The Discovery and comparative expression analysis of three distinct type 1 interferons in the perciform fish, meagre (Argyrosomus regius)"

D.J. Milne[a], C. Campoverde[b], K.B. Andree[b], X. Chen[c], J. Zou[a], C.J. Secombes[a]

a Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, United Kingdom

b IRTA, Centro de San Carlos de la R_apita, San Carlos de la R_apita, 43540 Tarragona, Spain

c College of Animal Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, PR China

Abstract

Type I interferons (IFN) play an important role in anti-viral responses. In teleost fish multiple genes exist, that are classified by group/subgroup. That multiple subgroups are present in Acanthopterygian fish has only become apparent recently, and 3 subgroups are now known to be expressed, including a new subgroup termed IFNh. However, the potential to express multiple IFN subgroups and their interplay is not well defined. Hence this study aims to clarify the situation and undertook the first in-depth analysis into the nature and expression of IFNc, IFNd and IFNh in the perciform fish, meagre. Constitutive expression was analysed initially during larval development and in adult tissues (gills, mid-gut, head kidney, spleen). During early ontogeny IFNc was the highest expressed IFN, and this was also the case in adult tissues with the exception of gills where IFNd was highest. However, comparison between tissues for individual isoforms showed that spleen had high transcript levels of all three IFNs, IFNd/ IFNh were also highly expressed in gills. The expression of each sub-group was increased significantly in the four tissues following injection of poly I:C, however, this increase was only seen in the mid-gut for IFNh. Following in vitro stimulation with poly I:C again all three isoforms were upregulated, although with differences in kinetics and the cell source used. For example, early induction was seen for IFNc/ IFNh in gill cells, IFNd/ IFNh in splenocytes and all three isoforms in head kidney cells. Induction was sustained in splenocytes and head kidney cells, but in gut cells only a late induction was seen. These results demonstrate a complex pattern of regulation between the different IFN isoforms present in meagre and highlights potential sub-functionalisation of these IFN subgroups during perciform anti-viral responses.

1. Introduction

Meagre (*Argyrosomus regius*) is a newly emerging species in aquaculture, due to its fast growth rate, large size, high feed conversion ratio and high processing yield (Monfort, 2010). It is native to the Mediterranean sea, Black sea and eastern Atlantic coast and has been cultured in France, Spain, Portugal, Italy, Greece and Croatia (FAO, 2012). However, several threats to the sustainable production of farmed meagre have been identified, with disease being one of the most prominent and potentially devastating. As meagre culture has intensified there has been a corresponding increase in the number of pathogens that have been reported, ranging from viral to monogenean in nature (Toksen et al., 2007; Merella et al., 2009; Ternengo et al., 2010). It is therefore critical that a greater understanding of the meagre immune response is achieved to aid the development of effective strategies to prevent or control future pathogen outbreaks.

Type I interferons (IFNs) play a major role in anti-viral responses by activating a number of immune cells, such as natural killer cells and $CD8^+$ T-cells, and inducing a wide range of IFN-stimulated genes (ISGs) to interfere with viral replication in host cells. They are produced in response to a variety of viral pathogen associated molecular patterns (PAMPs), including viral nucleic acids, that are recognised by pattern recognition receptors (PRRs) such as Toll-like receptors and RIG-1-like receptors (McNab et al., 2015). In teleost fish seven sub groups of type I IFN have been identified to date, IFNa-f and IFNh. These sub groups can be further divided into group 1 IFNs which contain 2 conserved cysteines (subgroups a, d, e and h) and group 2 IFNs which contain 4 conserved cysteines (subgroups b, c and f) (Secombes and Zou, 2017). Salmonids are capable of expressing IFN subgroups a-f (Zou et al., 2014) and cyprinids IFN sub groups a, c and d (Zou and Secombes, 2011), but until recently the Acanthopterygian fish (spiny finned fish such as perciformes, pleuronectiformes and gasterosteiformes) where thought to express only IFN subgroup d.

Hints that this was not the case were first discovered in turbot (*Scophthalmus maximus*) (Pereiro et al., 2014), where two distinct IFN types were found (IFN1 and 2). IFN1 clustered with group 2 IFNs, especially IFNc that had been unknown in Acanthopterygians previously, and had the typical 4 conserved cysteines. The second IFN was of less clear identity although seemed to be a group 1 IFN. Intriguingly IFN2, unlike IFN1, was not able to induce protection when administered prior to VHSV infection and this was linked to a lack of induction of a number of ISGs (eg Mx, ifi56, isg15), but it could induce IL-1β expression. More recently a new IFN subgroup, IFNh, has been identified in large yellow croaker (*Larimichthys crocea*) and in various other perciform species (Ding et al., 2016). IFNh is produced in response to poly I:C stimulation and the recombinant protein induces the expression of ISGs such as Mx and PKR (as well as itself) and has anti-viral activity. However, it does not induce the expression or phosphorylation of IRF3 or IRF7 and it has been hypothesised that downstream signalling may occur solely through the Jak-STAT pathway (Ding et al., 2016). Further analysis of IFN2 from turbot reveals it is in fact an IFNh, confirming this subgroup is more widely present in Acanthopterygian species. Thus IFNh is present in species that also express IFNc (turbot) or IFNd (large yellow croaker), but whether all three isoforms could exist within a single species was unknown until earlier this year, when Hu et al. (2017) showed that Japanese flounder (*Paralichthys olivaceus*) possess four IFN genes, that cluster with IFNc (jfIFN3), IFNd (jfIFN1 and jfIFN2) and IFNh (jfIFN4) in phylogenetic analysis. Poly I:C injection induced a transitory (detectable at 3h post-

