Deliverable Report

Deliverable No:	D24.3	**Delivery Month:**	26
Deliverable Title	Cloning of key marker genes of innate and adaptive immune responses in meagre.		
WP No:	24	**WP Lead beneficiary:**	P5. UNIABDN
WP Title:	Fish Health– Meagre		
Task No:	24.5	**Task Lead beneficiary:**	P5. UNIABDN
Task Title:	First characterization of the immune system		
Other beneficiaries:	P3. IRTA		
Status:	Delivered	**Expected month:**	26
……..			

Lead Scientist preparing the Deliverable: Secombes, C.J. (UNIABDN)

Other Scientists participating: Andree, K. (IRTA), Estévez, A. (IRTA)

Objective: The objective of this Deliverable was to develop quantitative PCR assays for investigating the development of the immune system during grow-out of meagre, *Argyrosomus regius*.

Table of Contents

1. Introduction

The diversification of aquaculture requires consideration of social, economic, ecological and biological factors to facilitate continued success of this fast-growing industry. Industry expansion requires species that are suitable for commercialization. Meagre (*Argyrosomus regius*) is a species recognized as having a high potential for aquaculture diversification in the European region, because of its good adaptation to captivity, rapid growth, and high quality flesh (Poli *et al.*, 2003; Monfort 2010; Grigorakis *et al.*, 2011). However, intensive rearing efforts are for the most part incipient and, therefore, the health maintenance issues that accompany intensive rearing are also mostly still unknown.

It is anticipated that future management of disease issues in meagre will require vaccines as part of the arsenal of approaches used. For this approach to be useful, there will be a need to understand more completely the chronology of events that occur –within the context of the immune system- during grow-out. Ontogeny of the immune system can be somewhat variable among species and quite distinct when comparing marine versus freshwater species (Magnadottir *et al.*, 2005). This deliverable outlines the work undertaken towards the characterization of the immune system to identify key immune molecules, as potential markers of immune system development and induction of antiviral and antibacterial responses (by P5. UNIABDN, P3. IRTA). For markers of the adaptive immune system, a number of key genes were chosen for cloning, including RAG1/2, Ig and TcR genes, to allow an analysis of when to vaccinate as the immune system matures. In addition, marker genes of inflammation (IL-1β, TNFα), antibacterial responses (antimicrobial peptides, such as piscidins and defensins) and the antiviral response (interferon, Mx) genes were also chosen. These latter genes require prior stimulation of fish/cells, since these genes show low constitutive expression, but are markedly induced upon infection. Pathogen Associated Molecular Patterns (PAMPS) were used for this purpose and included bacterial LPS, polyI:C (a synthetic double stranded RNA) and β-glucan. For all of the above-described genes, qPCR assays were subsequently established for future gene expression profiling following vaccination or immune-stimulant treatment.

This deliverable -*D24.3 - Cloning of key marker genes of innate and adaptive immune responses in meagre*, is a pre-requisite for the next part of this task, in which tissue samples collected during grow-out will be used in RT-qPCR for stage specific evaluation of immune gene expression profiling to understand at which stage post-hatch full immunocompetence is achieved.

2. Materials and methods

2.1 Biological material and experimental design

Spawning of meagre was carried out as previously described (Duncan et al. 2008; Fernandez-Palacios et al. 2014). Larvae were monitored during the entire grow-out period to monitor specific growth rate (SGR) and survival. Samples for this task were collected twice weekly during the pre-weaning phase and once per week thereafter (**Fig. 1**). The samples from whole larvae were assayed initially (*i.e.* RNA of entire animals). This contained transcripts of all representative tissues. As animals achieved significant size for dissection, individual tissues were collected to obtain samples enriched for transcripts of specific and relevant cell/tissue types, and enhance the ability to obtain reliable relative gene expression results. The tissues chosen for dissection were gills, spleen, kidney and intestine, due to their specific roles in immune stimulation and regulation.

Samples collected post-weaning were used for this deliverable for obtaining cDNA to isolate immune markers of interest. All samples were stored at -80ºC until use. Processed samples in the form of RNA or cDNA were also stored at -80^{o}C.

