. Begin characterisation of the immune system, with a focus on mucosal (skin/gill) defences,
4. Develop anti-monogenean parasites infection rearing protocols.
5. Develop diagnostic-prevention-treatment methods for diseases in greater amberjack.

Summary of progress towards objectives and details for each task:

Task 25.1. Study of Epitheliocystis during larval rearing (led by HCMR, Pantelis Katharios).

Epitheliocystis is a fish disease characterised mainly by small intracellular inclusions in the gills of the fish. Although it is considered benign in large fish it can be lethal in larvae and juveniles. According to the literature, the disease is caused by chlamydia or chlamydia-like organisms however our experience with the disease in HCMR facilities indicate the implication of a variety of bacteria belonging to beta and gamma proteobacteria responsible for the disease with chlamydia contributing only to small and not important lesions (complicating the pathology assessment of the fish).

The aim of the task is to investigate the aetiological agents of epitheliocystis in larval rearing systems in Greece and to develop and test tools for the early diagnosis of the disease. In order to study the disease we are relying exclusively on natural infections, since the bacteria responsible for epitheliocystis are not culturable.

The experimental design included samplings of water and fish during larval rearing for the detection of the pathogens using molecular tools developed earlier with the collaboration of Prof. Lloyd Vaughan of the Institute of Veterinary Pathology of the University of Zurich. Fractions of water samples (filtered through serial filters ranging from 0.22 μm to 250 μm) will be assessed for the presence of the pathogenic microorganisms in order to elucidate whether the pathogens enter the system autonomously or using a planktonic vehicle. The presence of the disease will be assessed following visual and histological examination but also with PCR using specific primers.

One 5-m^3 mesocosm tank receiving unfiltered natural sea water was stocked with 5,000 greater amberjack eggs on 25th June 2014. The environmental parameters of the tank water were monitored daily. Following mouth opening the fish were provided live rotifers while fresh microalgae *Chlorella minutissima* were added in the rearing water daily.

Fish were visible at the beginning of the culture however their population density was decreasing daily which together with water turbidity due to the microalgae made fish sampling extremely difficult. At 20 dph (14th July) the rearing was terminated due to the extremely low number of fish present in the tank.

Five samplings were made during the course of the experiment. At each sampling 10 l of rearing water was passed through serial filters of 250, 120, 53, 25 µm and 1 l of the last filtrate was passed through 0.22 µm filters using sterile syringes. All filtrates were collected in 3 replicate 50-ml vials and stored in -80°C, or RNA*later* and phosphate buffered formalin following centrifugation at 3,000 rpm for 5 min.

DNA from each filtrate and from the filters of 0.22 µm was extracted on the same day of the sampling and was subjected to PCR using universal chlamydia primers but also specific primers for bacteria which were associated with epitheliocystis previously. The primers used in the PCR analysis are shown in **Table 25.1.1**. DNA from positive controls was also included, using previously studied epitheliocystis agents from Crete and other places in Greece.

Table 25.1.1. Primers and PCR conditions used for the detection of epitheliocystis agents in greater amberjack larvae.

Endozoicomonas specific primers PCR with annealing temperature of 60°C, extension time 30 secs. Product = 400bp. Positive control is extracted Diplodus larva from 2012.	
Endo_sp_F	AGT AGG GAG GAA AGG TTG AAG G
Endo_sp_R	CCC AGA ATA CAA GAC TCC GGA C
Ichthyocystis specific primers (this is a novel unpublished genus identified in Sparus aurata in Greece with the collaboration of the University of Zurich-Prof. Lloyd Vaughan). PCR with annealing temperature of 60-62°C, extension time 1 min. Product = 900bp. Positive control is from infected Sparus	
Ichthyo_sp_F	AAC TAR GAT GGT GGC GAG TG
Ichthyo_sp_R	CGC ACA TGT CAA GGG TAG G

No epitheliocystis disease nor agent was detected in this experiment in any of the samples checked. The cause of fish mortality is unknown and the experiment will be repeated next year.

Task 25.2. Promoting resistance to parasitic incidence on greater amberjack (led by FCPCT, Daniel Montero).

During this first annual period, mass production of greater amberjack juveniles to be used for studies of promoting the resistance to parasitic incidence has been conducted. A production of more than 10,000 juveniles was conducted and animals were stocked at 10 m^3 tanks. Mass production begun in September 2014 and animals are being fed three times per day, during 6 days a week. Animals (~3 g at the time of writing this report) will be used in a first trial to determine the mucus production in terms of quality and quantity at different culture conditions. This has started in November 2014.

In addition, samples of different tissues (spleen, skin, gill, liver, kidney) for identification of immune markers (Task 25.3) have been taken, and sent to P5. UNABDN in September 2014.

Figure 25.2.1 Mass production of greater amberjack

Task 25.3. Identification of immune markers (led by UNIABDN, Chris Secombes).

Primers have been designed to the key immune genes to be cloned from amberjack for future studies (**Table 25.3.1**). Preliminary PCRs have been carried out using the samples received from P2 and promising products for both IL-17A/F (first primer set) and IgT (second primer set) have been obtained (**Fig. 25.3.1**). These will be sequence confirmed shortly.

Table 25.3.1 Primer sequences to be tested for amplification of amberjack immune genes.

Species - Target	Name	Sequence	bp	G/C	Tm°C
Seriola dumerili IL-17 A/F (sense)	IL-17F	GCGCCAACATGTCTCTGTC	19	57.9	56.5
Seriola dumerili IL-17 A/F (anti-sense)	IL-17R	GACTCTGTGGAGGACCAGGA	20	60	58.1
I. Seriola dumerili IL-22 (sense)	IL-22F	GCCAACATCCTCGACTTCTA	20	50	54.2
Seriola dumerili IL-22 (anti-sense)	IL-22R	AGTCTTCAGGTCCTCGC	17	58.8	53.9
Seriola dumerili IgM (sense)	IgMF	CGTTTATACGGGAGTCAGTCAAA	23	43.5	54.6
Seriola dumerili IgM (anti-sense)	IgMR	TCAAGGCTGAGCGATAACT	19	47.4	53.5
Seriola dumerili IgT (sense)	IgTF	GGTCACTCTGTTGTGTCTG	19	52.6	52.9
Seriola dumerili IgT (anti-sense)	IgTR	GTGGTGTAAAGACTCGTAAC	20	45	50.5
Seriola dumerili beta –defensin (sense)	DefF	ACATGAAGGGACTGAGCTTG	20	45	52.9
Seriola dumerili beta –defensin (anti-sense)	DefR	ACAGTTACACATCTGCTGCA	18	44.4	49.3
Seriola dumerili piscidin (sense)	PisF	GATGGTCGTCCTCATGGCTG	20	60	57.9
Seriola dumerili piscidin (anti-sense)	PisR	CTTTCAGATGAACCGCCATAGAT	23	43.5	54.9

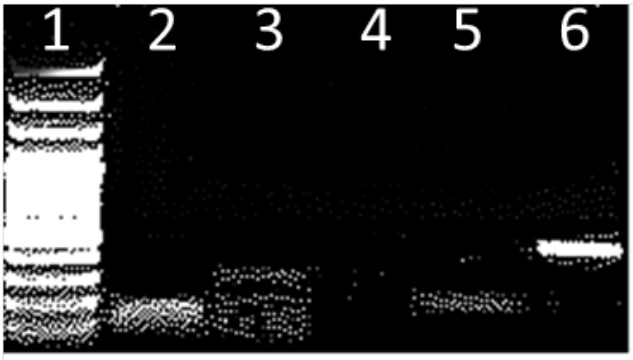


Figure 25.3.1 A PCR gel showing initial products obtained for amberjack IL-17A/F and IgT. Lane 1 = mw ladder. Lane 2 = IL-17A/F (1st primer set). Lane 3 = IL-17A/F (2nd primer set). Lane 4 = IgT (1st primer set). Lane 5 = IgT (2nd primer set). Lane 6 = beta actin (positive control).

Task 25.4. Effectiveness of stocking density and anti-oncomiracidia attaching substances in the control of monogenean parasites (led by IEO, J.R. Cejas).

The study of the efficacy of the baths with lectin-type substances as a treatment against monogenean parasites, which includes carrying out several trials with juveniles of greater amberjack, is scheduled later in the project. However we have done some preliminary work and obtained relevant information that will be useful for the planned trials and studies.

We have designed and tested a collector device and method to detect and quantify the level of infestation of monogenean parasites in the fish rearing tank without the need to manipulate the fish. The method relies on the counting of the monogenean eggs that are present in the culture tank when laid by the adult parasites that are infesting fish. The level of infestation is estimated based on the number of parasite eggs collected by a device that is suspended in the fish tank and removed for observation and counting periodically. The collector device (vertical support with nylon mesh discs) was designed considering the characteristics of the monogenean eggs, exhibiting filamentous appendages which cause that eggs get entangled in the nylon mesh discs (**Fig. 25.4.1**).

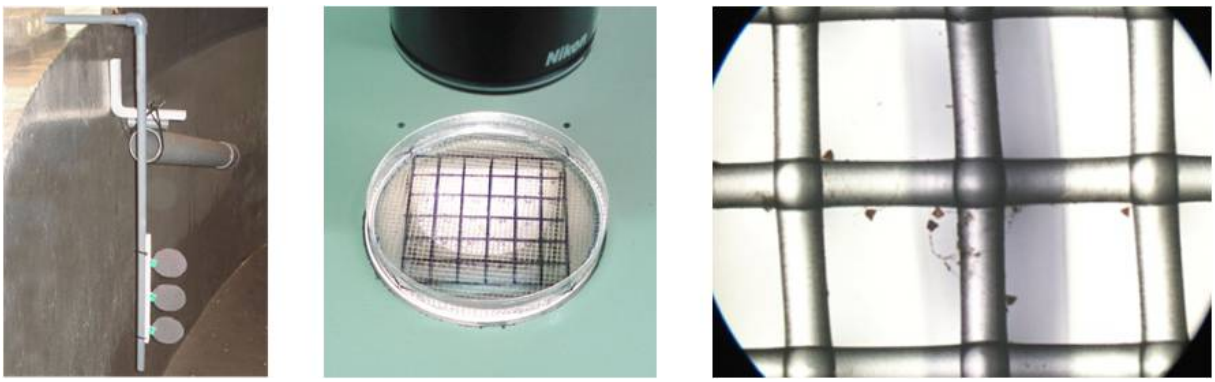


Figure 25.4.1 Nylon mesh discs removed from the vertical support submerged in the fish tanks are placed in Petri dishes with filtered seawater for observation and egg count under a stereomicroscope.

This simple device and method has proved to be effective to control the level of infestation of several species of monogenean parasites including *Benedenia seriolae, Neobenedenia melleni* and other species with eggs having a tetrahedral capsule and a single long filamentous appendage, and also species such as *Zeuxapta seriolae*, which lay chains of eggs joined by filaments.

The nylon mesh size, the position of the mesh traps in the tank in relation to the water inlet and outlet, and the number of days that the mesh traps should be kept in the tank, showed a significant effect on the number of eggs collected by the device. In our experimental conditions, the most practical and effective method to estimate the parasite population was the use of nylon mesh discs of 65 mm in diameter, with a mesh size of 1.5 mm, suspended near the water surface overflow of the tank, during 3 days. These values can be easily adjusted according to the requirements of each culture facility.

The method described has been used to control and estimate the level of infestation by monogeneans in several tanks of greater amberjack broodstock maintained at the IEO facilities, since January 2014 to now, with a weekly frequency. Thus, we have started to collect relevant information about the monogenean species that may infest groups of greater amberjack broodstock, and about the evolution of the infestation through the year. Further studies and more data are necessary to establish a possible relationship between the level of infestation and some environmental parameters such as water temperature, photoperiod and/or light intensity, as well as other culture condition parameters.

We have found that adult fish can support a certain level of infestation without any signs of disease over a long period of time. As an example, **Fig. 25.4.2** shows the evolution of the number of monogenean eggs collected in a tank with a group of infested greater amberjack broodstock over 14 weeks between January and April 2014. Although the number of eggs varies between a minimum of 61 ± 11 and reaches a maximum of 837± 66 eggs, fish did not show any pathological signs during this period. Monitoring carried out in other tanks (data not shown) confirm that the fish may appear asymptomatic even with some level of infestation. This shows the importance of carrying out a regular monitoring of the occurrence and population of these parasites in the fish tanks, in order to improve the effectiveness of any therapeutic treatment, especially bath treatments for which timing is critical.

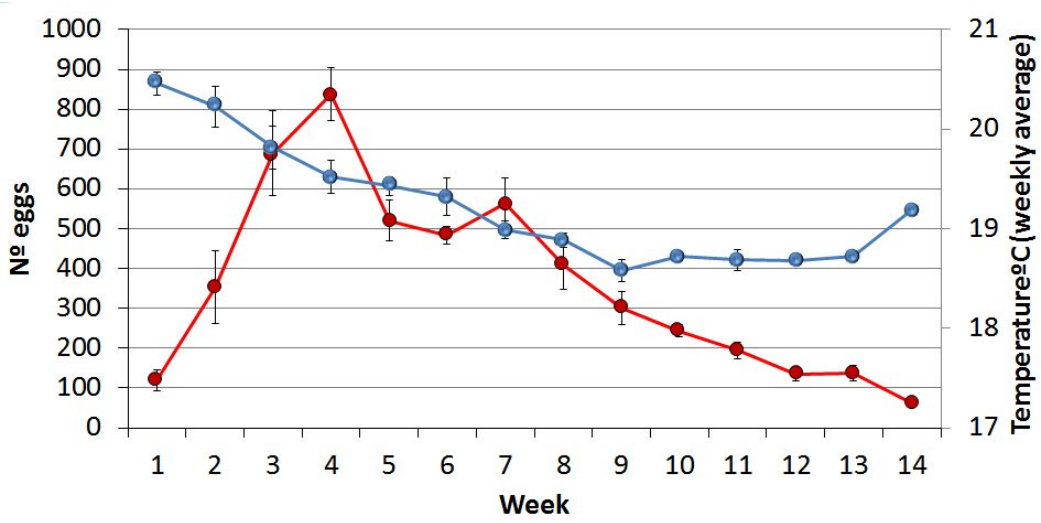


Figure 25.4.2 Number of monogenean eggs collected in the mesh discs (mean ± standard deviation) (n=3) over 14 weeks in greater amberjack broodstock tank F7 (red line) and temperature (blue line).

To date we have identified two species of monogenean ectoparasites infesting greater amberjack broodstock maintained at the IEO facilities.

The first species infests the skin of the fish and was preliminarily identified as *Benedenia seriolae* on the basis of its morphological characteristics. However, the genetic identification indicated that it was *Neobenedenia melleni*. These two species belongs to the same family *Benedeniiae* and are almost indistinguishable given their similar morphological characteristics and clinical signs on fish (stick to the skin, feed on mucus and epithelial cells and causing skin lesions). The main difference between the two species is their host specificity, because *B. seriolae* exclusively infests species of amberjack, while *N. melleni* has low specificity, being able to parasitise a wide range of species. In fact, unlike the vast majority of

monogeneans, which have very strict host specificity, *N. melleni* is notorious for a broad host range (more than 100 species in wild and cultivated specimens and a wide geographical distribution).

The second species infests the gills and has been identified as *Zeuxapta seriolae* by its morphological characteristics and clinical signs on fish (genetic identification will be done as soon as possible).

Mesh discs removed from the tank can be kept for several days in Petri dishes with filtered seawater under natural light to study the development and hatching of the monogenean eggs. Preliminary trials showed that the duration of egg development until hatching at 20°C is 7-9 days for *Neobenedenia melleni* and 4-6 days for *Zeuxapta seriolae*. However, we are currently conducting further studies to determine the effect of water temperature and/or photoperiod on the duration of the egg development and the hatching rates. The effect of the time of day / light intensity on the number of eggs emitted by the adult parasites is also been studied. All these data will provide information about the biology and behaviour of these parasites and may help the development of protocols against monogeneans through the management of environmental culture conditions.

Some of the preliminary results described here were presented at the IV International Symposium on Marine Sciences (Las Palmas de Gran Canaria, 11-13 June 2014). They were also the topic of a Master Thesis directed by S. Jerez and J.R. Cejas presented in the ULL in September 2014.

Task 25.5. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in greater amberjack (led by FCPCT, Daniel Montero).

Some analysis has been conducted to monitor amberjack to establish the seasonality of the potential diseases during the whole project. External morphology, necropsies and samples of liver, spleen kidney and gills are being studied for bacterial content.

The samples obtained were cultured in (BHIB) supplemented with 1.5% NaCl at 25ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl, and the bacteria grown in the culture media were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16S RNA.

Routine Samples obtained during the first sampling period:

1. **January 2014**

Juvenile greater amberjack, stocked at PCTM facilities, 600 g body weight. Symptoms were skin ulcers in the lateral to ventral zone (**Fig. 25.5.1**)

Figure 25.5.1 Skin ulcer in a 600 g greater amberjack.

Microbiology. Three different strains of bacteria were isolated from the ulcers and organs. The strains were sent to the Spanish Type Culture Collection (CECT) for identification. They were identified as bacteria belonging to *Vibrios* of the *harveyi* clade (**Fig. 25.5.2**).

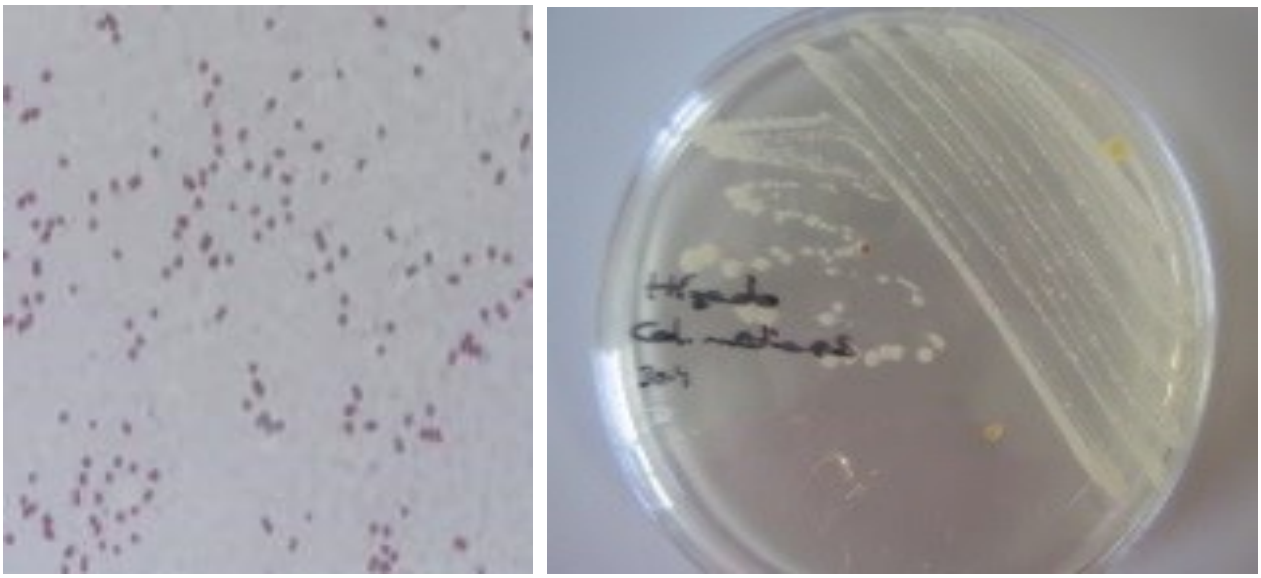


Figure 25.5.2. *Vibrio harveyi* isolated from greater amberjack juveniles

2. April 2014

Juvenile greater amberjack, stocked at PCTM facilities, 900 g body weight. Symptoms were skin ulcers in the Lateral zone and over the lateral line (**Fig. 25.5.3**).

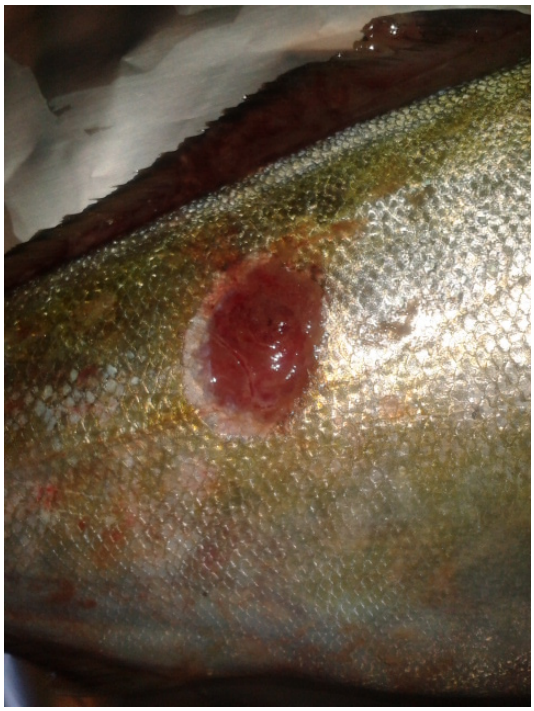


Figure 25.5.3 Skin ulcers over the lateral line in greater amberjack.

Microbiology: Four different strains of bacteria were isolated (**Fig. 25.5.4**). Those isolated strains of bacteria have been kept in the lab to check if their incidence is repeated in time and then to proceed to their identification.

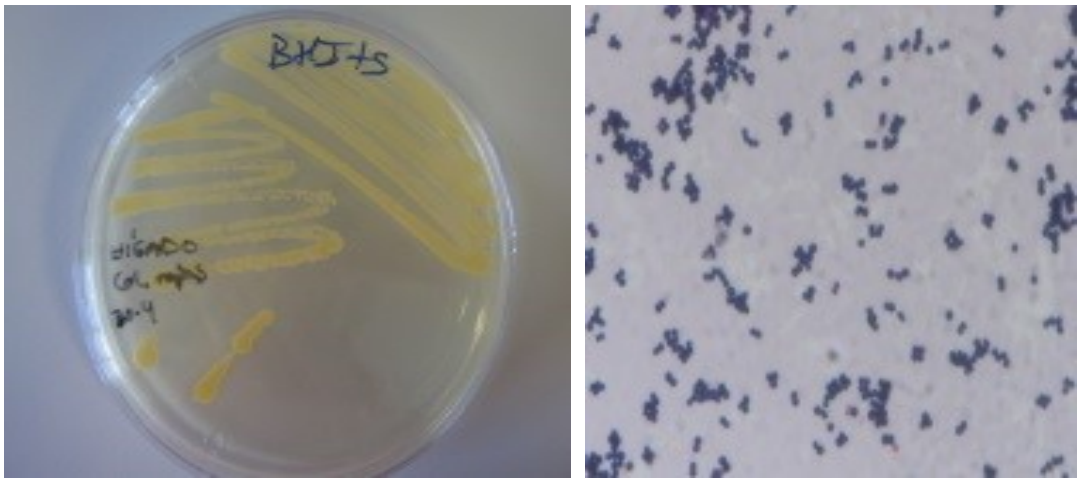


Figure 25.5.4. *Staphylococcus* gram+ isolated from skin ulcer in greater amberjack.

3. June 2014 .

Juvenile greater amberjack, stocked at P2. FCPCT facilities, 1,100 g body weight. Symptoms were skin ulcers, located in ventral zone, close to gills were detected (**Fig. 25.5.5**)

Figure 25.5.5 Skin ulcers of greater amberjack, located in the ventral zone.

Microbiology: Two different strains of bacteria were isolated from ulcers and organs: 1. - Catalase + g + Coco identified as *Staphylococcus epidermidis* . 2.- Coco + g cat - sent to the CECT identified as *Staphylococcus epidermidis*

Challenge test: Sublethal dose of opportunistic bacteria to greater amberjack fry (n=30).

Bacteria/dose: *Photobacterium demselae* subsp. *piscicida*/10^3cfu/fish, by intraperitoneal injection.

Mortality/microbiology: No fish died/ no bacteria recovered. The sublethal dose did not produce losses and seems to be useful for future immunological studies in this species.

Task 25.6 Diagnostic-recommendation manual for greater amberjack health (led by HCMR, Pantelis Katharios).

During the first reporting period HCRM staff visited all farms located in Greece with greater amberjack broodstock for implementing work related to the reproduction WP. During this survey, health status of the broodstock was also assessed and we have identified two important parasitic disease problems which were further investigated.

We examined more than 140 fish in 4 different locations, namely P1. HCMR inland facilities in Heraklion, HCMR cage farm in Souda, Galaxidi Aquaculture and Argosaronikos. All fish examined were affected by gill parasites. We identified the monogenean *Zeuxapta seriolae* in big numbers as well as cysts which were attributed to eggs of the digenean parasites *Paradeontacylix* sp. The monogenean parasite is a blood-feeding worm which causes anaemia while the digenean parasite is a blood fluke the eggs of which are released in the circulation and progressively block the gill blood vessels resulting in focal necrosis. Analysis of these pathologies is still in progress however experimental treatment of the disease using the anthelmintic Praziquantel was extremely effective as verified after visual examination of the treated fish in Souda. In **Fig. 25.6.1** we present some selective pictures from these incidences. Photographic material has already been uploaded to the Project site and the analysis will be included in the recommendation manual.

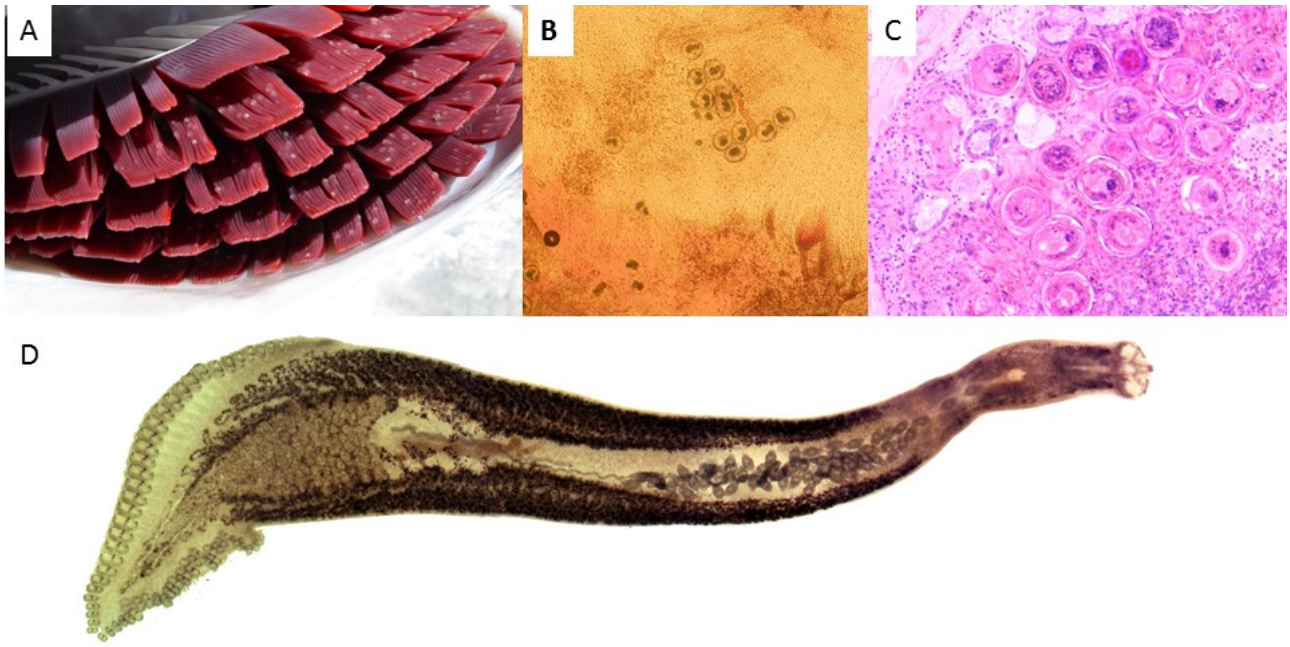


Figure 25.6.1 (A) Massively infected gill of greater amberjack showing white cysts from interlamellar eggs of the digenean *Paradeontacylix* sp. but also ectoparasitic monogenean, *Zeuxapta seriolae*. (B) Fresh preparation of gills showing the digenean eggs. (C) Histological section of the gills infected with the digenean eggs and (D) whole specimen of *Zeuxapta seriolae* from greater amberjack gills.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP No:	26	WP Lead beneficiary:	P7. IMR
WP Title (from DOW):	Fish Health – Atlantic halibut		
Other beneficiaries (from DOW):			
Lead Scientist preparing the Report (WP leader):	Sonal Patel		
Other Scientists participating:	n/a		

Objectives

1. Determine the effect of delivering recombinant capsid protein during late larval stages on protection to nodavirus (VNN).

Summary of progress towards objectives and details for each task:

Task 26.1 Production of VNN capsid protein (led by IMR).

For expression in *E.coli,* the viral ORF encoding the capsid protein was cloned behind an inducible T7-promotor. This gives a rather high expression where the recombinant protein aggregates in the bacterial cells as inclusion bodies, which are relatively easy to purify (**Fig. 26.1.1**).

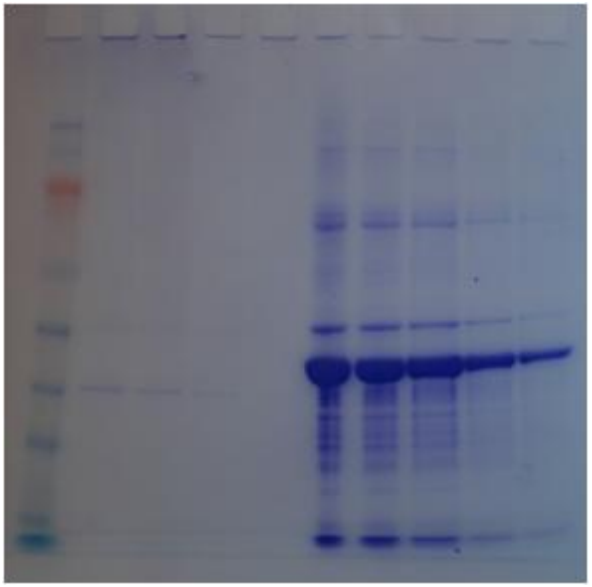


Figure 26.1.1 Commassie Blue stained PAGE for titration of the concentration of recombinant protein. The first lane is the protein standard; the next 4 lanes a 2-fold dilution of recombinant proteins expressed in tobacco plants; and the last 5 lanes a 2-fold dilution of recombinant proteins expressed in E.coli. The recombinant protein expressed in E.coli has got a His-tag at the end, explaining the larger size of the protein.

For expression in tobacco plants the same ORF was cloned behind the CaMV promoter (Cauliflower mosaic virus) for transient expression in chloroplasts, and a method for purification of the recombinant protein has been established (John Innes Centre, Norwich, UK). However, we still have to scale up and optimize the system.

For expression in *Leishmania tarentolae* the viral ORF was cloned into a plasmid vector where it was flanked by sequences of the protozoan 18S gene. The plasmid was propagated in *E.coli*, linearized by a restriction enzyme and introduced into *L.tarantolae* cells by electroporation, where it inserts into one of the 18S genes in the genome. We are now working with optimization of the system and a method for purification of the recombinant protein (**Fig. 26.1.2**)

Figure 26.1.2 Western-blot using anti-sera recognizing the VNNV capsid protein. The first lane is the protein standard. The next 3 lanes are 3 different *L.tarentolae* clones expressing the viral capsid protein. The last lane is the recombinant protein expressed in tobacco plant.

In conclusion, we have managed to express the VNNV capsid protein in all three systems, but optimization of the plant and protozoan systems still remains.

Task 26.2 Monitor and assess immune response and protection (led by IMR).
No work was planned during this period.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

Group Work Packages

Socioeconomics

In the EU, a substantial share of the aquaculture fish that is available in the market is imported from outside the EU. The objective of DIVERSIFY is exploring the biological and socioeconomic potential of new and emerging fish species for the expansion of the European aquaculture industry. Within this objective the socio-economic work package has three main objectives: a) Identifying the market opportunities for these candidate fish species, b) Developing new products made of the candidate fish species and c) Developing business models for marketing of the new species.

In Y1, the activities have been focused on identifying the institutional and organizational context in which the new species can be introduced and on the first steps of identifying consumer segments for the candidate fish species. The macro-environmental context analysis indicated that the political, economic, social, environmental and legal environmental factors support introduction of new species in the market. This growth can only be realized at the expense of other protein sources, since the protein market in the EU has stabilized the last few years. So, market share development or growth for the candidate fish species can be realized only by competing out other fish species – for example imported species – or other protein products such as meat. For production, macro-economic developments suggest that the costs of production are expected to rise in the next 10 years, due to increasing electricity costs and commodity prices.

Concentration of consumer buying has also impact on consumption patterns and consumer choices. While consumption of sea food originally is concentrated in the catch regions, nowadays due to better logistics and preservation methods, consumption is spreading all over countries. The southern EU countries eat more whole fish, while northern EU countries prefer processed fish. With the growth of minority groups in the EU consumption patterns change. Ethnic products are gaining more importance all over the EU, since these groups grow faster than the natives. For the future it is expected that products related to health and well-being will be preferred in food choices, next to price/quality relationships. In most countries the supply and consumer preference will change from preserved and frozen products to fresh products and to more value added products like marinated fish.

This competitive analysis indicated that most of the species included in DIVERSIFY are not well known yet as aquaculture products. The production, if it exists, is relatively small in relation to the wild catch. Meagre is the only one of the candidate species with a substantial production volume. However, in relation to other species, all candidate species are relatively new and unknown species.

In the market development of the species a lot has to be done on brand awareness and brand recognition, while competing species such as tuna, hake, sole, turbot, Pangasius, carp, sea brass and sea bream are well known. Only grey mullet is very well known for people with an Arabic background. This competitive analysis learns that if the objective of the candidate fish species is to penetrate the market with value added products, the added value of, *e.g.*, greater amberjack in relation to the cheap alternative canned tuna must be clear. Or the traditional choice for carp must be challenged by, *e.g.*, pikeperch. What is most difficult for new species is production development in accordance with the market development. In choosing buyers and selecting a market segment this should be taken into account.

Industrial buyers state that consumer preferences concerning farmed fish seem to converge, although certain regional differences still exist within the European market. Consumers are increasingly looking for convenience, which is reflected in the assortment of retailers and foodservice providers. Although certain preferences of consumers still differ between regions; most consumers in Southern countries perceive pre-seasoned fish as being of lesser quality, while consumers in the Netherlands and the UK increasingly purchase these products. Furthermore, consumers in most countries perceive frozen fish as of lesser quality than fresh, which is why most retailers innovate mainly in the fresh fish category. These consumer preferences influence buying preferences within the industry, which implies that international buying

preferences will continue to become more similar. Already, an important trend is the movement towards more standardized products like fish fillets, portioned meals, and processed foods. That this standardization is caused by the increasing influence of a few large international retailers and foodservice providers seems plausible. Many industrial buyers mentioned the importance of sustainability – either as environmental impact, animal wellbeing, , waste management, social standards or feed ingredients. These were all considered important for buying new fish products. But as consumers demand fish to be sustainable but are often not willing to pay more for these products, sustainability is no market driver. Locality (*i.e.*, region) is also becoming more important – something which will benefit EU farmed fish. Overall, product quality and price are consistently important buying criteria.

The overall farmed fish perception of the industry is positive, many buyers see the potential of farming fish to provide reliable supplies, standardized product forms, and relieve pressure from wild stocks. Due to previous media reports on farming practices in Asia and Africa, consumers have become skeptical of farmed fish. Especially in France, Spain, and Italy – also countries where they have a higher preference for locality. Buyers are overall moderately aware of the DIVERSIFY species. Buyers in the UK, the Netherlands, and Germany consider most fish to be more or less the same and require a product to add sufficient novelty to their existing product range. Buyers in France, Spain, and Italy consider differences in species as an asset, which is also related to the way fish is presented to consumers.

In approaching industrial buyers, farmers should to be able to provide full information on their entire production process, feed, and logistics. Most buyers do not have a logistics system to transport fish, as fish transportation is very specialized. Furthermore, buyers need to be able to sample the product and see where the product would fit. Approaching smaller parties might be an interesting option for suppliers who have their own logistics system, as smaller parties are often more willing to cooperate and innovate. An encouraging trend is that buyers are increasingly looking to source directly from farmers. Probably the best place to start selling new species would be at fresh fish counters, where customers interact with sales people who are able to inform and persuade them, and display a new type of fish in full sight.

Overall, what ultimately determines the success of establishing markets for new fish species is the cooperation between farmers to deliver a certain industry standard, to aggregate resources to build a positive image, and market their product together. Farmers thus need to recognize that, although they are inherently competitors, they need to cooperate when they aspire to establish new markets in a Europe increasingly dominated by large buying cooperations.

Consumer survey show that there are differences between the five countries that are selected for the study (*i.e.* UK, Germany, Spain, France and Italy) in values and costs attached to a fictitious new fish species. Consumers in Germany are relatively giving higher scores to functional value, while the southern European countries (Spain and Italy) place more weight on the social values. German consumers tend to provide higher scores on price, whereas Italian consumers give higher scores to performance risk and safety risk. In terms of outcomes (satisfaction, word of mouth and intention to buy) it looks like France and UK are comparatively less enthusiastic, given their scores. Overall, farmed fish is not perceived as significantly better or worse than wild fish. In general, most consumers in the five countries are open to find out more about a new fish species.

A first cluster analysis has given more insights in the market potential for new species in general. This analysis shows that three segments of consumers can be identified:

- Involved traditional consumers (29%): know more about fish and buy traditional fish products;
- Involved innovators (36%): know more about fish and have an open mind to buy new fish products;
- Ambiguous indifferent (35%): know less about fish and are less open to buy new fish products.

Based on the first findings more than 1/3 of the consumers in the five selected countries belong to the segment of 'Involved innovators' and could therefore potentially be open to buy new species. More in-depth analysis in the upcoming year must give insights in the opportunities in the consumer market for the new species and more specific in the five countries.

WP27 Socioeconomics – Institutional and organizational context

WP No:	27	**WP Lead beneficiary:**		P6. DLO
WP Title (from DOW):	Socioeconomics – Institutional and organizational context			
Other beneficiaries (from DOW):	P10. TU/e	P11. AU	P12. APROMAR	
Lead Scientist preparing the Report (WP leader):	Gemma Tacken			
Other Scientists participating:	Rik Beukers (P6), Victor Immink (P6), Machiel Reinders (P6), Olga vd Valk (P6), Michel vd Borgh (P10), Ed Nijssen (P10), Athanasios Krystallis (P11), Javier Ojeda (P12)			

Objectives

1. To give insight in the competitive field and market developments in the European aquaculture market with a focus on the species selected in DIVERSIFY (meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet),
2. To assess the obstacles for growth in the current aquaculture production chains and for these selected species,
3. To identify market opportunities for future growth of the European aquaculture sector for the selected species,
4. Propose a certification framework for the species addressed in DIVERSIFY.