injection) induction of jfIFN4 in spleen and kidney, but not jfIFN1-3, leaving uncertainty as to the relative function of the three isoforms in antiviral defence in this fish lineage.

As the potential to express multiple IFN subgroups is not well defined in perciforms this study aims to clarify the situation and provide further insight into the nature and expression of IFNc, IFNd and IFNh in the perciform fish, meagre. Initially the three isoforms were cloned by homology PCR, and the sequences analysed for relatedness to known teleost IFN genes. Their constitutive expression was then comparatively analysed during ontogeny and in individual tissues in larvae. Lastly, the potential differential modulation of expression was examined in gills, mid-gut, head kidney and spleen after injection with different PAMPs, and in isolated cells from these tissues stimulated *in vitro* with the same immunostimulants.

2. Materials and Methods

2.1 Fish husbandry

Healthy meagre (*Argyrosomus regius*) were supplied by the Institute for Agri-Food Research and Technology (IRTA), San Carlos de la Rapita, Spain. The fish were reared from fertilised eggs, using a mesocosm system, at 20^{o}C (Milne et al., 2017). From two days post hatch (dph) to 8 dph enriched rotifers, *Brachionus plicatilis*, were added to the tanks. From 9 to 31 dph enriched Artemia, *Artemia salina*, were provided as a food source and from 21 dph an inert pelleted diet was given. Before sample collection, all fish were anaesthetized in MS222 (Sigma Aldrich) and then killed.

2.2 PAMP stimulation and sample collection

Samples of whole larvae (10 per time point) were taken at 8, 15, 29, 40, 47 and 60 dph for determining IFN expression during early ontogeny. Gills, mid-gut, head kidney and spleen were also taken from healthy 20 g juvenile meagre to determine constitutive IFN expression in individual tissues.

For *in vivo* PAMP stimulation, 10 healthy 20 g juvenile meagre received a 100 µl intraperitoneal (ip) injection of phosphate buffered saline (PBS, Sigma Aldrich) or 100 µl ip injection of PBS containing poly I:C (100 µg, Sigma Aldrich), LPS (400 µg, Sigma Aldrich) or β-glucan (100 µg, Sigma Aldrich). 24 h later the gills, mid-gut, head kidney and spleen were harvested for IFN gene expression analysis.

For *in vitro* PAMP stimulation, the gills, mid-gut, head kidney and spleen of 6 healthy 20 g juvenile meagre were collected and pressed through a 70 µm nylon mesh (Greiner) with 10 ml of L15 media (ThermoFisher) containing penicillin (1,000 units/ml), streptomycin (1,000 µg/ml) (P/S, ThermoFisher) and 2% foetal calf serum (FCS, Sigma Aldrich). The cell suspension was then centrifuged for 10 min at 400g, the supernatant discarded, the pellet suspended in 10ml L15 media plus 2% FCS and P/S, and centrifuged for 10 min at 400g. The supernatant was again discarded and the pellet re-suspended in 30 ml of fresh media. 5 ml aliquots of the cell suspension where then transferred to the wells of a 12 well plate (Greiner). 250 µl of PBS (control) or PBS containing poly I:C, LPS or β-glucan was added to the appropriate wells giving final concentrations of 100 µg/ml, 50 µg/ml and 50 µg/ml respectively. . After 4, 12 and 24 h cells were collected, centrifuged for 10 min at 400g, the supernatant removed and the pellet re-suspended in 1.5 ml of RNA later (Sigma Aldrich). All samples were stored at -20^{o}C until use.

2.3 Molecular cloning of IFN isoforms

Total RNA was extracted from a pool of meagre gill, mid-gut, head kidney and spleen homogenate in TRI reagent (Sigma Aldrich), following the manufacturer's standard protocol. The total RNA was then reverse transcribed using SuperScript III (ThermoFisher) with Oligo dt (T_{26}VN) as the primer. Partial sequences were then obtained by PCR using MyTaq DNA polymerase (Bioline) and consensus primers **(Table 1)** were designed to conserved regions of the gene of interest in closely related species. The amplicon produced by the PCR reaction was ligated into pGEM-T easy vector (Promega) and transformed into *Escherichia coli* RapidTrans TAM1 competent cells (Active motif), which were plated onto MacConkey agar plates (Sigma Aldrich) containing ampicillin at 100 µg/ml (Sigma Aldrich) and incubated overnight at 37^{o}C. Plasmid DNA was extracted from positive colonies using a QIAprep Spin Miniprep Kit (Qiagen) and sent to Eurofins Genomics for sequencing. Next, 5' and 3' RACE was performed using primers designed from the partial sequence **(Table 1)**, as described by Hong et al. (2013), with the resulting amplicons undergoing the same cloning and sequencing protocol as described above. Finally, full coding sequence was confirmed by sequencing of the amplicon generated from a PCR reaction using Pfu DNA polymerase (Promega) and specific consensus primers to the 5' and 3' ends **(Table 1)** of the IFN isoforms.