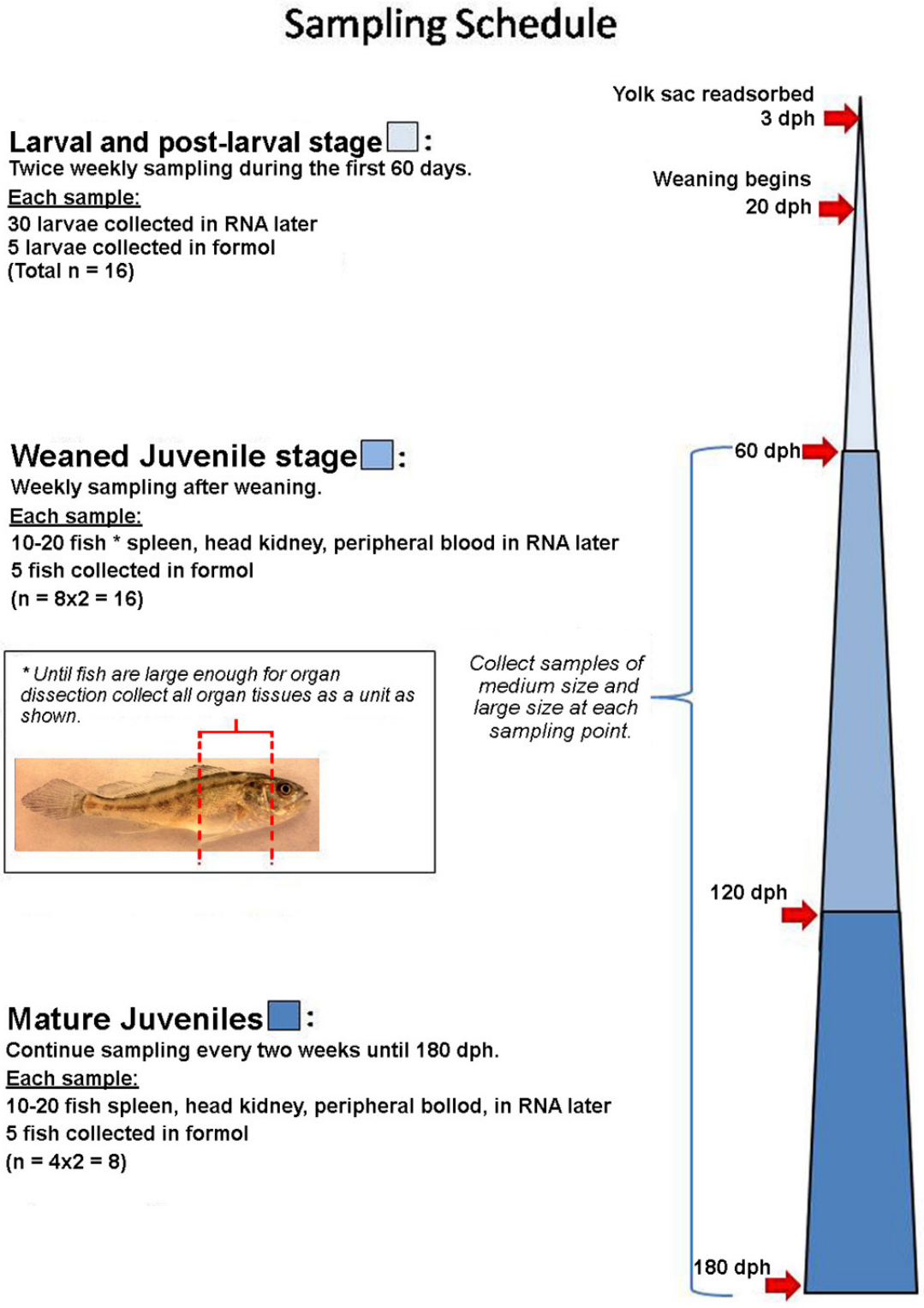

Figure 1. Scheme for sample collection during grow-out of larvae of meagre. Duplicate samples were collected at each time point with one set collected in RNA later for the purpose of molecular biological analyses and the second set collected in neutral buffered formalin for histological analyses.

2.2 Cloning immune gene fragments

As there is no annotated genome yet published for meagre, it has been necessary to recover particular genomic fragments of the genome that encode the relevant immune marker genes. At the outset of this work specific genes were agreed upon as being markers of immune system development and also representative of different immune functions. This list of genes that were targeted is shown in **Table 1**. The genes that were targeted were unknown for meagre and for this reason recovery of the sequences of interest was needed prior to designing the specific quantitative PCR assays for the completion of the task (Task 24.5). For this purpose, alignments of homologs of the target genes were prepared using sequences from extant species already annotated in GenBank. These alignments were used for the purpose of identifying the regions of the genes of interest that were conserved among the various species represented in the alignments. When they were available, sequences from marine species were used for preparation of the alignments and the species more phylogenetically related to meagre were given priority.

Table 1. Target genes divided into functional groups to be obtained by degenerate /consensus-primed PCR.