Summary of progress towards objectives and details for each task:
In this work package the institutional and organizational context was analysed. This was done by looking at the macro-environmental and micro-environmental (competitive) factors that influence supply and demand in the aquaculture production chains in general, and the chains of the considered species that are currently in production (meagre, Atlantic halibut, pikeperch and grey mullet) or are supplied by the capture fishery (wreckfish and greater amberjack). This analysis is the basis of WPs 28, 29 and 30, since they define the opportunities and threats for product development, market development and the development of successful new product marketing strategies and the feasibility study.

Task 27.1 External environmental analysis (led by DLO, Gemma Tacken)

Sub-task 27.1.1 (led by DLO, Arie van Duyn,)

Many factors in the macro-environment will influence decision-making of managers of any organization, like in the European aquaculture sector. The methodology used in this Sub-task, the PESTEL-model (Gillespie, 2011) distinguishes 6 macro-environmental factors the social, technological, economic, ecological and political context of European aquaculture that influence for decision-making. PESTEL enables managers, policy makers, traders and researchers to consider which factors are most likely to change and which ones will have the greatest impact as each actor must identify the key factors in his or her own environment. The factors may overlap since particular issues may fit in several categories. PESTEL provides long-lists with factors that might be relevant in the production chain, in this case of the production chains of the specific aquaculture species greater amberjack, Atlantic halibut, meagre, grey mullet, pikeperch and wreckfish.

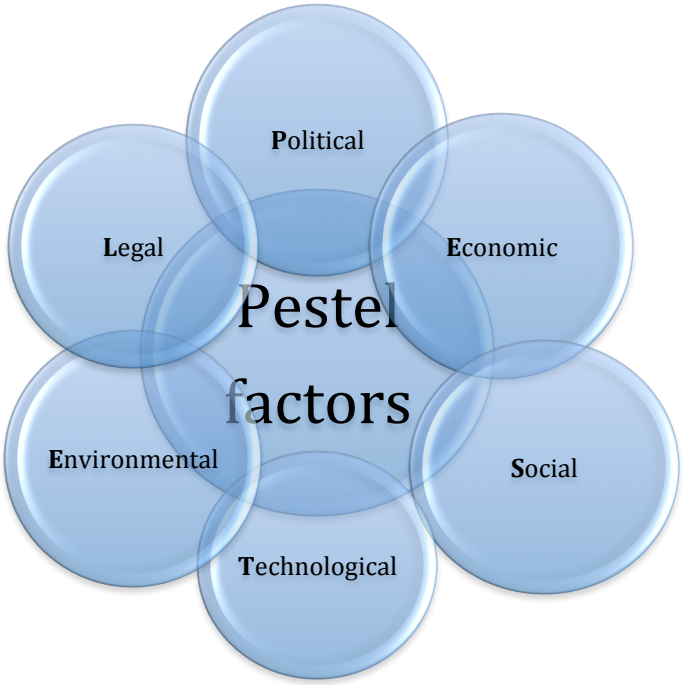


Figure 27.1.1 The PESTEL approach.

The PESTEL approach[3] distinguishes between:

Economic factors. These include interest rates, taxation changes, economic growth, inflation and exchange rates. These factors may have a major impact on a firm's behaviour. For example:

- higher interest rates may deter investment because it costs more to borrow
- a strong currency may make exporting more difficult because it may raise the price in terms of foreign currency
- inflation may provoke higher wage demands from employees and raise costs
- higher national income growth may boost demand for a firm's products

Political factors. These refer to government policies such as the degree of intervention in the economy; for our aim specifically in the import and export of aquaculture fish. What extent does governments subsidise firms? What are its priorities in terms of business support? Political decisions can impact on many vital areas for business such as the education of the workforce, the quality of the infrastructure of the economy such as the road and rail system.

Social factors. Changes in social trends can impact on the demand for a firm's products and the availability and willingness of individuals to work. Food and diet preferences, linked to social status or other factors (such as age) are changing and may affect the preference for certain aquaculture fish.

Technological factors. New technologies create new products and new processes. The logistics of aquaculture fish may become more efficient; the sanitary conditions may improve; which may have an impact on the costs of aquaculture production, transport, food safety etc.

Environmental factors. Environmental factors include the weather and climate change. Changes in temperature can impact on many industries. With major climate changes occurring due to global warming and with greater environmental awareness this external factor is becoming a significant issue for firms to consider. The growing desire to protect the environment is having an impact on many industries and the

[3] Based on Gillespie (ibid)

general move towards more environmentally friendly products and processes is affecting demand patterns and creating business opportunities. Sustainability in aquaculture is one of these issues that has received much attention.

Legal factors. These are related to the legal environment in which firms operate. Changes in trade-related legislation, in particular non-trade measures (NTMs), are important factors to consider.

For this project the following macro-economic factors are relevant to be addressed.

Table 27.1.1 Relevant PESTEL-factors for aquaculture production**.**

PESTEL	Relevant PESTEL-factors for aquaculture production of the new species	Relevant PESTEL-factors for the selected potential markets for the new species (UK, F, ES, It, D)
Political Factors	Aquaculture in politics in the EU affecting the production country now or in the future.	Government policy regarding fish consumption / health now and in the future (e.g. government campaigns to increase fish consumption)
	EU aquaculture sector policies / subsidies / tax policies / environmental laws now and in the future and how this affects the production country .	
	Domestic legal factors that affect how a fish farming company operates, its costs, and the demand for its products in the production country	
Economic Factors	Economic development of the production country now and in the future. This includes economic growth, national income, interest rates (for investment), exchange rates (for imports and exports) and inflation rate.	Economic development of the country now and in the future. This includes economic growth, national income, consumption rate food of household income, interest rates (for investment), exchange rates (for imports and exports) and inflation rate
	Energy prices and/or dependence on imports now and in the future	The current production (fisheries and aquaculture) per species
	(General) Fish meal and fish oil prices, grain prices (e.g. soy & corn) now and in the future.	Developments in retail (concentration) and foodservice (concentration)
Social Factors	None	Consumers preferences: Dietary trends
Techno-logical Factors	Technological capability. Established techniques for hatchery production, grow out husbandry, feeds and culture systems now and in the future	
Environmental Factors	Environmental issues that affect the farming of production of the specific species in the production country now and in the future, such as pollution and water quality now and in the future.	Sustainability: Consume awareness concerning sustainability
		Sustainability: Sustainability certification and eco labelling
Legal Factors	EU legal factors that affect how a company operates, its costs, and the demand in the production country, now and in the future.	None
	Domestic legal factors that affect how a fish farming company operates, its costs, and the demand for its products, now and in the future.	

As indicated above, the factors presented have a different scope in terms of applicability. Some factors apply to more than one country, and some factors apply to several fish species. Having carried out an analysis on the most important PESTEL factors for the production process and export of the six fish species, these are our main findings:

PESTEL for production

Political factors

- As from the start of 2014, the new Common Fisheries Policy has been in effect. At the end of January 2014, the EU institutions also reached a political agreement on the European Maritime Fisheries Fund (EMFF), the policy's financial instrument for 2014-2020. This fund will finance the new fisheries policy and support the sector to adapt to the new policy objectives such as the discard ban. Moreover, it will also support Europe's blue growth policy through the maritime strand of the fund.
- The European Commission intends to boost aquaculture through the Common Fisheries Policy reform and has published Strategic Guidelines presenting common priorities and general objectives at EU level. Four priority areas have been identified in consultation with all relevant stakeholders: reducing administrative burdens, improving access to space and water, increasing competitiveness, and exploiting competitive advantages due to high quality, health and environmental standards.

Economic factors

- Current domestic production (capture and aquaculture) of the six fish species is generally low in all importing markets (Italy, France, Spain, Germany and United Kingdom).
- The economic outlook for 2014 and 2015 generally is moderately positive to positive for both the production countries and the importing markets. The countries that were in dire straits, in particular Greece, Spain and Italy, show the first signs of recovery. In France, recovery remains slow, whereas Germany, United Kingdom and Norway have favourable economic growth expectations.
- Power generation costs significantly increase by 2020 relative to 2010. Average electricity price in the period 2010-2020 increases by 31%. Beyond 2020, average electricity prices remain broadly stable up to 2035 and then are projected to moderately decrease up to 2050.
- Commodity prices are currently high by historical levels. In the first years of the Outlook, crop and livestock prices are expected to diverge, reflecting different supply situations. Meat, fish and biofuel prices are projected to rise more strongly than primary agricultural products.

Social factors

- Not applicable

Technological factors

- Based on the outcomes in DIVERSIFY the established techniques for hatchery production, growout husbandry, feeds and culture systems may alter. This relates to the rearing environment, the feeding system, the diet, husbandry practices, environmental factors, the domestication level and geographical origin, among other things.

Environmental factors

- All cultures require high quality water, so it is crucial that the water quality of both surface and groundwater and the marine environment meets high standards. In Greece, France and Italy, around 25 to 40% of the surface water had a good or better ecological status in 2009. Since EU member states are committed to implement measures under the EU Water Framework Directive (WFD), the water quality is expected to improve dramatically towards 2015 in France and Italy. Ground water quality was moderate in Italy (53% of the ground water had a good or better ecological status in 2009) and good in France (89%) and is expected to improve due to the WFD. Greece has no plans to improve water quality. For Spain, no relevant data were available.
- Sustainability certification for fish species is becoming increasingly important and is expected to become a market access requirement throughout Europe.

Legal factors

- There is a large set of EU policies and rules to be applied, both regarding the EU market and other European issues. The rules and policies can be both limiting, what with competition rules and consumer protection, and stimulating, such as free movement of goods and services and cross-border mobility of workers.
- All production countries have some kind of domestic legislation regarding aquaculture, on the one hand establishing guidelines and basic principles and on the other hand promoting the profitability and competitiveness of the aquaculture industry.

PESTEL for consumption markets

Political factors

- Governments in France, Italy, Spain, Germany and the United Kingdom are running campaigns promoting the health benefits of fish and seafood.

Economic factors

- Retail concentration is low to medium in Italy and medium to high in France, Spain, Germany and the United Kingdom. This means that the retail market in these countries is dominated by a few large retailers.

Social factors

- There is an increasing demand for ready-to-eat and easy-to-cook, value-added fish products such as microwave products or fish fingers. The demand for convenience foods is expected to increase still further over the long term.
- Fish is marketed as a healthy product in northern, western and southern Europe.

Technological factors

- Not applicable

Environmental factors

- Consumer awareness will continue to grow over the short to long term, and the responsible sourcing of fish will become even more important for exporters wishing to export to the European market.
- Sustainability certification for fish species is becoming increasingly important and is expected to become a market access requirement throughout Europe.

Legal factors

- Not applicable

In conclusion, this study shows that for production political, economical and environmental factors dominate the future competitive power. From consumer perspective, there is attention for all themes but no dominant factor can be identified yet.

Sub-task 27.1.2 (led by DLO, Victor Immink and Rik Beukers)

Analysis of current certification schemes and standards and their business dynamics in the different domestic and international supply chains (P6. DLO, P12. APROMAR). In order to get insight in the level playing field of the selected species in relation to wild fish and similar species from other world regions, insight in certification schemes and standards are necessary. This Sub-task has led to Delivarable D27.2 Report on

current certification schemes and standards and their business dynamics in the fish supply chain and on potential interesting certification schemes for the species in Diversify.

Standards and certification schemes for aquaculture are often directly related to capture fishery certification schemes. Although, aquaculture products are in essence different to captured fish and fish products, fish from both the capture fishery and aquaculture share similar morphological characteristics and are offered to consumers in comparable market spaces. Standards and certification schemes in aquaculture address critical issues about environmental and animal health/welfare matters as these are increasing stakeholder concerns driving the creation of powerful certification organizations. Standards and certification schemes are especially useful where there is *information asymmetry* on safety and quality as well as sustainability issues, that is, where buyers and consumers cannot easily judge certain quality aspects of products or production processes. These aspects include what are termed *credence goods* (FAO, 2001). This also explains why certification schemes and standards are increasing rapidly their impact on trading practices in aquaculture marketing channels to bridge societal concerns about aquaculture. These standards and certification schemes build upon national (and international) binding legal requirements that address basic food safety and quality requirements, and imply additional requirements for producers and chain parties.

In certification, 2 types of standards can be identified:

- **Public requirements**

The international regulatory framework for aquaculture and fish safety and quality takes its origin in the following international regulatory frameworks:

- The International Organization for Standardization (ISO) Guide 2: Standardization and related activities – General vocabulary ISO, 2004;
- Binding agreements of the WTO – the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), and the Agreement on Technical Barriers to Trade (TBT Agreement); and
- Relevant food standards, guidelines and codes of practice issued by the Codex Alimentarius Commission (Codex, or CAC).

In addition, specific technical directives were developed for aquaculture, such as:

- HOLMENKOLLEN GUIDELINES FOR SUSTAINABLE AQUACULTURE (1998)
- TECHNICAL GUIDELINES ON AQUACULTURE CERTIFICATION (FAO 2011)
- AQUATIC ANIMAL HEALTH CODE (World Organization for Animal Health 2009)

- **Private standards and certification schemes for aquaculture and fish products**

We distinguish between the following types of private standards and certification schemes:

- Baseline private standards and certification schemes, for example HACCP, ISO, BRC, IFS, GLOBALGAP. These set basic requirements often about production processes and processing of fish.
- NGO-driven standards and certification schemes: for example ACC, ASC, Friend of the Sea, Bioland/ Naturland that focus very much on the farm level.
- Private in-house standards and certification schemes of large retail firms, for example Carrefour.
- EU standards and certification schemes.
- Other standards such as Label Rouge (France) and producer standards and certification schemes, as for example Crianza del Mar (Spain).

Private standards and certification schemes are increasing rapidly their impact on trading practices in aquaculture marketing channels. Although private standards and certification schemes by definition are voluntary, they may in practice become de facto mandatory where compliance is required for entry into markets (FAO, 2011). This is becoming an increasingly common practice both inside and outside the EU. In this way, retailers and food service expand their decision making backward in the channel to include products, food safety, animal welfare and sustainability.

The cost of certification to private standards and certification schemes could range from several thousands to hundreds of thousands of Euro's, depending on the selected standard, the size of the company, the type of operation, and the gap between the current production process and the one required by the private standard. Some costs are direct (licensing fees, audit fees to certification companies) while others are indirect, e.g. management time spent in planning and implementing any improvements required, developing new systems, and the costs of actual plant or gear upgrades. In general, fish farmers and processors bear a disproportionate share of the costs of certification compared with those at the retail end of the supply chain where demands for certification generate. The costs of compliance are disproportionately higher for small operators where they have fewer economies of scale (FAO, 2011).

Considerable geographical differences exist throughout Europe in the importance of private standards and certification schemes, especially with respect to sustainability. In general, large retailers and food service companies in North-West Europe have more requirements than in countries in Southern Europe, where sustainability issues are less dominant, or work indirectly through quality and origin of the product. In North-West Europe a higher proportion of seafood is sold in supermarkets, and there is a greater predominance of processed and value-added products (FAO, 2011).

Although there has been significant expansion in the aquaculture sector, there are still relatively few options for simple certification. Most private standards and certification schemes are specie-specific, meaning that before a new species can be labeled with a particular standard for that fish, standard setting will be necessary. This procedure can be lengthy and expensive for both the certifying body and for the fish farmer delaying its creation until a sufficiently large critical mass of fish are produced and placed on the market. The use of some type of official EU Ecolabel for responsibly farmed fish could be of high interest, also for the DIVERSIFY species. However, this Ecolabel does not yet exist and the European Commission has shelved such possibility, for the moment.

The pressure on fish farmers and processors of farmed fish to comply with private standards depends on the market, how that market is structured, and on the type of product being sold. Large retailers and food firms may not be equally demanding of all their suppliers or product lines. Retail-buyers specifications differ by retail organization, with retailers demanding baseline requirements versus compliance to third party private standards and certification schemes. The pressure on suppliers to conform to stringent private standards and certification schemes depends on the market and the type of product in question. For example, requirements are more stringent for private label that contribute highly to the reputation of the retailer and high-risk processed fish products that require large efforts on food safety and quality, than for basic commodity fish. Therefore, in the follow-up of this research in DIVERSIFY it is important to understand buyers opinion on the use of private standards and certification schemes for the species under study. These additional insights are important as the proliferation of standards causes confusion for fish farmers and processors trying to decide which standard will bring the most market returns.

Furthermore, fish marketers still face safety and quality control regimes that vary from one jurisdiction to the next, as well as a growing proliferation of private standards and certification schemes being introduced by the private sector. Therefore, throughout Europe there are differences in countries or even regions based on cultural values and how standards are embedded in the local technical requirements.

Task 27.2 Competitive analysis (led DLO, Gemma Tacken)

Sub-task 27.2.1 (led by DLO, Victor Immink)

With the Porter five forces model (Porter, 1985; Porter, 1998), a competitive analysis will be carried out for the selected species. For each production chain the current suppliers, customers and markets, substitutes and

potential entrants in the market will be described. These five forces provide insight in the market structure and competitive situation of the five selected species in the market (incl. different products and market segments). This Sub-task will result in deliverable D27.3 Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.

For each species we have performed a Porter five forces analysis (**Fig. 27.2.1**). This is a framework to analyse the level of competition within an industry and business strategy development. It draws upon industrial organization (IO) economics to derive five forces that determine the competitive intensity and therefore attractiveness of a market. This analysis is associated with its principal innovator Michael E. Porter of Harvard University.

Figure 27.2.1 A graphical representation of Porter's five forces.

The five forces, as depicted in figure 1, are:

Bargaining power of suppliers - The bargaining power of suppliers of the fish and fish products is based on supplier volume of supplies (the larger the volume the higher the bargaining power), supplier strategies (strategic skills are suppliers in negotiation), level of differentiation (difference the products from other products in the market).

Bargaining power of buyers/customers – Describes the ability of buyers to put the supplier firm(s) under pressure. The higher the buyer concentration and buyer volume, the higher the bargaining power of buyers. The more product differences and decision maker incentives the lower the bargaining power. Next to that buyer bargaining power is high if the buyer has many alternatives.

New entrants - Profitable markets that yield high returns will attract new firms. The threat of new entrants will be higher if the product can't be protected and thresholds to start production are low. This results in many new entrants, which eventually will decrease profitability for all firms in the industry.

Substitute products – The more differentiated the product, the less the chance that buyers or consumers perceive other products as a substitute. The candidate fish species enter a market with successful and less successful products. Every new candidate will be existence of current fish or fish products increases the propensity of customers to switch to new species based on relative price performance.

Intensity of competitive rivalry - For most industries the intensity of competitive rivalry is the major determinant of the competitiveness of the industry through factors such as innovation, level of advertising expense, powerful competitive strategy and firm concentration ratio.

All these factors together determine the competition in a sector. Below the conclusions over the species and per species are summarised as far as analysed now. More analysis is necessary.

The bargaining power of suppliers

The bargaining power of suppliers for all fish at this moment is low because of absent/small production volumes. Status of production and favourable conditions of access are keys to industry competitiveness. Growth in production and sales will depend upon the capacity of producers and processors to offer products in demand, namely fillets, at good prices. The strategy the producers will deploy are pivotal for their competitiveness. The question upon is, for which markets do suppliers want to produce? Are these local markets and offer a unique product, supplying traditional fishmongers, or are producers aiming at selling throughout Europe. Some species can be market throughout Europe, were others will remain local. What does this mean for production size, facilities, quality, product assortment and promotions.

The buyer power of buyers

The buyer power of buyers / customers is high for all fish because the buyer has many alternatives, all fish have substitutes that currently fulfil consumer demand. Getting access to markets at the 'right' price with the right product is a key factor in the success of any commercial aquaculture project (FAO, 2007). The supermarket is nowadays the pivotal market throughout Europe for marketing industrial farmed products. For the higher end products hotels and restaurants offer a dynamic outlet for high-value farmed species that can find their way to upper-class tourist hotels and restaurants.

Key success factors for supplying retail are good logistic infrastructure (continues supply) and the efficiencies of the handling, transporting and marketing operations as was shown for Seabass (FAO, 2007). This was shown by other aquaculture species recently introduced in the retail market. Price competitiveness is probably the primary enticing factor, as it was for the success of Nile perch as well as has it been for the boom in pangasius sales. Most communication efforts of retailers is advertised of advantageous prices, the weekly sales promotions. A well organized and advertised price promotions can generate a three-to-five times sales increase. Furthermore, as is the case with seabass, sea bream, salmon and others, these are often distributed under the umbrella of the retailers' quality brands. Other deliverables answer the question the terms of conditions that producers need to fulfil and the necessity to market under such quality label.

Substitute products

These potential new entrants of DIVERSIFY species enter an existing and saturated market. In the case of a species, a well-structured industry with industrialized tools and products is developed and the species get well known than these still have substitutes. Three segments could be potentially occupied by these species given the price and volume: large volume with low price, medium volume and medium price, the exclusive segment with high margins (**Fig. 27.2.2**). For each species the key question is, what volume and for what prices can it deliver to a certain defined market. For the producer prices are merely determined by production costs. To what segments the species will deliver depends on the development of production and processing as well as efficiency in organizing supply chains. As an entry strategy it can choose to deliver one retail chain against competitive prices, but then it will bare great risk of a single market. Therefore, the preferred strategy is to diversify in market segments and products by processing these or other options to added value. This way producers and industry work on a portfolio of product to attack substitutes and spread risk.

Intensity of competitive rivalry

For most industries the intensity of competitive rivalry is the major determinant of the competitiveness of the industry through factors such as innovation, level of advertising expense, powerful competitive strategy, firm concentration ratio. Now for the new species of DIVERSIFY these can be offset against market performance of the rivalry products. Former analyses showed that performance can be offset against status of the species

at the moment of introduction in the market, availability in volume throughout the year, overall appetite for fish in the specific country (FAO, 2007). These characteristics differ for each species. Market performance of other farmed species is good for Nile perch, Pangasius, Salmon and Trout. Where market performance is moderate for farmed species Sea bass, Sea bream, Tilapia, and market performance is low for Cod, Turbot.

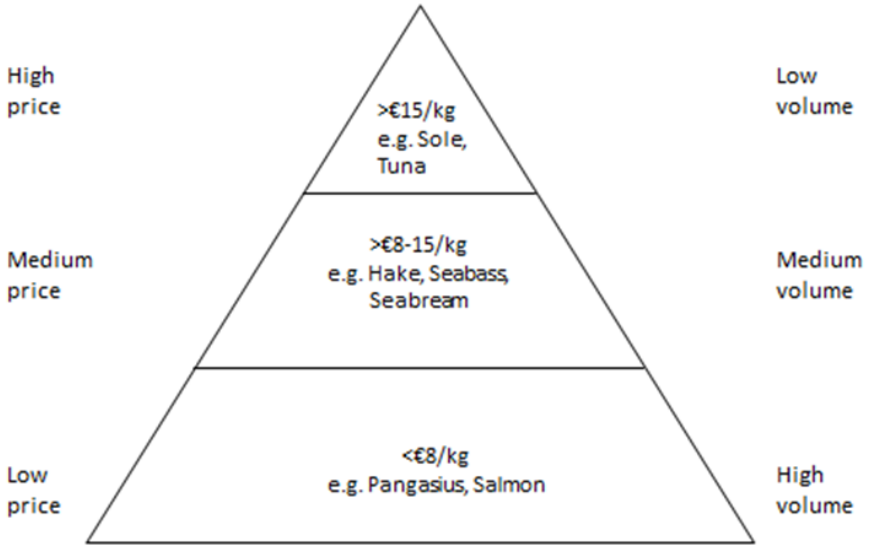


Figure 27.2.2 European retail market for fresh fish divided in three segments

The following actions should be considered for the specific species:

Meagre

Meagre is a large fish with excellent taste, but it is not well known by consumers and the European market is still a niche product. Substitute fish are Sea bass and Sea bream. Sea bass is market throughout Europe and has built market share at price promotions. If Meagre producers and wholesalers develop a marketing plan it should consider how to profit from the reputation of sea bass. If competing on price it will become difficult, therefore additional consumer aspects need to be exposed. Meagre is a fish that can follow the major markets for sea bass and sea bream that are located in southern Europe, where both species belong to fishing and culinary traditions and where domestic production does not satisfy the appetite of the population for the species (FAO, 2007). Therefore these fish should start marketing first in Italy, Spain and France, but if supply is available can lift with the reputation of sea bass in the northern European countries. In northern Europe consumers have little appetite for whole fish, and producers probably aim at less exotic species or filets.

Market orientation for meagre needs to be developed starting for Italy and Spain. Besides retail, commercial catering, medium class restaurants and ethnic restaurants including sushi eating places. Production should focus on fish of around 1 – 2 kg. Small-sized meagre (400–700 g) are not considered suited for marketing as this size fish have a large head, large bones, little flesh, not very tasty, and considered too dark (negra), and consequently not very attractive.

Product development is also recommended as Meagre is excellent raw material for the seafood processing industry as it has excellent fish growth and conversion ratio and is year round available. New product development could support market development. Reputation building needs to start preferably with a

common European name to create scale efficiencies in marketing. Meagre should consider its positioning with regard to gilthead sea bream and European sea bass for both fresh fish and processed.

Greater amberjack

The greater amberjack is a large fish with high flesh quality and market value. Substitute fish is Tuna that is offered at all segments like fresh, frozen and canned. The challenge for greater amberjack is to differentiate as a Tuna plus fish in order to compete on price.

In Europe, there has been interest from the aquaculture sector, but production levels are miniscule. Therefore, a consumer oriented market introduction of cultured amberjack is necessary. A very limited commercial exists in Malta and further aquaculture production needs to be developed. Market orientation could facilitate demand-driven production by developing markets insight in potential countries like Spain, Greece, Italy and Cyprus. Also, market development is necessary for growth with preservation of the added value and price, once production increases.

Pikeperch

There is already a market for Pikeperch in Europe and North America, showing strong demand. Substitute fish are Pangasius and Carp. Where Pangasius is getting well know and affordable Pikeperch is acknowledge for being more tastefull but expensiver as well. Carp is an east-european product and availability is seasonal.

To keep up the high market value, product development and market development is necessary for coordinated growth. Therefore, potential markets and consumer segments have to be identified to maintain or increase the added value for further development.

The main growth market of pikeperch is for countries of Western Europe, such as Germany, Austria and France, where surplus demand is met mostly with frozen goods from Russia, this could be supplied by European farms as well. Already, the production capacity of this fish is expected to grow fast in the coming years.

Atlantic Halibut

Atlantic Halibut is a large fish with a very good reputation in the north European market and a high market value. Demand exceeds the current production capacity. Therefore, product availability needs to have the first priority. Substitutes are Sole and Turbot. These are typical products served at luxery top segment of the markets.

A market development strategy for the long run is necessary. The UK consumption of atlantic halibut is accounting for most of the world reported consumption. There seems a good niche for cultivated halibut in the foodservice sector who like to use fresh fish, therefore market orientation should be focused on the UK food service market as a start. Atlantic halibut has a good fillet yield for a common edible marine fin fish species, so also for processing this is a promising species.

Wreckfish

Wreckfish is a large fish with excellent flesh, but not available as a cultured fish. Substitute with Hake. Market orientation needs to be developed parallel production. Market positioning in relation to other species is necessary for the short run, and for the long run the market potential will be identified.

Grey mullet

Grey mullet is a medium size herbivorous fish for which the market is well developed around the Mediterranean. Substitutes with whitefish. Grey mullet is very well known for people with an Arabic

background and is regarded an important segment to start distribution of these fish. The potential market is all over Europe, especially within segments of population of North African, Middle Eastern or Asian origin. Market and new product development are necessary for growth in the middle-long run in the native European market and the immigrant market.

Sub-task 27.2.2 (led by DLO, Rik Beukers)

The objective of this deliverable has been to identify and map the main trends that are relevant for the European seafood market in general, and also more specifically for aquaculture markets in the selected countries within DIVERSIFY. The identified trends and developments reveal an increasing competition for animal proteins on the long term, which will increase fish prices, and the prices of other proteins. Prices of protein sources will also increase, because of growing production cost. For captured fish, fuel prices are an important cost driver while for aquaculture and other proteins feed prices will have an important effect. At the same time the EU market for animal proteins is facing increasingly higher requirements with respect to quality, traceability, sustainability and animal welfare. Although some of these developments show some differences per country, the main trends are comparable.

The implications of the main trends for the DIVERSIFY project is that SME's could take these trends into account in developing their marketing strategy. In some of the countries included in this study a large share of the consumers have the intention to decrease their meat consumption. This holds opportunities for fish products. Also fish products benefit from being marketed as healthy products, especially compared with meat products. Demographic developments such as the ageing population in the EU will make health issues much more important. In the marketing of the six species of DIVERSIFY, the health aspects should therefore be taken into account. Although aspects such as sustainability, traceability and animal welfare can be seen as requirements that one must meet, they can also be interpreted as an opportunity to give the species within DIVERSIFY an identity. This identity can be used in the positioning the fish products in the market. In some countries, groups of consumers prefer to eat fish that was produced in their own country or region, rather than being imported. This holds both for native consumers and for immigrants. This preference can also contribute to the identity of the fish species.

Overall, key aspects that could be taken into account when marketing the species considered in DIVERSIFY are healthiness, an alternative for meat products, sustainable and local or regional. These characteristics make the species suitable for being marketed as a fresh product for fishmongers and local restaurants. For SMEs it is difficult to sell to large retailers with strict requirements and who demand stable quantities of fish. Fishmongers and restaurants are more interested in fresh fish products that are marketed as local or regional. Selling to fishmongers and restaurants, however, requires short supply chains in order to maintain the quality.

Sub-task 27.2.3 (led by DLO, Gemma Tacken)

International survey in selected countries (UK, France, Spain, Germany and Italy) to investigate the industrial buyers preferences (i.e., purchase managers of up to 10 retailers and food service companies will be interviewed in each country), in order to gain insights into their buying criteria, cultured fish perception (DLO, TU/e). More specific, industrial buyers' preferences in terms of country of origin, suppliers, production circumstances, types of products, pricing and quality will be covered in the survey. These insights have to identify dimensions for expansion of the category. Deliverable D27.5 presents the results of the international survey on industrial buyers' attitudes and perceptions regarding cultured fish.

All interviewed respondents indicated that they had an interest in expanding the fish category (new species, new product forms). There is definitely a growing demand for fish products among consumers and within the industry. Most of the time, new products are introduced within the fresh fish category, and maybe later transferred to frozen. Interviewees indicated that consumers receive their information through the media and look for fish (products) because of cooking shows and restaurants. As fish is promoted as a healthy product, it is bought as an alternative to meat. The **image of farmed fish** is currently an issue, as several bad media reports have stressed the conditions at fish farms in Asian and African countries.

All interviewees noted that there is a definite need for farming fish species to relieve pressure from the wild stocks and to provide more reliable and controlled supplies. European aquaculture can have an advantage when it is 1: able to deliver **high quality fresh fish** instead of frozen (to compete with China, Vietnam etc.), 2: able to provide **a cheaper product than currently available** or 3: able to compete through higher **availability and predictability**. **Sustainability** labels and **certifications** are increasingly used as quality indicators within industry, as there seems to be low (though growing) consumer awareness on the different certification schemes but larger parties face NGO scrutiny when selling unsustainable fish. It is thus also important for suppliers to be able to prove all information ensuring traceability of their products and their ecological footprint. **Certification, Farm management, Supply chain management,** and **Continuity** are mentioned by most interviewees as important criteria to select their suppliers on.

However there are also differences between buyers and countries. These are described below.

General market preferences (A): The ***taste and quality*** of fish products is more important for consumers in France and Italy, compared to the Northern European countries. Furthermore, consumers in France, Italy and Spain ***do not appreciate pre-seasoned fish*** – as can be illustrated by a Spanish buyer's comment: *"They think that it is not fresh when it is already processed, they like to see the whole fish itself so if it's already in plastic they don't want that"*. In the UK, the Netherlands and Germany, consumers prefer to buy ***convenience food,*** pre-seasoned and also innovatively packaged so they need as little preparation time as possible. Consumers in these countries are also ***less aware of the different species existing***. Both of these statements are expressed in the following statement made by a retail buyer in the Netherlands: "*Our customers are looking for convenience, no matter what type of fish it concerns"*. Consumers in the Southern European countries tend to be more ***aware*** of the differences between fish species. Also, clear differences exist for preferences on ***salt water fish*** (as for the UK and France) and countries where they eat ***both fresh – and salt water fish*** (Germany, Italy, Spain)

Category management & range (B): Retailers and foodservice companies in the UK and the Netherlands have a different approach in managing the fish category: they use ***extensive market research*** and evaluate sales data. This means that they manage their product category market driven, or as a UK buyer stated: "*We do a lot of research and we're trying to produce for the market"*. Industrial buyers in France, Italy, and Spain are more driven by their suppliers (i.e., a sellers' market), and tend to follow the Northern European countries on trends and developments in the category. Retailers in the Netherlands, France, and Spain indicated that they use price promotions to compete and promote new products, compared to Germany and the UK which also use branding, storytelling, and seasonal marketing. Note that in Germany there is a shift in the market as discounters like the Lidl are now also moving upward in the fish market and start competing on quality.

Buyers in all countries indicated that they were adding more fillets and processed fish to their ranges, while they ***traditionally prefer whole fish***. Even in France retailer buyers stated: *"we have focused most of our development on processed products, like fillets, steaks and smaller cut pieces"*. ***Preseasoned fish*** seems to be mainly sold in the Netherlands, Germany, and the UK, because consumers in Spain and Italy perceive those as less fresh.

Buying preferences & process (C): Despite of German buyers indicating not to do extensive market research, they too mentioned ***market demand*** as most important for their buying decision. Certification is mainly used by the UK, the Netherlands, and Germany as a main buying preference, although environmental concerns also influence the buying decisions in France.

Price and quality are important to all industrial buyers, but nuances in the term "quality" should be made. To most buyers, quality is definitely related to freshness and consistency. But in France and Italy, quality is also related to the taste of the product. Where buyers in the Netherlands refer to Pangasius as *"Cheap, easy to prepare, and good base quality"*, French buyers say "*You don't buy Panga for the taste"*. Important to mention is also that in the UK, the Netherlands and Germany, the start of the buying process can be ***initiated by both the suppliers and the retailer or foodservice provider***, whereas buyers in France and Spain indicated that this was mostly ***initiated by a supplier approaching them***.

Supplier selection (D): Retailers in France and Italy select their suppliers based on ***price,*** leaving very little to no margins to their suppliers. As one Italian wholesaler noted: *"They allow suppliers to deliver but they*

strictly demand very low prices; no margins left". Buyers in France and Spain value ***local suppliers***, whereas category managers and buyers in the UK, Germany, and Spain select their suppliers also based on their ***innovative outlook***, or as one buyer in the UK formulated this: *"So we would look for any supplier going forward, and that is also an important part of our buying criteria"*.

Farmed fish perception (E): Overall, **the image of farmed fish is positive** among buyers, but remains a difficulty as this image is ***not positive among consumers*** in France and Spain. There is a great demand for Amberjack in all countries, this is considered a high-quality fish and would sell for a high price. Halibut is said to be established, although buyers indicate that they would benefit from more farms. Meagre is also established, buyers would benefit from more farms but not all buyers consider this necessary. Pikeperch is currently established as wild catch in Germany and France, there buyers indicated that they would benefit from more farms for a continuous supply – although they would only consider EU farmed Pikeperch. In France, the image of farmed Pikeperch struggles, this is an issue which needs to be overcome. The interest for Grey Mullet is especially in France, Spain, and Italy, in the Northern countries this fish is relatively unknown. None of the respondents was able to identify a category for Wreckfish, only an interviewee in the UK stated that it would not fit well on a UK menu.

Task 27.3 Opportunities and barriers for growth (led by DLO, Gemma Tacken)

Sub-task 27.3.1 (led by DLO, Gemma Tacken)

A success-failure study of comparative cases will be carried out in order to identify critical success factors for market acceptance, given the legal, organizational, competitive and trend context as analysed in Tasks 27.1 and 27.2. (P6. DLO, P12. APROMAR, P11. AU). This Sub-task will result in D27.6 List of critical success factors for market acceptance. This deliverable is still in preparation and will be ready at the end of Dec 2014..

Sub-task 27.3.2 (led by DLO, Gemma Tacken)

Using the Business Model Canvas approach (Osterwalder & Pigneur, 2010), a business model and supply chain analysis of the participating SME's will be made, by organizing a workshop, in order to identify the presence or absence of the identified critical success factors and opportunities for improvement (DLO, TU/e). At the end of the project (in WP30), this analysis will allow an assessment of the contribution of project innovations, to the competitiveness of the aquaculture sector (DLO, APROMAR, AU). The final outcome of WP 27 will be made available for decision-making purposes in the activities provision further in WP 28 and 29 and will be presented in a report on the analysis of the business models and supply chains of the participating SME's (Deliverable D27.7)

In the next sections we describe in detail each business model for candidate fish species along the 4 pillars of the business model; *product, customer interface, infrastructure management, and financial aspects*. For Greater amberjack and wreckfish this was very difficult, since there is hardly any production yet.

Current Business Model for Grey Mullet

Product. Grey mullet is a species that has been farmed for centuries in ponds in many countries, among others in the Mediterranean region. Grey mullet is a diurnal feeder consuming mainly zooplankton, dead plant matter, and detritus (*i.e.*, low trophic level). The grey mullet is considered an easy to cook cheap fish that has a versatile range of preparations. In addition, a premium product made from grey mullet is Bottarga. Bottarga is an expensive Mediterranean delicacy of salted, cured fish roe (fish eggs), typically from grey mullet or tuna. The product is similar to the softer cured mullet roe, karasumi from Japan and East Asia. Bottarga is a hand-made product that is massaged by hand to eliminate air pockets, then dried, and cured in sea salt for a few weeks. Market prices for this product easily go up to €100,-/kg.

Customer interface. Whole mullet is usually marketed fresh or chilled to a traditional mass market, but gutted mullet is also accepted. Older and frozen mullet are considered of inferior quality in its main markets: the southern and eastern Mediterranean region. Most of the mullet is sold in the country of origin, where demand is increasing (no export). Farmers have close relationships with their buyers and communicate with dealers and Bottarga makers face-to-face or by telephone.

Infrastructure management. Farmers focus mainly on breeding, farming, and processing of the grey mullets. Most of the grey mullet fry is collected from the wild (coastal waters and estuaries). Then the fry is nursed to fingerlings in grow-out farms and brought to ponds for further on growing. Often these stages are carried out by different companies. The future expansion of flathead grey mullet farming is expected to be limited because it depends mainly on wild fry or fingerlings (which is cheaper than hatchery produced fry or fingerlings). Key resources for farmers money/capital for the production facilities, knowledge of the farming process, good water quality, oxygen, temperature, and energy. Farmers work closely with hatcheries, other farmers, feed suppliers, scientists, and processing companies.