Primer	Sequence (5' to 3')	Purpose
IFNc F	GCGGCGTTTCCTGTCCACTGCC	Partial cloning
IFNc R	GCCTCCAGCCACAGGCAGCACTGTCC	Partial cloning
IFNd F	TGGATCATAAATTCAGACAGTACAG	Partial cloning
IFNd R	TCCCAGGATTCAGCACTGT	Partial cloning
IFNh F	GGCTGAGGTGCAGTCTCAGTTGG	Partial cloning
IFNh R	GCTCCCAGGACGCAGGACTGCC	Partial cloning
IFNc 5N1	ATGCACCACCCATAATGCTTTACGGC	5' RACE nested PCR
IFNc 5N2	GGATGATTGGCTGTGGCAGCAGGG	5' RACE nested PCR
IFNc 3N1	CATCCTCAGTGCCGTAAAGCATTATGGG	3' RACE nested PCR
IFNc 3N2	ATTCCAGGACAATGACATACCTGTCGG	3' RACE nested PCR
IFNd 5N1	CAGTGGTGTTAGTGGAGTTATTAGCC	5' RACE nested PCR
IFNd 5N2	GATCCAAAGAATTTTCACTGTAC	5' RACE nested PCR
IFNd 3N1	GCTGCACATGTATTTCAAGAGACTGTCG	3' RACE nested PCR
IFNd 3N2	GAGCCACAGTGCTGAAGCCTGGG	3' RACE nested PCR
IFNh 5N1	CCGCCCATCTGCTGGAGGAG	5' RACE nested PCR
IFNh 5N2	GTTGACCGTAGTATCTGAGCCAATCAC	5' RACE nested PCR
IFNh 3N1	CTCACCTCTGTTTCCTGGGACACCG	3' RACE nested PCR
IFNh 3N2	GACAGAAGAACTCAACACCTGTGTG	3' RACE nested PCR
IFNc FULL F	ATGACACTTCAGTCCTCTTCAGTCCTC	Full sequence cloning
IFNc FULL R	TTAGCGGACACCTCTCCAGGTAAAGC	Full sequence cloning
IFNd FULL F	ATGCTCAGCAGGATCTTGTTTGTGTGCC	Full sequence cloning
IFNd FULL R	TTAGTTGGTGTTGAGTAGAGATGAAACCAGC	Full sequence cloning
IFNh FULL F	ATGGTTAACTGGACCGGCGTGCTC	Full sequence cloning
IFNh FULL R	TCAGTGCTGCCGTCCACTCGCTGCAGAG	Full sequence cloning
IFNc qPCR F	CAACGCCAACGTCTCCTTTC	Gene expression
IFNc qPCR R	TCATGCACCACCCATAATGC	Gene expression
IFNd qPCR F	CTTCATGGGAGGAGAACACAGTGGAG	Gene expression
IFNd qPCR R	CAGGATTCAGCACTGTGGCTCATTTTC	Gene expression
IFNh qPCR F	GACACCGTCAAGACCGAACA	Gene expression
IFNh qPCR R	CAGCAGGTCCAACTGATCCA	Gene expression

GAPDH qPCR F	CCAGTACGTGGTGGAGTCCACTG	Gene expression
GAPDH qPCR R	AGCGTCAGCGGTGGGTGCAGAG	Gene expression

Table 1. Primers used for gene discovery and qPCR. This table gives the primer names, sequence (5' to 3') and what they were used for.

2.4 Sequence analysis of IFN isoforms

Amino acid sequences were determined by the translation of cDNA sequence by the ExPASy translate tool (http://web.expasy.org/translate) and subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov). Protein similarity and identity were calculated using MatGAT 2.0 software (Campanella et al., 2003). A phylogenetic tree was generated by subjecting the amino acid sequence to a Neighbour-Joining test and Jones-Taylor-Thornton (JTT) model, with 10,000 bootstrap repetitions using MEGA 6 software (Kumar et al., 2004). The properties of the proteins were determined using various software programs; Compute pI/Mw tool (http://www.expasy.ch/) for the isoelectric point and molecular mass, TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM/) for determining transmembrane domains and ExPASy Prosite (http://prosite.expasy.org) for identifying conserved domains and signatures.