Immune Ontogeny Marker Genes
Endogenous Controls
EF-1a (elongation factor-1 alpha)
GAPDH (glyceraldehyde phosphate dehydrogenase)
18S
Beta actin
HPRT (hypoxanthine phosphoribosyl transferase)
Innate Immunity
Piscidin
Hepcidin
Beta - defensin
Lysozyme
Metallothionein
Transferrin
MX protein
NOD3 (nucleotide oligomerizing domain)
Adaptive Response
RAG1 (recombination activating gene)
IgM (immunoglobulin M)
IgT (immunoglobulin T)
TcR (T-cell receptor)
C3 (complement)
TNFa (tumor necrosis factor)
IFN (type 1 interferon)
IFN gamma (type II interferon)
IL-1beta (interleukin)
IL-4/13
IL-10
IL-17
IL-22
Inflammatory Response
COX (cyclooxygenase/prostaglandin synthase)
Myd88 (myeloid differentiation primary response gene)

Tissue samples collected from gills, spleen, kidney and intestine were used for RNA extractions. These samples for RNA isolation were prepared from post-weaning sub-adult individuals to optimize recovery of all targeted genes including those for which expression is initiated later in development. Extraction of RNA was performed using Trizol® (Ambion), followed by salting out of solution the resulting nucleic acid. Purity was assessed by spectrophotometry (A_{260}/A_{280}), followed by a visual quality assessment via agarose gel electrophoresis on 2% agarose gels stained with ethidium bromide. After quality control of the RNA, aliquots were treated with DNAse I to remove traces of genomic DNA that may have carried through the RNA purification process. Thereafter, from each of the different tissue samples 2 µg of RNA were used in a reverse transcription reaction for synthesis of first-strand cDNA, to be used for amplification of target gene fragments (SuperScript III First-Strand cDNA Synthesis Kit, Invitrogen).

Amplification of immune gene fragments required the design of gene "specific" primers in the absence of known meagre sequence data. Primers were designed from the conserved regions identified in the sequence alignments described above, such that amplified products were between 200 and 2000 bp to enable ease of amplification and subsequent cloning/sequencing. Primers designed for this purpose are shown in **Table 2**. Amplified products were sequenced bi-directionally using the primers from the initial amplification to confirm every nucleotide residue of the sequence obtained. 5´- 3´RACE reactions were performed for some of the recovered sequences to identify the entire open reading frame.

Table 2. Degenerate and consensus primers based upon sequence alignments of gene homologs from extant fish species annotated in GenBank. These are for the purpose of amplifying meagre specific sequences. Size of expected fragments to be obtained is shown; when two possible pairs of primers are listed two sizes are also shown.

Degenerate /Consensus Primers

Species - Target	Name	Sequence	Size (bp)
Piscicidin 1 degenerate (sense)	dgPisc1F	GRATGAGGCTGYRTCRTTTCCC	100/110
Piscicidin 1 degenerate (antisense)	dgPisc1R	ACWRGAATCCCTTKCCACAGCC	110
Piscicidin 1 degenerate (antisense)	dgPisc1 R2	CTTKCCACAGCCRAYVGGTCCAAAG	100
C3 degenerate (sense)	dgC3F	ACTGGAGGCCACAGCTTAYGCTC	1201
C3 degenerate (antisense)	dgC3R	GCCAGTACTCYATCCAGGTTCTC	1201
COX2 degenerate (sense)	dgCOX F	CACCAGTTCTTCAAATCTGATATGAAG	400/1000
COX2 degenerate (antisense)	dgCOX R1	CCTCRATCACGATCTTRATGGTCTC	400
COX2 degenerate (antisense)	dgCOX R2	TGCAGCGAGGCTGTGTTGATGATG	1000
MX Protein degenerate (sense)	dgMXPF	GACATAGCAACCACAGAGGCYYTGA	570
MX protein degenerate (antisense)	dgMXPR	GTCTTGTAGTTGARGAABCCDGGKAG	570
Lysozyme degenerate (sense)	dgLysoF	CTGGTGTTTCTGCTYCTGGTGGC	220/250
Lysozyme degenerate (antisense)	dgLysoR	CCAKRAGCGYCTYTTYATCTGYAAYATG	220
Lysozyme degenerate (sense)	dgLysoF2	CAGCCTGGCSRAYTGGGTKTGYC	250
Lysozyme degenerate (antisense)	dgLysoR2	CCASGCCACCCASGCGCSGATGC	250
Lysozyme degenerate (antisense)	dgLysoR3	GTCAGAAGCTCGCTGCADCTGATG	250
EF-1α degenerate (sense)	dgEF1F	GACTTCATCAAGAACATGATCACTG	230
EF-1α degenerate (antisense)	dgEF1R	GATCTTCTTGATGTAGGTGCTCAC	230
GAPDH degenerate (sense)	dgGAPDHF	GGASTACATGGTCTACATGTTCAAGTA	239
GAPDH degenerate (antisense)	dgGAPDHR	TGGTTGACYCCCATGACYAACATG	239
MET degenerate (sense)	dgMETF	AARASTGGRACCTGCAACTGCGGWG	70/400
MET degenerate (antisense)	dgMETR1	GCAGCCAGAGGCGCARTTGSTGC	70