Financial aspects. The cost model is cost-driven (i.e., lowering production costs). Labour, feed, and transport take up 65% of the total costs of production. Market prices are mainly determined by market prices of wild catch (i.e., availability of fish).

Current Business Model for Atlantic Halibut

Product. The flatfish Atlantic halibut is among the largest bony fish and the largest flatfish in the world (up to 4.7 m in length and 320 kg.). Atlantic Halibut mainly consumes other fish (e.g., cod, haddock and herring) and therefore has a relatively high trophic level. The Atlantic halibut was formerly a popular food fish but due to overfishing and slow population growth its fishery has largely collapsed. In response to this, Atlantic Halibut has attracted investment in fish farming. As of 2006, five countries - Canada, Norway, the UK, Iceland, and Chile - were engaged in some form of Atlantic halibut aquaculture production. Atlantic Halibut is an endangered species and farming is considered a viable option. Farmed Atlantic Halibut is marketed as a branded fish (e.g., Sterling) with a high quality flesh. Low production volumes and relatively high demand keep market prices high. Atlantic Halibut is a difficult to prepare fish.

Customer interface. The market for farmed Atlantic Halibut is a niche market. Main customer segments for farmers are specialized fish dealers and chefs from restaurants. Farmers hold close relationships with the customers and aim to further develop the small set of relationships in a personal manner (face-to-face, telephone contact).

Infrastructure management. Farmers of the Atlantic Halibut have now mastered the breeding and farming of the fish species. But this was difficult as little was and still is known about the breeding and growth in wild. Farmers also carried out part of the processing of the fish, but given the high demands of customers and difficulty in processing the fish farmers increasingly outsource this to specialized partners or customers. Key resources for farmers money/capital for the production facilities, knowledge on the farming process, good water quality, oxygen, temperature, and energy. Farmers often manage a large part of the farming process themselves (i.e., from hatchery to mature fish). To support and improve this process they work closely with scientists. Next to that they increasingly collaborate with processing companies.

Financial aspects. The cost structure is cost-driven but in the near future can also move more towards a value-driven model with a focus on branded halibut. Labour, feed, and administration take up 62-80% of the total costs of production. Market prices are mainly determined by market prices of wild catch (i.e., availability of fish).

Current Business Model for Pike perch

Product. Pikeperch (a.k.a. zander) is a freshwater fish that has a long tradition as farmed species. It is considered one of the most valuable food fishes native to Europe. Pikeperch is especially well suited for fish fillets, sushi, and sashimi. It can also be served whole, baked, smoked, or cooked. In some culinary circles, pikeperch (or zander) is appreciated even higher than salmon. The fish is low in fat content (usually 1-2%)

and has highly assimilable protein, which makes pikeperch meat highly valued by dieticians. The fish is usually sold frozen as gutted whole fish, fillets with skin, or skinned fillets. The fillets are usually sold in the following weight categories: 120-170 g, 170-230 g, 230-300 g, 500-800 g, >800 g. Pike perch is less frequently sold fresh. Smaller pikeperch (total length >45 cm, body weight <1.0 kg) is perfect for frying, poaching, or grilling. The main producing countries are the Czech Republic, Denmark, Hungary, Romania, Tunisia, and Ukraine. In addition, pikeperch are also grown in the Netherlands and Poland. Currently most fish is imported from capture fisheries from countries such as Russia or Kazakhstan. But supply is characterized by large fluctuations. As a consequence, wholesale prices for pike-perch fluctuate significantly but usually range from USD 5.6-12.5/kg (whole fish) with a mean of about ~USD 8.3/kg. Pike perch farms can counter this by supplying fish year round (by controlling water temperature). In addition, these fish are of high quality and in the near future can be supplied in customized sizes. Finally, in some countries such as France it is advantageous to farm the fish in France itself (i.e., country of origin is important). However, to date the total volume of farmed fish is relatively small.

Customer interface. Pike-perch is mainly sold in countries of Western Europe, such as Germany and France. In these countries prices can be as high as USD 22.2/kg. The fish is sold as a high quality domestic fish through a small set of dealers and chefs. Farmers retain close relationships with their main customers.

Infrastructure management. In the last decades farmers have gained more knowledge on intense farming of pikeperch (as an alternative to farming in ponds). Farmers collaborated with scientists to develop methods for intense pikeperch aquaculture production, mainly in recirculation aquaculture systems (RAS). Only a few farms keep broodstocks implying that most farm facilities depend on these suppliers for supply of fry or fingerlings. Culturing pikeperch in the isolated environment of RAS facilities mostly use water from wells that in some cases deliver constant water temperature (~25 degrees Celsius). This ensures year round production capability but also lowers energy costs (i.e., no costs for heating water). One of the bottlenecks in this field remains the low effectiveness and high costs of rearing larval pikeperch in RAS.

Financial aspects. The cost structure is cost-driven but in the near future can also move more towards a value-driven model with a focus on branded pikeperch. The following contribute to the costs of producing fingerlings in RAS: labour 40%, energy 28 percent, feed 12%, fry 20%. The cost of producing 10 g fingerlings in RAS (2009) is ~ USD 0.6/individual. Labour costs are high due to small scale of production and low level of atomization of farming process. The cost of producing marketable pikeperch (final body weight 1.5 kg) is estimated to be around USD 6.2-7.0/kg. Market prices are benchmarked against the price for wild catch and import from outside Europe.

Current Business Model for Meagre

Product. The meagre (also known as shade-fish, salmon-basse, or stone basse) is found mainly in the Mediterranean and Black Sea, and along the coast of West Africa (Haffray et al., 2012). It has attractive attributes for the consumer market that include large size, good processing yield, low fat content, excellent taste and firm texture (Monfort, 2010). Meagre is a carnivore and therefore has a relatively high trophic level. The history of meagre in aquaculture is quite recent and the first commercial production (in France) was recorded in 1997 while the first commercial fry and juvenile production (Italy) was first reported in 2002. Meagre is mostly sold as a whole fish or in fillets. However, meagre farmers are trying to differentiate between products. Its size is very suitable for processing. Smaller fish (body weight from 600 g to 1 kg) are sold whole or filleted. Larger fish (body weight from 1 kg to 3-5 kg) are sliced or filleted and smoked. The smoking procedure is a relatively new technique and provides good results. Because of its very high content of polyunsaturated fatty acids meagre meat quality is considered very good. Production of farmed meagre is limited (produced in southern France, Corsica, and Italy) with 10,221 tonnes in 2012.

Customer interface. Meagre is mainly sold in southern France and Italy. Prices are around EUR 7-12/kg. Fish is supplied from both capture fisheries and aquaculture. However, demand for meagre is still low as it is relatively unknown to the consumer (sea bass and sea bream is more known and appreciated). Yet given the high quality of the flesh (also quality labels installed such as Label Rouge), diversity in processing, and the rapid production it may become an interesting alternative in the coming years. Currently the fish is sold to a close set of customers (e.g., dealers, restaurants).

Infrastructure management. The farming of meagre is similar to sea bass and sea bream. Also meagre feed is comparable. Reasons why farmers have not yet scaled up their production has several reasons. First, the number of hatcheries is limited. Second and relatedly, the market demand remains low, which also caused that firms do not invest in building hatcheries. Third, meagre is often produced in farms that also produce sea brass and sea bream causing internal competition for resources in favour of the latter. A major bottleneck in the production of meagre is limited knowledge about the rearing procedure (caused due to limited number of hatcheries and broodstocks). Collaboration with scientists therefore is important in the coming years to overcome this bottleneck in meagre production.

Financial aspects. The cost structure is cost-driven. Costs for the meagre production is difficult to provide as production numbers are still low. For land-based systems (ponds) costs depend mainly upon the size of the farm. For cage culture the major expense is the cost of juveniles (limited supply; can only be bought in the South of France). Generally, feed is another major cost (during grow-out) but lower than other comparable marine fish species given better FCR (FCR for meagre is 1.7:1). Market prices are based on total availability of wild catch and farmed fish, but has relatively low margin due to low demand.

Greater Amberjack (No current business model)

Product. The greater amberjack is a fast growing bony fish that is found in the Mediterranean Sea, the Atlantic Ocean, the Pacific Ocean and the Indian coasts. The greater amberjack is a carnivore and is a powerful hunter, which feeds on other fish and invertebrates having a high trophic level. Due to its excellent flesh quality it is considered an excellent eating fish (firm texture and rich flavour of their flesh). Its rapid growth (i.e., short time to market size), large size, worldwide market availability, and high consumer acceptability makes this fish very attractable for the aquaculture sector (Nakada, 2000). However, production of greater amberjack remains very low (Mediterranean production in 2012 was only ~2 t). Next to that, there is limited commercial activity with hatchery-produced individuals in Malta. Main bottlenecks are the lack of reliable reproduction and lack of production of juveniles.

Other amberjacks (*Seriola spp*), such as the Japanese yellowtail (*Seriola quinqueradiata*) are produced on a larger scale in Japan and Korea. For instance in Japan 120,000 tonnes are produced each year. Farmers are supplied with wild fry and feed the fish with extruded pellets. Most *Seriola spp* are produced in cages, but trials for land-based amberjack culture are also implemented. Costs for greater amberjack feed can be kept reasonably low by feeding them on fish of low commercial value (i.e., trash fish).

Wreckfish (No current business model)

Product. Wreckfish is a deep-water marine fish that can be found in both the Atlantic and Pacific oceans usually in water depths from 140 feet up to 3000 feet. It is a fast-growing fish that is easy to bone and has good quality flesh that is easy to cook. Wreckfish is also attractive for the aquaculture industry because of its high market price and limited fisheries landings, and easy of manipulation in captivity. However, lack of reproduction control and limited broodstock has inhibited the commercialisation of farmed wreckfish.

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Deviations from Annex I and their impact:

The majority of the proposed Deliverables has been submitted as planned. Deliverable 27.1 had some start-up problems, as it took some time to compose an experienced research team, but was ultimately delivered in the end of May 2014. Deliverable 27.2 also had some start-up problems but was delivered in the beginning of March 2015. Deliverable 27.3 had team problems, therefore the Deliverable didn't have the appropriate scientific quality at due date (Mo 12). Therefor, this deliverable will be submitted at the end of January. Deliverable 27.4 was delivered a week after due date, since revisions had to be made. Deliverable 27.5 was delivered in time, but the response in some countries was quit low. Retailers and food service buyers were not very willing to participate in the research, since they don't want to be held on earlier statements. Deliverable 27.6 is due at the end of January. Since not all deliverables before are finished, this Deliverable can't be finished earlier. Deliverable 27.7 was finished in time.

So, in general after some start-up problems delayed the first deliverable, lots of work has been done in the first year according the DOW.

WP No:	28	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Socioeconomics – New product development			
Other beneficiaries (from DOW):	P1. HCMR	P6. DLO	P10. TU/e	P11. AU
P15. ULL	P18. CTAQUA	P38. HRH		
Lead Scientist preparing the Report (WP leader):	Luis Guerrero			
Other Scientists participating:	Kriton Grigorakis (P1), Athanasios Krystallis (P11), Covadonga Rodriguez (P15), Rocio Robles (P18), Hellas - Maria Saltavarea (P38)			

Objectives

1. To develop new product concepts from selected species, by incorporating consumer and expert input,
2. To select product ideas and develop physical new products from the selected species,
3. To monitor the quality of new products in terms of organoleptic characteristics and nutrition-rearing history,
4. To make a technical assessment of the products.

Summary of progress towards objectives and details for each task:

Task 28.1 Product concept development: technical and consumer-driven (led by AU, Athanasios Krystallis)

Sub-task 28.1.1 (led by AU, Athanasios Krystallis)

This first sub-task concerns the execution of a series of focus group discussions with consumers and experts in the selected countries of the project (UK, D, ES, F, I). The objective of the focus group discussions is to generate input in the form of a set of ideas that will be screened out and further developed into product concepts for further testing in subsequent tasks in the new product development process that is the purpose of the entire WP28 and WP29.

More specifically, Sub-task 28.1.1 builds directly on the segmentation exercise and results of sub-task 29.1.2 that is completed in the first 12 months of the project. In sub-task 29.1.2, a number of consumer segments are identified in the five target-countries of the project based on a multitude of criteria incorporated in the large survey undertaken in Sub-task 29.1.1. These criteria include a) a number of psychographic constructs that impact on the value consumers perceive in relation to the new fish products (i.e. consumer involvement, innovativeness, subjective knowledge, etc.); b) perceptual trade-offs of gains and losses from the hypothetical consumption of the new fish products (*i.e.*, functional, hedonic, emotional etc. values; and price, effort, familiarity, evaluation etc. costs); and c) a series of socio-demographic and fish consumption-related behavioural characteristics.

Based on the segments identified with the above-described criteria, two focus groups per country with 6-8 consumers each, and personal interviews with 3 experts per country will be conducted in early year 2 of the project. Screening criteria for consumer recruitment will be based on the outcomes of the segmentation task above, so that each of the two focus groups represents one segment of high policy and strategy priority for the project across the 5 target-countries (i.e. early adopters vs. laggards in relation to the new fish products' adoption).

The experts will be consulted in depth interviews. For this consultation stakeholders from the industry will be selected, as well as researchers working for state or private agencies, policy makers and consumer representatives with strong impact on the micro- and macro-environment of the fish industry and market. Each expert participant will be recruited with the direct involvement of local partners in each of the 5 target-countries. Per country 3 stakeholders will be interviewed. In order to have different stimuli to show to the participants in the different focus groups an extensive search for new developed fish products have been carried out by means of the GNPD (Global New Products Database) from Mintel. Figure 28.1.1 shows some interesting products to be used for the proper development of the focus groups.

Figure 28.1.1 Example of some of the fish products to be used as stimuli during the focus group discussions.

Sub-task 28.1.2 (led by HCMR, Kriton Grigorakis)

No work done during this period

Task 28.2 New Product Development (led by IRTA, Luis Guerrero)

Sub-task 28.2.1 (led by HCMR, Kriton Grigorakis)

This sub-task focuses on the estimation of optimum fish sizes for developing the selected new products. The activities to perform include somatometric measurements for the five species of interest (meagre, greater amberjack, pikeperch, wreckfish and grey mullet) as well as their chemical, physical/mechanical and sensory characterization.

During this first year only pikeperch has been analysed for fatty acids (P1. HCMR) and somatometric measurements (P3. IRTA). Fish were slaughter according to custom commercial methods and transferred within a day to the lab for analyses. The somatic measurements included whole and gutted fish weights,

visceral weights, liver, gonad and fillet weights. These were used for the calculation of basic somatometric indices: dressing rate, viscerosomatic (VSI), hepatosomatic (HSI) and gonadosomatic (GSI) indices and filleting yield, all expressed as percentages of gutted weight, viscera weight, hepatic weight, gonads weight, respectively to fish total body weights. Also the Condition index was calculated as: Condition index (CI) = [100× body weight (g) / body length3 (cm3)]. Regarding fatty acid analysis 10 individuals of pikeperch and a sample of the feed received were sent to P1. HCMR for fatty acid analysis. All samples were transferred frozen and vacuum packed and remained so until analysis. Total lipids were extracted from 1 g of dorsal muscle and purified, based on the procedure of Folch et al. (1957). Subsequently, the extracted lipids were transesterified with anhydrous methanol containing 2% sulphuric acid for 16 h at 50°C under nitrogen (Christie, 1989). Fatty acid methyl esters analysis took place as previously described by Fountoulaki et al. (2003). A GC-FID (Varian 3300) was used for this purpose, equipped with a flexible fused silica Megabore column (Length: 30 m, Inner diameter: 0.32 mm, Film thickness: 1μm) with a bonded stationary phase of CP-WAX. Helium (purity 99.999%) was the carrier gas.

The somatometric indexes of the studied pikeperch are presented in Table 28.2.1. Feed and tissue analysis for the rest of the studied species are pending. Samples are already available. The fatty acid profile (%) and total contents (mg/g muscle tissue) of the feed and the fish (n=10) are shown in Table 28.2.2.

Table 28.2.1 Somatometric indexes of pikeperch (n=10, mean value± SD).

Dressing yield	VSI	HSI	GSI	Filleting yield	Condition index
93.5±1.26	5.73±1.30	0.81±0.18	0.23±0.26	36.2±4.42	0.71±0.13

Table 28.2.2 Fatty acid contents (mg/g feed or tissue) and profile (%) of feed of pikeperch fillets (n=10, mean value± SD).

	Feed		pikeperch	
	mg/g	%	mg/g	%
Saturates	33.5	29.2	1.41±0.15	29.7±1.72
Monounsaturates	37.3	32.5	0.59±0.15	12.3±1.86
n-9	33.5	29.2	0.50±0.14	10.3±1.81
n-6	12.6	11.0	0.26±0.04	5.40±0.46
n-3	29.7	25.9	2.00±0.21	42.0±1.83
EPA (20:5n3)	10.4	9.06	0.33±0.04	6.96±0.41
DHA (22:6n3)	12.6	11.0	1.64±0.17	34.5±1.87
ARA (20:4n6)	0.89	0.77	0.09±0.01	1.82±0.20

Regarding sensory analysis an extensive investigation of the most relevant descriptive terms to characterize fish and fish products have been carried out. Sensory descriptive methods comprise different steps depending on the technique chosen, but in general all of them have in common the selection of the appropriate sensory descriptors to assess (Murray et al., 2001). The quality and reliability of a descriptive profile is closely linked to the accurate selection of the attributes included in it (Montouto et al 2002).

In order to train a panel of assessors and to have a descriptive profile for the sensory evaluation of the selected species eighteen different fish species were selected at the fish market based on visual differences (size, colour, shape) and expected in-mouth characteristics and availability throughout the length of this project. Samples were acquired in fillet presentation and were vacuum-packed and stored frozen until evaluation. Twenty four hours before analysis, samples were thawed at 4ºC. In all cases, samples were cooked in an oven at 115ºC during 20 minutes inside individual transparent glass jars with caps to keep the odour from disappearing. These were then placed inside electrical heaters at 60ºC to keep them warm while being tasted. Eighteen panellists with more than four years of experience in sensory profiling of different food products (previously trained according to ISO regulations) were recruited for this study. In order to ensure the right selection of the sensory attributes that will constitute the final descriptive profile a Check All That Applies method (CATA) combined with a Free Choice Profiling (FCP) were carried out.

The number of elicited attributes in CATA and in FCP analysis is shown in **Table 28.2.3. Figs 28.2.1** to **28.2.4** display the relationship between the fish species and the selected sensory attributes. This information will constitute the basis for the development of sensory references during the training of the panellists.

Table 28.2.3. Attributes elicited by means of CATA and Free Choice Profiling.

	CATA		Free Choice Profiling	
	Number of attributes	%	Number of attributes	%
Appearance	19	25	8	27
Odour	21	28	8	26
Flavour	18	24	6	20
Texture	17	23	8	27
Total	75	100	30	100

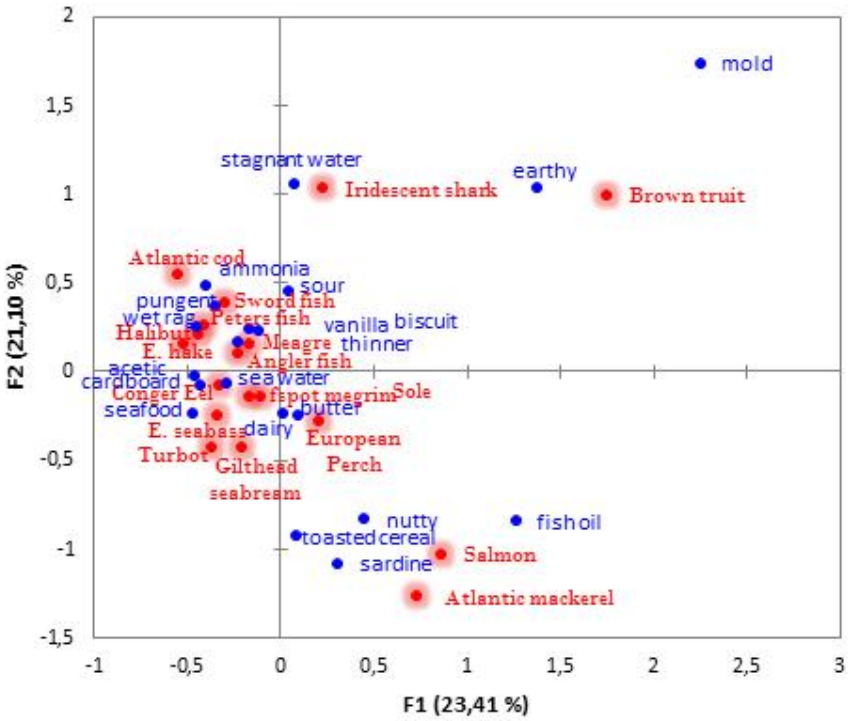

Figure 28.2.1 Fish species and Odour attributes in the first two dimensions of the simple correspondence analysis.

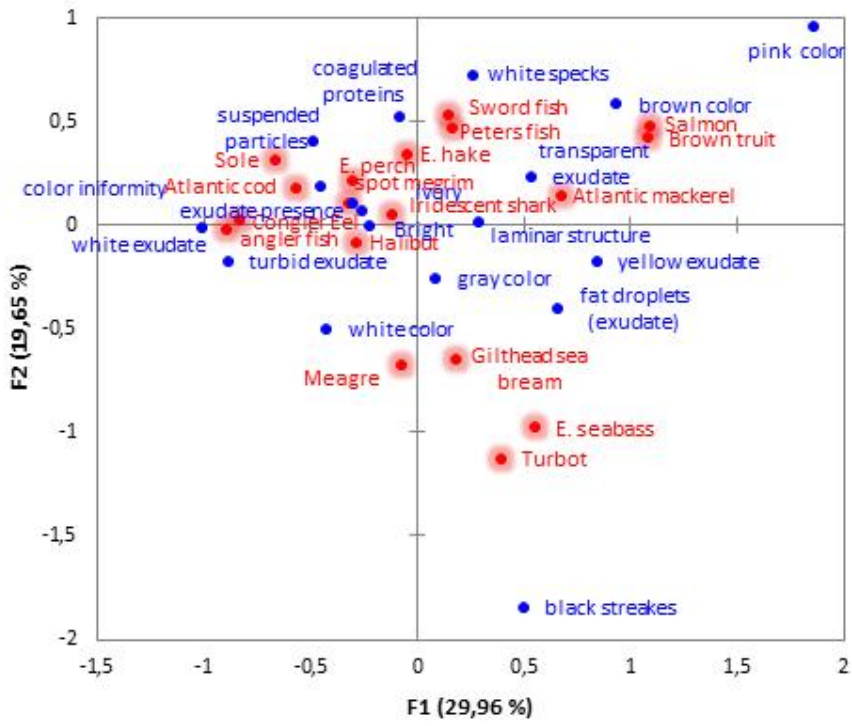

Figure 28.2.2 Fish species and Aspect attributes in the first two dimensions of the simple correspondence analysis.

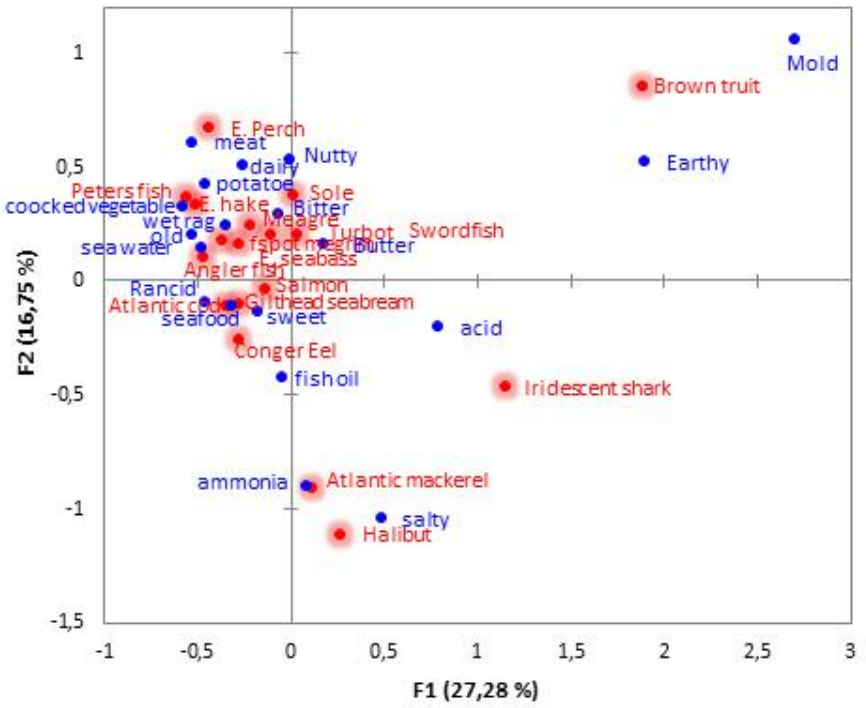

Figure 28.2.3. Fish species and Flavour attributes in the first two dimensions of the simple correspondence analysis.

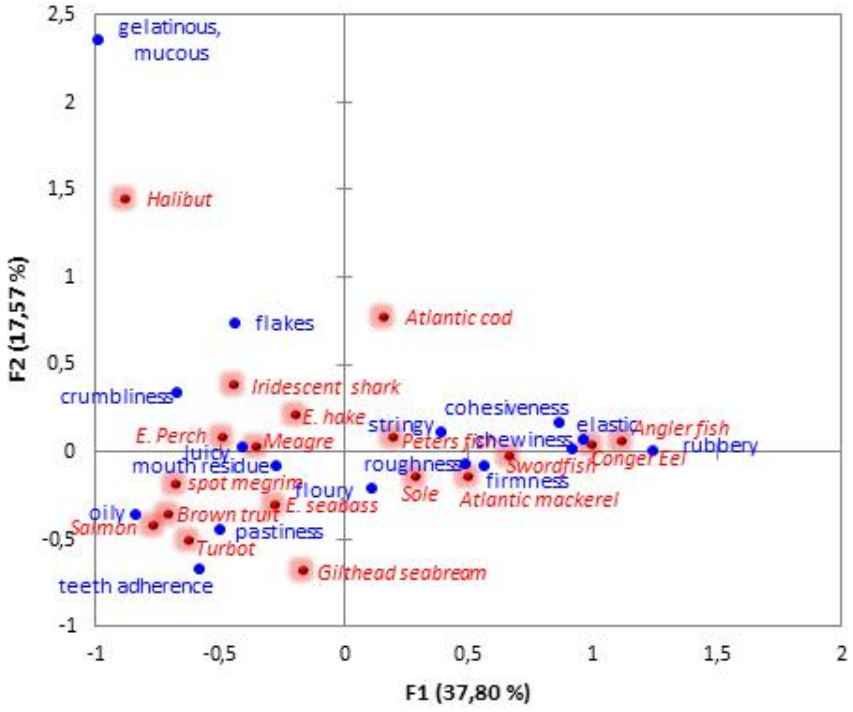

Figure 28.2.4. Fish species and Texture attributes in the first two dimensions of the simple correspondence analysis.

The description provided in these figures will be essential when selecting fish references to illustrate the different sensory descriptors to evaluate. Accordingly, for instance, Brown trout would be an excellent reference to exemplify a high intensity of the earthy character (both in odour and in flavour) or Halibut to demonstrate what a gelatinous/mucus texture is. According to Rainey (1986), sensory references are a key tool in order to train a panel in an efficient and reliable way and constitute the best method to make sensory profiles more objective and comparable. Based on the results obtained in this activity a scientific paper (Journal of Sensory Studies) is being prepared.

Sub-task 28.2.2 (led by IRTA, Luis Guerrero)

No work done during this period

Task 28.3 Monitoring technical quality of the products (led by HCMR)

Sub-task 28.3.1 (led by HCMR, Kriton Grigorakis)

No work done during this period

Sub-task 28.3.2 (led by ULL, Covadonga Rodriguez)

No work done during this period

Sub-task 28.3.3 (led by IRTA, Luis Guerrero)

No work done during this period

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Deviations from Annex I and their impact:

Deliverable 28.1 Results of focus groups with consumers and experts regarding idea for new products will be delayed by 3 months. The reasons for this are as follows:

1. Complicated multi-country analysis was required to decide on criteria for focus group participants' recruitment (analysis got completed before the ACM 2014 meeting in November 2014)
2. Time consuming task to develop the discussion guide in 5 languages

3. Data collection initially promised, now coincides with the Christmas season (given that the start of the project was set to Dec 2013 by the EU), which prohibits any field-work. As a rule of thumb, irregularities in consumer behavior because of the special conditions that prevail during this period, prevents us every year from data collection between early December and early January.

No impact is anticipated, however, on any of subsequent deliverables due to the fact that (a) the requested delay is small and (b) all preliminary actions (*i.e.*, finalization of the screening / recruitment criteria, development of draft and final discussion guides, contact with local field collaborators etc.) will unfold in parallel during the Christmas period, in order to accelerate the process immediately after that.

WP No:	29	**WP Lead beneficiary:**		P11. AU
WP Title (from DOW):	Socioeconomics – Consumer value perceptions and behavioral change			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P6. DLO	P18. CTAQUA
	P38. HRH			
Lead Scientist preparing the Report (WP leader):	Athanasios Krystallis			
Other Scientists participating:	Lluis Guerrero, (P3), Machiels Reinders, GemmaTacken (P6), Hellas-Maria Saltavarea (P38)			

Objectives

1. To analyse and understand overall value perceptions of consumers with regard to cultured fish in general and the DIVERSIFY fish species in particular, and undertake a value-based segmentation study,
2. To evaluate consumer sensory perceptions towards the newly developed DIVERSIFY species' products,
3. To optimize the DIVERSIFY species' newly developed products in terms of ideal extrinsic product attribute combinations that have the potential to generate ideal consumer value perceptions,
4. To determine the effectiveness of market communication in consumer behaviour change in relation to the DIVERSIFY species considered and the new raw and other value added products developed.

Summary of progress towards objectives and details for each task:

Task 29.1 Consumer value perceptions and segmentation (led by AU, Athanasios Krystallis).

As described in the DOW, WP29 starts with Task 29.1, which is relevant for the 1st Period Report of the project. Task 29.1 runs for the first 24 months of the project and corresponds to the first objective of the WP, as described above. Task 29.1 is further organized into sub-tasks 29.1.1 (months 1-9), and 29.1.2 (months 10-24).

Sub-task 29.1.1 (lead by DLO, Machiel Reinders)

This first sub-task concerns the designing and execution of an international online consumer survey in the 5 countries selected (i.e. UK, Germany, Spain, France and Italy), with (nationally representative) samples of n=500 consumers in each country. The purpose of the survey is to investigate consumers' associations with and perceptions of the new products developed, attitudes towards established and new aquaculture as opposed to wild fish, buying intentions, willingness to pay, and overall value perceptions of consumers in the five target countries. The information gathered determines consumer perceived value as a trade-off between beliefs about gains (i.e. benefits) and losses (i.e. sacrifices) from the purchasing/consumption of the examined new products.

More specifically, in the first 12 months of the project the following work has been completed:

1. Development by lead partners P11. AU and P6. DLO, after extensive consultation with P3. IRTA and P38. HRH of the research protocol for the execution of the online survey in the five target countries according to the objectives of the survey as described above;

This protocol operationalizes the following conceptual model call Customer Value model (**Fig. 29.1.1**), more information about which can be found in Deliverable D29.1.

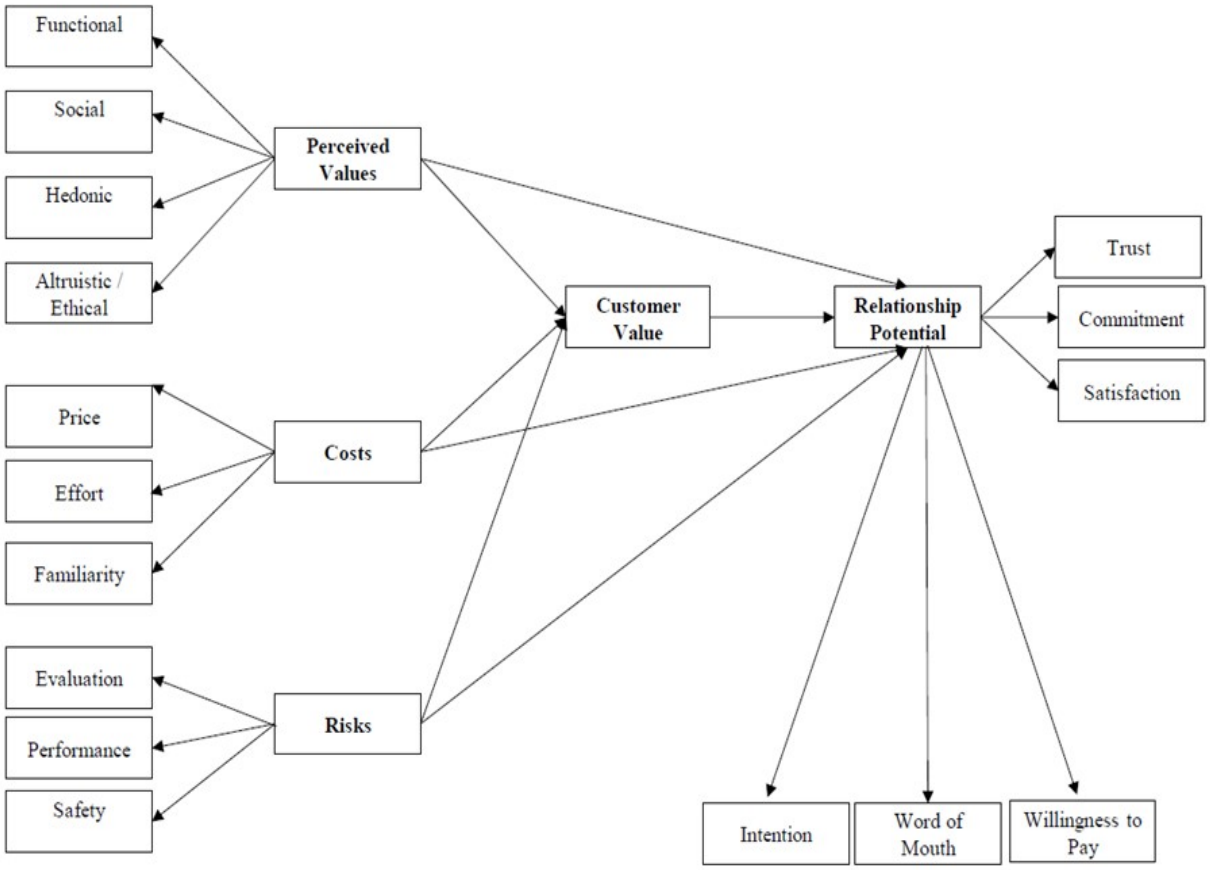


Figure 29.1.1 Customer Value model

2. Execution of a pilot online study with (approx.) n=100 consumers in each of the target countries in order to statistically test and validate the questionnaire that resulted from the research protocol and that will be used for the main data collection phase;

3. Analysis of the pilot data in order to make all necessary modifications to the questionnaire. This analysis completed in June 2014;

4. Execution of the main data collection phase with (approx.) n=400 consumers in each of the target countries. Main data collection completed in July 2014; and

5. Preliminary analyses of the datasets at both the country-level and the pooled data (merged country)-level. The analyses first concerned the testing of differences between the pilot and the main datasets per country. No statistically significant differences have been identified, so the pilot and the main datasets have been merged to formulate one master dataset per country.

Sub-task 29.1.1 consisted of an online consumer survey with minimum N=500 consumers for each country (nationally representative samples) was run in the five study countries (i.e., DE, FR, UK, ES and I). A conceptual model with various components (i.e. types of perceived values, perceived costs and risks, and evaluative outcomes) is operationalized in a questionnaire. The questionnaire is identical for all countries, created in English, translated into the different national languages and back-translated as appropriate. Respondents received a description of a fictitious fish species (i.e., a new marine finfish species, based on the specifications of the five species under consideration in the DIVERSIFY-project). Considering this description, respondents had to indicate the perceived values, perceived costs and risks, and evaluative outcomes. In addition, the questionnaire asked questions about personal characteristics of the respondents,

their beliefs about farmed fish, their knowledge about fish, their current fish consumption, and their awareness and evaluation of three different certification logos.

Sub-task 29.1.1 resulted in Deliverable D29.1 (expected date: month 9, actual date: month 7), a dataset of consumer perceptions, attitudes, buying intentions, willingness to pay and value perceptions towards the selected species. D29.1 includes a detailed overview of all preliminary analyses' results at the country level.

Following, we present below a summary of the most significant of those results based on the basis of a number of tables (**Table 29.1.1** and **Table 29.1.2**). The first part of the questionnaire focused on the values that respondents attributed to the fish species. As can be deducted from **Table 29.1.1**, the mean scores are mainly around or somewhat below the mid-point of the scale (=4), indicating that respondents tend to slightly agree with most of the items. Differences between countries are mainly observed for the items of functional value, social value, and hedonic value. The Cronbach's alphas indicate that the reliability of the items for the used constructs were good for all countries.