2.5 Real time quantitative PCR

Total RNA was extracted from each sample for IFN expression analysis using TRI reagent, following the manufacturer's standard protocol. The samples were then treated with TURBO DNase (ThermoFisher) to remove genomic DNA contamination prior to reverse transcription with SuperScript III and Oligo dT (T_{26}VN). Prior to analysis of the target genes in the *in vitro* stimulated samples an initial qPCR was undertaken to determine the GAPDH cycle number of the samples, as a means to assess the relative cell viability. This analysis confirmed that the GAPDH values for all 4 tissue cell suspensions had a similar GAPDH cycle number. Transcript level of IFN isoforms was then quantified using a Light Cycler 480 (Roche) and normalised to GAPDH, as described by Wang et al. (2011). A reference was placed on each plate that consisted of a serial dilution of equal molar quantities of each IFN isoform, to allow comparison of each isoform between plates.

2.6 Data transformation and statistical analysis

qPCR data was initially calculated as arbitrary units. These values were then used to show the expression of IFN isoforms during early and late ontogeny, and to examine relative constitutive expression between tissues. Data from *in vivo* and *in vitro* PAMP stimulation were transformed to a fold change relative to the respective PBS control samples. Time points during early and late ontogeny were interrogated using a general liner model and each time point had a sample size of N = 10. Differences in constitutive expression between tissues were determined by One-way ANOVA followed by the Tukey post hoc test. This was also the case for the PAMP stimulated groups in the *in vivo* and *in vitro* experiments. All statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) software, and were deemed significant when $P \leq 0.05$.

3. Results

3.1 Molecular cloning of IFNc, IFNd and IFNh

The meagre IFNc transcript (GenBank: MG489872) contains a 570bp open reading frame (ORF), the IFNd transcript (GenBank: MG489873) a 558bp ORF and the IFNh transcript (GenBank: MG489874) a 573bp ORF **(Figure 1)**. The putative proteins for IFNc, IFNd and IFNh consist of 188aa, 185aa and 190aa, with isoelectric points of 6.36, 6.13 and 9.36 and molecular weights of 20.9kDa, 21kDa and 21.8kDa, respectively. A signal peptide consisting of Met^{1} to Ala^{24} was identified in the IFNc putative protein, Met^{1} to Ser^{20} in the IFNd putative protein and Met^{1} to Phe^{21} in the IFNh putative protien. No transmembrane regions were detected for IFNc, IFNd or IFNh. An IFN alpha/beta domain was identified within each putative protein. This domain ranged from Leu^{41} to Arg^{164} in the IFNc putative protein, Met^{26} – Leu^{181} in the IFNd putative protein and Ser^{35} – Leu^{162} in the IFNh putative protein. All three meagre subgroups were predicted to contain several potential glycosylation sites. IFNc appears the least glycosylated with only 3 potential glycosylation sites compared to IFNd and IFNh which contain 4 potential glycosylation sites. Interestingly the glycosylation sites appear to be evenly distributed throughout the IFNc and IFNh putative proteins, however the glycosylation sites in the IFNd putative protein appear concentrated, all appearing in the 1^{st} half of the protein.