MET degenerate (antisense)	dgMETR2	TTTATTTCAACAHHGWARTGTRGTAAC	400
MYD88 degenerate (sense)	dgMYD88F	CCYGARCTSTTTGATGCCTTCATCT	130
MYD88 degenerate (antisense)	dgMYD88R	CACCTCRCTCRTCAATGAGTTCYC	130
TNFa degenerate (sense)	dg TNFaF	GGCGTTYGCTCAGGGCGGCTTC	250
TNFa degenerate (antisense)	dg TNFaR	GCTGAAACACVGCYCCCAGATAYATG	250
NOD3 (sense)	dgNOD3 F	CATSRGAACTGTGCTGACRAAGG	380
NOD3 (antisense)	dgNOD3 R	CCAGATGAGYGCAGAGGGAAG	380
transferrin (sense)	dgTRN F	CYGCRGGCTGGAACATYCCCATCG	800/1000
transferrin (antisense)	dgTRN R	CCATCNACMGMMATGGCATCWGCYTC	800
transferrin (antisense)	dgTRN R2	TGCCCAAGCCSGTGTGGCAMGAC	1000
transferrin (sense)	dgTRN F2	CKTGCCACACSGGCTTGGGCAG	500
transferrin (sense)	dgTRN F3	TACTACGGCTACGCTGGAGCC	300
transferrin (antisense)	dgTRN R3	CTTGGTGGAGTCCTTGAAGAGGAG	300/500
Interleukin-1b (sense)	IL1b F3	CTGTGGCTCTGGGCATC	222
Interleuklin-1b (antisense)	IL1b R2	CCGTGCTGATGTACCAG	222
Interleukin-4/13 (sense)	IL4/13 pF1	GGTCAGTCCGGCTCTG	131
Interleukin-4/13 (antisense)	IL4/13pR1	ACACGTCCTCCACAAA	131
Interleukin-10 (sense)	IL10 pF2	ATGACTCCTCGGTCTCT	348
Interleukin-10 (antisense)	IL10 pR3	GGACTCCATGTGAGGCTT	348
Interleukin-17 (sense)	IL17 F1	CTCGGTGGCCCCAGAG	111
Interleukin-17 (antisense)	IL17 R1	CCTGGTAGTAGATGGGTTGAGC	111
Interleukin-22 (sense)	IL22 pF1	GCCAACATCCTCGACTTCTA	115
Interleukin-22(antisense)	IL22 pR2	AGTCTTCAGGTCCTCGCT	115
TNFa (sense)	TNFa F1	GAGAGCAGCCATTCATT	228
TNFa (antisense)	TNFa R1	CTGTAAACAAAGTAGAGGC	228
IFN type 1 degenerate (sense)	dgIFN1 DF1	TGGATCATAAAT TCAGACAGYACAG	405
IFN type 1 degenerate (antisense)	dgIFN1 DR1	TCCCAGGMTTCARCACTGT	405
IFN gamma degenerate (sense)	dgIFNG DF1	GAGGGCAGTGRTYTGYCTGT	515
IFN gamma degenerate (antisense)	dgIFNG DR2	CAGCTCCCACARTGCTTTG	515
RAG (sense)	RAG F3	CGGTGATGAGGATGAATGG	451
RAG (antisense)	RAG R3	GGTGTAGAGCCAGTGATGTTT	451
IgM (sense)	IgM F3	AAGAGACAGGACTGGGA	827
IgM (antisense)	IgM R2	TTTCACAAAGCAAGTCAGGG	827
IgT (sense)	IgT F2	GGTCACTCTGTTGTGTCTG	159
IgT (antisense)	IgT R2	GTGGTGTAAAGACTCGTAAC	159
TCR beta chain (sense)	TCRB F1	CCAAGGAACCAAACTGACA	115
TCR beta chain (antisense)	TCRB R1	CGCCACACAAACCAAGG	115
Piscidin (sense)	Pisc F2	TGGTTGTTCTCATGGCTGAAC	153
Piscidin (antisense)	Pisc F3	GGTCATAAGAAAGTGAACGT	153
Hepcidin (sense)	Hep F5	ATGAAGACATTCAGTGTTGC	216
Hepcidin (antisense)	Hep R5	CAGCAACCGCAGCAAA	216
Beta defencin (sense)	Def F3	GTGCTTCTCCTGATGCTCGC	98
Beta defencin (antisense)	Def R2	CTGTATCTTCGAGGGCAAC	98

EF-1a (sense)	EF1a F2	GTCAACAAGATGGACTCC	548
EF-1a (antisense)	EF1a R2	GGTGGGTCGTTCTTGCTGTC	548
Beta actin (sense)	BACT F1	TGGACTTTGAGCAGGAGATGG	452
Beta actin (antisense)	BACT R1	GAGGGACCAGACTCGTCGTA	452
HPRT degenerate (sense)	dgHPRT F1	GACATGGGGGGVCACCA	632
HPRT degenerate (antisense)	dgHPRTR1	TCCTCCACARTCRAGACRTTCTT	632

Amplified gene fragments were purified using spin columns (QiaQuick PCR Purification Kit) and 4 µl aliquots were loaded onto a 2% agarose gel with a mass standard and separated by size electrophoretically to assess concentration of the purified material for sequencing (**Figure 2** in Results below). Concentrations were estimated visually by comparison to the mass standard. Aliquots of the purified material and the primers originally used in the amplification were sent to an outside contractor for sequencing (ie. Sistemas Genomicos, Valencia, Spain).