Table 29.1.1 Mean values for the constructs belonging to the VALUES-component of the conceptual model.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Mean difference-test (*F*-value)	Pooled sample (N = 2511)
Functional value	***α = .91***	***α = .95***	***α = .95***	***α = .96***	***α = .96***		***α = .95***
1. This fish would have consistent quality	2.92	3.28	3.30	3.17	3.07	5.61***	3.15
2. This fish would be well produced	3.04	3.29	3.19	3.23	3.12	2.03	3.17
3. This fish would be a tasty dish	2.85	3.19	3.20	3.12	3.03	4.84**	3.08
4. This fish would be a nutritious food choice	2.65	3.19	2.95	3.04	2.95	8.11***	2.96
5. This fish would be a healthy food choice	2.62	3.24	2.97	3.02	2.99	10.12***	2.97
Social value	***α = .88***	***α = .89***	***α = .89***	***α = .87***	***α = .88***		***α = .88***
6. This fish would be purchased by many people I know	3.65	3.66	3.90	3.54	3.32	9.16***	3.61
7. This fish would improve the way other people perceive me	4.29	4.26	4.28	4.05	3.97	3.57**	4.17
8. Buying this fish would make a good impression on other people	3.79	3.96	3.94	3.63	3.59	5.48***	3.78
9. This fish would give those who buy it social approval	4.16	3.77	3.85	3.49	3.73	10.28***	3.80
Hedonic value	***α = .89***	***α = .91***	***α = .90***	***α = .88***	***α = .90***		***α = .90***
10. I would like this fish	2.90	3.30	3.25	2.99	3.12	5.82***	3.11
11. I would feel relaxed consuming this fish	3.42	3.46	3.37	3.46	3.54	0.76	3.45
12. This fish would make me feel good	3.39	3.65	3.54	3.42	3.31	3.57**	3.46
Ethical value	***α = .79***	***α = .90***	***α = .89***	***α = .91***	***α = .90***		***α = .88***
13. Buying this fish is coherent with my ethical values	3.03	3.48	3.42	3.34	3.25	6.31***	3.30
14. Buying this fish would make good to the environment	3.09	3.25	3.31	3.20	3.06	2.18	3.18
15. Buying this fish would contribute to the survival of the aquaculture industry	3.02	3.27	3.20	3.13	3.10	1.92	3.14
16. Buying this fish would be beneficial to social groups in need (e.g. the children)	4.07	3.58	3.56	3.25	3.47	16.81	3.59
Emotional value	***α = .88***	***α = .92***	***α = .93***	***α = .92***	***α = .91***		***α = .91***
17. Buying this fish makes me feel excited	3.94	3.63	3.87	3.93	4.14	5.88***	3.90
18. Buying this fish makes me enthusiastic	3.64	3.71	3.74	3.76	3.65	0.55	3.70
19. Buying this fish makes me feel happy	3.85	3.88	3.63	3.77	3.71	1.98	3.77

Notes: Answer scales ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.

The second part of the questionnaire focused on the costs and risks that respondents attributed to the fish species. As can be deducted from **Table 29.1.2**, the mean scores are again around or somewhat below the mid-point of the scale (=4), indicating that respondents tend to slightly agree with most of the items. Differences between countries are observed for the items of all constructs. The Cronbach's alphas indicate that the reliability of the items for the used constructs were good for all countries, although they are lower than the Cronbach's alphas for the constructs belonging to the VALUES-component of the model.

Table 29.1.2 Mean values for the constructs belonging to the COSTS-component of the conceptual model.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Mean difference-test (*F*-value)	Pooled sample (N = 2511)
Price	***α = .74***	***α = .80***	***α = .80***	***α = .82***	***α = .86***		***α = .80***
20. This fish would not be reasonably priced	3.85	3.59	3.49	3.71	3.42	7.33***	3.61
21. This fish would not be as good a product as its price indicates	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]		n.a.[1]
22. This fish would have higher price than the average of farmed fish	2.84	3.15	3.11	3.36	3.02	7.42***	3.10
23. This fish would not be economical	2.97	3.42	3.80	3.55	3.21	23.87***	3.39
Effort	***α = .87***	***α = .84***	***α = .89***	***α = .88***	***α = .87***		***α = .87***
24. This fish would require too much time to find	3.58	3.55	3.68	3.68	3.39	3.28*	3.58
25. This fish would require too much effort to find	3.55	3.61	3.76	3.77	3.45	4.39**	3.63
26. This fish would be hard to find	3.38	3.57	3.50	3.53	3.12	7.67***	3.42
Unfamiliarity	***α = .77***	***α = 79***	***α = .84***	***α = .79***	***α = .81***		***α = .81***
27. I won't be able to understand everything about this fish	3.92	4.15	3.99	4.27	3.52	16.70***	3.97
28. I won't be able to know all I need about this fish	3.48	4.08	3.86	4.05	3.35	22.90***	3.76
29. I won't feel as familiar as I want with this fish	3.46	4.12	3.78	3.83	3.43	18.65***	3.72
Evaluation costs	***α = .83***	***α = .73***	***α = .84***	***α = .83***	***α = .80***		***α = .81***
30. It would be difficult to recognize this fish	3.41	3.72	3.71	3.80	3.16	15.86***	3.56
31. I could not afford the time to get the information to fully evaluate this fish	3.88	3.92	4.04	4.15	3.85	3.11*	3.97
32. Comparing the benefits of my previous preferred fish with this fish would take too much time and effort	3.82	4.21	3.94	4.13	3.55	15.45***	3.93
33. If I would change my previously preferred fish, I would have to search very much to find this fish	3.64	3.91	3.62	3.64	3.38	8.24***	3.64
Performance risk	***α = .81***	***α = .81***	***α = .84***	***α = .79***	***α = .84***		***α = .82***
34. There might be a chance that this fish would not taste properly	3.94	3.89	3.76	3.76	3.25	17.50***	3.72
35. There might be a chance that I lose money, e.g. if the taste of this fish would be too different from the fish I usually buy	3.96	3.92	3.76	3.79	3.54	6.11***	3.79
36. This fish would come from a production method that I cannot trust	4.11	4.13	4.16	4.27	3.56	16.74***	4.05
37. This fish would not have any extras to offer	4.30	4.58	3.93	4.20	4.03	14.27***	4.21
Safety risk	***α = .78***	***α = .71***	***α = .84***	***α = .75***	***α = .83***		***α = .79***
38. This fish would not be safe to consume	4.34	4.94	4.60	4.47	3.87	30.74***	4.44
39. Not enough experience is gained in this fish so as to ensure safety	3.73	3.56	3.81	3.71	3.40	5.67***	3.64
40. There might be a risk if the safety of consuming this fish is not warranteed	3.53	3.59	3.86	3.55	3.32	7.49***	3.57

Notes: Answer scales ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.

[1] Based on the outcomes of the pilot-test, this item was dropped in the analyses.

The third part of the questionnaire focused on the customer value and other evaluative outcomes that respondents attributed to the fish species. As can be deducted from Table 29.1.3, the mean scores are again around or somewhat below the mid-point of the scale (=4), indicating that respondents tend to slightly agree with most of the items. Differences between countries are observed for the items of all constructs, except customer value. The Cronbach's alphas are generally high, indicating that the reliability of the items for the used constructs were good for all countries.

Table 29.1.3 Mean values for the constructs belonging to the CUSTOMER VALUE and OUTCOMES-components of the conceptual model.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Mean difference-test (*F*-value)	Pooled sample (N = 2511)
Customer value	***α = .90***	***α = .86***	***α = .92***	***α = .91***	***α = .90***		***α = .90***
41. I would consider this fish to be good value for money	3.54	3.71	3.65	3.50	3.60	2.04	3.60
42. I would consider this fish to be a good buy	3.23	3.47	3.47	3.23	3.27	4.15**	3.33
43. The value of this fish to me would be high	3.38	3.43	3.48	3.34	3.46	0.88	3.42
44. Compared to what I would have to give up, the overall ability of this fish to satisfy my needs would be high	3.21	3.18	3.46	3.49	3.54	7.72***	3.38
45. This fish replace old fish products with new valuable products	3.34	3.45	3.41	3.52	3.54	1.63	3.45
46. This fish is a promising fish product	2.93	3.30	3.10	3.07	3.13	4.41**	3.11
Satisfaction	***α = .94***	***α = .94***	***α = .94***	***α = .93***	***α = .94***		***α = .94***
47. It would be a wise choice to buy this fish	3.16	3.51	3.49	3.25	3.35	5.42***	3.35
48. Overall, I would be satisfied with this fish	3.19	3.49	3.40	3.27	3.38	3.52**	3.35
49. It would be the right thing to choose this fish	3.18	3.47	3.38	3.40	3.30	2.89*	3.34
Trust	***α = .94***	***α = .94***	***α = .91***	***α = .95***	***α = .94***		***α = .94***
50. I would trust this fish	3.32	3.62	3.43	3.29	3.42	4.09**	3.42
51. I would rely on this fish	3.40	3.76	3.90	3.23	3.37	18.18***	3.53
52. I would consider this fish to be an honest product	3.22	3.46	3.31	3.28	3.42	2.63*	3.34
53. This fish would be safe to buy	3.35	3.59	3.29	3.29	3.46	4.16**	3.40
Word of Mouth	***α = .90***	***α = .91***	***α = .91***	***α = .86***	***α = .92***		***α = .90***
54. I would recommend this fish to my friends and family	3.43	3.69	3.72	3.42	3.48	4.54**	3.55
55. I would talk favorably about this fish	3.22	3.62	3.57	3.13	3.37	10.77***	3.38
Willingness to pay	***α = n.a.***	***α = n.a.***	***α = n.a.***	***α = n.a.***	***α = n.a.***		***α = n.a.***
56. I am willing to pay a premium price to buy this fish	3.59	4.05	4.01	3.97	4.05	6.96***	3.93
Intention to buy	***α = .86***	***α = .86***	***α = .88***	***α = .82***	***α = .82***		***α = .85***
57. I intend to purchase this fish next time I buy fish	3.51	3.78	3.88	3.42	3.52	7.65***	3.62
58. I intent to replace my current fish with this fish	3.94	4.11	4.21	3.98	4.03	2.44*	4.05

Notes: Answer scales ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.

The fourth part of the questionnaire focused on personal characteristics (psychographics) that could potentially moderate the scores on the variables of the model: 'involvement in the category', consumers' domain-specific innovativeness, subjective knowledge, optimistic bias, and social representations of novel food. **Table 29.1.4** shows that respondents generally attached lower scores to involvement (indicating that they tend to agree) and higher scores to domain-specific innovativeness. Except for social representations of novel food and domain-specific innovativeness, respondents' scores on the psychographic constructs differed significantly between countries. The Cronbach's alphas are again generally high, indicating a good reliability of the items for the used constructs.

Table 29.1.4 Mean values for the constructs belonging to the MODERATORS-component of the conceptual model.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Mean difference-test (*F*-value)	Pooled sample (N = 2511)
Consumer involvement	***α = .88***	***α = .94***	***α = .94***	***α = .95***	***α = .96***		***α = .94***
59. I am very concerned about what fish products I purchase	2.65	2.49	2.97	2.63	2.29	14.68***	2.61
60. I care a lot about what fish products I consume	2.14	2.46	2.78	2.62	2.44	14.44***	2.49
61. Generally, choosing the right fish products is important to me	2.26	2.42	2.75	2.57	2.28	11.21***	2.46
Domain specific innovativeness	***α = .88***	***α = .86***	***α = .90***	***α = .86***	***α = .86***		***α = .87***
62. In general, I am among the last in my circle of friends to purchase new fish products.	4.22	4.17	4.20	4.18	3.95	2.10	4.14
63. Compared to my friends, I do little shopping for new fish products.	4.19	4.00	4.25	4.23	3.97	3.04*	4.13
64. I would consider buying new fish products, even if I hadn't heard of it yet.	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]		n.a.[1]
65. In general, I am the last in my circle of friends to know the names of the latest new fish product trends.	4.17	4.09	4.17	4.14	3.99	1.02	4.11
66. I know more about new fish products than other people do.	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]	n.a.[1]		n.a.[1]
Subjective knowledge	***α = .93***	***α = .95***	***α = .94***	***α = .93***	***α = .94***		***α = .94***
67. I consider that I know more about fish than the average person	3.49	3.97	3.60	3.69	3.53	7.50***	3.66
68. I think that I know more about fish than my friends	3.39	3.92	3.48	3.54	3.43	8.96***	3.55
69. I have a lot of knowledge about how to prepare fish	3.12	3.85	3.50	3.36	3.25	16.33***	3.41
70. I have a lot of knowledge about how to evaluate the quality of fish	3.29	3.95	3.63	3.59	3.37	14.00***	3.57
Optimistic bias	***α = .81***	***α = .90***	***α = .88***	***α = .86***	***α = .85***		***α = .86***
71. Compared to the average person of my age and sex, the likelihood of me getting health problems when eating new product from a new farmed fish is *[-3/+3: much less/more likely than the average person]*	-0.73	-0.15	-0.27	-0.46	-0.51	12.95***	-0.42
72. The health risks associated with eating a new product from a new farmed fish to me personally are *[1=very low to 7=very high]*	2.87	3.57	3.11	2.95	3.10	16.73***	3.12
73. The health risks associated with eating a new product from a new farmed fish to the average [Spanish / ..... / ..... / ..... / .....] are *[1=very low to 7=very high]*	3.06	3.62	3.24	3.05	3.36	13.22***	3.27
Social representations of food	***α = .73***	***α = .74***	***α = .76***	***α = .74***	***α = .79***		***α = .75***
74. I value things being in accordance with nature.	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
75. I feel good when I eat clean and natural food.	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
76. I would like to eat only food with no additives.	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
77. Eating is very important to me	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
78. For me, delicious food is an essential part of weekends.	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
79. I treat myself to something really delicious.	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]		n.a.[2]
80. New foods are just a silly trend.	4.43	4.47	4.38	4.58	4.44	1.01	4.46
81. Consequences of eating new foods are unknown.	3.31	3.18	3.53	3.53	3.50	5.41***	3.41
82. I have some doubts about food novelties.	3.67	3.49	3.59	3.51	3.68	1.64	3.59

Notes: Answer scales ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.
[1]Based on the outcomes of the pilot-test, the reversely formulated items were dropped in the analyses; [2] Items A74-79 were dropped from the analysis; only items A80-82 ('novel food' dimension) are kept.

Respondents were also asked after their beliefs about farmed fish and their objective knowledge about fish. The results as presented in **Table 29.1.5** indicate that the scores on the belief-items are around the midpoint of the scale, indicating a relatively neutral stance with regard to the formulated items. The scores on most of the items differ significantly across the five different countries. However, more interesting are the scores on the objective knowledge questions. Almost all countries know that fish is a source of omega-3 fatty acids. Strikingly low is the percentage of correct answers to the (false) statement that more than half of the fish we buy is farmed fish. Country differences are also notable with regard to these questions: only 39% of the German respondents knows that cod is not a fatty fish, whereas about 71% of the Italian respondents provided the correct answer. And, with regard to salmon as a fatty fish, only 44% of the British respondents gave the correct answer as compared to 76% of the French respondents.

Table 29.1.5: Mean values for Beliefs about farmed fish and Objective knowledge about fish.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Country difference-tests	Pooled sample (N = 2511)
Beliefs about farmed fish							
1. Farmed fish is safer than wild fish	3.71	4.25	3.78	3.60	3.61	$F = 17.79^{***}$	3.79
2. Wild fish is more affected by marine pollution (spillages) than farmed fish	3.23	3.62	3.37	3.18	3.20	$F = 7.83^{***}$	3.32
3. Wild fish contains more heavy metals than farmed fish	3.70	3.80	3.70	3.36	3.39	$F = 10.64^{***}$	3.59
4. Wild fish contains more antibiotics than farmed fish	4.84	4.46	4.25	4.22	4.49	$F = 12.22^{***}$	4.45
5. Wild fish is more affected by parasites (anisakis) than farmed fish	3.68	3.87	3.70	3.36	3.50	$F = 9.87^{***}$	3.62
6. Farmed fish has a healthier diet than wild fish	4.08	4.50	4.01	3.94	4.01	$F = 11.21^{***}$	4.11
7. Farmed fish is healthier than wild fish	4.20	4.34	4.05	3.95	3.67	$F = 15.85^{***}$	4.04
8. Farmed fish is of better quality than wild fish	4.09	4.54	4.14	4.23	4.41	$F = 8.17^{***}$	4.28
9. Farmed fish is fresher than wild fish	4.06	4.48	4.07	4.06	4.12	$F = 7.48^{***}$	4.16
10. Farmed fish is more nutritious than wild fish	4.18	4.34	4.13	4.15	4.32	$F = 2.38$	4.22
11. Wild fish is more fatty than farmed fish	3.86	4.17	4.10	3.89	4.16	$F = 5.20^{***}$	4.04
12. Farmed fish tastes better than wild fish	4.31	4.61	4.17	4.38	4.55	$F = 7.24^{***}$	4.40
13. Farmed fish is firmer than wild fish	3.96	4.36	3.94	4.00	4.17	$F = 8.23^{***}$	4.08
14. Farmed fish is more controlled than wild fish	3.15	3.43	3.05	2.99	3.09	$F = 7.36^{***}$	3.14
15. Farmed fish is more handled than wild fish	2.99	2.86	3.39	3.16	4.32	$F = 81.83^{***}$	3.34
16. Wild fish is more artificial than farmed fish	5.01	4.73	4.56	4.89	4.83	$F = 5.08^{***}$	4.80
17. Farmed fish provides more guarantees than wild fish	3.57	4.19	3.55	3.49	3.56	$F = 21.24^{***}$	3.67
18. Farmed fish is easier to find than wild fish	2.91	2.91	2.93	3.02	2.87	$F = 0.76$	2.93
19. Farmed fish is cheaper than wild fish	3.14	3.11	3.23	3.27	3.03	$F = 2.20$	3.16
Objective knowledge about fish (% correct answers)							
20. More than half of the fish we buy in [country] is farmed fish (correct answer: FALSE)	15.4%	11.6%	16.4%	25.8%	15.6%	$X^2 = 49.49^{***}$	17.0%
21. Fish is a source of dietary fibre (correct answer: FALSE)	57.1%	31.6%	29.1%	22.8%	45.8%	$X^2 = 185.62^{***}$	37.3%
22. Cod is a fatty fish (correct answer: FALSE)	39.1%	45.6%	65.7%	57.8%	70.6%	$X^2 = 149.75^{***}$	55.8%
23. Fish is a source of omega-3 fatty acids (correct answer: TRUE)	92.3%	85.0%	91.7%	93.2%	91.8%	$X^2 = 33.16^{***}$	90.8%
24. Salmon is a fatty fish (correct answer: TRUE)	65.8%	76.4%	43.6%	70.2%	68.8%	$X^2 = 146.90^{***}$	64.9%

Notes: Answer scales for 'Beliefs about farmed fish' ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.

The results are presented in **Table 29.1.6** and show that for the pooled sample the consumption patterns differ not much across the different types of fish. Again, consumption frequencies differ significantly across countries. Generally, fish consumption is higher in the UK, Spain and Italy. Wild fish appears to be most popular in Italy, seafood is eaten most frequently in the UK, and whole fish is consumed most often in Spain.

Table 29.1.6 Mean values for Current Fish Consumption. Respondents were asked after their current fish consumption for different categories of fish.

Construct		Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Country difference-tests (Chi-square)	Pooled sample (N = 2511)
How often did you eat the following fish products in the last month? (in percentages)								
25. Farmed fish (aquaculture)	Never	12.3%	8.6%	5.5%	4.8%	10.0%		8.2%
	Once a month or less	40.7%	38.8%	32.5%	30.8%	30.6%		34.7%
	2-3 times a month	25.3%	30.8%	30.3%	29.6%	33.0%	135.78***	29.8%
	Once a week or more	10.1%	17.0%	23.2%	29.8%	23.8%		20.7%
	I don't know	11.7%	4.8%	8.5%	5.0%	2.6%		6.5%
26. Wild fish	Never	16.8%	13.6%	23.0%	20.2%	3.6%		15.5%
	Once a month or less	40.9%	40.2%	35.6%	26.4%	28.4%		34.3%
	2-3 times a month	25.3%	25.8%	20.2%	22.4%	34.6%	212.51***	25.6%
	Once a week or more	10.5%	15.0%	10.9%	19.6%	28.6%		16.9%
	I don't know	6.5%	5.4%	10.3%	11.4%	4.8%		7.7%
27. Seafood	Never	23.3%	12.2%	8.3%	6.8%	8.2%		11.8%
	Once a month or less	36.0%	47.0%	26.9%	44.4%	30.8%		37.0%
	2-3 times a month	26.9%	25.2%	31.5%	31.0%	38.8%	213.04***	30.7%
	Once a week or more	10.9%	13.8%	30.3%	17.0%	21.6%		18.7%
	I don't know	3.0%	1.8%	3.0%	0.8%	0.6%		1.8%
28. Frozen fish	Never	5.5%	10.0%	6.9%	5.0%	7.2%		6.9%
	Once a month or less	25.3%	30.8%	25.5%	22.0%	28.2%		26.4%
	2-3 times a month	41.3%	35.8%	35.8%	35.0%	33.6%	44.29***	36.3%
	Once a week or more	26.9%	22.6%	30.5%	37.0%	30.4%		29.5%
	I don't know	1.0%	0.8%	1.2%	1.0%	0.6%		0.9%
29. Whole fish	Never	21.9%	16.2%	12.7%	4.8%	9.6%		13.1%
	Once a month or less	36.6%	36.6%	35.6%	24.8%	29.4%		32.6%
	2-3 times a month	22.5%	27.0%	26.9%	32.2%	31.8%	169.92***	28.1%
	Once a week or more	14.2%	16.4%	21.6%	35.6%	27.8%		23.1%
	I don't know	4.7%	3.8%	3.2%	2.6%	1.4%		3.1%
30. Processed fish (e.g., fish-fingers)	Never	11.9%	14.8%	13.1%	18.6%	10.8%		13.8%
	Once a month or less	33.4%	32.8%	33.3%	29.0%	25.6%		30.8%
	2-3 times a month	31.8%	25.6%	28.5%	27.0%	34.8%	80.52***	29.5%
	Once a week or more	21.9%	25.6%	23.4%	19.8%	28.2%		23.8%
	I don't know	1.0%	1.2%	1.8%	5.6%	0.6%		2.0%

Notes: ***significant at p < .001; **significant at p < .01; *significant at p < .05.

As can be deducted from Table 29.1.7, the mean scores are generally below the mid-point of the scale (=4), indicating that respondents tend to be aware of the logos and evaluate these logos positively. Differences between countries are observed for all items.

Table 29.1.7: Mean values for awareness and evaluation of three different logos. In the questionnaire, respondents also had to give their assessment for three different logos.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Mean difference-test (*F*-value)	Pooled sample (N = 2511)
Awareness and evaluation of Friend of the Sea-logo							
31. I am aware of this logo	3.33	2.32	2.46	2.51	2.49	46.42***	2.62
32. The likely quality of products carrying this logo is extremely high	2.92	2.90	3.17	3.14	3.00	7.45***	3.03
33. Products carrying this logo would be my first choice	2.78	2.78	2.99	3.01	2.90	5.55***	2.89
34. I find this logo trustworthy	2.88	2.91	3.15	3.16	3.06	8.11***	3.03
35. I value this logo	2.80	2.75	3.08	3.09	3.06	11.87***	2.95
Awareness and evaluation of ASC-logo							
36. I am aware of this logo	2.62	2.15	2.82	2.60	2.46	17.22***	2.53
37. The likely quality of products carrying this logo is extremely high	3.14	2.95	3.28	3.20	3.11	7.23***	3.14
38. Products carrying this logo would be my first choice	2.96	2.75	3.13	3.04	3.01	8.70***	2.98
39. I find this logo trustworthy	3.11	2.95	3.35	3.26	3.13	10.71***	3.16
40. I value this logo	2.98	2.76	3.23	3.17	3.08	14.57***	3.04
Awareness and evaluation of Organic-logo							
41. I am aware of this logo	2.71	2.58	2.34	2.56	2.66	5.31***	2.57
42. The likely quality of products carrying this logo is extremely high	3.06	2.95	2.90	3.10	3.10	4.14**	3.02
43. Products carrying this logo would be my first choice	2.90	2.80	2.76	2.99	2.07	4.49**	2.89
44. I find this logo trustworthy	3.03	3.00	2.90	3.10	3.10	2.92*	3.03
45. I value this logo	2.93	2.78	2.82	3.04	3.05	6.76***	2.92

Notes: Answer scales ranged from 1 = 'strongly agree' to 7 = 'strongly disagree'; ***significant at p < .001; **significant at p < .01; *significant at p < .05.

Looking at the frequencies in Table **29.1.8**, no striking results are observed. An exception is the education level of the sample. It looks like the samples for most of the countries (except Germany) are higher educated than expected based on national statistics. Finally, except for age and gender, all countries differ significantly in their demographic profile of their respondents.

Table 29.1.8: Mean values for socio-demographic variables. Finally, respondent had to provide demographic information.

Construct	Germany (n = 506)	France (n = 500)	UK (n = 505)	Spain (n = 500)	Italy (n = 500)	Country difference-tests	Pooled sample (N = 2511)
Mean age (in years)	41.75	41.72	42.29	41.11	40.28	$F = 1.82$	41.43
Gender							
Male	49.2%	48.6%	51.1%	50.4%	46.8%	$X^2 = 2.23$	49.2%
Female	50.8%	51.4%	48.9%	49.6%	53.2%		50.8%
Education							
No formal education	0%	0.4%	0.2%	0%	0%		0.1%
Primary school	3.0%	1.2%	0%	1.4%	0.4%		1.2%
Secondary school	41.9%	25.0%	27.1%	18.0%	23.8%	$X^2 = 218.46***$	27.2%
Technical School	23.9%	19.8%	25.5%	28.6%	28.6%		25.3%
University Degree	23.7%	30.0%	33.9%	46.6%	34.4%		33.7%
Post-graduate Degree	7.5%	23.6%	13.3%	5.4%	12.8%		12.5%
Income level							
Lower than average	25.3%	25.0%	27.9%	26.4%	30.6%		27.0%
About average	53.8%	61.4%	55.4%	62.2%	64.0%	$X^2 = 60.67***$	59.3%
Higher than average	20.9%	13.6%	16.6%	11.4%	5.4%		13.6%
Socio-economic class							
Social Class A/B	11.7%	19.0%	12.5%	11.8%	20.0%		15.0%
Social Class C1	27.3%	31.6%	38.8%	34.4%	25.0%		31.4%
Social Class C2	45.3%	36.4%	32.7%	38.8%	41.2%	$X^2 = 59.47***$	38.9%
Social Class D	15.8%	13.0%	15.8%	14.8%	13.8%		14.7%
Social Class E	0%	0%	0.2%	0.2%	0%		0.1%
Who is responsible for doing the grocery shopping in your household?							
I am the main decision maker of the household	80.4%	78.0%	71.5%	62.6%	73.6%	$X^2 = 48.85***$	73.2%
I am the joint decision maker of the household	19.6%	22.0%	28.5%	37.4%	26.4%		26.8%
Marital status							
Single	34.4%	32.4%	35.2%	33.2%	35.8%		34.2%
Co-habiting	17.8%	9.2%	20.2%	18.0%	16.2%	$X^2 = 33.98***$	16.3%
Married	47.8%	58.4%	44.6%	48.8%	48.0%		49.5%
Are there children in your household?							
Yes	37.7%	55.2%	40.4%	49.6%	45.2%	$X^2 = 39.93***$	45.6%
No	62.3%	44.8%	59.6%	50.4%	54.8%		54.4%
Are you the main wage earner of household?							
Yes	68.4%	71.0%	71.9%	59.6%	51.0%	$X^2 = 69.51***$	64.4%
No	31.6%	29.0%	28.1%	40.4%	49.0%		35.6%

Notes: ***significant at p < .001; **significant at p < .01; *significant at p < .05.

Sub-task 29.1.2 (led by AU, Athanasios Krystallis)

This second sub-task concerns the segmentation of the data collected in 29.1.2 at the country as well as the cross-country levels. The segmentation study was based on the five moderators included in the survey: Consumer Involvement, Domain Specific Innovativeness, Subjective Knowledge, Optimistic Bias and Social Representations of Food. According to their reliability (Cronbach's Alpha) and uni-dimensionality the mean value for each construct was calculated and retained.

A two-step clustering analysis was carried out for each country and for the complete dataset (N=2511). Firstly an Agglomerative Hierarchical Cluster Analysis (AHCA) (Ward method and Euclidian distance) was applied over the standardised dataset per participant in order to block the idiosyncratic use of the scale. The final number of clusters to retain in each case was based on the percentage of within-cluster variance drop when adding a new cluster. Secondly a k-means clustering was performed (Determinant (W) criterion) after

selecting as initial cluster centres the centroids obtained in the previous AHCA. Table 29.1.1 shows the centroids obtained for each cluster, country and variable.

In general, the clusters identified differ significantly in all the constructs in the five countries analysed and for the whole dataset. These results reinforce the accurate selection of the final number of clusters made according to the parsimony rule (the simplest possible structure or low number of clusters that still represents homogenous groups assessed by the number of constructs that differed significantly between clusters).

Consumer Involvement and Domain Specific Innovativeness are the constructs with the higher discriminant ability between clusters, which may be explained by the aim of the study: the introduction of new products from new species (diversification). Overall, similar clustering patterns can be observed in the five countries studied (**Fig. 29.1.1**), thus indicating a relatively homogeneous European food-related culture.

Table 29.1.1 Centroids for each cluster, country and construct (Likert-type agreement scales with end-points 1=strongly agree and 7=strongly disagree). N indicates size of the samples in each country (i.e. number of consumers), as well as size of each cluster identified.

Cluster	C Involvement	DS Innovativeness	Sub Knowledge	Opt Bias	Social Rep F	N
OVERALL (N=2511)						
O1	1.95^{b}	2.81^{c}	2.84^{b}	3.89^{a}	3.00^{c}	728
O2	1.77^{c}	5.37^{a}	2.92^{b}	2.62^{c}	4.43^{a}	911
O3	3.77^{a}	3.94^{b}	4.78^{a}	3.57^{b}	3.87^{b}	872
GERMANY (N=506)						
D1	1.94^{c}	2.56^{c}	2.61^{c}	3.62^{a}	2.54^{c}	106
D2	1.74^{c}	5.82^{a}	2.81^{c}	2.39^{c}	4.40^{a}	171
D3	3.79^{a}	3.66^{b}	5.15^{a}	3.15^{b}	3.66^{b}	89
D4	2.50^{b}	3.78^{b}	3.34^{b}	3.42^{ab}	4.12^{a}	140
SPAIN (N=500)						
E1	4.38^{a}	4.25^{b}	4.85^{a}	3.76^{a}	4.06^{a}	107
E2	2.17^{b}	3.02^{c}	2.93^{c}	3.83^{a}	3.02^{b}	147
E3	2.09^{b}	4.85^{a}	3.35^{b}	2.54^{b}	4.30^{a}	246
FRANCE (N=500)						
F1	3.23^{a}	3.87^{b}	4.92^{a}	3.75^{b}	3.71^{b}	250
F2	1.50^{b}	5.18^{a}	3.19^{b}	4.77^{a}	3.15^{c}	68
F3	1.75^{b}	5.22^{a}	2.90^{bc}	2.30^{c}	4.78^{a}	107
F4	1.79^{b}	2.18^{c}	2.70^{c}	4.44^{a}	2.69^{c}	75
ITALY (N=500)						
I1	1.60^{bc}	2.13^{d}	2.38^{c}	3.65^{a}	4.71^{a}	38
I2	3.91^{a}	3.92^{b}	4.71^{a}	3.58^{a}	4.11^{b}	145
I3	1.95^{b}	2.85^{c}	3.18^{b}	3.80^{a}	2.76^{c}	147
I4	1.50^{c}	5.39^{a}	2.68^{c}	2.60^{b}	4.45^{a}	170
UK (N=505)						
GB1	2.33^{b}	2.67^{c}	2.88^{b}	4.22^{a}	2.95^{c}	121
GB2	2.12^{b}	5.10^{a}	2.95^{b}	2.74^{c}	4.24^{a}	226
GB3	4.24^{a}	4.09^{b}	4.93^{a}	3.58^{b}	3.93^{b}	158

a-d: centroids within country and construct with different letters differ significantly ($P \leq 0.05$).

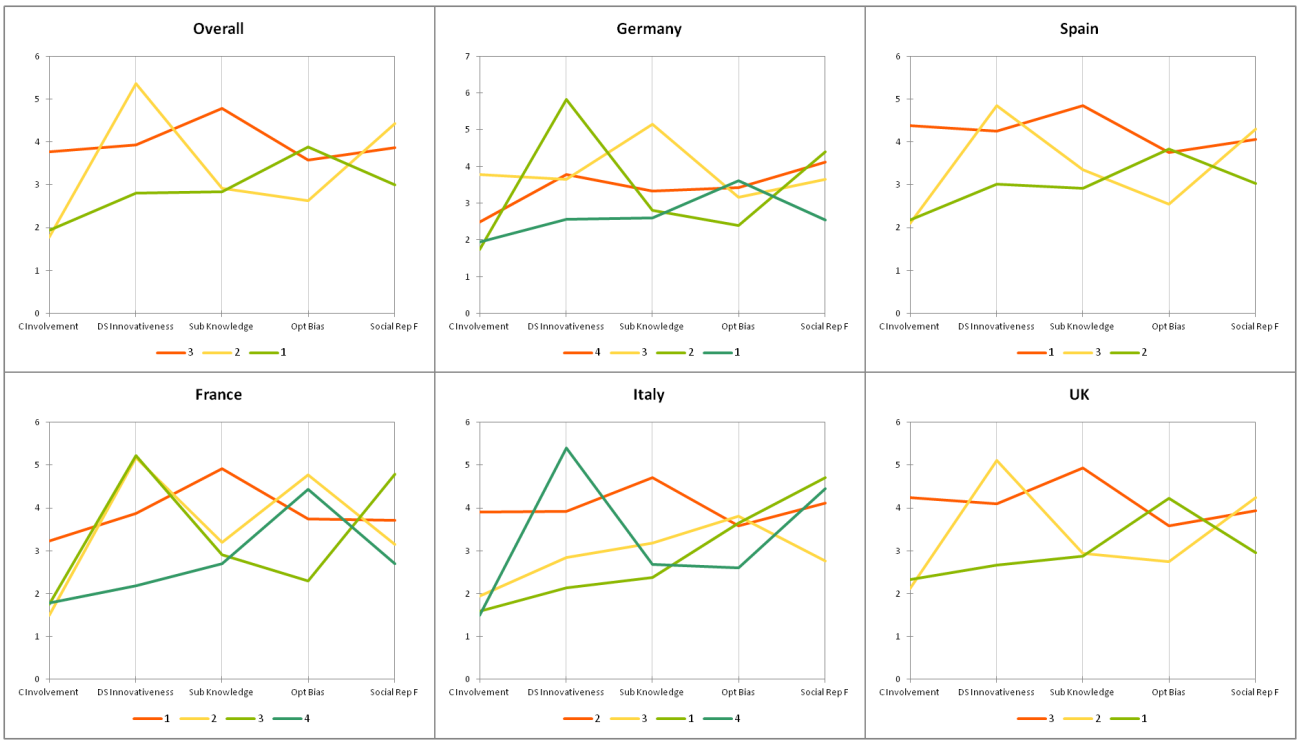


Figure 29.1.1 Construct profile for each country and for the pooled dataset (centroids).

Sub-task 29.1.2 will result to Deliverable D29.2 (due date: month 24, expected date: month 14), a report on the segmentation analyses based on consumer value perceptions about the new products from the selected species in the five target-countries. D29.2 will include a fully detailed profile of the consumer clusters identified in terms of a) psychographic profile in relation to the five moderators, b) perceived value, cost, and outcomes constructs (**i.e.**, satisfaction, trust, willingness to pay etc.), and c) socio-demographic and behavioral profile.

Task 29.2 Consumer sensory perceptions (led by IRTA, Lluis Guerrero).

No work done during this period

Task 29.3 Optimization of intrinsic-extrinsic attribute combinations (led by AU, Athanasios Krystallis).

No work done during this period

Task 29.4 Communication effectiveness in behavioural change (led by AU, Athanasios Krystallis).

No work done during this period

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

WP30 Socioeconomics – Business model and marketing strategy development

WP No:	30	**WP Lead beneficiary:**		P10. TU/e
WP Title (from DOW):	Socioeconomics – Business model and marketing strategy development			
Other beneficiaries (from DOW):	P3. IRTA	P6. DLO	P11. AU	P12. APROMAR
P18. CTAQUA	P23. ARGO	P24. ITICAL	P25. DOR	P28.CANEXMAR
P29. ASIALOR	P38. HRH			
Lead Scientist preparing the Report (WP leader):	E. Nijssen (P10), business modelling G. Tacken and M. Reinders (P6), end report			
Other Scientists participating:	To be determined later			

Objectives

1. To identify business models for sustainable profitability and improved competititveness of the sector for all the DIVERSIFY species.
2. To devise marketing strategies for the newly developed products from the DIVERSIFY species aiming to develop a market that is as large and profitable as possible
3. To come up with policy/strategy recommendations for further development and market expansion.

Summary of progress towards objectives and details for each task:

In the DOW, this WP is not planned to start until project month 43. However some activities were already initiated, because the work for this work package is highly dependent on work done in other work packages (*e.g.*, WP 27, WP 28 and WP 29). A PhD candidate was selected (Maren Vos) who will execute a large part of the work for WP30. Next to that, we consulted with P6. DLO in order to make sure that work executed in Sub-task 27.2.3 and Sub-task 27.3.2 is aligned with work to be executed in WP 30.

Deviations from Annex I and their impact:

Some activities were startd ahead of the DOW.

WP31 Dissemination

WP No:	31	**WP Lead beneficiary:**		P18. CTAQUA
WP Title (from DOW):	Dissemination			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P7. IMR	P8. IEO
P9. UL	P12. APROMAR	P13. UNIBA	P33. FGM	P34. BVFi
P35. MASZ	P36. ANF	P37. EUFIC		
Lead Scientist preparing the Report (WP leader):	Rocio Robles			
Other Scientists participating:	Constantinos Mylonas (P.1), Javier Ojeda (P.12), Aldo Corriero (P.13), Jessica Miller (P.37), Adrian Giordani (P.37), Laslo Varadi (P.35), Mathias Keller (P.34)			

Objectives

1. Disseminate the knowledge acquired to the scientific community, to promote further research,
2. Disseminate the knowledge acquired to the aquaculture sector, to enhance feed back acquisition,
3. Promote implementation of new husbandry methods, protocols and products developed by DIVERSIFY by the aquaculture industry and the seafood processors,
4. Enhance awareness of the diversification efforts of the project to the general public, with special attention to the food industry and consumer's organizations,
5. Promote investment opportunities making available the species feasibility studies to the industry,
6. Provide documented information to fish producers, fish processors and consumers on the new farmed aqua products from DIVERSIFY.

Summary of progress towards objectives and details for each task:

Task 31.1 Project website and brochure (led by CTAQUA, Rocio Robles).