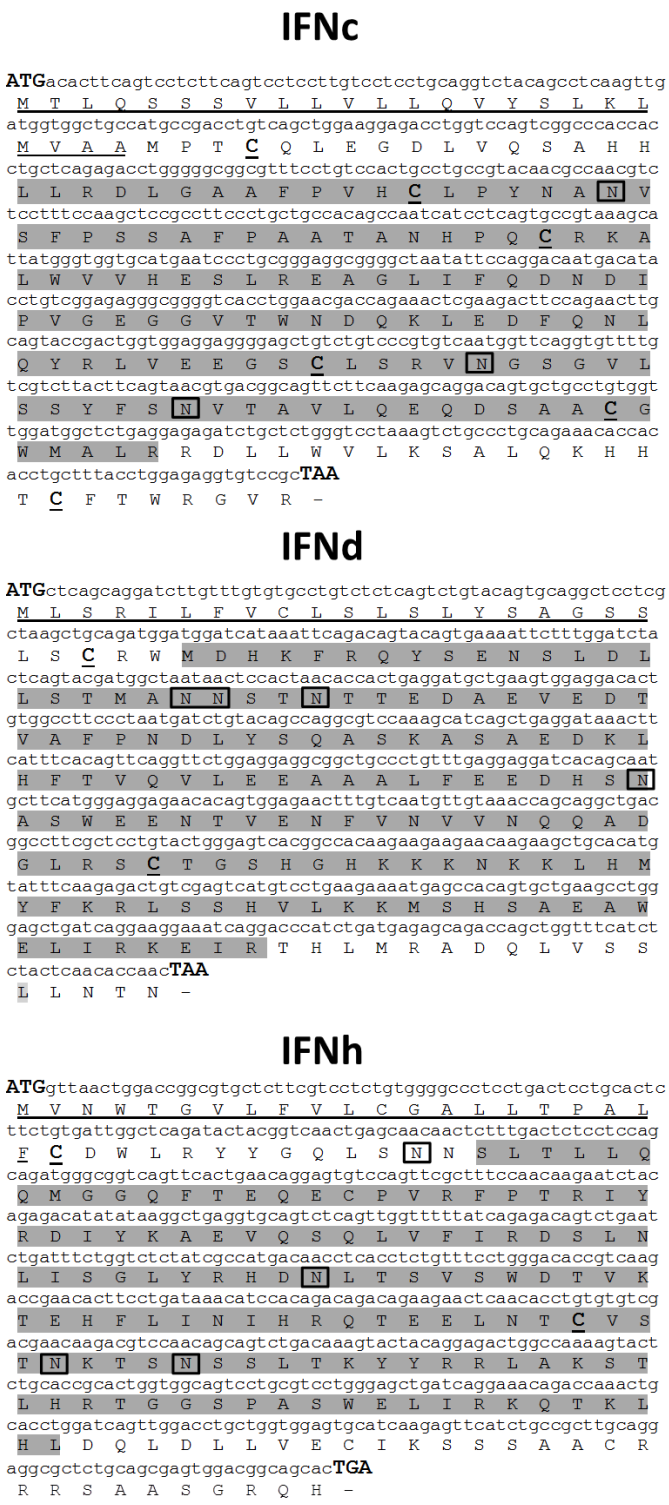

Figure 1. Nucleotide and deduced amino acid (aa) sequence of meagre IFNc (top), IFNd (middle) and IFNh (bottom). The putative aa sequence is shown under the triplet codon. Start and stop codons are in bold, the signal peptides are underlined and the interferon alpha/beta domains are highlighted in grey. N-glycosylation sites are boxed and conserved cysteines in bold and underlined.

3.2 Amino acid sequence and phylogenetic analysis of IFNc, IFNd and IFNh

The IFN putative proteins show high similarity and identity with Acanthopterygian IFNs and reasonable similarity to trout and human IFNs, as seen in **Table 2**. Meagre IFNc shows both high similarity (75.5 – 81.9%) and high identity (58.4 - 64.6%) with pleuronectiform IFNc and of the rainbow trout IFN subgroups shares the most similarity (59.6%) and identity (35.4%) with IFNc. Meagre IFNd shows both high similarity (81.6 - 98.9%) and identity (68.6 - 96.2%) with Acanthopterygian IFNd and of the rainbow trout subgroups has the most homology with IFNd, with a similarity of 64.9% and identity of 44.7%. Meagre IFNh also has high similarity with the recently identified large yellow croaker IFNh (93.2%), Japanese flounder IFNh (69.5%) and Nile tilapia IFNa3, a suspected IFNh (66.5%), as well as sharing a high identity of 89.5%, 56.3% and 54.1% respectively. IFNh has not been identified outside of the Acanthopterygii, but meagre IFNh shares the highest homology with rainbow trout IFNa, with a similarity of 56.3% and identity of 32.5%. Meagre IFNc and IFNd also have a slightly higher similarity and identity with human IFNa compared to human IFNb. Interestingly, IFNh is equally similar to both human IFNa and IFNb with a similarity of 43.7% but shows a higher identity to human IFNa.

Alignment of these IFN protein sequences **(Figure 2)** shows that all teleost IFNs retain at least 2 conserved cysteines, the first almost immediately after the signal peptide and the other approximately 80% through the IFN sequence. These cysteines align with those present in human IFNa which form a di-sulphide bond essential for the correct folding of the protein (Kontsek, 1994). Group 2 IFNs have 4 conserved cysteines and when looking at the sequence of trout IFNb, IFNc and IFNf it can be seen that the additional 2 cysteines in this group, which come 2nd and 4th in the sequence, are found 25-35 amino acids after the 1st cysteines and 30 – 35 amino acids from the end of the sequence. Interestingly, Acanthopterygian IFNc, including meagre IFNc, appear to have 6 conserved cysteines, with these additional cysteines being found 24 amino acids after what is considered the 2nd group 2 cysteine and 23 amino acids after the 4th group 2 cysteine. Interferon alpha/beta domains where identified in all meagre IFN sequences and **Figure 2** shows the domain covers a similar region in the IFN subgroups, within teleost and human IFNs. There are a number of conserved glycosylation sites, which differ between the IFN sub groups. Whilst two glycosylation sites are found in rainbow trout IFNc present towards the end of the sequence, in Acanthopterygian IFNc molecules three sites are present and are focused more in the middle of the sequence. Interestingly two of these putative glycosylation sites are conserved between species, and perhaps influence protein conformation (through the prevention of di-sulphide bond formation) (Meng et al., 2007). The Acanthopterygian IFNd sequences have conserved N-glycosylation sites near the beginning of the mature protein but no glycosylation sites are found in the corresponding rainbow trout sequence. Lastly, a conserved glycosylation site is present in IFNh molecules, between the 2nd and 3rd cysteines. Additionally, the meagre IFNc sequence contains a CxW motif that is conserved in group 2 type I IFNs in other teleosts and type I IFNs in other vertebrates, further solidifying its identity (Zou et al., 2007).

Phylogenetic analysis **(Figure 3)** shows that all three IFN subgroups have been identified in a number of Acanthopterygian fish previously, however due to differences in nomenclature the subgroup assignment was not immediately obvious. The meagre putative proteins for IFNc, IFNd and IFNh group strongly with their respective homologs in other teleost species and demonstrate clearly that both group 1 and group 2 IFNs are present in perciform fish, confirming data from pleuronectiformes.