2.3 Design and validation of qPCR assays for immune genes of meagre

Using the meagre sequences obtained, new primers were designed to have approximately the same melting temperatures (Tm ºC), to optimize the efficiency of primer binding during amplification reactions. This would also allow the multiplexing of different reactions on the same qPCR experimental plate in future studies. Primers developed for the qPCR assays are shown together with amplification efficiency values determined empirically in **Table 3** (in Results below). Whenever possible, primers were designed to span exon/intron junctions to diminish the possibility of amplification of any trace genomic DNA contamination that might still be present in cDNA samples. Efficiency of amplification was established by preparing multiple five-fold dilutions of cDNA from individual tissues, or cDNA samples prepared from a pool of RNA extracted from each individual tissue. Samples from each dilution were loaded into the plates in triplicate. After amplification outliers were removed.

Amplification efficiency (E%) was calculated following the equation: $E\% = 10^{(-1/slope)} - 1$, where the "slope" is that calculated from the regression line of the standard curve. Calculations of efficiency for each immune gene qPCR assay will be used in calculation of relative gene expression of the target genes relative to the endogenous control genes.

2.4 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3. Results

3.1 Isolation of immune gene fragments

There are now sequences obtained from 5 endogenous control genes and 22 target genes for evaluation of the immune system functions and ontogenetic development. Sequences have been confirmed and for some genes the entire open reading frame (ORF) has been obtained. These sequences will be uploaded into GenBank following the completion of the Task and all deliverables, to provide open source material for other investigative teams working on this species.

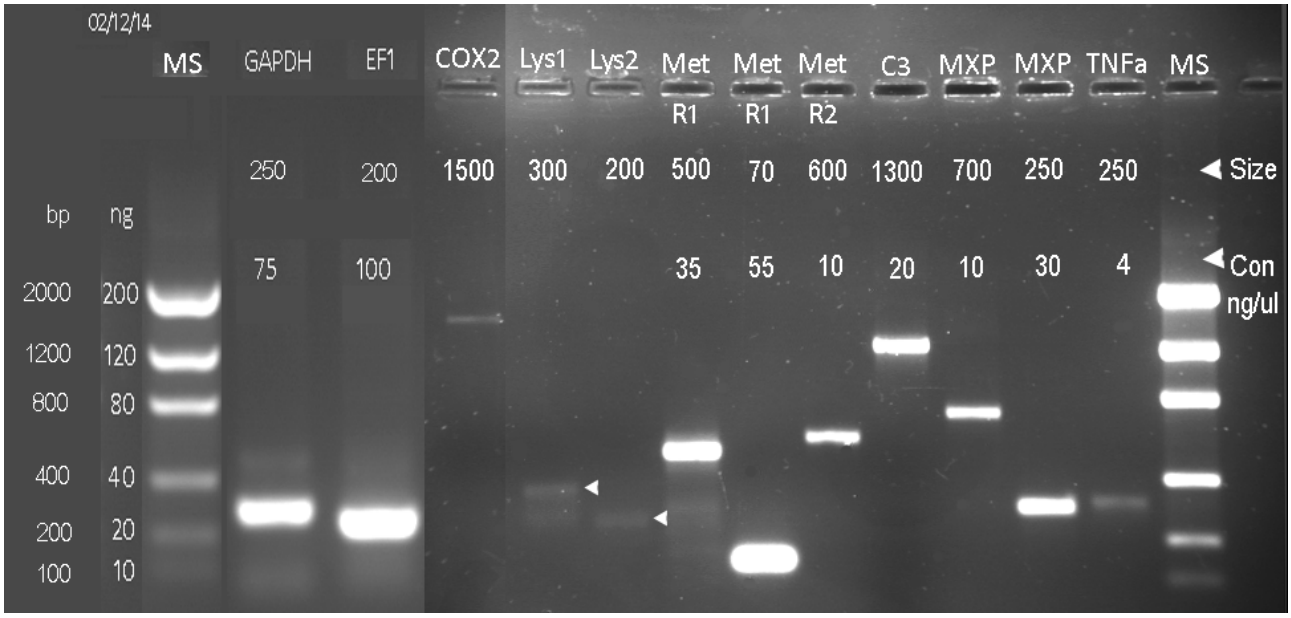

Figure 2. Examples of the purified amplified gene fragments prepared for sequencing shown alongside a mass ladder standard (MS). Where concentration was sufficient for purposes of sequencing the estimated concentrations for each purified fragment are shown. GAPDH = glyceraldehyde phosphate dehydrogenase; EF1 = elongation factor-1 alpha; COX2 = cyclooxygenase 2; Lys = Lysozyme; Met = Metallothionein; C3 = Complement C3 protein; MXP = MX protein; TNFa = Tumor necrosis factor alpha.