WEBSITE

Construction of the web site began already in M1 of the project using the www.weebly.com platform. Hosting of the site was then moved to its final location at www.diversifyfish.eu in Mo 2. The website was already used extensively to provide up-to-date information on the organization, agenda and logistics of the kick off meeting, which was hosted by P1. HCMR at its facilities in Heraklion, Crete, Greece on 29-30 January 2014. The website was designed to provide fast access to the project information, which is included in seven main pages. The structure of the web site follows the following scheme:

1. News: This page includes the most recent activities on the initiation of specific tasks from the work plan, providing brief descriptions of various actions, with graphic support. This page is updated often (monthly), with the objective of providing up-to-date information to partners, but also to interested people visiting the web site, on the ongoing work of the project. Major results and findings will also be provided to speed up dissemination and hopefully implementation of the outcomes of the project by relevant stakeholders. (**Fig 31.1.1**)

2. Summary: The page includes the full title of the project, a brief description of its objectives and contact details of the Project Coordinator, geographical distribution of the partners, general structure of the work to be implemented (Pert diagram) and organization of the consortium and its governing bodies and research area groups (Management structure) (**Fig. 31.1.2**)

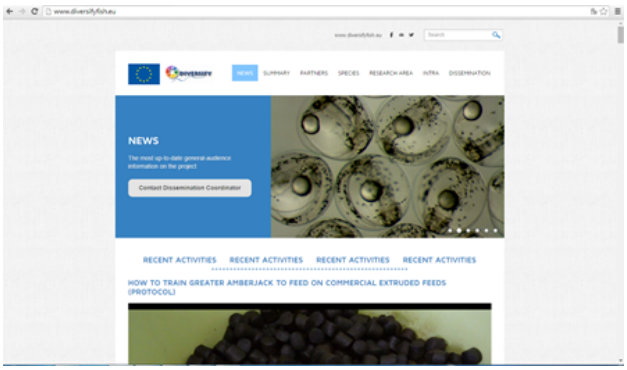


Figure 31.1.1 The "News" page of the DIVERSIFY website hosted at www.diversifyfish.eu

Figure 31.1.2 The "Summary" page of the website www.diversifyfish.eu/summary.html

3. Partners: These pages are grouped in RTDs, SMEs, Large enterprises, and Associations and NGOs, providing the contact information of each partner and the Principal Investigator (PI), including the logo of the partner. The main "PARTNERS" page includes also a gallery of photos from all involved researchers in the consortium. (**Figs 31.1.3** and **31.1.4**)

Figure 31.1.3 The "Partners" main page of the website www.diversifyfish.eu/partners.htm

Figure 31.1.4 Gallery of photos at the "Partners" pages of the website www.diversifyfish.eu/rtd-organizations.htm

4. Species: In these pages, a full description is provided of the six species included in the project (**meagre** *Argyrosomus regius,* **greater amberjack** *Seriola dumerili*, **pikeperch** *Sander lucioperca,* **Atlantic halibut** *Hippoglossus hippoglossus*, **wreckfish** *Polyprion americanus* and **grey mullet** *Mugil cephalus*). This section includes biological information per species and the reasoning behind the species selection for the project. A photo gallery is also included, providing photos (when available) of the species at various stages and from research activities of the partners. (**Figs 31.1.5** and **31.1.6**)

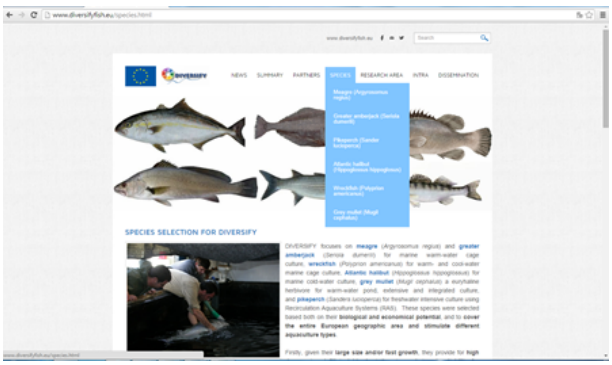


Figure 31.1.5 The "Species" main page of the website www.diversifyfish.eu/species.html

Figure 31.1.6 The wreckfish section in the "Species" pages in the website www.diversifyfish.eu/wreckfish-polyprion-americanus.html

5. Research area: These pages indicate how the research tasks are designed to address the identified bottlenecks in each selected species .The different tasks have been separated by scientific discipline (along the organization of Group Work Packages of the Description of Work, DOW), so separate WPs address work

in a specific discipline and species. There are six (6) disciplines including Reproduction and genetics, Nutrition, Larval husbandry, Grow out husbandry, Fish health and Socioeconomics (which includes final product and quality) (**Fig. 31.1.7** and **31.1.8**). The specific Work Packages that are include in the project and address work in the selected species are:

- Reproduction and genetics: Meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.
- Nutrition: Meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.
- Larval husbandry: Meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.
- Grow out husbandry: Meagre, greater amberjack, pikeperch and grey mullet.
- Fish health: Meagre, greater amberjack and Atlantic halibut.
- Socioeconomics: Institutional and organizational context, new product development, consumer value perceptions and behavioral change and business model and marketing strategy development.

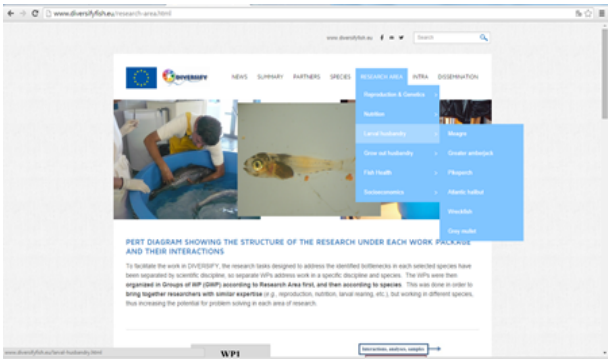


Figure 31.1.7 The "Research area" main page of the website www.diversifyfish.eu/research-area.html

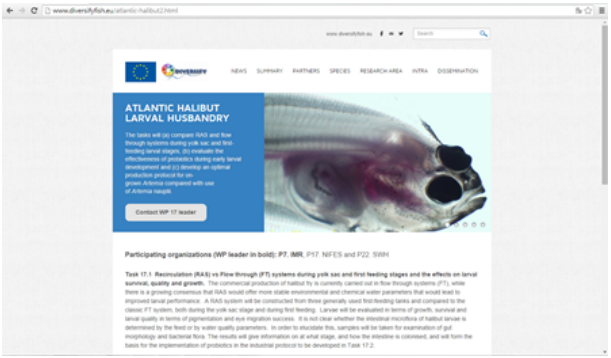


Figure 31.1.8 The Atlantic halibut larval husbandry section in the "Research area" pages of the website www.diversifyfish.eu/atlantic-halibut2.html

6. Intranet: This section includes information relevant to the partners and most of its pages are password protected (Figures 31.1.9 and 31.1.10). It is intended to provide specific information to the consortium, and different official documents and other internal project documents can be found here such as:

- Information about the annual meetings and activities
- A copy of the DOW, Grant Agreement and Consortium Agreement

• EU supporting documentation (CORDIS, use manuals on reporting, financial guide, etc.)

• Management documents, such as a list of all researchers in the consortium and their contact information, the minutes of the Annual Coordination Meetings, etc.

• Forms and protocols (logos, dissemination rules, templates for expenses justification, Deliverable reporting, etc.).

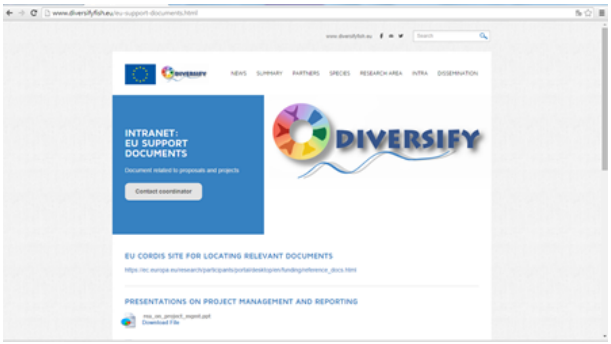


Figure 31.1.9 The "Intranet" main page of the website www.diversifyfish.eu/-intra.html

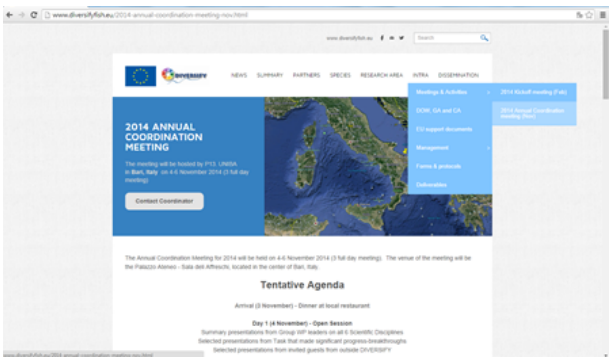


Figure 31.1.10 Annual coordination meeting 2014 posted at the website "Intranet" pages www.diversifyfish.eu/2014-annual-coordination-meeting-nov.html

7. Dissemination: These pages include all activities related to the broadcasting of project activities. (Figures from 31.1.11. to 31.1.14). Several sections are incorporated:

   - Newsletters: First issue already available at the website www.diversifyfish.eu/newsletter.html. Second one is in progress and will be uploaded in month 13.
   - Articles in magazines and internet with project information in other languages of the consortium; publications included are the following:
     - English: Links to all the articles and press releases published http://www.diversifyfish.eu/articles-in-magazinesinternet.html
     - Greek: One video where PC gave an interview (via skype) to Ant1 Cyprus, explaining the objectives of DIVERSIFY and the benefits for aquaculture industry from the introduction of new/emerging species. (www.youtube.com/watch?v=gZIjfZY3JEI); A seven pages article in Fishing News (Greek news magazine on fishing and aquaculture news), issue 385, pages 38-45; Press release of Kickoff Meeting of DIVERSIFY in Crete, Greece. It was published at least in 4 national and 1 local (Cretan) news websites.

- Spanish: A Master thesis related to sexual determination, reproduction, embryonic progress and larval husbandry of wreckfish is uploaded, as well as several press releases and articles in national websites related to aquaculture, economy and agriculture, one article at the Spanish Aquaculture Observatory magazine (OESA) (Figure 31.1.37) and a presentation in Spanish about the work done at IRTA in meagre larval husbandry.
- German: A brief description of DIVERSIFY project and the German version of the article published in Aquaculture Europe magazine. Vol. 39 (1) March 2014.
- Italian: A brief description of DIVERSIFY project and one press release at an Italian website related to food and technology (http://www.foodandtec.com/it/notizia.php?id_news=749)

- Scientific articles: in this section, no scientific article has been published so far, due to the early stage of the experiments
- Presentations and posters: The presentation about DIVERSIFY project made by the PC (C.Mylonas) in Aquaculture Europe 2014 (Figures from 31.3.2 to 31.3.7) and one Poster about the progress done in growth of the Wreckfish http://www.diversifyfish.eu/presentationsposters.html
- Photo gallery
- A Blog, to provide a discussion forum about DIVERSIFY and related topics for everyone visiting the web
-

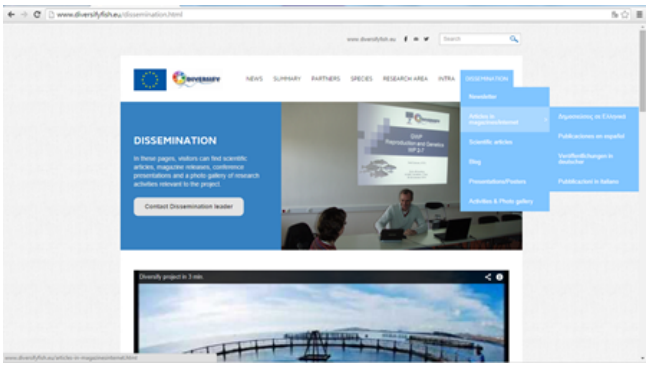


Figure 31.1.11 The "Dissemination" main page of the website www.diversifyfish.eu/dissemination.html

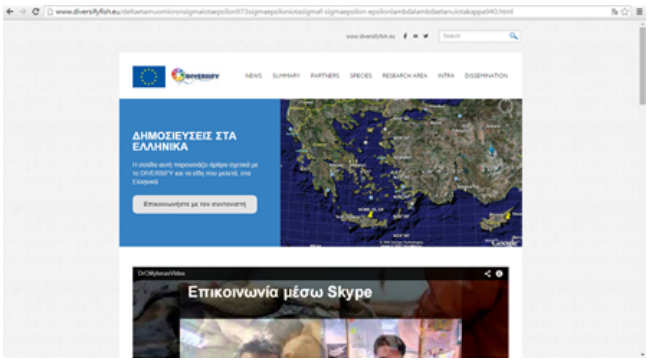


Figure 31.1.12 Greek section in the "Dissemination" pages of the website www.diversifyfish.eu/deltaetamuomicronsigmaiotaepsilon973sigmaepsiloniotasigmaf-sigmaepsilon-epsilonlambdalambdaetanuiotakappa940.html

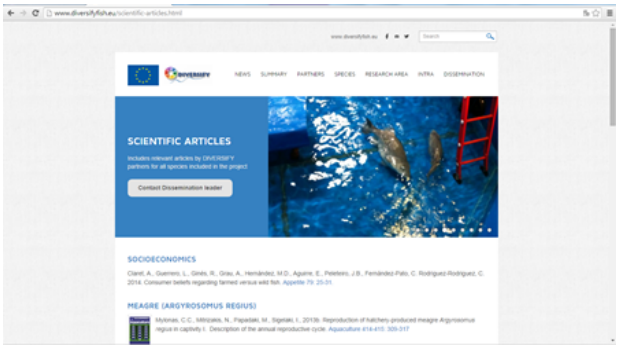


Figure 31.1.13 Posters and presentations section in the "Dissemination" pages of the website www.diversifyfish.eu/presentationsposters.html

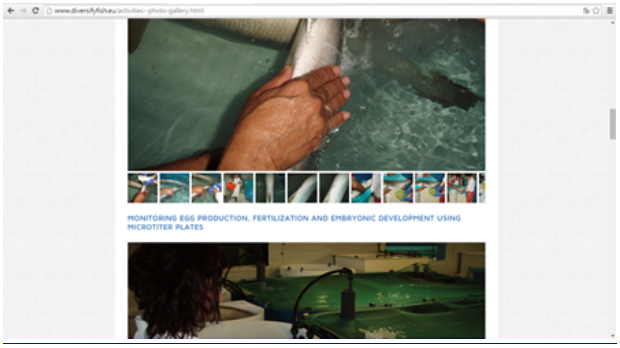


Figure 31.1.14 Photo gallery in the "Dissemination" pages of the website www.diversifyfish.eu/activities--photo-gallery.html

Also to be mentioned that P.37 EUFIC has included in its web page, within the section EU initiatives, a section about DIVERSIFY , which is specifically dedicated to providing information about the EU projects EUFIC are involved in. This webpage also helps drive traffic to the DIVERSIFY website. The article linked to the section introduces the background to the DIVERSIFY project, including its goals and the need to expand the EU aquaculture sector; it also introduces the six finfish species that are going to behave been selected for use by the project. The webpage outlines the main research objectives of DIVERSIFY and also the expected outcomes and results. Finally, a link to the project website is included at the bottom of the page. Web link to the page on eufic.org: http://www.eufic.org/article/en/show/eu-initiatives/rid/diversify/ (**Fig. 31.1.36**).

Impact: The average number of page views between being published and September 30th 2014 was 203 and the average time spent on the page was one minute and 51 seconds (source: Google Analytics).

DIVERSIFY LOGO

The project logo has been created from the first month of the project, taking into account the different anatomic characteristics of the six fish species included in the project. In particular, the different fish tails have been included in the logo as identity print of each species (**Fig. 31.1.15**). The application of different color range has contributed to the diversification concept of the project. Dr. Karl Andree, a fish health researcher from Partner 3 IRTA, Spain, created the logo. The logo is currently used in all the pages and subpages of the website (**Fig. 31.1.17**), in the profiles of the social networks, Facebook (Figure 31.1.19) and twitter (**Fig. 31.1.18**), in all dissemination material (brochure – **Fig. 31.1.20** and **31.1.21**- and bookmark – **Fig. 31.1.22**-) and as part of the signature of the partners. A short version including only the tails has become the easiest way to recognize any dissemination material related to the project (**Fig. 31.1.16**).

Figure 31.1.15 The full project logo including the acronym as designed by Dr. Karl Andree, P3. IRTA and Dr Pantelis Katharios, P1. HCMR, Spain.

Figure 31.1.16 The short version of the logo of DIVERSIFY as designed by Dr. Karl Andree, P3. IRTA, Spain.

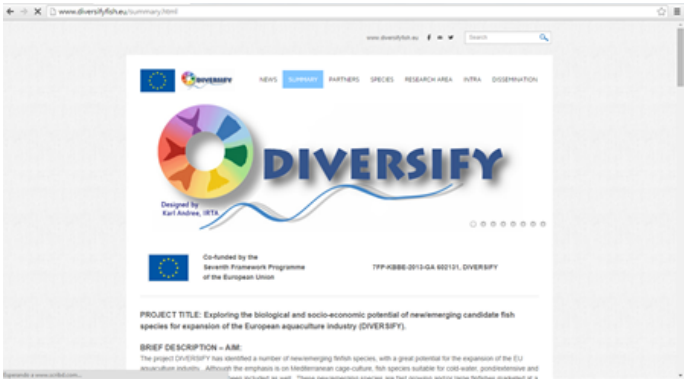


Figure 31.1.17 Desktop capture of the "Summary" page in the website www.diversifyfish.eu/summary.html

Figure 31.1.18 Desktop capture of the Home page in twitter twitter.com/diversifyfish

Figure 31.1.19 Desktop capture of the home page of the project in Facebook www.facebook.com/diversifyfish

DIVERSIFY BROCHURE

A project brochure has been prepared as printed dissemination material of DIVERSIFY. The information provided in this document gives a summarized overview of the scientific and socioeconomic research that is going to be carried out, and the expected results and products from the project (Figures 31.1.20 and 31.1.21).

The design of the brochure (coordinated by EUFIC) has focused on the description of the objectives, aims, background and expected results and products of DIVERSIFY. The text and images included in the brochure have been selected to present the information in an easy, clear and attractive way to all kinds of audiences.

The brochure includes five sections:

- • About diversify (including the selection criteria for the fish species of the project)
- • Main objectives
- • Expected outcomes
- • Research areas
- • List of partners

Relevant information about the financing source and administrative identification data of DIVERSIFY is also incorporated in the front page of the brochure.

A total of two thousand units of the brochure have been printed. The brochure has been distributed through the partners of the consortium, including the producer and processor associations such as APROMAR, BVFi, ANFACO, MASZ, and FGM. These organizations agglomerate members from the production sector (fingerling producers and grow out farmers), feed manufacturers, equipment suppliers, fish and shellfish processors, distributors and consumers. The partners have regular contacts with governmental organizations, regional and national policy decision makers, fishing industry representatives, NGO's and the media, which allow the broadcasting of the project advances and results.

Distribution of DIVERSIFY dissemination printed material has been also done at the occasion of conferences and other events of interest for the project, such as AQUA EUROPE meeting (Donostia – San Sebastián, Spain, October 14-17, 2014), project seminars, promotional workshops and any other event considered of interest for sharing project information.

Impact: So far 487 folders have been disseminated to aquaculture farms in Cyprus, Greece, Spain, Portugal and Turkey. Additionally, 50 bookmarks and leaflets were disseminated to various agencies in Greece and Spain, and 30 leaflets and bookmarks were distributed at the 79th International Fair of Thessaloniki (under the Greek General Secretariat for Research and Technology). Another 700 leaflets were distributed among the 38 project partners with the occasion of the ACM in Bari, Italy. As it is mentioned further in this report (Task 31.7, Dissemination to the food industry and consumers), association partners APROMAR, BVFi, ANFACO, MASZ, and FGM, have also distributed folders and bookmarks among their associates to promote project dissemination.

Figure 31.1.20 The DIVERSIFY brochure design (outer), produced by EUFIC, in collaboration with CTAQUA and HCMR.

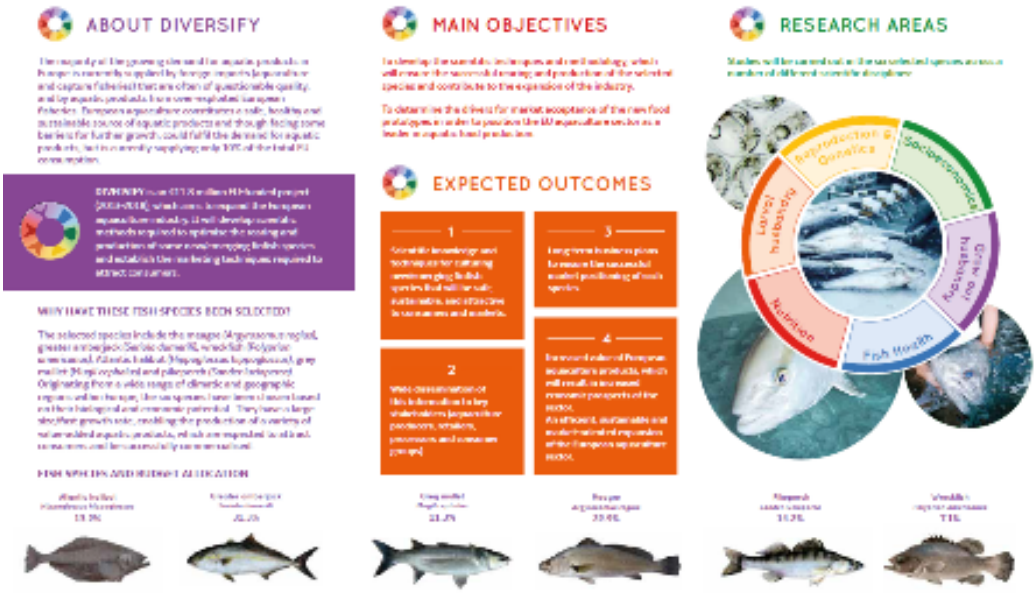


Figure 31.1.21 The DIVERSIFY brochure design (inside), produced by EUFIC, in collaboration with CTAQUA and HCMR.

DIVERSFY BOOKMARK

Another dissemination tool has been designed to increase the spreading of DIVERSIFY project. A bookmark with small concentrated project information has been created and printed (4000 units) and, as in the case of the brochure, distributed to the sector through the partners (Figure 31.1.22). Likewise, the bookmark has been distributed during EAS Conference in San Sebastian, Spain, October 2014. This material will be also distributed in any other event of interest for the project and during DIVERSIFY own organized events (full-day seminars, promotional workshops and annual meetings). A quick response (QR) code has been included in the strip, to allow easy and fast access to the DIVERSIFY website when scanned with a smartphone, tablet or any other authorized electronic device.

Figure 31.1.22 The DIVERSIFY bookmark design, produced by EUFIC, in collaboration with CTAQUA and HCMR

Impact: 590 bookmarks have been distributed so far to aquaculture farms, ministries, regional governmental institutions, AE 2014 and other international fairs, as well as the distribution done by the association partners of the project. Another 760 bookmarks were distributed among the 38 project partners with the occasion of the ACM in Bari, Italy.

PRODUCTION AND RELEASE OF DOWNLOADABLE AUDIOVISUAL MATERIAL

An initial promotional video has been produced and uploaded in the DIVERSIFY web (www.diversifyfish.eu/dissemination.html) and in YouTube (www.youtube.com/watch?v=Gk-_BhQjvCk). The video presents an overview of the project, including background, objectives, main research areas, and expected results and products from the project. (**Fig. 31.1.23**)

Additionally, short video recordings and a photo gallery are being regularly uploaded in the website of the project, in the NEWS section (www.diversifyfish.eu/) presenting the activities of DIVERSIFY within WP 2

Reproduction & Genetics - meagre, WP 3 Reproduction & Genetics – greater amberjack, WP 5 Reproduction & Genetics - Atlantic halibut, WP 6 Reproduction & Genetics – wreckfish and WP 9 Nutrition – greater amberjack which are the most active WPs at this initial moment of the project. (**Figs** from **31.1.24 to 31.1.29**).

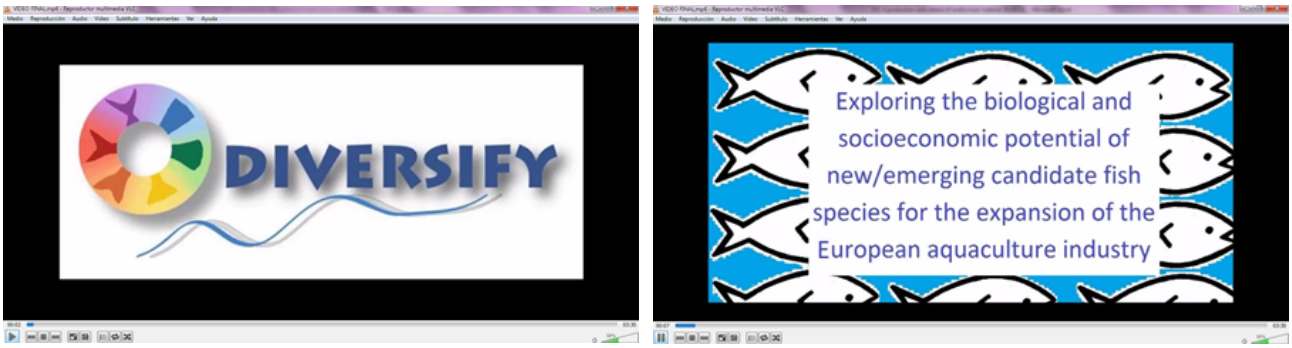


Figure 31.1.23 Desktop capture of the first two sequences of the initial DIVERSIFY video.

Figure 31.1.24 Desktop capture of the activities done on greater amberjack feeding protocols in the NEWS page of the website www.diversifyfish.eu/

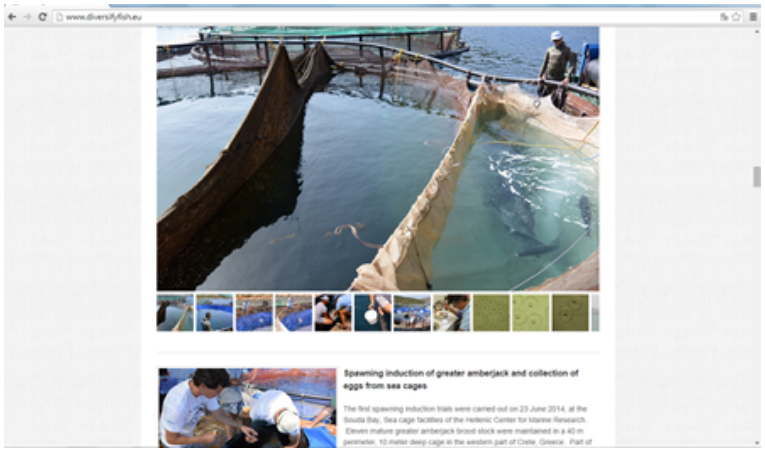


Figure 31.1.25 Desktop capture of the activity on greater amberjack reproduction in the NEWS page of the website www.diversifyfish.eu/

Figure 31.1.26 Desktop capture of the activity on meagre reproduction in the NEWS page of the website www.diversifyfish.eu/

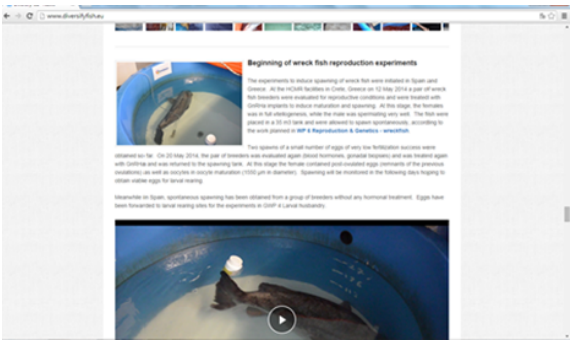


Figure 31.1.27 Desktop capture of the activity on wreckfish reproduction the NEWS page of the website www.diversifyfish.eu/

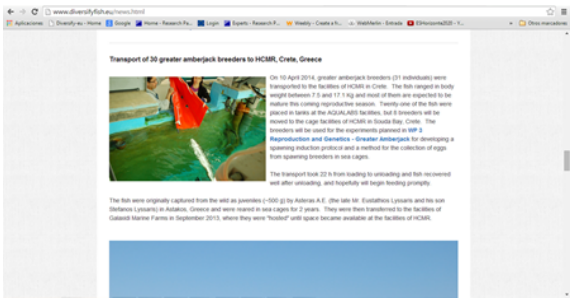


Figure 31.1.28 Desktop capture of greater amberjack broodstock transport activity in the NEWS page of the website www.diversifyfish.eu/

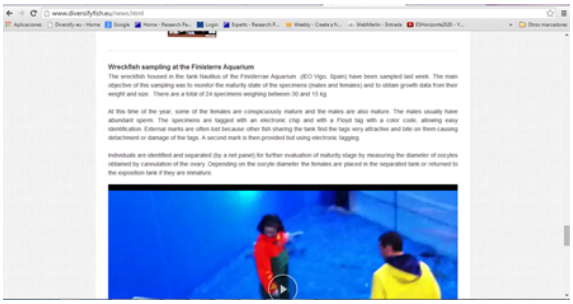


Figure 31.1.29 Desktop capture of wreckfish broodstock sampling activity in the NEWS page of the website www.diversifyfish.eu/

A new video reporting on the experiments carried out with wreckfish by P.32 MC2 and P.8 IEO is being elaborated at the time of preparing this annual report.

ADDITIONAL DISSEMINATION ACTIVITIES

Various dissemination activities were undertaken during the first year of the project, in order to make the kick-off of this important initiative known to the scientific community and the aquaculture industry, as well as the general public (**Table 31.1.1**).

Table 31.1.1 Dissemination activities of DIVERSIFY from the beginning of the project, including Press Releases, Internet-based articles, Interviews, Newspaper and Magazine articles. This information has been uploaded on the Dissemination activities site of the SESAM application of the Participants Portal.

DIVERSIFY
KBBE2013
Dissemination articles

No	Date	Discipline	WorkPackage	Title	Type	Language	Link	File name
1	6-Νοε-13	All	All	Ctaquac will help diversify EU aquaculture	web	english	fis.com http://www.mispeces.com/nav/actualidad/noticias/noticia-detalle/La-diversificacin-pisccola-espaola-a-travs-de-la-corvina-el-lenguado-y-la-seriola/#.Up7ok9H-LWR	20131106 fis.com
2	28-Νοε-13	All	All	La diversificatcion piscicola espanola a traves de la corvina, el lenguado y la seriola	web	spanish		201312 "La diversificatcion piscicola espanola a traves de la corvina, el lenguado y la seriola"
3	15-Δεκ-13	All	All	Enhancing the European aquaculture production by removing production bottlenecks of emerging species, producing new products and accessing new markets	magazine	english	http://www.theparliament.com/	20131211 Parliament magazine DIVERSIFY
4	5-Φεβ-14	All	All	The fish that will feed Europe in the future	newspaper	greek	hardcopy	20140205 Patris-Crete.pdf
5	5-Φεβ-14	All	All	IMBBC of HCMR in a research programme for aquaculture	web	greek	http://www.flashnews.gr/page.ashx?pid=3&aid=162746&cid=312	20140205 flashnews, 20140205 Flashnews.gr - Σε ερευνητικό πρόγραμμα ιχθυοκαλλιεργειών το ΙΘΑΒΒΥΚ ΕΛΚΕΘΕ
6	5-Φεβ-14	All	All	Kickoff meeting of the research programme DIVERSIFY at IMBBC/HCMR	web	greek	http://www.2810.gr/index.php/news-texnologia-epistimi/39952-enarktiria-synantisi-tou-erevnitikoy-programmatos-diversify-sto-ithavvyk-elkethe	20140205 www.2810
7	5-Φεβ-14	All	All	Inauguration of the programme DIVERSIFY for the production of aquaculture fish	web	greek	http://www.agrocapital.gr/Category/Thalassis/Article/8761/xekina-to-programma--diversify-gia-tin-paragwgi-psariwn-ichthyokalliergeias-	20140205 Agrocapital, 20140205 Προϊόντα Θαλάσσης Ξεκινά το πρόγραμμα Diversify για την παραγωγή ψαριών ιχθυοκαλλιέργειας
8	5-Φεβ-14	All	All	HCMR/IMBBC in a large research project for aquaculture	web	greek	http://www.cretalive.gr/culture/view/to-elkethe-ithabbuk-se-megalo-ereunhtiko-programma-gia-ichthuokalliergeies/140175	20140205 CRETALIVE
9	6-Φεβ-14	All	All	Kickoff meeting of the research programme DIVERSIFY	web	greek	http://www.kathimerini.com.cy/index.php?pageaction=kat&modid=1&artid=161776	20140206 Η ΚΑΘΗΜΕΡΙΝΗ Εναρκτήρια συνάντηση του ερευνητικού προγράμματος DIVERSIFY
10	7-Φεβ-14	all	all	Project DIVERSIFY started	web	spanish	http://www.mispeces.com/nav/actualidad/noticias/noticia-detalle/Expertos-europeos-dan-inicio-al-proyecto-europeo-Diversify/#.UxYVdPldNqU	mispeces noticias 07022014
11	11-Φεβ-14	All	All	Arranca DIVERSIFY	web, interview	english	http://aquicat.blogspot.com.es/2014/02/arranca-diversify.html	20140211 Arranca DIVERSIFY
12	11-Φεβ-14	All	All	EC works to diversify aquaculture	web, blog	spanish	http://www.worldfishing.net/news101/industry-news/ec-works-to-diversify-aquaculture	20140211 World Fishing & Aquacult...'diversify' aquaculture
13	14-Φεβ-14	All	All	Diversify, a new project for the Mediterranean Aquaculture	radio/TV interview	greek	http://www.ant1iwo.com/ellada/2014/02/15/nea-eidh-cariwn-stis-ixuyokaliergeies-ky/	20140216 Ant1 iwo
14	21-Φεβ-14	All	All	Dr Mylonas speaks to AgroTypos for the largest research project in aquacultrue that he is coordinating	web, interview	greek	http://www.agrotypos.gr/index.asp?mod=articles&id=84765	20140221 Αγροτυπος.gr
15	7-Μαρ-14	All	meagre	El proyecto Diversify tratará de dar respuesta a las preocupaciones del sector de la corvina	web	spanish	http://www.mispeces.com/nav/actualidad/noticias/noticia-detalle/El-proyecto-Diversify-tratar-de-dar-respuesta-a-las-preocupaciones-del-sector-de-la-corvina/#.Uxm_gNFuRfR	20140307 El proyecto Diversify tratará de dar respuesta a las preocupaciones del sector de la corvina
16	28-Μαρ-14	All	All	Βελτίωση της Ευρωπαϊκής παραγωγής ιχθυοκαλλιέργειας με την άρση των περιοριστικών παραγόντων για την παραγωγή νέων/αναδυόμενων ειδών, την παραγωγή νέων προϊόντων και την πρόσβαση σε νέες αγορές	magazine	greek	Fishing News (Αλιευτικά Νέα), 385, Feb 2014	20140328 Αλιευτικά Νέα
17	3-Απρ-14	All	All	Removing production bottlenecks of emerging species for European aquaculture	magazine, web	english	http://www.easonline.org/publications/eas-magazine (Aquaculture Europe, Vol 39(10 March)	20140403 Aquaculture Europe vol 39
18	7-Απρ-14	All	All	11.8 million to diversify EU aquaculture sector	magazine, web	english	http://www.intrafish.com/ffi/epaper	20140407 INTRAFISH (FFI)
19	8-Απρ-14	All	All	Fish Diversify: The Hellenic Center for Marine Research, Greece, leads a project to enhance the European aquaculture production with new and emerging fish species	magazine, web	english	http://www.paneuropeannetworks.com/H2/#90	20140408 H2020 Portal
20	29-Απρ-14	All	All	New EU project aims to expand the production, marketing and consumption of European finfish species	web	english	http://www.eufic.org/article/en/page/FTARCHIVE/artid/New_EU_project_aims_to_expand_the_production_marketing_and_consumption_of_European_finfish_species/	20140429 Food today
21	15-Μαϊ-14	All	All	Acquacoltura europea: è arrivato Diversify	web	italian	file://localhost/1.%20PROGRAMS/13.%20DIVERSIFY/Dissemination/Articles/2014/20140501 6%20food%20&%20tec%20%20Notizie%20%20Acquacoltura%20europea%20%20e%CC%80%20arrivato%20Diversify.html	20140516 food & tec
22	4-Ιουν-14	All	All	VIDEO DIVERSIFY	web	english	https://docs.google.com/file/d/0BwJmB6K6-pZXY2t3ODRFNExUNTQ/edit	
23	1-Αυγ-14	All	All	EU aquaculture boosted through research into emerging species	web	english	http://fis.com/fis/worldnews/worldnews.asp?l=e&country=0&special=&monthyear=&day=&id=70344&ndb=1&df=0	20140801 "FIS.com"

PRESS RELEASE

On the occasion of the Kick-off meeting, several press releases were made in Greek (Kathimeri, Flash news, Cretalive, Agrocapital, Agrotypos, Fishing news, etc.), Spanish (Anfaco, ipac, Mispeces, La voz digital, Ctaqua, Finanzas.com, Besana, Faro de Vigo, La Mar Salao, etc.), Italian (Food & Technology) and international (World & Fishing, Aquafeed, Eufic, Fis, etc.) press and web-based media organizations that follow aquaculture activities (**Figs** from **31.1.30** to **31.1.36**).

Figure 31.1.30 Desktop capture of Greek press release about DIVERSIFY project at Flash News newspaper www.flashnews.gr/page.ashx?pid=3&aid=162746&cid=312

Figure 31.1.31 Desktop capture of Greek press release about DIVERSIFY project at Agrocapital website www.agrocapital.gr/Category/Thalassis/Article/8761/xekina-to-programma--diversify-gia-tin-paragwgi-psariwn-ichthyokalliergeias-

Figure 31.1.32 Desktop capture of Spanish press release about DIVERSIFY project at Ctaqua website www.ctaqua.es/140206-reunion-lanzamiento-diversify-ctaqua.aspx#.VDUdtfl_te9

Figure 31.1.33 Desktop capture of Spanish press release about DIVERSIFY project at the ipac website www.ipacuicultura.com/noticias/en_portada/33016/da_comienzo_diversify_el_objetivo_convertirse_en_un_pilar_de_apoyo_para_la_diversificacion_acuicola_en_europa.html

Figure 31.1.34 Desktop capture press release about DIVERSIFY project at the World&Fishing website www.worldfishing.net/news101/industry-news/ec-works-to-diversify-aquaculture

Figure 31.1.35 Desktop capture press release about DIVERSIFY project at the Aquafeed website www.aquafeed.com/read-article.php?id=5203&sectionid

Figure 31.1.36 Desktop capture of the press release about DIVERSIFY project posted at EUFIC website www.eufic.org/article/es/show/eu-initiatives/rid/diversify/

ARTICLES

On 30th October 2014 a 5 pages article was published in the electronic magazine of the Spanish Aquaculture Observatory (OESA), (Figure 31.1.37). DIVERSIFY project was selected as project of special interest to be included their electronic publication. .

Concerning International press, a full page article was published in the 16th December 2013 issue of the "The Parliament Magazine", a publication on politics, policy and people in the European Union (**Fig. 31.1.38**).