Similarity/Identity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. Meagre IFNc		64.6	58.4	20.5	21	19.9	19.4	19.4	19.8	21.5	20.4	18.1	17.1	21.8	25.9	35.4	20.2	18	21.5	22.3	17.3
2. Turbot IFNc	81.9		72.8	21.5	21	18.4	19.4	21	20.4	18.7	17.2	21.6	18.5	19.3	24	34.4	20.6	19.1	20.6	21.7	21.0
3. Flounder IFN3	75.5	82.6		17.7	18.2	17.3	17.8	16.8	18.7	15.8	14.7	21.1	18.2	18.9	22.5	27.9	22.6	17.5	17.3	22	18.3
4. Meagre IFNd	43.1	44.3	40.5		96.2	86.6	83.9	73	68.6	28.2	27.7	29.8	26.3	31.7	28.8	24.7	44.7	24.2	17.6	25.5	23.7
5. Croaker IFNd	42	43.8	40	98.9		84.4	82.8	69.7	67.6	27.2	27.7	28.8	26.3	31.7	28.8	24	45.7	23.7	18.2	26.2	24.2
6. Seabass IFNd	44.1	47.3	40.3	91.9	91.4		89.8	72.6	67.2	31.4	29.9	29.7	24.8	34.2	28.9	24.6	42.3	26.8	21.6	25.8	25.5
7. Seabream IFNd	43.1	44.6	38.7	91.4	91.4	95.7		69.9	63.4	32.4	30.9	31.3	26.1	31.6	26.8	24	41.5	26.3	21.1	24.4	25.9
8. Flounder IFN1	46.8	47.8	42.4	84.3	83.8	83.3	81.7		69.2	28.4	27.9	30.4	27.9	33	27.6	26	39.9	27.8	23	23.1	22.7
9. Flounder IFN2	45.2	51.9	41.8	81.6	81.1	81.2	81.7	83.2		27.4	28.4	30.8	27.4	31.9	27.7	24.1	40.8	24.7	23.4	25.9	22.6
10. Meagre IFNh	41.1	42.6	35.8	48.9	48.4	51.1	52.1	50	50		89.5	56.3	54.1	32.5	23.6	25.4	28.9	26.1	21.8	20.9	18.8
11. Croaker IFNh	40	41.6	37.4	48.9	48.9	50	51.1	50.5	52.6	93.2		55.3	52.7	33	23.4	25.2	27.4	25.1	20.7	22.1	19.6
12. Flounder IFN4	35.1	44.2	40.8	50.3	49.7	47.8	49.5	52.2	53.1	69.5	66.3		45.1	36.4	22.8	27.2	31.2	27	21.6	21.9	24.0
13. Tilapia IFNa3	36.9	35.9	36.4	47.1	47.1	45.1	45.1	48.5	44.7	66.5	66	55.8		27.1	22.9	20.5	25.8	22.4	19.9	20.8	19.9
14. Trout IFNa1	43.6	43.1	39.7	54.1	54.6	53.8	53.8	56	55.9	56.3	55.8	57.7	49.5		30.3	26.4	36.5	27.6	23	28.2	18.9
15. Trout IFNb1	44.1	48.1	48.4	49.7	49.7	48.4	47.3	53.3	52.5	48.4	49.5	45.9	45.6	51.4		35.2	32.1	21.3	24.9	26.2	25.8
16. Trout IFNc1	59.6	57.2	52.9	45.5	44.9	43.9	43.9	48.1	49.7	44.2	46.3	46.5	41.3	46.5	56.7		27.6	21.9	22.8	29.4	28.4
17. Trout IFNd1	43.6	42.5	40.2	64.9	65.4	63.4	61.3	64.1	67.6	47.4	47.4	48.3	41.3	59.6	55.2	46		32.5	22.8	27.3	24.1
18. Trout IFNe1	42	39.8	37.1	50	48.4	51.1	51.6	52.2	50	47.9	47.4	48.9	41.3	51.6	43.5	41.7	51.1		19.1	26.6	22.3
19. Trout IFNf1	43.1	43.1	43.5	36.8	40.5	39.2	39.8	43.5	46.4	43.2	44.2	41.8	37.4	40.1	43.7	43.3	44.9	40.3		21.2	19.5
20. Human IFNa	44.1	41.5	39.9	48.9	48.4	47.9	46.3	47.3	48.9	43.7	45.8	40.4	38.3	44.1	52.1	49.5	47.3	51.6	41.5		33.7
21. Human IFNb	38.8	43.9	40.6	43.9	44.4	45.5	45.5	47.6	46	43.7	43.2	44.9	36.9	42.8	44.9	50.8	46	45.5	42.2	52.7	

Table 2. Amino acid (aa) similarity (bottom, left) and identity (top, right) of meagre IFNc, IFNd and IFNh with other known aa sequences of teleost fish IFN and both human IFNα1 (AAB59402.1) and IFNβ1 (NP_002167.1).