3.2 Validation of the qPCR assays

The assays were evaluated for amplification efficiency using serial dilutions of cDNA. The E% was near 100% for all the assays ranging from 96.1% to 104.3%. These E% values will be used in the calculations of relative gene expression. Specificity of each assay was also confirmed using analysis of the melt curves to confirm that only one amplification peak was present. For a few gene amplifications where melt curves displayed slight deviations, confirmation of the uniqueness of the product amplified was also confirmed by running an aliquot on agarose gels to confirm the presence of only one DNA amplicon.

Table 3. Primers designed from specific meagre sequences obtained are shown below together with the expected size of the amplified product and the empirically derived E% values.

Quantitative PCR Primers

Species - Target	Name	Sequence	Size (bp)	E%
18S rRNA (sense) qPCR	**qpAr18S F**	CACACCGCCCGTCGCTACTACC	140	100.1%
18S rRNA (antisense) qPCR	**qpAr18S R**	TACGACTTTTACTTCCTCTAGATAGTC		
elongation factor 1a (sense)	**qpEF1a F**	CTCTCAGGCTGACTGCGCCGTG	186	100.8%
elongation factor 1a (antisense)	**qpEF1a R**	CTCGAAACGGGCCTGGCTGTATG		
elongation factor 1a (sense)	**qpEF1 F2**	AAC ATG CTT GAG GGC AGT GAC AA	189	97.75%
elongation factor 1a (antisense)	**qpEF1 R2**	TAC GGT TCC GAT ACC GCC G		
glyceraldehyde phosphate dehydrogenase (sense)	**qpGAPDH F**	CCAGTACGTGGTGGAGTCCACTG	109	100.0%
glyceraldehyde phosphate dehydrogenase (antisense)	**qpGAPDH R**	AGCGTCAGCGGTGGGTGCAGAG		

Beta Actin (sense)	**qpBACT F**	TGG GGG AGC AAT GAT CTT GAT CTT CA	212	100.05 %
Beta Actin (antisense)	**qpBACT R**	AGC CCT CTT TCC TCG GTA TGG AGT C		
Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (sense)	**qpHPRT F**	CATGGACTCATCTTGGACAGGACAGA	137	100.95 %
Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (antisense)	**qpHPRT R**	GCCTTGATGTAGTCCAGCAGGTC		
Metallothionein (sense)	**qpMet F**	GATCCTGCAATTGCAAAGACTGTTC	70	102.2%
Metallothionein (antisense)	**qpMet R**	CCGGATGGGCAGCATGGGCAG		
C3 complement (sense)	**qpC3 F**	AACCCATACGCTGTTGCCATGACG	120	100.1%
C3 complement (antisense)	**qpC3 R**	CACGTCCTTTAGGTACTGGGCCAG		
MX protein (sense)	**qpMXP F**	AGTCAGTGGTTGACATTGTTCATAATG	187	98.2%
MX protein (antisense)	**qpMXP R**	AACAGTGGCATGACCGTCATTGTAG		
tumor necrosis factor 1a (sense)	**qpTNF1a F**	CACCTCTCAGCCACAGGATCTGG	104	99.2%
tumor necrosis factor 1a (antisense)	**qpTNF1a R**	TTGTCCTCCTGAGCTGTGTTCTGG		
Myeloid differentiation primary response 88 (sense)	**qpMYD88 F**	GCTACTGCCAGAGTGACTTCGAGT	120	102.2%
Myeloid differentiation primary response 88 (antisense)	**qpMYD88 R**	TCCATACACACGAACCCGGGAGG		
COX2 degenerate (sense)	**qpCOX2F**	GGAAGTTGGTGTTGACATGCACTAC	211	100.5%
COX2 degenerate (antisense)	**qpCOX2R**	CAATCAGGATGAGCCGTGTGGTC		
Lysozyme degenerate (sense)	**qpLysoF**	GATGGATCCACTGACTACGGCATC	148	100.0%
Lysozyme degenerate (antisense)	**qpLysoR**	ACACGTTTGGCACAGTTGATCGCC		
NOD 3 (sense)	**qpNOD3 F**	CAGCTTGGTGGAACTTGTTCATCAC	154	100.8%
NOD 3 (antisense)	**qpNOD3 R**	TAACATCAGTCAGGATCTCAGTGTTG		
Ig M (sense)	**qpIGM F**	AAA CTC TAT GAA AGG AGT ATT GGA GGA CA	304	99.85%
Ig M (antisense)	**qpIGM R**	CAG ACT CGT GGT GAA CTA CAC AG		
IgT (sense)	**qpIGT F**	CCAACCAAGTCCCAAAGAAA	104	100%
IgT (antisense)	**qpIGT R**	GCCATGTGATTCTGCTTTCA		
T Cell Receptor B heavy chain (sense)	**qpTCRB F**	CAA TCA CCA GCA GGC TGA GG	281	103.15 %
T Cell Receptor B heavy chain (antisense)	**qpTCRB R**	CTT TCC AGT CGA ACC CTG AAG C		
RAG (sense)	**qpRAG F**	CCGGTAATGAGGATGAATGG	144	100.4%
RAG (antisense)	**qpRAG R**	CACAGGCTTCATCTGCAAGTAG		
Interleukin 1b (sense)	**qpIL1B F**	GATTGCCTGGATTTTCCACTGTCTCCA	103	99.6%
Interleukin 1b (antisense)	**qpIL1B R**	GTGGCTCTGGGCATCAAGGG		
Interleukin 4/13 (sense)	**qpIL4/13F**	TCA GTC CGG CTC TGA CCA CTC CT	107	99%
Interleukin 4/13 (antisense)	**qpIL4/13R**	CTG AGC GAG AGA TCT ATT GTA TTT GTC AGC C		
Interleukin 10 (sense)	**qpIL10F**	ACTCCTCGGTCTCTCCTCGTATCCGC	187	102.15 %
Interleukin 10 (antisense)	**qpIL10R**	CTGTGTCGAGATCATCGTTGGCTTCATAA AAGTC		
Interleukin 17 (sense)	**qpIL17F**	CTC TGG CTG TCT GAG CCT GC	91	96.1%
Interleukin 17 (antisense)	**qpIL17R**	CCT GTG GAG GAC CAA AAC CTG G		