An extensive, 10-page feature article was published by the European Aquaculture Society in its 1st Semester issue of 2014, presenting information about DIVERSIFY in terms of the selected species and the justification for their selection, and describing the various scientific areas of research (Figure 31.1.39). A two-page article was also included in the April issue of the Pan European Network Portal "Horizon 2020" (**Fig. 31.1.41**). On April 2014 another article was published of the EUFIC Magazine "Food Today" (**Fig. 31.3.42**). DIVERSIFY has been also included in the CommNet Catalogue 2014 (**Fig. 31.1.43**) launched in June 2014. With occasion of CommNet annual conference, DIVERSIFY has been presented by Javier Ojeda (P12. APROMAR) during the CommNet Workshop held in Brussels last June 2014 (see page 19) .

The latest article about DIVERSIFY project has been published in the magazine Hatchery International (http://hatcheryinternational.com/topics/diversify-project/) on 13th October 2014 (**Fig. 31.1.40**).

Figure 31.1.37 Desktop capture of the article about DIVERSIFY published on OESA Foundation website http://www.fundacionoesa.es/proyectos-i+d-destacados/diversify

Figure 31.1.38 Full page Article about DIVERSIFY project published in "The Parliament Magazine" (Dec. 2013).

Figure 31.1.39 Cover page of the article published on the European Aquaculture Society magazine. Aquaculture Europe vol. 39 (1) March 2014.

Figure 31.1.40 Article about DIVERSIFY published on Oct 2014 in Hatchery International website http://hatcheryinternational.com/research/greek-research-centre-to-focus-on-early-rearing-in-european-/

Figure 31.1.41 Desktop capture of the full article about DIVERSIFY posted at the Pan European Network Portal "Horizon 2020" www.horizon2020publications.com/H2/#90

Figure 31.1.42 Desktop capture of the article about DIVERSIFY published on "Food Today" and posted at the EUFIC website www.eufic.org/article/en/page/FTARCHIVE/artid/New_EU_project_aims_to_expand_the_production_marketing_and_consumption_of_European_finfish_species/

WORKSHOPS

On Jun 12Th 2014, Javier Ojeda from APROMAR (P.12) presented DIVERSIFY project with the occasion of the Workshop organized by CommNet: "New fish to feed the world. DIVERSIFY identifies the potential of new species for fish farming".

Figure 31.1.43 CommNet catalog presenting DIVERSIFY project (pages from 8 to 10)

Task 31.2 Annual Coordination Meetings (led by HCMR, Constantinos Mylonas).

The Kick-off meeting was held at the Hellenic Centre of Marine Research (P1. HCMR) in Heraklion, Crete, Greece on the 29 and 30 January 2014. The 2-day meeting was attended by 73 persons: 21 coming from the Project Coordinator's (PC) organization (HCMR) and 52 coming from the other 35 Partners. The first part of the meeting was dedicated to presentations by the Project Coordinator, Dr. C.C. Mylonas relating to WP 1 Project management (Figure 31.2.1), including a description of the governing bodies, the structure of the Annual Coordination Meetings, Reporting, the use of the Participants Portal, Financial reporting, the use of the DIVERSIFY's web site INTRANET facilities, instructions on publications and communications, and approval of a few amendments that need to be made in the DOW. Then the Group Work Package leaders made presentations of the work to be implemented in each area of Scientific Research. No dissemination activities took place during this first meeting.

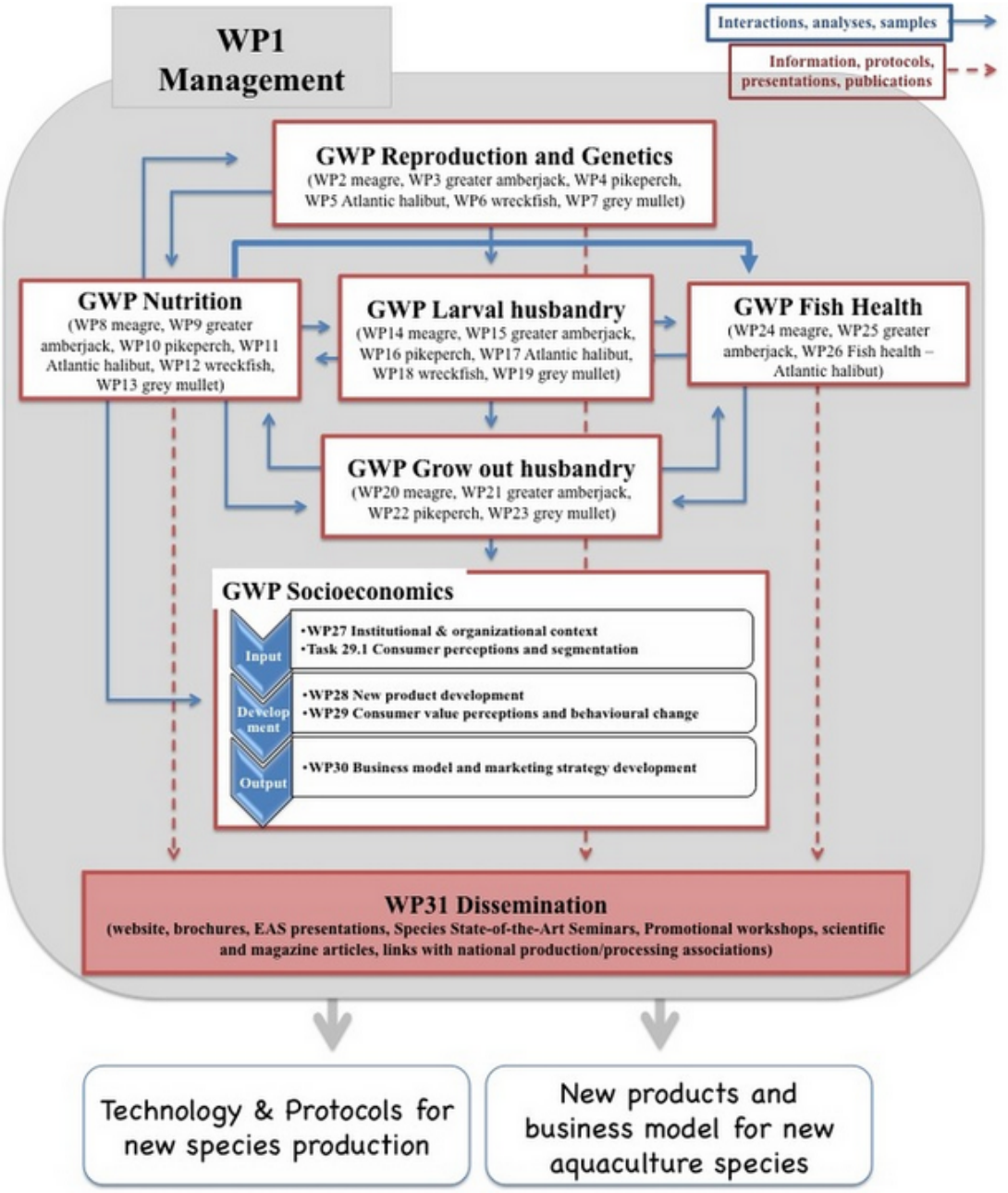


Figure 31.2.1 Graphic presentation of the organization of research and management activities.

The Annual Coordination Meeting 2014 took place in Bari, Italy, from 4 to 6 November 2014 and was hosted by P. 13, University of Bari (Dr. Aldo Corriero). As planned, the first day was dedicated to summary presentations by the six Group work package leaders as well as some short presentation from selected tasks implemented during the first year of the project. Detailed minutes of the meeting have been prepared by the PC and submitted to the EU office, Dr Marta Iglesias, and a report was also prepared and uploaded in the Participants Portal as Deliverable D1.3.

Task 31.3 Presentation of DIVERSIFY at the AQUA EUROPE meetings (led by HCMR, Constantinos Mylonas).

A presentation has been made at the European Aquaculture Society's annual meeting (AQUACULTURE 2014) at San Sebastian, Spain during 14-17 October 2014 (**Fig. 31.3.1**). The 20-min presentation was given in the "Species Diversification" Sessions and was titled "**DIVERISFY-Exploring the biological and socioeconomic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry**". The session was chaired by Dr. Benito Peleteiro from P8. IEO and Dr. Rocío Robles, the WP31 Dissemination leader, from P12. CTAQUA. The presentation included relevant information regarding the project, such as the selected new/emerging species, the scientific disciplines in which work is going to be done, the partnership and structure of the consortium and its funding. A few slides were dedicated to the project's website, in order to introduce the audience to the extensive content of the site and encourage them to utilize the site to keep updated about the progress of the project, but also to provide feedback to the partners. At the end, some of the early results of the project, mainly in the area of Reproduction & Genetics have been presented.

Since this was the first of such presentations and a limited number of results were available at the time, it was decided to make an introductory presentation of DIVERSIFY, familiarizing the aquaculture community with the project. An estimated 120 persons attended the presentation.

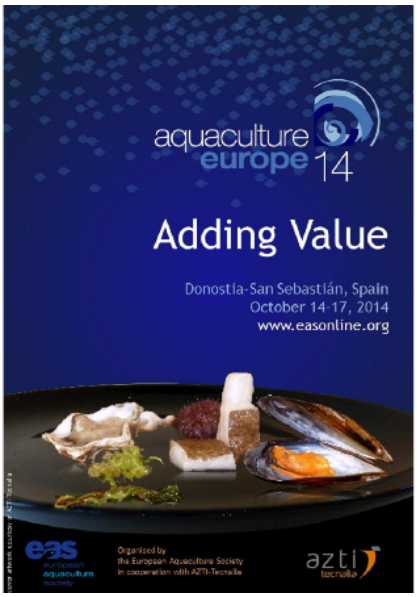


Figure 31.3.1 The announcement poster of the AQUACULTURE EUROPE 2014 conference that is organized every year by the European Aquaculture Society, and a panoramic view of the bay of San Sebastian.

The objective of the project were presented, together with the justification of the project's species selection, which includes meagre (*Argyrosomus regius*) and greater amberjack (*Seriola dumerili*) for warm-water marine cage culture, wreckfish (*Polyprion americanus*) for warm- and cool-water marine cage culture, Atlantic halibut (*Hippoglossus hippoglossus*) for marine cold-water culture, grey mullet (*Mugil cephalus*) a euryhaline herbivore for pond/extensive culture, and pikeperch (*Sander lucioperca*) for freshwater intensive culture using recirculating systems (**Figs. 31.3.2** and **31.3.3**).

Figure 31.3.2 Opening slide of the DIVERSIFY presentation at AQUACULTURE EUROPE 2014.

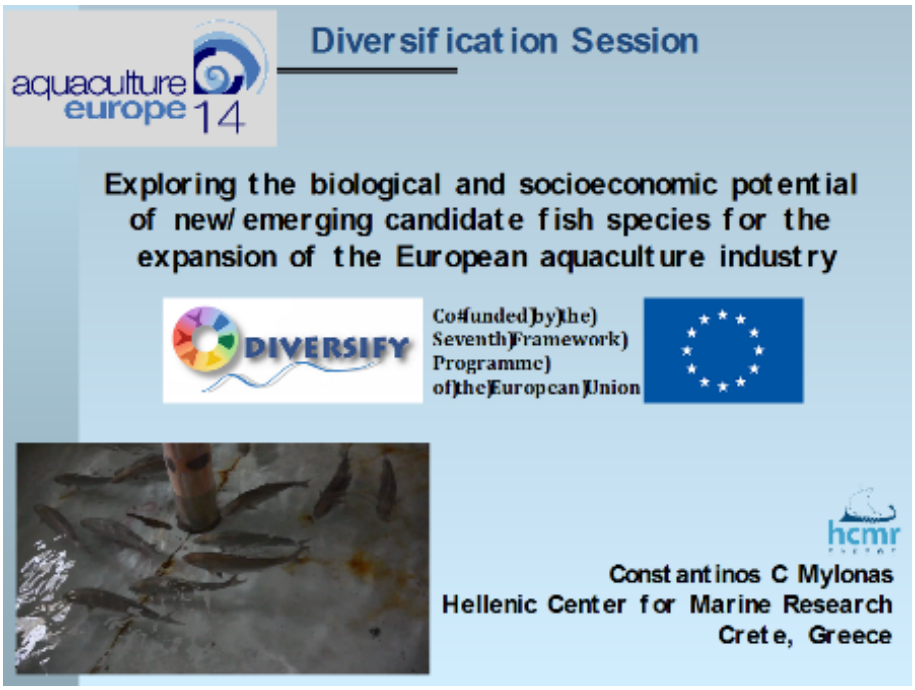


Figure 31.3.3 Slide with the justification of the species selection from the DIVERSIFY presentation at AQUACULTURE EUROPE 2014

Then there was a brief description of the identified bottlenecks of each of the selected species (**Fig. 31.3.4**), and a brief outline of the work planned in the six scientific disciplines, which are Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health and Socioeconomics, including final product quality (**Fig. 31.3.5**).

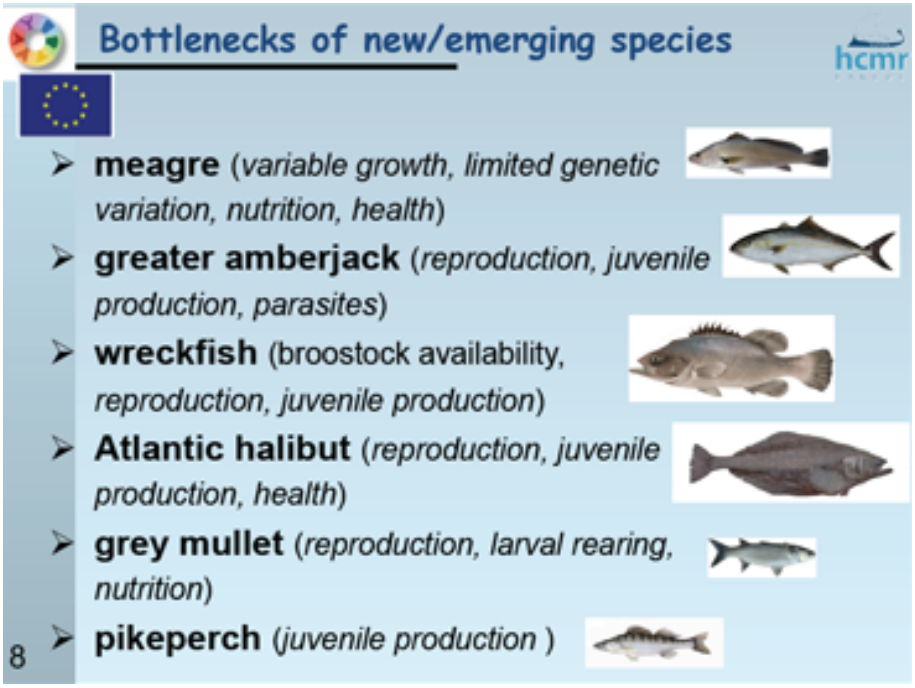


Figure 31.3.4 Slide with the bottlenecks identified for each species from the DIVERSIFY presentation at AQUACULTURE EUROPE 2014.

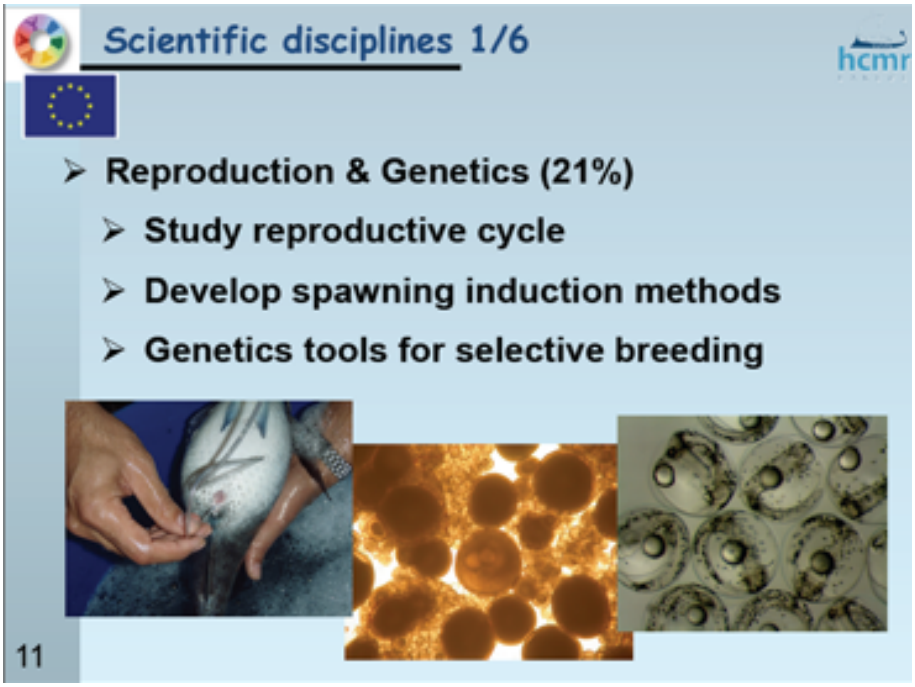


Figure 31.3.5 Slide with the work planned for the scientific disciplines --*e.g.*, Reproduction and Genetics from the DIVERSIFY presentation at AQUACULTURE EUROPE 2014.

Afterwards, a presentation of the website (www.diversifyfish.eu) and its organization was provided, encouraging people to seek information on the project, either according to species of interest or of scientific discipline (**Fig. 31.3.6**). At the end of the presentation, the Coordinator informed the audience about the next Annual Coordination Meeting and the possibility to attend for interested researchers and industry managers willing to participate during the open day of the meeting. The attendees were also handed out the DIVERSIFY brochure and bookmark produced recently to introduce the project (**Figs 31.1.21, 31.1.22** and **31.3.7**). Brochures were also made available to all conference participants at specific literature distribution tables and relevant sessions. Also, the Project Coordinator and the WP31 Leader handed out DIVERSIFY brochures to researchers and industry managers with whom they discussed the project and the potential for dissemination and collaboration with other National initiatives.

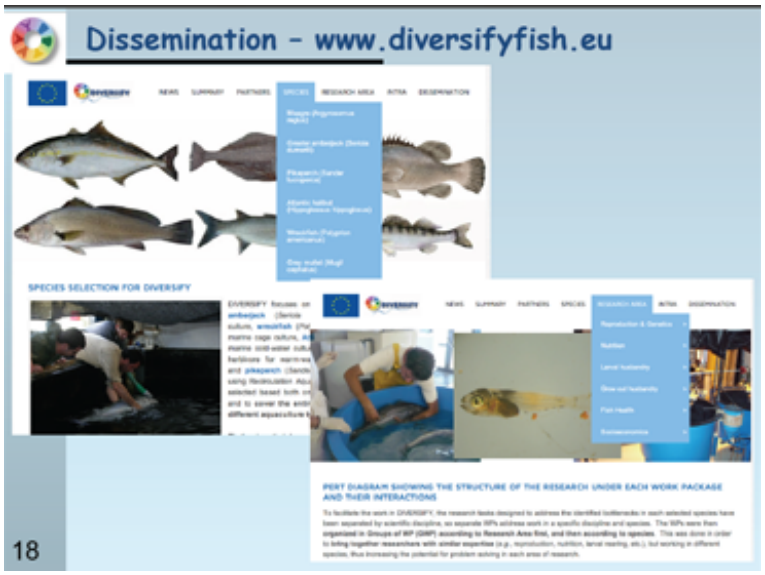


Figure 31.3.6 Slide with a presentation of the project's webpage from the DIVERSIFY presentation at AQUACULTURE EUROPE 2014.

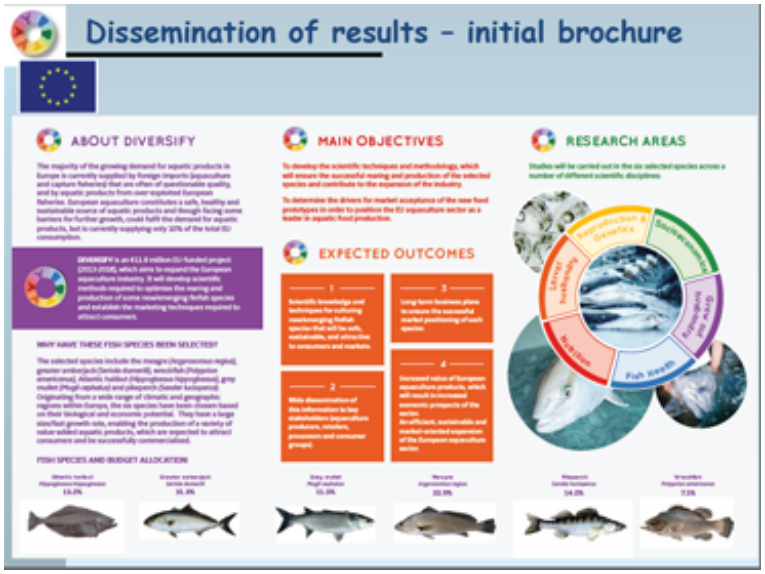


Figure 31.3.7 Slide with the brochure produced recently and distributed to the people attending the presentation, as well as the conference at large from the DIVERSIFY presentation at AQUACULTURE EUROPE 2014.

During the conference, there were many opportunities to publicize DIVERSIFY and inform numerous colleagues on the project's objectives and planned work. People were encouraged to follow the website of the project, where it is intended to upload brief, concise but meaningful updates on implemented work and the obtained results, on a regular basis (monthly or more often). Interested researchers and industry managers were also informed of the open component of the Annual Coordination Meetings, and various researchers expressed already the interest to attend our meetings, in an effort to benefit from the knowledge produced by the project, but also to investigate the potential of coordinating their research activities with DIVERSIFY and perhaps carry out joined experiments.

Also, a meeting was arranged with the Mr Nigel Balmforth of Wiley - Blackwell, who expressed an interest in publishing the final results of DIVERSIFY in the form of a book or a series of books, probably specific to each species. As the Project Coordinator edited a book recently (2012, Sparidae: Biology and Aquaculture of Gilthead Seabream and Other Species) with this publisher, there is an established relation with the company representative responsible for the Aquatic Sciences titles of Wiley - Blackwell, which will facilitate any effort at producing such books from DIVERSIFY.

Overall, the presentation in the Diversification Session and the presence of both the Project Coordinator and the WP31 Dissemination leader was considered successful, resulting in increased awareness for DIVERSIFY and developing various contacts for future collaboration within its frame.

Task 31.4 Scientific presentations and submission of manuscripts (led by HCMR, Constantinos Mylonas).

No work done during this period.

Task 31.5 Full-day seminars on "Know-how Transfer" of the aquaculture for each of the studied species (led by CTAQUA and the Species Leader Partner)

No work done during this period.

Task 31.6 Promotional workshops (led by CTAQUA, Rocio Robles). No work done during this period

Task 31.7 Dissemination to the food industry and consumers (led by APROMAR Javier Ojeda and EUFIC, Jessica Miller and Adrian Giordani).

COLLABORATION AGREETMENTS

The first objective was to stablish collaborations agreements with the European Association of Supermarkets (COOPERNIC), the European consumer organization (EUROCONSUMERS) and the platform Global Initiative for Life and Leadership through Seafood (GILLS) to create awareness of the project among their associates and to establish collaboration agreements in terms of including DIVERSIFY results and updates in their websites.

Due to difficulties encountered to reach collaboration agreements with the initially proposed organizations (COOPERNIC, EUROCONSUMERS, BEUC and GILLS) the list of potential partners was widened. The following organizations have been contacted:

- EUROCOMMERCE (European Organization of retail, wholesale and trading companies) http://www.eurocommerce.be
- ASEDAS (Spanish member of Eurocommerce) http://www.asedas.chil.org
- BEUC (European Consumer Organisation) http://www.beuc.org

- EUROCONSUMERS (European Consumers Organization) http://www.euroconsumers.org
- OCU (Spanish Consumers Organization) http://www.ocu.org
- GILLS (Global Initiative for Life & Leadership through Seafood)
  https://www.facebook.com/GILLSEAFOOD
- COOPERNIC (European Cooperative Organization of Independent Retailers)
- FEAP (Federation of European Aquaculture Producers) http://www.feap.info
- EATiP (European Aquaculture Technology and Innovation Platform) http://www.eatip.eu
- AIPCE-CEP (European Fish Processors Association (Association des Industries du Poisson de l´UE) and CEP, European Federation of National Organizations of Importers and Exporters of Fish), http://www.aipce-cep.org/

Collaboration Agreements have been signed, so far, between DIVERSIFY and OCU (**Fig. 31.7.1**), FEAP (**Fig. 31.7.2**) , EATiP (**Fig. 31.7.3**) and AICEP (**Fig. 31.8.4**).

EUROCOMMERCE, ASEDAS, BEUC and EUROCONSUMERS were not interested in the collaboration and declined the offer. They suggested redirecting DIVERSIFY's efforts to other organizations.

GILLS (Global Initiative for Life & Leadership through Seafood) has merged with the Association of International Seafood Professionals (http://seafoodprofessionals.org) and an agreement has not been reached yet. Because of the high interest of DIVERSIFY to collaborate with this organization, the efforts to reach an agreement with them will continue for as much time as necessary.

COOPERNIC (European Cooperative Organization of Independent Retailers), now renamed CORE, is a purely commercial organization directed to improving the buying power of its members. They have communicated to us that they are not interested in disseminating DIVERSIFY's activities.

COLLABORATION AGREEMENT

DIVERSIFY is a European research project that explores the biological and socio-economic potential of new/emerging candidate aquaculture fish species. This initiative is an ambitious 5-year project (2013-2018) that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07). The DIVERSIFY consortium includes 38 partners from 12 European countries. Further information can be obtained from the project's website at www.diversifyfish.com.

DIVERSIFY includes a strong effort on dissemination of its results. For this reason, and to formalise relationships, it establishes individual collaboration agreements with relevant European food industry and consumer organisations.

The ORGANIZACIÓN DE CONSUMIDORES Y USUARIOS (OCU) is a Spanish, independent, private & non-profit association that defends consumer's rights since 1975 and works to help them to assert their rights. OCU has more than 300.000 members that pay the fees that support the organisation (www.ocu.org/organizacion). OCU is member of EUROCONSUMERS (www.euroconsumers.org) and BEUC (www.beuc.eu).

The DIVERSIFY Consortium and OCU agree on the value of sharing the main results of the DIVERSIFY project.

To achieve this objective, OCU will communicate to its members through its website the main relevant results of DIVERSIFY. OCU will decide which of the news offered by DIVERSIFY are finally communicated and how.

The Consortium of DIVERSIFY will offer OCU permanent information on the development of the project, will answer any question that OCU might have on the subject and will periodically update OCU with the results of the project.

This agreement includes no economic obligations between the two parties.

This agreement has no further obligations for either side.

This agreement will begin at the date of its signature and will terminate with the ending of the DIVERSIFY project in December 2018.

Dated: September 10th, 2014

By the DIVERSIFY Consortium
Mr Dinos Mylonas

By OCU
Mr. David M. Ortega Peciña

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

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from www.diversifyfish.com.

Figure 31.7.1 Collaboration agreement signed by OCU

COLLABORATION AGREEMENT

DIVERSIFY is a European research project that explores the biological and socio-economic potential of new/emerging candidate aquaculture fish species. This initiative is an ambitious 5-year project (2013-2018) that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07). The DIVERSIFY consortium includes 38 partners from 12 European countries. Further information can be obtained from the project's website at http://www.diversifyfish.eu.

DIVERSIFY includes a strong effort on dissemination of its results. For this reason and to formalise relationships, it establishes individual collaboration agreements with relevant European food industry and consumer organisations.

The FEDERATION OF EUROPEAN AQUACULTURE PRODUCERS (FEAP) is the united voice of the European fish farming industry. FEAP has 26 members drawn from 22 States across the European continent. FEAP continuously supports and promotes the responsible development of European aquaculture and, through diversified support actions, develops and provides the common positions and opinions of the European sector (http://www.feap.info).

The DIVERSIFY Consortium and FEAP agree on the value of sharing the main results of the DIVERSIFY project.

To achieve this objective, FEAP will communicate to its members through its website the main relevant progress and results of DIVERSIFY. FEAP retains the right to decide the content of the news offered by DIVERSIFY that are finally communicated and how.

The Consortium of DIVERSIFY will offer FEAP, on a regular and permanent basis, information on the development of the project, will answer any question that FEAP might have on the subject and/or content and will periodically update FEAP with the results of the project.

This agreement includes no economic obligations between the two parties.

This agreement has no further obligations for either party.

This agreement will begin at the date of its signature and will terminate with the ending of the DIVERSIFY project in December 2018.

Dated: September 24th, 2014

By the DIVERSIFY Consortium
Mr Dinos Mylonas

For the FEAP
Mr Arnault Chaperon
President

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

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from www.diversifyfish.eu.

Figure 31.7.2 Collaboration agreement signed by FEAP.

COLLABORATION AGREEMENT

DIVERSIFY is a European research project that explores the biological and socio-economic potential of new/emerging candidate aquaculture fish species. This initiative is an ambitious 5-year project (2013-2018) that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07). The DIVERSIFY consortium includes 38 partners from 12 European countries. Further information can be obtained from the project's website at http://www.diversifyfish.eu.

DIVERSIFY includes a strong effort on dissemination of its results. For this reason and to formalise relationships, it establishes individual collaboration agreements with relevant European food industry and consumer organisations.

The EUROPEAN AQUACULTURE TECHNOLOGY AND INNOVATION PLATFORM (EATiP) has been established by the European aquaculture sector to reinforce the research and innovation processes that are required within a modern and developing Europe. EATIP works on developing measures and structures that will improve the research, development and innovation conditions so as to support the sustainable development of European aquaculture (http://www.eatip.eu/).

The DIVERSIFY Consortium and EATiP agree on the value of sharing the main results of the DIVERSIFY project.

To achieve this objective, EATiP will communicate to its members through its website the main relevant progress and results of DIVERSIFY. EATiP retains the right to decide the content of the news offered by DIVERSIFY that are finally communicated and how.

The Consortium of DIVERSIFY will offer EATiP, on a regular and permanent basis, information on the development of the project, will answer any question that EATiP might have on the subject and/or content and will periodically update EATiP with the results of the project.

This agreement includes no economic obligations between the two parties.

This agreement has no further obligations for either party.

This agreement will begin at the date of its signature and will terminate with the ending of the DIVERSIFY project in December 2018.

Dated: September 24th, 2014

By the DIVERSIFY Consortium
Mr Dinos Mylonas

By EATiP
Mr Gustavo Larrazábal
Chairman

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

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from www.diversifyfish.eu.

Figure 31.7.3 Collaboration agreement signed by EATiP.

COLLABORATION AGREEMENT

DIVERSIFY is a European research project that explores the biological and socio-economic potential of new/emerging candidate aquaculture fish species. This initiative is an ambitious 5-year project (2013-2018) that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07). The DIVERSIFY consortium includes 38 partners from 12 European countries. Further information can be obtained from the project's website at www.diversifyfish.com.

DIVERSIFY includes a strong effort on dissemination of its results. For this reason, and to formalise relationships, it establishes individual collaboration agreements with relevant European food industry and consumer organisations.

AIPCE-CEP (European Fish Processors Association (Association des Industries du Poisson de l´UE) and **CEP, European Federation of National Organisations of Importers and Exporters of Fish)** are professional associations established in 1959 and working together on the basis of a Co-operation Agreement. They are integrated by the EU Fish Processors and Traders Association.

.The DIVERSIFY Consortium and AIPCE-CEP agree on the value of sharing the main results of the DIVERSIFY project.

To achieve this objective, AIPCE-CEP will communicate to its members through its website the main relevant results of DIVERSIFY. OCU will decide which of the news offered by DIVERSIFY are finally communicated and how.

The Consortium of DIVERSIFY will offer AIPCE-CEP permanent information on the development of the project, will answer any question that AIPCE-CEP might have on the subject and will periodically update AIPCE-CEP with the results of the project.

This agreement includes no economic obligations between the two parties.

This agreement has no further obligations for either side.

This agreement will begin at the date of its signature and will terminate with the ending of the DIVERSIFY project in December 2018.

Dated: 5th December, 2014

By the DIVERSIFY Consortium
Dr Dinos Mylonas

By AIPCE-CEP
Mr. Guus Pastoor

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

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from www.diversifyfish.com.

Figure 31.7.4 Collaboration agreement signed by AICEP-CEP.

BVFi (P.34) after the kick-off meeting has provided a German text with the project description to be included in the project website. BVFi informed its members via a regular newsletter about the project and has informed the PC of a German new website for aquaculture information (www.aquakulturinfo.de) with the future option to report regularly news from Diversify. During the ACM, BVFi gave support to some research institutes to revise the market information reports from Germany and provided names for expert interviews. Finally BVFi informed the EU-umbrella organization of fish processors and importers (AIPCE-CEP) about the progress of Diversify and asked the board to sign a collaboration agreement, which is included in this document (**Fig 31.7.4**).

The Hungarian Aquaculture Association MASZ (P.35), following the kick-off meeting in January, has displayed on its web (www.masz.org) Diversify project's logo with the contact details, where visitors link on directly to the project's website (www.diversifyfish.eu) (Figure 31.7.5). The association has also distributed the project brochure to its members. The first quarterly Newsletter of the Association gave information to the members about the project's progress (March 2014). During this year the President of the MASZ participated in several professional forums, in which he had presented the role of MASZ in the Diversify project. Hungarian and Central Eastern European farmers took part in these events. The project description was translated to Hungarian, which available in the Association's website. The above mentioned paper via e-mail and also in printed form was distributed to the Hungarian farmers (May 2014). The feedback was positive and several farmers have interested in the project progress. The first bookmark and leaflet was also translated to Hungarian and it was distributed to our members (October 2014).

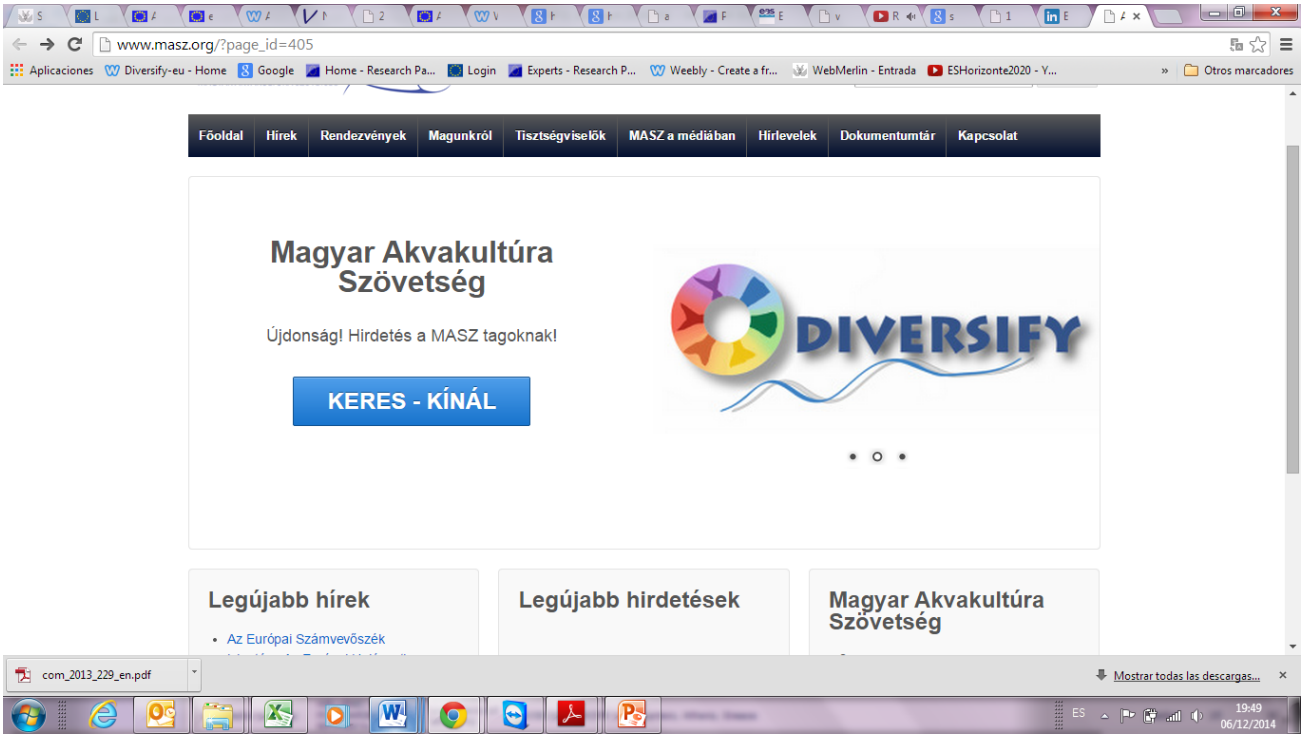


Figure 31.7.5 Desktop view of the initial page of MASZ (P.35) website.

APROMAR, FGM and ANFACO have also distributed the project dissemination material among their associates (leaflet and bookmarks) as well as informed their members about the project with the occasion of general assemblies.

ADDITIONAL DISSEMINATION ACTIVITIES TO JOURNALIST

A presentation was also given during a workshop at the headquarters of HCMR on 30 September 2014, to a group of 30 journalists from all over the Europe, who were in Athens on the occasion of the 40 years of the "Regional Seas" programme of the United Nations Environmental Programme. The workshop presented some of the large research projects coordinated by HCMR in the area of Marine Research. A 30-min presentation titled "**DIVERISFY-Exploring the biological and socioeconomic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry**" presented the journalists with information regarding the project, such as the selected new/emerging species, the scientific disciplines in which work is going to be done, the partnership and structure of the consortium and its funding. An effort was made to highlight the relation of DIVERSIFY to food security, human health, competitiveness, fisheries sustainability, environmental protection, etc. (**Fig. 31.7.4**)

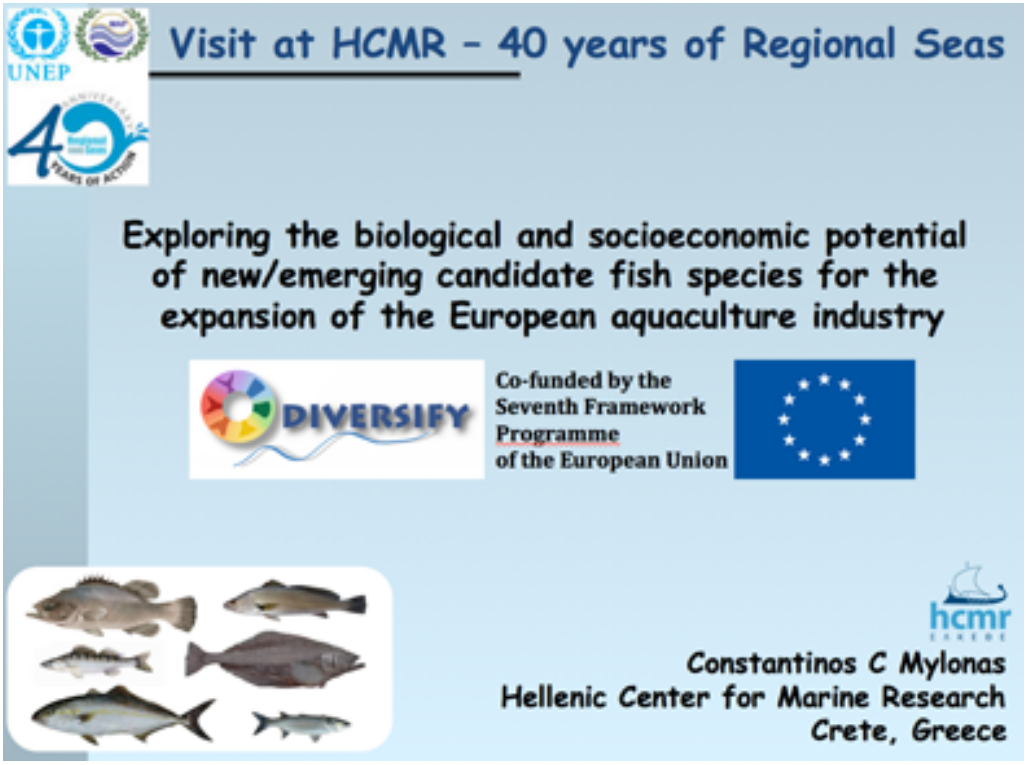


Figure 31.7.4 Opening slide of the presentation given to European, African and American journalists at the HCMR headquarters, on the occasion of the UNEP meeting of the Regional Seas programme in Athens, Greece in October 2014.