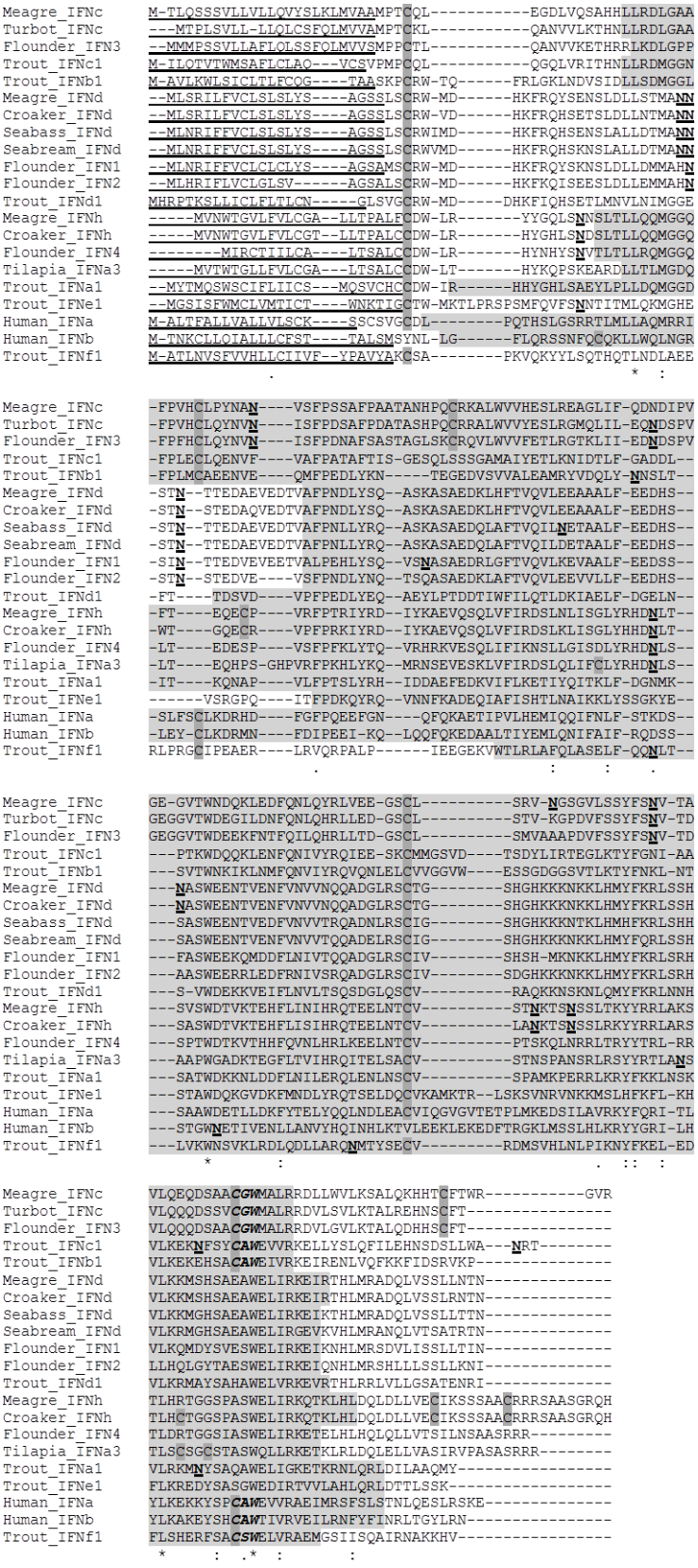

Figure 2. A multiple alignment of IFN subgroups in bony fish species, with human IFNα and IFNβ sequence as references. The multiple alignment was produced using MAFFT alignment software https://mafft.cbrc.jp/alignment/server/index.html. The signal peptide is underlined, conserved cysteines are highlighted in dark grey, N-glycosylation sites are in bold and underlined, the interferon alpha/beta domain is in light grey and the CxW motif is in bold and italics. * = conserved amino acids (aa), : = largely conserved aa and . = slightly conserved aa.