Interleukin 22 (sense)	**qpIL22 F**	GCC AAC ATC CTC GAC TTC TAC CTG AAC	146	101.75 %
Interleukin 22 (antisense)	**qpIL22 R**	TGG TCG TGG TAG TGA GTC ACA TTG C		
TNFa (sense)	**qpTNFA F**	CACAAGAGCGGCCATTCATTTACAAGGA G	173	103%
TNFa (antisense)	**qpTNFA R**	GGAAAGACGCTTGGCTGTAGATGG		
Interferon type 1 (sense)	**qpIFN F**	CTTCATGGGAGGAGAACACAGTGGAG	178	100.1%
Interferon type 1 (antisense)	**qpIFN R**	CAGGATTCAGCACTGTGGCTCATTTTC		
Interferon gamma (sense)	**qpIFNG F**	GCCAGCGATCCTCAGGTGG	171	99.85%
Interferon gamma (antisense)	**qpIFNG R**	CTGAACGACAGAGTCATTCATCTGGATG TG		
Beta Defensin (sense)	**qpDEF F**	GGGAACGAAGATCCAGAGATGCAGTATT GGAC	138	102.3%
Beta Defensin (antisense)	**qpDEF R**	CTAAGACCTCACAGCACAGCACCTG		
Piscidin (sense)	**qpPISC F**	CAATGATCCATGGGCTTATCC	111	101.95 %
Piscidin (antisense)	**qpPISC R**	TTCAGTCTCGCCATTGAAGC		
Hepcidin (sense)	**qpHEPC F**	CCG TCA TGC TCG CCT TCG	140	104.3%
Hepcidin (antisense)	**qpHEPC R**	CTC ACG CAT GTA ATA CGG AAT CTT GCA TG		

3.3 Results presented for dissemination of this Deliverable in 2015

Preliminary results obtained on the progress for this deliverable were presented at the European Aquaculture Symposium (EAS) in Rotterdam, The Netherlands in October 2015 (**Fig. 3**).

4. Summary

All of the genes initially targeted for this work have been obtained with the exception of transferrin. The expression assays have been established for these genes, which have currently been obtained for the study of immune ontogeny in meagre. Immune markers are now established for the innate, adaptive and inflammatory responses of the immune system as originally proposed. In total we have 28 assays developed for genes of interest for the study of immune function in this species, and this will be of interest for other groups as well who may be studying this species outside of the DIVERSIFY consortium.