Deviations from Annex I and their impact:

There were no deviations from Anex I during this reporting period.

2.3 Project management during the period

Please use this section to summarise management of the consortium activities during the period. Management tasks are indicated in Articles II.2.3 and Article II.16.5 of the Grant Agreement.

Amongst others, this section should include the following:

- *Consortium management tasks and achievements;*
- *Problems which have occurred and how they were solved or envisaged solutions;*
- *Changes in the consortium, if any;*
- *List of project meetings, dates and venues;*
- *Project planning and status;*
- *Impact of possible deviations from the planned milestones and deliverables, if any;*
- *Any changes to the legal status of any of the beneficiaries, in particular non-profit public bodies, secondary and higher education establishments, research organisations and SMEs;*
- *Development of the Project website, if applicable;*

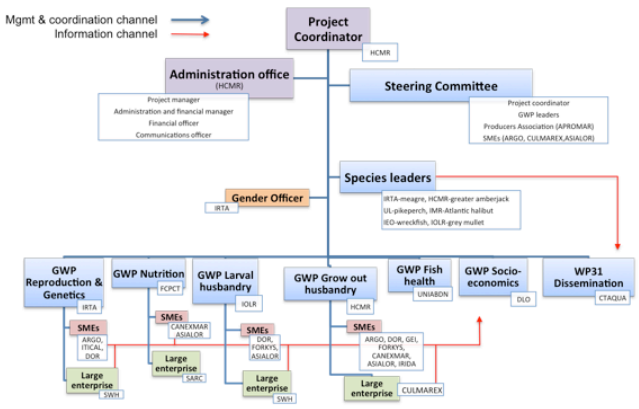
The section should also provide short comments and information on co-ordination activities during the period in question, such as communication between beneficiaries, possible co-operation with other projects/programmes etc.

Objectives

- Coordinate and implement the Technical Annex and Grant Agreement in a timely, efficient and successful manner,
- Provide the periodic reporting to the EU for the evaluation of the implementation of the programme, ensuring that correct and consistent financial and technical progress reports are submitted by participants and presented to the coordinator and submitted to the European Commission on time and in accordance with relevant guidelines,
- Organize and coordinate the work and exchange of information, samples and protocols among Partners involved in the same or different WPs,
- Organize and coordinate the work and exchange of information among Partners involved in work with the same species, but different work packages.

Establishment of management bodies

The first order of business for the management of this large-scale project –*i.e.*, involving many Partners, long duration, work on various species and activities in different disciplines- was the establishment of the necessary management bodies, as follows:



Project coordination team: To assist Project Coordinator (PC), a full-time staff (Executive Secretary or Project Manager) was recruited by P1. HCMR through an open call publicized in the Local and National presses (Greece). The appointed Project manager is Mr Yanis Fakriadis, M.Sc. who joined in May 2014 (month 6). Mr. Fakriadis has been involved in various management activities (see later) as well as in the RTD activities of the Group WP Reproduction (10-20%). Mr Stelios Kastrinakis (Special Account of Research Office) and Mr Manolis Dramitinos (Project Management Office) were also part of the coordination team, dealing with the financial issues of the project. Mrs Maria Papadaki is involved in the preparation and the management of the project's

website. Together, this team is responsible for the overall management of the project and communications with the EC, and will also work together with the WP31 Dissemination leader for the maintenance of the project's website and the preparation of dissemination activities and materials.

Group Work package Leaders (GWPL): The people leading the groups of related RTD WPs in the areas of Reproduction & Genetics, Nutrition, Larval husbandry, Grow out husbandry, Fish health and Socioeconomics were selected from the stage of the proposal preparation, again given their expertise and excellence in research in the scientific discipline. The GWPLs are:

Dr. Neil Duncan (P3. IRTA) for all Reproduction & Genetics WPs,

Dr. Marisol Izquierdo (P2. FCPCT) for all Nutrition WPs,

Dr. Bill Koven (P4. IOLR) for all Larval husbandry WPs,

Dr. Nikos Papandroulakis (P1. HCMR) for all Grow out husbandry WPs,

Dr. Chris Secombes (P5. UNIABDN) for all Fish health WPs, and

Drs. Gemma Tacken (P6. LEI/DLO) for all Socioeconomics WPs.

During the implementation of DIVERSIFY, GWPLs are responsible for coordination with each WP Lead Beneficiary (LB, see below) for (a) the timely execution of all planned research activities in their WPs, (b) the compilation of all results and preparation of the periodic reports and (c) liaising with the PC, WP31 Dissemination leader and the Species Leaders (see below) for the preparation of the dissemination material for the project (*e.g.*, web information, brochures, presentations and articles).

Work package Lead Beneficiaries (LB): The people leading each WPs have the responsibilities for (a) the timely execution of all planned research activities in their specific WP, following the projects time schedule (Table 1.3ii Gantt chart), (b) the compilation of all results and preparation of the periodic reports and (c) liaising with the PC, WP31 Dissemination leader and the Species Leaders. Similarly, due to the participation of many Partners in most research activities, for each Task and Action in the WPs, a Partner has been identified who is responsible for the implementation of the planned work and the preparation of the relevant reports (See Annex I-DOW, WT 3).

Species Leaders (SL): The SLs were selected from among the consortium based on their involvement, expertise and excellence in research with the selected species (one per species). At the phase of the proposal preparation, these Partners were responsible for identifying and prioritizing the main bottlenecks for the aquaculture production of each species. During the implementation of the program, the SLs have been involved in overseeing, and in the following years with compiling and disseminating the work done in the various RTD WPs of the project. The SLs are:

Dr. Alicia Estevez (P3. IRTA) for meagre,

Dr. Nikos Papandroulakis (P1. HCMR) for greater amberjack,

Dr. Pascal Fontaine (P9. UL) for pikeperch,

Dr. Birgitta Norberg (P7. IMR) for Atlantic halibut,

Dr. Jose Benito (Tito) Peleteiro Alonso (P8. IEO) for wreckfish, and

Dr. Bill Koven (P4. IOLR) for grey mullet.

Steering Committee (SC): The SC consists of the PC, the GWPLs, three SME representatives (P30. CULMAREX, P23. ARGO and P29. ASIALOR) and one representative from the professional associations (P12. APROMAR). The SC will be involved with the overall decision making of the project in matters of Technical Annex implementation and modification, evaluation of project progress, knowledge management and any conflict resolutions.

Coordination meetings

Kick off meeting. The Kick off meeting was held at the facilities of P1. HCMR in Heraklion, Crete, Greece on the 29 and 30 January 2014. The 2-day meeting was attended by 73 persons: 21 coming from the Project Coordinator's (PC) organization (HCMR) and 52 coming from the other 35 Partners. No representative was sent from three Beneficiaries (P20. SARC, P30. MAREMAR and P36. ANFACO). Beneficiaries SARC and ANFACO were unable to attend the meeting due to prior commitments that could not be changed, while Beneficiary MAREMAR has been removed from the consortium and was substituted with another Beneficiary (CULMAREX) in March 2014. The Group Work Package (GWP) leader for Nutrition, Dr. Marisol Izquierdo did not attend the meeting and was represented by Dr Lidia Robaina from FCPCT..

Information regarding the meeting was uploaded continually on the project's web site to ensure that all participants had access to the most updated information. The Agenda (**Table 3.2.3.1**) consisted of a common session for all participants during DAY 1, and 6 Workshop Sessions running in parallel (2 sessions at a time) during DAY 2. The DAY 1 session addressed management and dissemination issues, and provide beneficiaries with an idea of the scientific work planned. The DAY 2 workshops were organized according to Research Areas (GWP) in order to plan the work to be implemented in the various scientific WPs, and to address issues of harmonization of protocols, exchange of visits and samples among beneficiaries.

Table 3.2.3.1. Agenda of the Kickoff meeting, taking place on the 28-29 January 2014, at HCMR, Crete Greece.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenta

DAY 1	HCMR Auditorium				
Start	End	break	Title	Details	
9.00	9.30		Welcoming from A. Magoulas	Meeting logistics, agenda	Presentations of participants
9.30	10.00		Presentations of participants	Presentation of participants	
10.00	10.30		WP1 Management	Governing bodies, Annual meetings, interactions	
10.30	11.00		WP1 Management	Reporting, Participants Portal, Deliverables, Dissemination	
11.00	11.30	coffee			
11.30	12.00		WP1 Management	Financial issues, web site, communications, amendments	
12.00	12.30		WP1 Management	Consortium Agreement	COST
12.30	13.00		WP31 Dissemination	Web site	
13.00	13.30	Lunch	Creta Aquarium		
13.30	14.00	Lunch	Creta Aquarium		
14.00	14.30		GWP presentations 2 Repro		
14.30	15.00		GWP presentations 3 Nutrition		
15.00	15.30		GWP presentations 4 Larvae		
15.30	16.00	coffee			
16.00	16.30		GWP presentations 5 Grow out		
16.30	17.00		GWP presentations 6 Health		
17.00	17.30		GWP presentations 7 Socio		
17.30	18.00		Wrap up	Agenda for next day	Room allocations
20.00			Dinner at Parasies Restaurant		

DAY 2	Conference rooms				
Start	End	break	ROOM 1	ROOM 2	Library
9.00	9.30		GWP 4 Larva l husbandry	GWP 7 Socioeco	
9.30	10.00		GWP 4 Larva l husbandry	GWP 7 Socioeco	
10.00	10.30		GWP 4 Larva l husbandry	GWP 7 Socioeco	
10.30	11.00		GWP 4 Larva l husbandry	GWP 7 Socioeco	
11.00	11.30	coffee			
11.30	12.00		GWP 3 Nutrition	GWP 2 Repro & Genetics	
12.00	12.30		GWP 3 Nutrition	GWP 2 Repro & Genetics	
12.30	13.00		GWP 3 Nutrition	GWP 2 Repro & Genetics	Available for any group to
13.00	13.30		GWP 3 Nutrition	GWP 2 Repro & Genetics	have a meeting
13.30	14.00	Lunch	Creta Aquarium		
14.00	14.30	Lunch	Creta Aquarium		
14.30	15.00	Lunch	Creta Aquarium		
15.00	15.30		GWP 5 Grow out Husbandry	GWP 6 Fish health	
15.30	16.00		GWP 5 Grow out Husbandry	GWP 6 Fish health	
16.00	16.30		GWP 5 Grow out Husbandry	GWP 6 Fish health	
16.30	17.00		GWP 5 Grow out Husbandry	GWP 6 Fish health	
17.00	17.30	coffee			
17.30	18.00		Wrap up in Auditorium	Wrap up in Auditorium	

During DAY1, a presentation was made by the PC and a brief discussion followed on the Consortium Agreement (CA), which had already been circulated among beneficiaries in the previous weeks via email, and many had the chance to make their modifications/additions to the basic draft (**Fig. 3.2.3.1**). It was agreed that the second version of the CA that included the modifications of the beneficiaries would be circulated once more for final approval, before producing a pdf file and sending it for signatures by the Legal representatives of each beneficiary. This second version of the CA was sent to the Beneficiaries for final approval on the 4th of February 2014, and it was approved, signed and submitted to the EU (Deliverable 1.2 Consortium Agreement) on 20/03/2014.


DIVERSIFY Consortium Agreement

DIVERSIFY

Consortium Agreement

DIVERSIFY

Project full title:	Enhancing the European aquaculture production by removing production bottlenecks of emerging species, producing new products and accessing new markets.
Project acronym:	DIVERSIFY
Grant Agreement Identifier:	FP7-KBBE-2013-7 single stage
Grant Agreement number:	603121
Type of funding scheme:	Collaborative Project, Large-scale integrating project targeted to SMEs

Figure 3.2.3.1. The Consortium Agreement of DIVERSIFY that was approved, signed and submitted to the EU (Deliverable 1.2 Consortium Agreement) on 20/03/2014.

Also in DAY 1, the WP31 Dissemination LB (Dr Rocio Robles, P18. CTAQUA) presented some of the new beneficiaries that joined the consortium during the negotiations face, which includes professional associations such as P33. FGM (Greece), P34. BFVi (Germany), P35. MASZ (Hungary), P36. ANFACO (Spain) and the consumer NGO P37. EUFIC (Belgium). There was also a presentation of the project's web site, which was launched already during Mo 1, and was under construction at the time, with continual inclusions of new material. The web site (www.diversifyfish.eu) contains information according to the

Species studied in DIVERSIFY, as well as according to the scientific disciplines (Research Area). Detailed information on the web site can be found in the Description of WP31 Dissemination, in this report.

Annual Coordination Meeting 2014. The ACM 2014 was hosted by Dr. Aldo Corriero from the University of Bari Aldo Moro, Bari, Italy (P13. UNIBA) and was held at the Palazzo Ateneo, Salone Degli Affreschi on the 4-6 November 2014. The 3-day meeting was attended by 73 persons: 65 coming from the DIVERSIFY consortium and 8 invited guests from outside the consortium. No representative from four Beneficiaries (P26. GEI, P27. FORKYS, P35. MASZ and P37. EUFIC) attended the meeting. Beneficiaries P12. APROMAR and P36. ANFACO were unable to attend the first two days of the meeting, but attended the third day.

As for the kickoff meeting, information regarding the meeting was uploaded continually on the project's web site (www.diversifyfish.eu/INTRA/Meetings & Activities/2014 Annual Coordination Meeting) to ensure that all participants had access to the most updated information. The Agenda (**Tables 3.2.3.2 and 3.2.3.3**) was developed with assistance from GWP leaders and consisted of:

(a) DAY 1: a common session for all participants and invited guests during DAY 1 (including summaries of the work implemented in all six Scientific Disciplines, specific presentations from various Work packages or tasks, and presentations from invited guests),
(b) DAY 2: Six (6) parallel Scientific Discipline-specific workshops, and
(c) DAY 3: common session dealing with Dissemination, and Scientific and Financial Reporting. A meeting of the Steering Committee was also held at the end of the session.

Table 3.2.3.2. Agenda of DAY 1 of the Annual Coordination Meeting, which took place on the 4-6 November 2014, at the University of Bari Aldo Moro, Bari, Italy.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda — 2014 Annual Coordination Meeting — Bari 4-6 November 2014 — Palazzo Ateneo

DAY 1 Start	End	4-Nov	Tuesday Title	Salone degli Affreschi Presenter	Details
9,00	9,30		Welcoming	Constnantinos Mylonas (HCMR), Aldo Corriero (UNIBA)	Meeting logistics, agenda, welcoming from U Bari rector
9,30	10,00		GWP presentation 2 Repro & Genetics	Neil Duncan (IRTA)	
10,00	10,15		Greater amberjack spawning in Greece	Constantinos Mylonas (HCMR)	Reproduction & Genetics
10,15	10,30		Atlantic halibut spawning	Birgitta Norberg (IMR)	Reproduction & Genetics
10,30	11,00		GWP presentation 3 Nutrition	Marisol Izquierdo (FCPCT)	
11,00	11,30	coffee			
11,30	12,00		GWP presentation 4 Larval husbandry	Bill Koven (IOLR)	
12,00	12,15		The effect of dietary taurine on grey mullet larval performance at different stages of development	Bill Koven (IOLR)	Larval rearing
12,15	12,30		Effect of feeding regimes and probiotics in larval rearing of greater amberjack	Marisol Izquierdo (FCPCT)	Larval rearing
12,30	13,00		GWP presentation 5 Grow out husbandry	Nikos Papandroulakis (HCMR)	
13,00	13,30	Lunch	Catering at the site of the meeting		
13,30	14,00	Lunch			
14,00	14,15		Meagre growout problems	Marilo Lopez (Culmarex)	Grow out
14,15	14,30		Presentation of Andromeda and potential for collaborations	Mr Costas Tsokas	Andromeda S.A. (Greece, Spain)
14,30	15,00		GWP presentation 6 Fish Health	Chris Secombes (UNIABDN)	
15,00	15,15		Epitheliocystis and parasites in greater amberjack	Pantelis Katharios (HCMR)	Fish Health
15,15	15,30		Meagre Production and Selective Breeding	Mr Remi Ricoux	Le Poisson du Soleil (France)
15,30	15,45		Presentation of GMF and Involvement in DIVERSIFY	Mr Nikos Papaioannou	Galaxidi Marine Farms (Greece)
15,45	16,00		Amberjack Aquaculture Research in Malta - Advances and Bottlenecks	Dr Robert Vassallo-Agius	Malta Aquaculture Research Center (Malta)
16,00	16,30	coffee			
16,30	17,00		GWP presentation 7 Socio economics	Gemma Tacken (LEI/DLO)	
17,00	17,15		Consumer behaviour in fish consumption	Athanasios Krystallis (AU)	Socioeconomics
17,15	17,30		Buyer preference at retail and foodservice	Michel v.d Borgh (TU/e)	Socioeconomics
17,30	18,00		Wrap up	Constantinos Mylonas (HCMR)	Agenda for next day
20,00			Dinner at Local Restaurant		

Throughout DAYs 2 and 3, there were also one-to-one training sessions of interested Partners with the Project Manager from P1. HCMR, on the use of the Participants Portal. This was done to ensure that all partners were familiar with the Participants Portal, its various functionalities and the way both Scientific and Financial reporting need to be done. This was especially useful to the Partners that had no previous experience with EU Framework Program funding (*e.g.*, some of the SMEs), but was also very useful to more experienced Partners that did not have to use the Participants Portal before in their EU projects.

Table 3.2.3.3 Agenda of DAYs 2 and 3 of the Annual Coordination Meeting, which took place on the 4-6 November 2014, at the University of Bari Aldo Moro, Bari, Italy.

DAY 2		5-Noε	Wednesday		
Start	End		Salone degli Affreschi	ROOM 2	ROOM 3
9.00	9.30		GWP 4 Larval husbandry	GWP 2 Repro & Genetics	GWP 7 Socioeco
9.30	10.00		GWP 4 Larval husbandry	GWP 2 Repro & Genetics	GWP 7 Socioeco
10.00	10.30		GWP 4 Larval husbandry	GWP 2 Repro & Genetics	GWP 7 Socioeco
10.30	11.00		GWP 4 Larval husbandry	GWP 2 Repro & Genetics	GWP 7 Socioeco
11.00	11.30	coffee			
11.30	12.00		GWP 3 Nutrition	GWP 2 Repro & Genetics	GWP 7 Socioeco
12.00	12.30		GWP 3 Nutrition	GWP 2 Repro & Genetics	GWP 7 Socioeco
12.30	13.00		GWP 3 Nutrition	GWP 2 Repro & Genetics	GWP 7 Socioeco
13.00	13.30		GWP 3 Nutrition	GWP 2 Repro & Genetics	GWP 7 Socioeco
13.30	14.00	Lunch	Catering at the site of the meeting		
14.00	14.30	Lunch			
14.30	15.00	Lunch			
15.00	15.30		GWP 5 Grow out	GWP 6 Fish health	GWP 7 Socioeco
15.30	16.00		GWP 5 Grow out	GWP 6 Fish health	GWP 7 Socioeco
16.00	16.30		GWP 5 Grow out	GWP 6 Fish health	GWP 7 Socioeco
16.30	17.00		GWP 5 Grow out	GWP 6 Fish health	GWP 7 Socioeco
17.00	17.30	coffee			
17.30	18.00		Wrap up in Auditorium	Wrap up in Auditorium	
DAY 3	Auditorium	6-Noε	Thursday		
Start	End		Title	Presenter	Details
9.00	9.30		WP31 Dissemination	Rocio Robles	
9.30	10.00			Constantinos Mylonas	
10.00	10.30		Reporting, Participants Portal, Deliverables	Constantinos Mylonas	
10.30	11.00				
11.00	11.30	coffee			
11.30	12.00		Reporting, Participants Portal, Deliverables	Constantinos Mylonas	
12.00	12.30				
12.30	13.00		Financial issues, web site, communications, amendments	Constantinos Mylonas	
13.00	13.30				
13.30	14.00	Lunch	Catering at the site of the meeting		
14.00	14.30	Lunch			
14.30	15.00	Lunch			
15.00	15.30		Steering Committee meeting	Coordinator, GWP leaders, SME representatives (ARGO, ASIALOR, CULMAREX), APROMAR	
15.30	16.00				
19.00	22.00		Visit Eataly, Bari	Eataly is a high-end Italian food market/mall chain comprising a variety of restaurants, food and beverage stations, bakery, and retail items.	A bus will transport all interested to the site and bring them back at a predetermined time

The summary presentations started with the one from Dr. Neil Duncan (P3. IRTA), the Group Work Package (GWP) leader for Reproduction & Genetics, presenting the overall objectives of the 6 Work Packages (WP) in this Scientific Discipline, highlighting the important data obtained this first year of the project from each of the different WPs (**Fig. 3.2.3.2**). The summary presentation (30 min) was followed by two 15 min presentations on the results obtained from WP 3 Reproduction & Genetics – greater amberjack (Dr. C.C. Mylonas, P1. HCMR) and WP 5 Reproduction & Genetics – Atlantic halibut (Dr. B. Norberg, P7. IMR). Likewise, all the presentations from the GWP leaders explained the objectives of the WPs in each Scientific Discipline and provided an extensive summary of the work implemented during the first year of the project, and the significant results obtained. The specific presentations from various other WP Lead Beneficiaries or task leaders, allowed a more detailed presentation of the work that was carried out, for example the "Consumer Behaviour in Fish Consumption", presented by Dr. A. Krystallis (P11. AU) and the "Buyer Preference at Retail and Foodservice" presented by M. v.d. Borgh (P10. TU/e).

The presentations from the invited guests, which followed the presentations from consortium GWP leaders and Partners, demonstrated the involvement of other companies in the implementation of DIVERSIFY, such as the provision of tissue samples from the commercial operations of Galaxidi Marine Farms (Greece), Les Poisson du Soleil (France) and Andromeda Group (Greece and Spain) for the study of genetic diversity of

captive broodstocks of meagre, which is implemented in WP 2 Reproduction & Genetics – meagre (Dr. J. M. Afonso, P2. FCPCT). In addition, Galaxidi Marine Farms (Greece) provided their valuable broodstocks for the successful implementation of Task 3.2 Development of optimized spawning induction protocol for captive greater amberjack in the Mediterranean (WP3) and Task 3.5 Spawning induction of greater amberjack and egg collection in cages (WP3). The eagerness of these three very important EU aquaculture companies to collaborate in DIVERSIFY as non-partners and at no cost to the project, underlines the relevance of the proposed tasks of DIVERSIFY to the EU Aquaculture industry, and the important contribution the project can make to the enhancement of the Aquaculture industry in the coming years. In addition, it ensures that expensive infrastructures and resources from outside the consortium may become available to DIVERSIFY at no extra charge, and dissemination of the results obtained to the relevant stakeholders will be prompt and effective.

Figure 3.2.3.2 The opening slides from some of the presentations of DAY 1 of the ACM 2014, including one presentation from an invited guest from outside the consortium (Andromeda Group, Greece).

As planned in the DOW (WP1 Management), the invited scientists from outside the consortium will act in a way as a **Project Advisory Board** for the proposal (See also Part B, Section 2.1 Management of the consortium), providing critical assessments of the results and planned tasks for the following period. Such assessments and criticism have been provided by two of the invited quests (*e.g.*, more interactions of the Socioeconomics group with the industry), and will be taken into consideration for the improvement of the project

During DAY 3 of the meeting, the presentation of WP 31 Dissemination begun with a brief reiteration of the WP's many objectives, emphasizing the need for all Partners to participate actively in the preparation of dissemination materials and activities. To that effect, there was a presentation of the DIVERSIFY website (www.diversifyfish.eu), which is now fully functional, and an explanation of its organization both according to the Species studied in the project, but also according to Scientific Discipline. Then, there was a presentation of the Deliverables submitted so far, which included the development of the website, brochure and logo, audiovisual material, presentation to the European Aquaculture Society's annual meeting (Aquaculture 2014) in San Sebastian, Spain, and dissemination to the aquaculture industry, food industry and

consumers. All these dissemination activities have been already registered in the Participants Portal. The Dissemination leader underlined the need to implement the recent instructions by the EU to include the appropriate funding acknowledgements in all dissemination activities and material (**Fig. 3.2.3.3**).

The following statement should be included in all Dissemination material (press releases, interviews, web material, etc.)

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

This 5-year-long project (2013-2018) has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY). The consortium includes 38 partners from 12 European countries –including 9 SMEs, 3 Large Enterprises, 5 professional associations and 1 Consumer NGO- and is coordinated by the Hellenic Center for Marine Research, Greece. Further information may be obtained from the project site at "www.diversifyfish.eu".

The following statement should be included in all Scientific presentations (Posters, Oral presentations and scientific articles)

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

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY).

Figure 3.2.3.3 Instructions for the acknowledging of the EU funding to all dissemination activities, as prepared by the PC and uploaded in the website of the project.

During DAY 3, a presentation was given by the PC to explain how the Scientific reporting needs to be done for the project. This included both Deliverables and Periodic Reports. The presentation had begun with a reiteration of the roles of the PC, the Species Leaders (SL) the GWP leaders and the WP leaders (Lead Beneficiaries), in an effort to clarify their responsibilities and to remind the information that must be provided by the rest of partners to the WP leaders and GWP leaders. Regarding the Deliverables, it was emphasized again to the Partners that they must be uploaded on time electronically in the Participants Portal. To ensure that all Deliverables are uniform throughout the consortium –in terms of appearance, format, and content quality and extent, the PC has prepared a specific format file, as well as explicit instructions on the preparation of the Deliverables which are included in our website.

The session continued with the presentation dealing with the upcoming Periodic Report (Period 0-12 months, due January 2015). As for the Deliverables, special format files have been produced by the PC for each Work Package and were sent to the Lead Beneficiaries of each Work Package to help them compile the results and data from each Task. It was stressed that the Periodic Report must include the work carried out during the reporting period with enough detail, but without excessive and unnecessary information. This will allow the Consortium members to follow the major achievements as well as problems encountered during the 1st period, and will enable both the EU Scientific Officer and the Mid-Term Evaluation committee to evaluate the work in relation to the DOW, and be able to make any necessary recommendations.

After a short coffee break, the PC gave another presentation on the Financial Reporting for the 1st Period of DIVERSIFY. The objective of the presentation was to reiterate the need for all Partners to become familiarized with the Participants Portal, especially the Principle Investigators from the SMEs who are most likely the ones responsible for entering the financial information (Form C) into the system. After a brief mentioning of the functions of the Participants Portal, the Partners were reminded of some important aspects

relating to EU funding schemes and the budget of DIVERSIFY for each Partner. Specifically, it was reminded that there are different types of "Activities" (*e.g.*, RTD, Management and Other), which are handled separately and differently by the EU. All partners in DIVERSIFY have at least two types of Activities (RTD and Management), while many are also involved in Dissemination activities ("Other").

Partners were reminded that each Partner has requested a specific budget, and allocated the EU support to clearly defined and described types of expenses, such as Personnel, Subcontracting, Travel, Consumables, Durable Equipment and Other types of expenses. It was emphasized that all Partners must do their utmost to **abide by the budget allocation as described and agreed in the DOW,** as relates to both the types of expenses, but also the items that will be charged to the project. The objective is to avoid unnecessary modifications that would require transfer of budget between types of expenses, or spending of the money in items not indicated in the DOW. Of course, it is recognized that there is always the possibility of unforeseen costs, but the Partners were encouraged to keep this to the absolute minimum.

At the end of the third day, a meeting of the Steering Committee (SC) was held, as planned in the DOW. The SC members are the PC, the six GWP leaders, three representatives of SMEs and one representative from a professional organization. The people attending this meeting were Mylonas, C.C. (PC, P1. HCMR), Duncan, N. (GWP leader, P3. IRTA), Izquierdo, M. (GWP leader, P2. FCPCT), Koven, W. (GWP leader, P4. IOLR), Papandroulakis, N. (GWP Leader, P1. HCMR), Secombes, C. (GWP leader, P5. UNIABDN), Tacken, G. (GWP leader, P6. DLO), Daniil, M. (P23. ARGO), Lopez, M. (P30. CULMAREX), Deves, K. (P29. ASIALOR) and Ojeda, J. (P12. APROMAR). No official agenda was prepared for the meeting, but the PC addressed the following issues:

1. **Modifications**
    a. A significant problem was created by the loss in April 2014 of the wild-caught, greater amberjack broodstock at P24. ITTICAL due to a parasitic infection (*Amylodinium spp*). This stock was required for Task 3.2 Description of the reproductive cycle of greater amberjack. To address this problem, P23. ARGO was requested and accepted to provide a similar broodstock that they acquired for their activities (outside DIVERSIFY). Covering the cost of (a) replacing and maintaining this stock for the period required by the project (Dec 2013 to April-June 2015) by P23. ARGO, and (b) traveling for sampling the fish during the reproductive season by P1. HCMP will be done through redistributing funds from the P24. ITTICAL budget.
    b. A problem was also encountered in the activities of P8. IEO in Tenerife, Spain under WP9 Nutrition – greater amberjack and WP15 Larval husbandry – greater amberjack, due to the failure of their greater amberjack to spawn fertilized, high quality eggs. As a result, the work was not implemented and will have to be done next year, with one year delay. Such problems should be avoided in the future, and the PC asked the GWP leaders to be more "pushy" with managing the activities of their GWPs and the function of the Work Package Lead Beneficiaries.
2. **Dissemination** – GWP leaders need to be a bit more active in preparing dissemination activities. In addition, the PC asked the GWP leaders to encourage more contacts with national and private initiatives making similar research to DIVERSIFY, and to identify potential invited guests from International experts, according to the guidelines explained in the DOW (WP 1 Management and WP 31 Dissemination). Some members suggested that video-conferencing should be considered to include presentations from invited speakers during DAY 1 of the ACMs.
3. **Socioeconomic** – more focus of the Partners and better communication with other consortium members that can provide essential information (especially P12.APROMAR, P34.BVFi) was encouraged.
4. **Management** - Substitution of the leader for GWP Nutrition Dr. M. Izquierdo.
    a. The PC expressed his dissatisfaction on the commitment of this GWP leader to the project (*e.g.*, attendance to organized meetings) and the limited responsiveness to the PC's communications regarding the management and acquisition of information of the GWP, which are probably caused by a heavy workload of the GWP leader with other commitments,

b. The GWP leader responded that she proposes Dr. Hipolito Fernandez Palacios to take this role, since this scientist has had prompt and effective communications with the PC so far (as member of the GWP Reproduction & Genetics). However Dr. Izquierdo, should remain as the leader of WP 9 and Task leader in various WPs, and will assist Dr. H. Fernandez Palacios in the management of the GWP Nutrition, as much as possible.

c. The PC accepted the suggestion and both will try to work with this arrangement. Dr. H. Fernandez Palacios was contacted at the end of the meeting and presented with this development, and he accepted to assume the role of the GWP leader for Nutrition, with the support of Dr. M. Izquierdo.

Next Annual Coordination Meeting. The next ACM will be held later than planned in the DOW (*i.e.*, every 12 months), in order to be closer to the end of the next Reporting Period (30 month, May 2016). This will allow the Partners to use the occasion of the ACM for the preparation of the next Scientific Report, while at the same time there will be no loss of coordination of activities, since the next reproductive season for most of the fish in the project (and hence most of the subsequent activities of the project) will begin after February. So, the ACM 2016 will be held in Jan-Feb 2016, and will be hosted by Dr. Pascal Fontaine of P9. UL (France).

Communication with the European Commission

As planned in the DOW, the EC's project Scientific Officer has been invited to all the project meetings and was sent the detailed minutes of both the Kick off meeting (Feb 2014) and the Annual Coordination Meeting 2014 (Nov 2014), as soon as they were released. Deliverables reporting on the meetings have also been uploaded on the Participants Portal. The consortium, through the PC, has made every possible effort to keep the EU Scientific Officer informed in a timely manner, about any important developments, problems and major dissemination activities. We believe that a good communication exists between DIVERSIFY and the EU Scientific Officer (Dr Marta Iglesias), as well as the Financial Officer (Mr. Carlo Panella), who have been very responsive to all requests for information, in a promptly and very effective way.

Changes in the consortium

A change took place in the consortium, as P30. MAREMAR was bought over by CULMAREX S.A. during the contract negotiation period of DIVERSIFY. The new company expressed an interest of continuing to participate in the project, but moving the activities to a different site of the company, where they considered it more appropriate for the implementation of the Task described in the DOW (WP 20 Grow out husbandry – meagre). Through discussions between the WP leader Dr. Neil Duncan (P3. IRTA), the GWP leader for Grow out Dr. Nikos Papandroulakis (P1. HCMR) and Dr. Constantinos Mylonas, the PC (P.1 HCMR) with the person in charge of RTD at CULMAREX (Dr. Marilo Lopez), it was decided that the company was indeed a good alternative for the project and the process begun for the official change of the partner. This was done through Amendment 1 of the DOW, approved by the DG R&I of the European Commission on 15 May 2014.

Interactions with other projects

As planned in the DOW, in order to stimulate synergies and complementarities, links were established between the DIVERSIFY project and other relevant national and EU ongoing projects when the appropriate opportunities occurred. Fro example, links have been formed with the project "KRANIOS" coordinated by P1. HCMR, a Greek National project on the production of meagre. Also, links have been formed with

"KRIPIS", also a Greek National project coordinated by P1. HCMR on the reproduction, larval rearing and cage grow of greater amberjack. There was also a link with the coordinator of Targetfish (EU 7FP) who confirmed there interest and willingness to collaborate with DIVERSIFY in the area of vaccine development of VNN (WP 26).

Development of project website

Construction of the web site began already in Mo 1 of the project using the www.weebly.com platform. Hosting of the site was then moved to its final location at www.diversifyfish.eu in Mo 2. The website was designed to provide fast access to project information, included in seven main pages. The structure of the web site follows the following scheme, after some modifications done over time to make it more effective:

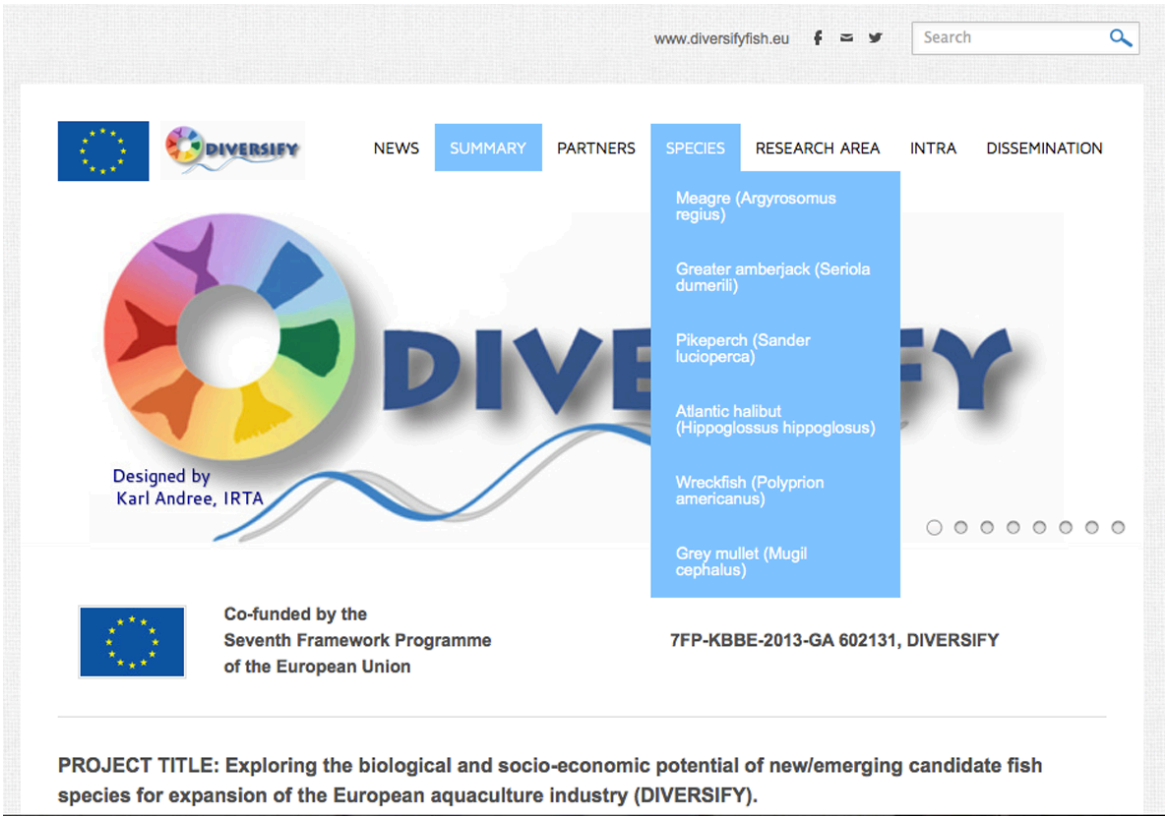


Figure 3.2.3.4 The web site of DIVERSIFY hosted at www.diversifyfish.eu.

1. NEWS: This page includes the most recent activities on the initiation of specific tasks from the work plan, providing brief descriptions of various actions, with graphic support. This page has be updated often (monthly), with the objective of providing up-to-date information to partners, but also to interested people visiting the web site, on the on-going work of the project. Major results and findings are also provided, in order to speed up dissemination and hopefully implementation of the outcomes of the project by relevant stakeholders.
2. SUMMARY: The page includes the full title of the project, a brief description of its objectives and contact details of the PC, geographical distribution of the partners, general structure of the work to be implemented (Pert diagram) and organization of the consortium and its governing bodies and research area groups (Management structure).

3. PARTNERS: These pages are grouped in RTDs, SMEs, Large enterprises, and Associations and NGOs, providing the contact information of each partner and the Principal Investigator (PI), including the logo of the partner. The main "PARTNERS" page includes also a gallery of photos from all involved researchers in the consortium.
4. SPECIES: In these pages, a full description is provided of the six species included in the project. This section includes biological information per species and the reasoning behind the species selection for the project. A photo gallery is also included, providing photos (when available) of the species at various stages and from research activities of the partners.
5. RESEARCH AREA: These pages indicate how the research tasks are designed to address the identified bottlenecks in each selected species. The different tasks have been separated by scientific discipline (along the organization of Group Work Packages of the Description of Work, DOW), so separate WPs address work in a specific discipline and species. There are six (6) disciplines including Reproduction and genetics, Nutrition, Larval husbandry, Grow out husbandry, Fish health and Socioeconomics (which includes final product and quality). The specific Work Packages that are include in the project and address work in the selected species are:
    - Reproduction and genetics: meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet
    - Nutrition: meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet
    - Larval husbandry: meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet
    - Grow out husbandry: meagre, greater amberjack, pikeperch and grey mullet
    - Fish health: meagre, greater amberjack and Atlantic halibut
    - Socioeconomics: Institutional and organizational context, new product development, consumer value perceptions and behavioral change and business model and marketing strategy development.
6. INTRANET: This section includes information relevant to the partners and most of its pages are password protected. It is intended to provide specific information to the consortium, and different official documents and other internal project documents can be found here such as:
    - Information about the annual meetings and activities
    - A copy of the DOW, Grant Agreement and Consortium Agreement
    - EU supporting documentation (CORDIS, use manuals on reporting, financial guide, etc)
    - Management documents, such as a list of all researchers in the consortium and their contact information, the minutes of the Annual Coordination Meetings, etc
    - Forms and protocols (logos, dissemination rules, templates for expenses justification, Deliverable reporting, etc)
    - Deliverables, where all submitted deliverables can be found by all partners
    - Scientific and Financial Reporting, where the periodic reports will be uploaded
7. DISSEMINATION: These pages include all activities related to the broadcasting of project activities. Several sections are incorporated:
    - Newsletter area, where a new version is uploaded every 6 months
    - Articles in magazines and internet (with project information in other languages of the consortium)
    - Scientific articles
    - Presentations and posters
    - Activities and photo gallery

More details about the DIVERSIFY webpage can be found at the description of the activities of WP31 Dissemination, in this report.

Deviations from the DOW

Some deviations from the DOW have been made, either as a necessity or a choice, in order to achieve the objectives of the project. Overall, we consider the modifications as minor, and with either no effect or a positive effect on the quality of the work to be done.

WP1 Project management.

The ACMs were planned in the DOW to consist of 2-days of open presentations and 1 for consortium activities. Instead, the ACM 2014 contained only 1 open day and 2 days reserved for consortium activities. This was considered necessary because of the large number of Work Packages in the project, and the need for as much time as possible to be allocated to the discussion of obtained results and future planning of the work. Based on the progression of ACM 2014, we believe this arrangement is adequate to both disseminate the most important information obtained in the previous period by the consortium, and also to benefit from presentations and the interaction with the invited guests. So, we intend to maintain this planning for the following ACMs.

The next ACM will be held later than planned in the DOW (*i.e.*, every 12 months), in order to be closer to the end of the next Reporting Period (30 month, May 2016). This will allow the Partners to use the occasion of the ACM for the preparation of the next Scientific Report, while at the same time there will be no loss of coordination of activities, since the next reproductive season for most of the fish in the project (and hence most of the subsequent activities of the project) will begin after February. So, the ACM 2016 will be held in Jan-Feb 2016.

Also, the ACM 2014 was held in Bari, instead of one of the originally planned locations. This was done because it was considered that Bari is more centrally located in Europe than some of the selected locatios (*e.g.*, Canary Islands, Spain), which would result in reducing the cost of traveling of the participants. The cost of hosting the meeting by P13. UNIBA will be covered by a transfer from the budget of P2. FCPCT that is not likely to host a meeting (located in Canary Islands, Spain), but this will decided at the very end of the project.

A discrepancy/error was noticed in the "Staff Effort" of P2. FCPCT, which is listed as having 5 PM for WP1 Project management, although it has not claimed any Personnel budget for this WP (and hence has not Personnel budget for the Management tyoe of activity). Therefor, not personnel costs/PMs will be claimed by this Partner under WP1.

WP2 Reproduction & Genetics - meagre.

In addition to the paired-spawning induction trials that have and will be undertaken by P3. IRTA (as per DOW) under Task 2.1, P1. HCMR has carried out a similar experiment and will also carry out another one in 2015 in order to obtain more relevant information for this deliverable. The additional data will also facilitate the publication of the results, since it will contain a mirrored experiment undertaken in two different facilities, in different geographic locations, but under the exact same protocol. This work is **without any extra cost** to DIVERSIFY, and it is undertaken because spawning induction is carried out anyway at P1. HCMR in order to produce eggs for juvenile production for other WPs, in which the partner is involved.

WP3 Reproduction & Genetics – greater amberjack.

Task 3.1 description of the reproductive cycle in captive greater amberjack will no longer be carried out at P24. ITTICAL (Italy), but at P23. ARGO. Partner 24. ITTICAL purchased a stock of fish from Greece in February 2014, and was moved to Italy in April 2014 and was supposed to be killed in April-May-June of 2015 to describe the reproductive cycle of the fish in captivity. Unfortunately, the stock died in May 2014,

due to a parasitic infection (*Amyllodinium spp.*). Fortunately, P23. ARGO that is involved in the spawning induction activities (Task 3.2 and 3.5) had purchased another stock of fish from the same source, and have offered to make this population available to DIVERSIFY. The fish will be killed in the farm and the scientists from Italy responsible for this task (P13. UNIBA, P4. IOLR and P14, IFREMER) will travel for some of the sampling times, but P1. HCMR will be responsible to travel there from Crete to help them with the sampling.

Deliverable 3.1 Establishment of quantitative PCR assays for target genes in greater amberjack (LHβ, FSHb, Leptin, Vg and VgR) will be delayed by 4 months. This is due to the late arrival of tissue samples derived from wild greater amberjack to P.7 IOLR (Eilat, Israel), which delayed the cloning of target genes (*i.e.*, pituitary gonadotropin beta subunits and liver leptin) still in progress. We expect to finalize this task and establish the respective quantitative real-time PCR assays, by March 2015. Therefore, Deliverable 3.1 will be delayed by 4 months, but this delay is not expected to have any impact on other tasks or deliverables.

WP5 Reproduction & Genetics – Atlantic halibut.

Due to a prolonged sick leave (August 2014 - December 2014) of the Principal Investigator of this WP (Dr Birgitta Norberg), planned analyses of data collections and egg samples from Task 1 have been put on hold. We expect to start the analyses in the first months of 2015 and there will be no delay in the deliverable for this task, which is due in month 30.

For the same reason, Task 3 was also delayed. Preliminary samples for histology and molecular biology were taken in 2014 and will be processed in the first half of 2015. The main sampling will start in the January 2015 reproductive season and will go on until 2016. The deliverable for this task is due in month 36 and should not be affected.

WP6 Reproduction & Genetics – wreckfish.

There was no deviation to the work in this WP. However, a limited number and low quality of fertilised eggs were obtained under **Task 6.3 Development of spawning induction procedures**, which had a negative impact on WP18 Larval husbandry – wreckfish, as indicated by the poor results obtained in **Task 18.2 Development of feeding methodology**. In 2015, work will continue aimed to ensure more and better-quality fertilised eggs. In particular, the broodstock diets will be improved, and spawning trisals will be focused both on spontaneous spawning (P32. MC2) that has given tentative promising results, and *in vitro* fertilisation spawning (P1. HCMR, P8. IEO, P19. CMRM, P32. MC2) that when fully developed offers complete control to obtain of good quality gametes and eggs.

WP10 Nutrion – pikeperch.

There were no deviations from Annex 1, but the experimental study was 1 month delayed due to late spawning of pikeperch breeders, the experimental study will be redone in December, where no activities are planned as so the expected work carried out during year 1 is within the expected time frame.

WP11 Nutrion – Atlantic halibut.

Although according to the DOW work should start from the beginning of the project period, there are no results yet. This has to do with the relatively few activities in this WP and the need to organize the work in a practical way. Furthermore, the spawning season of the halibut in late winter/spring determines when the activities can be executed. However, we do not expect any delays in the deliverables

WP12 Nutrion – wreckfish.

Task 12.2 was scheduled to begin in December of 2014, but in order to have information about the biochemical composition of the wild wreckfish and connected with Task 6.2 (describe reproductive cycle), samplings of wild wreckfish were performed to know some parameters as weight, size, the stomach content, perivisceral fat content, GSI, HSI, VSI etc. Furthermore, samples of some tissues (muscle, liver and gonad) were analysed to know the nutritional status of wild fish. The bibliography about feeding habits of this specie is scarce; therefore, the information obtained will be very useful for the development of a specific formulation for wreckfish broodstock feeding.

WP14 Larval husbandry – meagre.

Due to the high cannibalism observed in all the groups, particularly the groups fed the microdiet from day 12 onwards, we are planning to repeat the experiment in 2015 in order to increase the survival rate and dietary performance, and obtain more data to address the objective of the WP. We do not expect any delays in the submission of the planned deliverable and milestone.

WP16 Larval husbandry – pikeperch.

Deliverable 16.1 - Determine the effect of environmental factors on pikeperch larval rearing, will be delayed by 8 months. The experiment of Task 16.1 planned for April-June 2014 has been postponed for December 2014-February 2015. Partner P9. UL has initially scheduled the experiment first in spring 2014, because this period corresponds to the natural pikeperch spawning season in France and contacts have been previously established with the fish farm "Domaine de Lindre" to supply pikeperch larvae. As planned, 500,000 larvae were purchased from the company. To do this experiment our laboratory was going to use the brand-new and modern experimental platform (with many ARS, at a cost of €3 million. The facility was completed and delivered in late April 2014 (3 months later than scheduled, after a long period of technical tests by companies (to test cooling, ventilation and warming systems). That meant that regardless of our efforts, such as introduction of nitrifying bacteria in commercial solution (AQUACLEAN N), the ARS did not operate with mature biofilters. Then as the biofilter was not balanced, very high levels of total ammonia nitrogen and nitrite (more than 10 mg L^{-1} for each substance) were measured and larvae died five days after introduction. From that moment, we have continued to manage our larval rearing station in order to obtain a well-balanced biofilter; that was achieved in July 2014 only. We have contact several pikeperch farms (Aquapri in Denmark, Excellence Fish Farm in the Netherlands) to buy a new batch of larvae, but they had no larvae available at that period. Consequently, we have decided to postpone this experiment to late 2014 because P29. ASIALOR will be able to supply us with larvae produced out-of-season spawning.

WP17 Larval husbandry – Atlantic halibut.

Development of the DGGE method took longer than expected. However, the method is now up and running in the laboratory but results are delayed as a consequence of the methodological problems. This should not affect the Deliverable for Task 17.1, which is due month 36.

A mistake was made when preparing the Gannt Chart for Task 17.3 (Development of a production protocol for on-grown *Artemia*). This task should have been set to start in the last quarter of year 1 (November-December 2014), due to overlap in personnel and facilities with task 17.1. The work is now well underway and the deliverable is expected to be on time.

WP18 Larval husbandry – wreckfish.

It was not possible to perform Task 18.1, since as explained earlier, , the egg quality was very poor, compared to the previous year (P32. MC2). Also, the female that spawned at P1. HCMR in response to hormonal therapy (WP6 Reproduction & genetics – wreckfish), produced very low fertilization percentages (<11%). This did not allow us to perform this task. Although this task began during this reporting period, the

deliverable for this task is not until month 36, consequently we believe that this task deliverable will still be on time.

It was not possible to complete Task 18.2, due to low availability and poor quality of the larvae. Even though mortality was 100% at 20 days after hatch, larval development was completed and documented. Improvement of breeders' parental diets and hormonal induction planned for 2015 provides make us optimistic regarding the availability of good larval quality for next year. Although this task began during this reporting period, the deliverable for this task is not until month 36, consequently we believe that this task deliverable will still be on time.

WP18 Larval husbandry – grey mullet.

In general, there were no deviations from the work plan as grey mullet is a fall spawner. Consequently, the mullet experiments are only now underway and at the writing of this report, the larvae were 23 dph and the studies are proceeding as planned including the use of resources and person months. The only deviation was the closing of the company Zoopt. However, the deliverable of this task is not due until month 36, therefore there is time to resolve this issue and find a suitable substitute. The budget for subcontracting has not been used. There are no deliverables or milestones due in this reporting period.

WP20 Grow out husbandry– meagre.

Task 20.3.4 has not started and will be started in 2015. Poor performance of existing meagre stocks at P30. CULMAREX indicated that the planned autumn stocking in the DOW should be avoided until the cause of the poor performance is understood. Therefore, a spring-summer stocking period would be preferred. This deviation will not delay the deliverable.

WP21 Growout husbandry – greater amberjack.

The main deviation from the work plan is the short delay in the implementation of Sub-tasks 21.2.2 and 21.3.2, due to the lack of greater amberjack juveniles at P8. IEO, as already explained. Partner 8. IEO, however, has already acquired some juveniles and is going to acquire more, so that a sufficient number of juveniles from a commercial hatchery (Futuna España SL) will be used for the planned work. These juveniles born in captivity will be acquired in sufficient number and adequate size to allow conducting trials related to stocking density and feeding strategies. Thus, although the implementation of few tasks is slightly behind schedule, they will be initiated at the end of 2014 and completed during 2015 without any significant impact in the implementation of the project or the deliverables of this WP.

WP22 Growout husbandry – pikeperch.

The Milestone 48 that was for month 18 will be delayed for month 22. Indeed, the multifactorial study was planned to start between month 8 and 12, but it appeared necessary to adapt the rearing conditions of the UL facilities to the protocol requirements of the multifactorial design for a better implementation concerning the identification of the major stress factors for pikeperch juveniles. This adaptation took more time than expected. Due to limited information on stress responsiveness for pikeperch, it was also necessary to standardize some methodological aspects, especially concerning the physiological and immune analyses as well as the bacterial LC50 doses for the evaluation of the disease resistance of stressed fish. As a consequence, the multifactorial trial is postponed and is planned to start by early June 2015, but should be completed by November 2015. Therefore, Task 22.1 will be completed in time through preliminary methodological refinement, without any major delay on the Deliverables associated with this task (except some data on immune responses to bacterial challenge tests) since the only Deliverable is expected on Mo24.

WP27 Socioeconomics – Institutional and organizational context.

Deliverable 27.3 Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet and ***Deliverable 27.6 List of critical success factors for market acceptance*** will be delayed by 2 months (Mo14). The time required for the completion of these deliverables was probably underestimated in the DOW. In addition, the partners responsible were involved in the preparation of 3 more deliverables that were due at the same time (Mo 12), which made working on all deliverables at the same time difficult. The delay of the above two deliverables will not affect the implementation of other Tasks in the DOW.

WP28 Socioeconomics – New product development.

Deliverable 28.1 Results of focus groups with consumers and experts regarding idea for new products will be delayed by 3 months. The reasons for this are as follows:

4. Complicated multi-country analysis was required to decide on criteria for focus group participants' recruitment (analysis got completed before the ACM 2014 meeting in November 2014)
5. Time consuming task to develop the discussion guide in 5 languages
6. Data collection initially promised, now coincides with the Christmas season (given that the start of the project was set to Dec 2013 by the EU), which prohibits any field-work. As a rule of thumb, irregularities in consumer behavior because of the special conditions that prevail during this period, prevents us every year from data collection between early December and early January.

No impact is anticipated, however, on any of subsequent deliverables due to the fact that (a) the requested delay is small and (b) all preliminary actions (*i.e.*, finalization of the screening / recruitment criteria, development of draft and final discussion guides, contact with local field collaborators etc.) will unfold in parallel during the Christmas period, in order to accelerate the process immediately after that.

Budget mofications

P1. HCMR, P13. UNIVA, P14. IFREMER, P23. ARGO and P24. ITTICAL

Task 3.1 description of the reproductive cycle in captive greater amberjack will no longer be carried out at P24. ITTICAL (Italy), but at P23. ARGO. This means that we will move a significant amount of the budget from P24. ITICAL to P23. ARGO, to cover the cost of these additional fish (24-36, we need to decide if we will kill the original number from the DOW or reduce it to save fish, given the problem encountered), as well as to HCMR and UNIBA for additional traveling. At the end, the task will be completed and delivered as proposed in the DOW (except that it will most likely be done with n=4 fish for each sampling as opposed to n=6), within the budget agreed (*i.e.*, no more money will be required, but with some reallocation of resources among partners and types of expenses). We may also be able to buy fish from another farm in Greece, to "replenish" the stock of P23. ARGO, in which case the reallocation of funds will also go towards this purchase, and P23. ARGO will only charge the cost of maintenance of the fish (personnel, feed, etc), but not the original purchase. We do not expect any delay in any of the deliverables associated with this task.

The considered budget allocation will not affect the realization of the other tasks planned for the partner (P24. ITTICAL) in this or other WPs. The total budget for the company was to:

a) acquire and maintain a stock of amberjack for sampling them during the reproductive season
b) acquire and maintain a stock of amberjack for spawning induction
c) acquire and maintain a stock of grey mullet juveniles for maturation and sampling

The allocation of the transferred budget will be in the 100,000 EU contribution range, as follows:

a) ARGO: additional personnel (person months) and consumables for the cost of the additional fish, feeds, and husbandry and sampling
b) UNIBA/IFREMER: additional traveling expenses and consumables for the sampling that needs to be done in Greece (ARGO) at three different times in 2015

c) HCMR: additional personnel and travel expenses for the sampling that needs to be done in Greece (ARGO) at three different times in 2015; additional personnel and consumables for carrying out the CASA development for greater amberjack in our facilities (together with IFREMER staff), and perhaps additional consumables to purchase another stock of fish, which will be maintained at ARGO. These fish will replace the ones that will be killed, which were planned for additional spawning induction experiments.

P23. ARGO

In the DOW, this partner requested to purchase a "small fish feed machine for the production of moist food used for the rearing of wild agreater amberjack broodstock", since it was expected that the fish would not accept commercial feed. However, P1. HCMR has developed a procedure during the project for weaning wild greater amberjack onto a semi-moist commercial feed (re-hydrated extruded broodstock pellets), so it is no longer necessary to use such a machine. The company will then purchase commercial broodstock feed for the fish. Therefor, the cost of the requested equipment will be shifted to the purchase of broodstock feed.

Also, for the management of the greater amberjack broodstock (smapling, biopsying, treating with spawning induction thereapies, etc.) it was necessary to tag all acquired fish individually using Passive Integrated Transponders (PIT tags), so the company purchased a "Power tracker" (AVID, UK) to be able to indentify the fish. This equipment was not forseen in the proposal (erroneously!) and was not included in the DOW.

P8. IEO

The purchase of greater amberjack juveniles was necessary to complete Sub-task 21.2.2 and 21.3.2 by the partner, but was not included in the DOW. It was expected that juvenile production by the partner would be enough to implement these tasks, but this was not possible due to the failure of spawning during 2014. The cost of this purchase (~7,500 €) will be transferred from the consumable budget assigned to the partner.

3 Deliverables and milestones tables

Deliverables

The deliverables due in this reporting period, as indicated in Annex I to the Grant Agreement have to be uploaded by the responsible participants (as indicated in Annex I), and then approved and submitted by the Coordinator. Deliverables are of a nature other than periodic or final reports (ex: "prototypes", "demonstrators" or "others"). ***The periodic reports and the final report have NOT to be considered as deliverables****. If the deliverables are not well explained in the periodic and/or final reports, then, a short descriptive report should be submitted, so that the Commission has a record of their existence. If a deliverable has been cancelled or regrouped with another one, please indicate this in the column "Comments". If a new deliverable is proposed, please indicate this in the column "Comments". The number of persons/month for each deliverable has been defined in Annex I of the Grant Agreement and cannot be changed. In SESAM, this number is automatically transferred from NEF and is not editable. If there is a deviation from the Annex I, then this should be clearly explained in the comments column. This table is cumulative, that is, it should always show all deliverables from the beginning of the project.*

TABLE 1. DELIVERABLE

Del. no.	Deliverable name	Version	WP no.	Lead beneficiary	*Nature*	Dissemination level[4]	Delivery date from Annex I (proj month)	Actual / Forecast delivery date Dd/mm/yyyy	Status Not submitted/ Submitted	Comments
1.1	Kick-off meeting and Annual coordination meeting for Y1	1	1	1	Other	RE	31/01/2014	10/02/2014	Submitted	Due to the project starting in December, it was not possible to have the meeting

[4] **PU** = Public
PP = Restricted to other programme participants (including the Commission Services).
RE = Restricted to a group specified by the consortium (including the Commission Services).
CO = Confidential, only for members of the consortium (including the Commission Services).
Make sure that you are using the correct following label when your project has classified deliverables.
EU restricted = Classified with the mention of the classification level restricted "EU Restricted"
EU confidential = Classified with the mention of the classification level confidential " EU Confidential "
EU secret = Classified with the mention of the classification level secret "EU Secret "

										during month 1 of the project.
1.2	Consortium Agreement	1	1	1	Other	CO	28/02/2014	20/03/2014	Submitted	A delay was due to one Partner not being able to sign the CA (P32. MC2)
1.3	Annual Coordination Meeting for Y2	1	1	1	Other	RE	31/12/2014	28/11/2014	Submitted	
1.4	Periodic Report, including financial and administrative reports for Mo 1-12	1	1	1	Report	RE	31/01/2015	30/12/2014	Partly Submitted	The Financial Report will be submitted later than the Scientific Report (January), due to the closing of many organizations during Christmas
2.2	Genetic characterization of different meager captive broodstocks and evaluation of available variability	1	2	2	Report	PU	30/11/2014	15/12/2014	Submitted	
3.1	Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (*i.e.*, LHβ, FSHβ, leptin, Vg and Vg receptor)		3	4	Report	PU	30/11/2014	31/03/2015	Delayed	It was not possible to send the pituitary samples (at -80°C) to P4. IOLR until the end of 2014.
4.1	Genetic analysis of domesticated pikeperch broodstocks	1	4	1	Report	PU	30/11/2014	19/11/2014	Submitted	
7.1	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of	1	7	14	Report	PU	30/11/2014	12/12/2014	Submitted	

	grey mullet sperm									
16.1	Determine effect of environmental factors on pikeperch larval rearing	1	16	9	Report	PU	30/11/2014	31/12/2015	Delayed	The experiment will be repeated in Y2 due to technical problems (see WP 16, Deviations)
27.1	Report on external environmental factors that affect or will affect the production chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet	1	27	6	Report	PP	28/02/2014	22/05/2014	Submitted	The time required for this Deliverable was under-estimated
27.2	Report on current certification schemes and standards and their business dynamics in the fish supply chain	1	27	6	Report	PP	28/02/2014	7/03/2014	Submitted	
27.3	Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet	1	27	6	Report	PU	30/11/2014	31/01/2014	Delayed	More analysis is necessary.
27.4	Report on trend mapping for the European aquaculture, seafood sector and protein market in the (near) future	1	27	6	Report	PU	30/11/2014	5/12/2014	Submitted	
27.5	Report with results of international survey on industrial buyers' attitudes and perceptions regarding cultured fish	1	27	6	Report	PU	30/11/2014	28/11/2014	Submitted	
27.6	List of critical success factors for market acceptance	1	27	6	Report	PU	30/11/2014	31/01/2014	Delayed	The deliverable depends on D27.4,

										which is delayed
27.7	Report on the analysis of the business models and supply chains of the participating SME's	1	27	6	Report	PU	30/11/2014	28/11/2014	Submitted	
29.1	Dataset of consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the selected species in the five	1	29	6	Report	PU	31/08/2014	28/08/2014	Submitted	
31.1	Establishment of website (www.diversifyfish.eu)	1	31	18	Report	PU	31/03/2014	02/04/2014	Submitted	
31.2	Project logo and brochure	1	31	18	Report	PU	31/05/2014	24/06/2014	Submitted	
31.3	Publication of the first of two articles in Food Today	1	31	37	R	PU	31/05/2014	30/05/2014	Submitted	
31.4	Production and release of audio-visual material	1	31	18	R	PU	31/05/2014	24/06/2014	Submitted	
31.5	Collaboration agreement with food industry and consumer organization; linkage of websites	1	31	18	R	PU	31/08/2014	18/11/2014	Submitted	It proved to be much more difficult than expected to convince these organization to sign an agreement
31.6	Annual presentation of DIVERSIFY (Y1) at a relevant conference (mainly Aquaculture Europe, EU Forum (by the Project	1	31	1	R	PU	31/08/2014	27/10/2014	Submitted	

	Coordinator)									
31.7	Production and release of audiovisual material	1	31	18	R	PU	31/08/2014	31/01/2015	Delayed	Waited for the 1st Periodic Report (Jan 2015) in order to select material for the presentation

Milestones

Please complete this table if milestones are specified in Annex I to the Grant Agreement. Milestones will be assessed against the specific criteria and performance indicators as defined in Annex I.

This table is cumulative, which means that it should always show all milestones from the beginning of the project.

TABLE 2. MILESTONES

Milestone no.	Milestone name	Work package no	Lead beneficiary	Delivery date from Annex I dd/mm/yyyy	Achieved Yes/No	Actual / Forecast achievement date dd/mm/yyyy	Comments
1	Kickoff meeting and Annual coordination meeting for Y1	1	1	31/12/2013	Yes	30/01/2014	P1. HCMR, Crete, Greece
2	Consortium agreement	1	1	31/01/2014	Yes	20/03/2014	
3	Annual coordination meeting for Y2	1	1	31/01/2015	Yes	6/11/2014	P13. UNIBA, Bari, Italy
17	Database of genetic variability of pikeperch	4	1	30/11/2014	Yes	30/11/2014	Excel database completed
19	Basic diet formulation for meagre grow out studies	8	2	30/11/2014	Yes	30/11/2014	Established
21	Basic diet formulation for greater amberjack grow out studies	9	2	30/11/2014	Yes	30/11/2014	Established
22	Definition of reproductive quality parameters to be studied in amberjack	9	2	30/11/2014	Yes	30/11/2014	Literature search completed
23	Definition of parameters for skeleton study in pikeperch	10	21	30/11/2014	Yes	30/11/2014	Established

27	Definition of methodology to study cost-benefit of grey mullet weaning studies	13	4	30/11/2014	No (Delayed)	30/11/2015	IOLR larval-juvenile experiments to determine carnivorous -omnivorous feeding shift still on-going. Results will enable IRTA to do weaning cost-benefit experiments
29	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack producing good quality eggs	15	2	31/5/2014	Yes	30/6/2014	Provision of eggs for larval nutrition and rearing experiments in Greece and Spain.
34	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2014	No	31/5/2015	Eggs were produced both in Greece and Spain, but their quality was poor and did not allow implementation of larval rearing experiments.
38	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2014	Yes	31/10/2015	Millions of eggs of high quality were produced, allowing the start of larval rearing experiments.
51	Design of primers for amplification of meagre target gene DNA sequences	24	5	30/11/2014	Yes	30/11/2014	
63	Insights in the consumer and B2B market for cultured fish	29	11	30/11/2014	Yes	30/11/2014	
70	Agreement on project logo for website and publications, this will provide a recognizable image of DIVERSIFY	31	1	01/06/2014	Yes	01/06/2014	

71	Design and printing of project brochure (hard-copy) including the project logo, inserts with project	31	18	01/06/2014	Yes	24/06/2014	
72	Agreements with food industry and consumers associations for web linkage	31	18	31/08/2014	Yes	20/11/2014	Not all originally planned organizations agreed to sign with DIVERSIFY, but others were solicited successfully.

4 Explanation of the use of the resources and financial statements (Staff effort only)

The financial statements have to be provided within the Forms C for each beneficiary (if Special Clause 10 applies to your Grant Agreement, a separate financial statement is provided for each third party as well) together with a summary financial report which consolidates the claimed Community contribution of all the beneficiaries in an aggregate form, based on the information provided in Form C (Annex VI of the Grant Agreement) by each beneficiary.
The "Explanation of use of resources" requested in the Grant Agreement for personnel costs, subcontracting, any major costs (ex: purchase of important equipment, travel costs, large consumable items) and indirect costs, have now to be done within the Forms (user guides are accessible within the Participant Portal)[5].

When applicable, certificates on financial statements shall be submitted by the concerned beneficiaries according to Article II.4.4 of the Grant Agreement.

The use of the resources is explained in detail in the submitted Forms C from each Beneficiary. However, for the convenience of the potential reviewer, we include the staff effort in **Tables 4.1a** and **b** below. The staff effort numbers for some Partners may be modified slightly, since at the time the Scientific Report was submitted, the Financial Report (Form Cs) was not submitted officially from all Partners.

[5] In the past, the explanation of use of resources requested in the Grant Agreement was done within a table in this section. The merge of this table within the Forms C was a measure of simplification aimed at avoiding duplication and/or potential discrepancies between the data provided in the table 'Explanation of use of resources' and the data provided in the Forms C.

Table 4.1a Staff effort per Partner and WP, during the 1st Reporting Period (0-12 Mo). The "Total Project" effort included in the DOW is shown as well.

Calculation of staff effort per parter and sub WP				RTD partners											RTD partners										RTD partners				
Work Package (Proposal)	**Title**	**DOW WP**	**Total**	1. HCMR	2. FCPCT	3. IRTA	4. IOLR	5. UNIABDN	6. DLO	7. IMR	8. IEO	9. UL	10. TU/e	11. AU	12. APROMAR	13. UNIBA	14. IFREMER	15. ULL	16. FUNDP	17. NIFES	18. CTAQUA	19. CMRM	20. SARC	21. DTU	22. SWH
		Total Project	**1559,30**	320,60	256,00	97,00	72,00	38,00	35,85	28,66	31,50	34,00	27,50	37,50	20,49	41,00	9,00	52,90	34,50	8,50	32,30	13,60	9,50	19,50	17,50
		0-12 months	234,42	32,95	20,47	21,46	19,40	3,93	13,46	6,71	4,25	3,30	6,91	2,96	9,68	5,00	3,10	6,16	2,00	1,20	3,10	5,25	0,14	2,42	5,20
WP1 Management	Management	**1**	11,23	7,50	0,00	0,07	0,10	0,12	0,58	0,08	0,10	0,20	0,22	0,11	0,12	0,20	0,10	0,06		0,10	0,10			0,22	
WP2 Reproduction and Genetics			69,06	9,40	4,00	6,78	5,50	0,00	0,00	1,59	2,80	0,60	0,00	0,00	0,00	4,80	3,00	0,56	0,00	0,00	0,00	3,15	0,00	0,00	3,20
2.1	Reproduction - meagre	**2**	13,32	2,50	4,00	6,72											0,10								
2.2	Reproduction - amberjack	**3**	33,78	3,20			3,20				0,80					4,80	0,40	0,21							
2.3	Reproduction - pikeperch	**4**	1,60	1,00								0,60													
2.4	Reproduction - halibut	**5**	5,09	0,30						1,59															3,20
2.5	Reproduction - wreckfish	**6**	9,70	2,10		0,04					2,00						1,70	0,20				3,15			
2.6	Reproduction - mullet	**7**	5,57	0,30		0,02	2,30										0,80	0,15							
WP3 Nutrition			27,75	0,00	8,24	0,00	9,30	0,00	0,00	0,30	0,35	0,00	0,00	0,00	0,00	0,00	0,00	2,66	0,00	1,10	0,00	2,10	0,00	1,70	0,00
3.1	Nutrition - meagre	**8**	7,82		7,60													0,22							
3.2	Nutrition - amberjack	**9**	2,37								0,30							2,07							
3.3	Nutrition - pikeperch	**10**	3,89															0,19						1,70	
3.4	Nutrition - halibut	**11**	1,58							0,30								0,18		1,10					
3.5	Nutrition - wreckfish	**12**	2,79		0,64						0,05											2,10			
3.6	Nutrition - mullet	**13**	9,30				9,30																		
WP4 larval husbandry			23,48	0,25	0,90	3,03	4,50	0,00	0,00	3,55	0,80	2,40	0,00	0,00	0,00	0,00	0,00	2,08	0,00	0,00	0,00	0,00	0,00	0,25	2,00
4.1	Larval husbandry - meagre	**14**	2,93			2,74												0,19							
4.2	Larval husbandry - amberjack	**15**	3,84	0,25	0,90						0,80							1,89							
4.3	Larval husbandry - pikeperch	**16**	6,44			0,29						2,40												0,25	
4.4	Larval husbandry - halibut	**17**	5,55							3,55															2,00
4.5	Larval husbandry - wreckfish	**18**	0,22																						
4.6	Larval husbandry - mullet	**19**	4,50				4,50																		
WP5 Grow out husbandry			22,65	3,00	2,90	3,75	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,33	2,00	0,00	0,00	0,00	0,00	0,25	0,00
5.1	Grow out husbandry - meagre	**20**	9,04	3,00		3,24																			
5.2	Grow out husbandry - amberjack	**21**	4,85		2,90													0,33							
5.3	Grow out husbandry - pike perch	**22**	4,25																2,00					0,25	
5.4	Grow out husbandry - mullet	**23**	4,51			0,51																			
WP6 Fish health			23,25	7,80	4,43	4,53	0,00	3,81	0,00	1,19	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,23	0,00	0,00	0,00	0,00	1,16	0,00	0,00
6.1	Fish health - meagre	**24**	13,72	4,60	1,53	4,53		1,90															1,16		
6.2	Fish health - amberjack	**25**	8,34	3,20	2,90			1,91			0,10							0,23							
N/A	Fish health - halibut	**26**	1,19							1,19															
WP7 Socioeconomics			49,78	4,00	0,00	3,30	0,00	0,00	12,88	0,00	0,00	0,00	6,69	2,85	9,02	0,00	0,00	0,24	0,00	0,00	0,00	0,00	0,00	0,00	0,00
7.1	Institutional and organizational context	**27**	26,41						10,70				6,69		9,02										
7.2	New Product Development	**28**	7,54	4,00		1,80												0,24							
7.3	Consumer value perceptions and behavioral change	**29**	15,83			1,50			2,18					2,85											
7.4	Business model and marketing strategy development	**30**	0,00																						
WP8 Dissemination	Dissemination	**31**	7,22	1,00							0,10	0,10			0,54						3,00				

Numbers in red may be modified slightly in the Partner's Form Cs.

Table 4.1b Staff effort per Partner and WP, during the 1st Reporting Period (0-12 Mo). The "Total Project" effort included in the DOW is shown as well.

Calculation of staff effort per parter and sub WP				SME partners									New Partners						
Work Package (Proposal)	**Title**	**DOW WP**	**Total**	23. ARGO	24. ITICAL	25. DOR	26. GEI	27. FORKYS	28. CANEXMAR	29. ASIALOR	30. CULMAREX	31. IRIDA	32. MC2	33. FGM	34. BVFi	35. MASZ	36. ANF	37. EUFIC	38. HRH
		Total Project	**1559,30**	65,10	29,40	18,60	12,50	30,50	57,60	42,60	26,50	3,25	3,32	2,75	0,58	3,25	3,00	3,25	19,70
		0-12 months	234,77	16,20	6,05	4,30	2,10	0,00	1,72	7,50	2,10	0,35	0,78	0,55	0,22	0,45	0,16	1,22	11,00
WP1 Management	Management	**1**	11,33	0,10	0,18	0,30	0,10		0,10			0,10	0,05	0,05	0,06		0,07	0,04	0,20
WP2 Reproduction and Genetics			69,06	15,30	5,87	2,00	0,00	0,00	0,00	0,00	0,00	0,00	0,51	0,00	0,00	0,00	0,00	0,00	0,00
2.1	Reproduction - meagre	**2**	13,32																
2.2	Reproduction - amberjack	**3**	33,78	15,30	5,87														
2.3	Reproduction - pikeperch	**4**	1,60																
2.4	Reproduction - halibut	**5**	5,09																
2.5	Reproduction - wreckfish	**6**	9,70										0,51						
2.6	Reproduction - mullet	**7**	5,57			2,00													
WP3 Nutrition			27,75	0,00	0,00	0,00	0,00	0,00	0,00	2,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
3.1	Nutrition - meagre	**8**	7,82																
3.2	Nutrition - amberjack	**9**	2,37																
3.3	Nutrition - pikeperch	**10**	3,89							2,00									
3.4	Nutrition - halibut	**11**	1,58																
3.5	Nutrition - wreckfish	**12**	2,79																
3.6	Nutrition - mullet	**13**	9,30																
WP4 larval husbandry			23,48	0,00	0,00	0,00	0,00	0,00	0,00	3,50	0,00	0,00	0,22	0,00	0,00	0,00	0,00	0,00	0,00
4.1	Larval husbandry - meagre	**14**	2,93																
4.2	Larval husbandry - amberjack	**15**	3,84																
4.3	Larval husbandry - pikeperch	**16**	6,44							3,50									
4.4	Larval husbandry - halibut	**17**	5,55																
4.5	Larval husbandry - wreckfish	**18**	0,22										0,22						
4.6	Larval husbandry - mullet	**19**	4,50																
WP5 Grow out husbandry			22,90	0,80	0,00	2,00	2,00	0,00	1,62	2,00	2,00	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00
5.1	Grow out husbandry - meagre	**20**	9,04	0,80							2,00								
5.2	Grow out husbandry - amberjack	**21**	4,85						1,62										
5.3	Grow out husbandry - pike perch	**22**	4,25							2,00									
5.4	Grow out husbandry - mullet	**23**	4,76			2,00	2,00					0,25							
WP6 Fish health			23,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
6.1	Fish health - meagre	**24**	13,72																
6.2	Fish health - amberjack	**25**	8,34																
N/A	Fish health - halibut	**26**	1,19																
WP7 Socioeconomics			49,78	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	10,80
7.1	Institutional and organizational context	**27**	26,41																
7.2	New Product Development	**28**	7,54																1,50
7.3	Consumer value perceptions and behavioral change	**29**	15,83																9,30
7.4	Business model and marketing strategy development	**30**	0,00																
WP8 Dissemination	Dissemination	**31**	7,22								0,10			0,50	0,16	0,45	0,09	1,18	

Numbers in red may be modified slightly in the Partner's Form Cs.