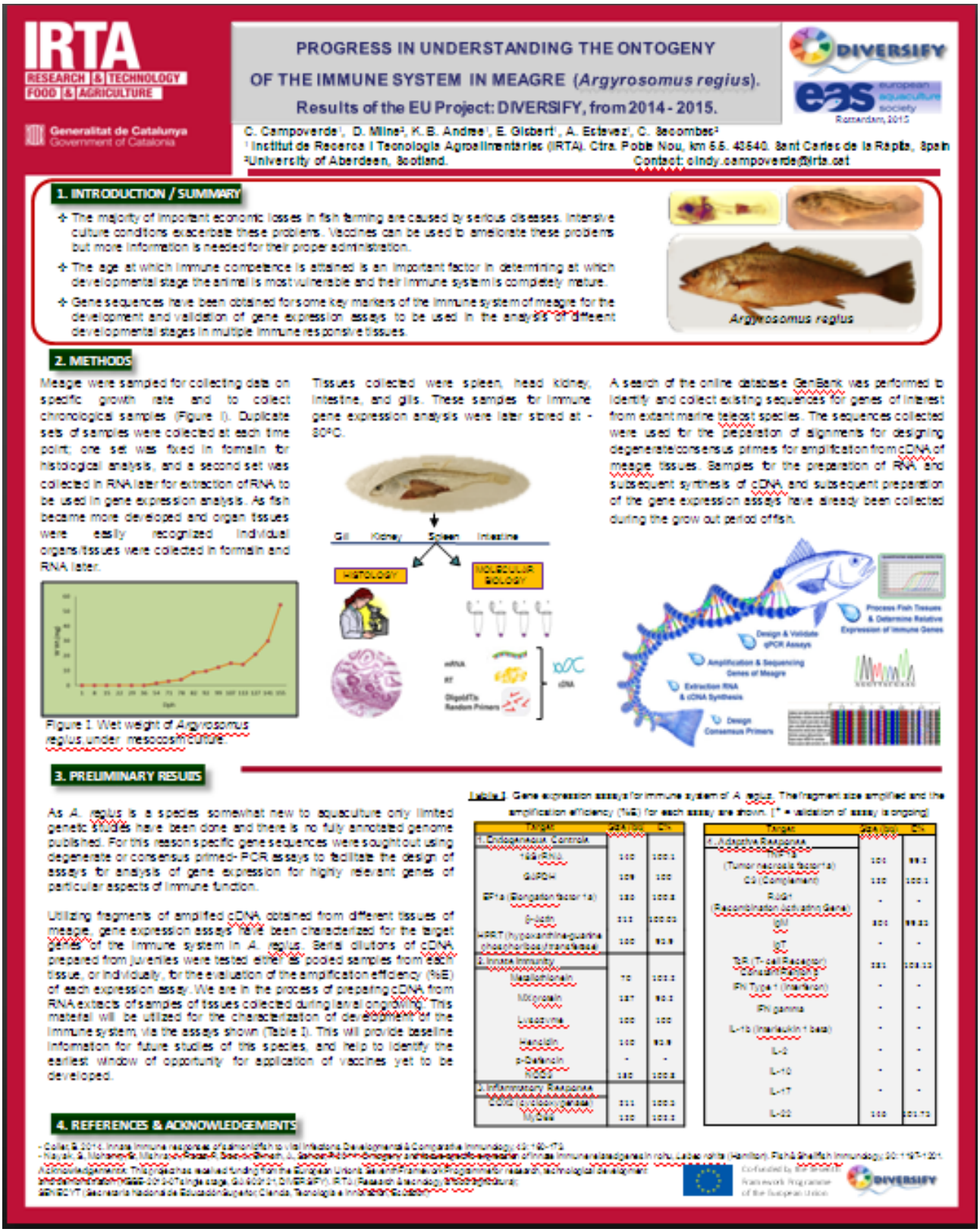

Figure 3. Poster with the work resulting from this Deliverable, presented at the European Aquaculture Society (EAS) annual conference in Rotterdam, The Netherlands in October 2015.

5. References

Duncan, D., Estevez, A., Padros, F., Aguilera, C., Montero, F.E., Norambuena, F., Carazo, I., Carbo, R., Mylonas, C. (2008) Acclimation to captivity and GnRHa-induced spawning of meagre (*Argyrosomus regius*) *Cybium*, 32(2) suppl.: 332-333.

Fernandez-Palacios, H., Schuchardt, D., Roo, J., Izquierdo, M., Hernandez-Cruz, C., Duncan, N. (2014) Dose-dependent effect of a single GnRHa injection on the spawning of meagre (*Argyrosomus regius*) broodstock reared in captivity. *Spanish J. Agric. Res.* 12(4): 1038-1048.

Grigorakis K., et al. (2011) Lipid quality and filleting yield of reared meagre (*Argyrosomus regius*). Int, J. Food Sci. Tech. 46: 711–716.

Magnadottir, B., Lange, S., Gudmundsdottir, S., Bøgwald, J., Dalmo, R.A. (2005) Ontogeny of humoral immune parameters in fish. *Fish Shellfish Immun.* 19: 429-439.

Monfort M. C. (2010) Present market situation and prospects of meagre *(Argyrosomus regius),* as an emerging species in Mediterranean aquaculture. Studies and Reviews, General Fisheries Commission for the Mediterranean No. 89. Roma: FAO.

Poli, B. M., Parisi, G.,Zampacavallo, G., Iurzan, F., Mecatti, M., Lupi, P., Bonelli, A. (2003) Preliminary results on quality and quality changes in reared meagre (*Argyrosomus regius*): body and fillet traits and freshness changes in refrigerated commercialsize fish. Aquacult. Int., 11: 301–311.


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