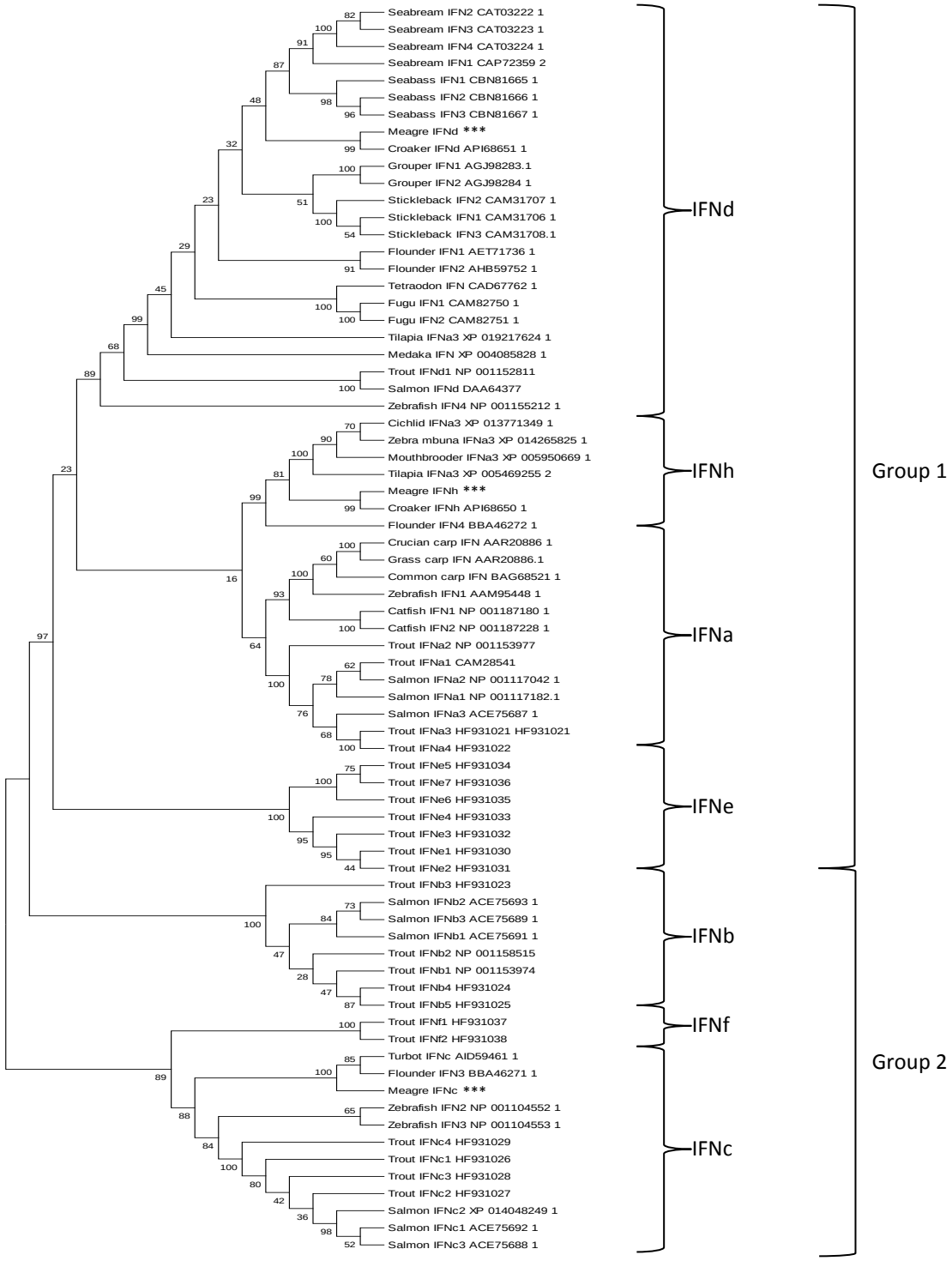

Figure 3. An unrooted phylogenetic tree of currently known teleost IFN sequences, constructed using amino acid multiple alignment software CLUSTALW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) and the neighbour-joining method in MEGA6. Node values represent the bootstrap percentage confidence following 10,000 runs. Groupings of families and IFN sub groups are indicated on the right. Meagre sequences are highlighted by a triple asterisk.

3.3 Expression of IFNc, IFNd and IFNh during early ontogeny

To determine the importance of the IFN subgroups in the larval defence against pathogens whole meagre larva were collected and IFN expression monitored. As seen in **Figure 4**, IFNc was the highest expressed IFN gene during the early development of meagre larvae, with expression reaching approximately 400 times that of IFNd and IFNh, which were both expressed at similar levels. IFNc, IFNd and IFNh generally followed the same expression profile, where expression levels were similar at 8 and 15 dph, increased at 29 dph and then returned to baseline levels by 60 dph. Interestingly, 29 dph was the first time point sampled following the switch from a live feed (Artemia) diet to an inert pelleted commercial feed.

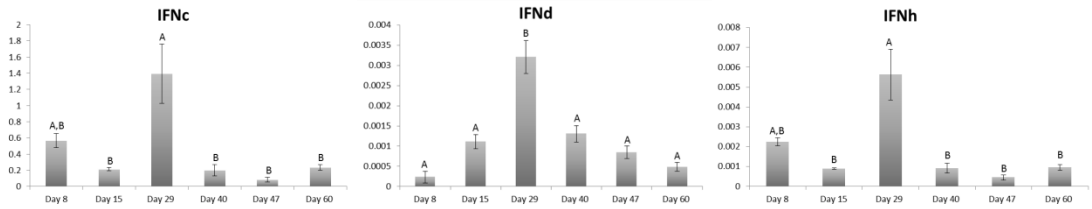

Figure 4. The expression of meagre IFN subgroups during larval development. Whole fish were homogenised and total RNA extracted at 8, 15, 29, 40, 47 and 60 days post hatch. Subsequently IFN transcripts were detected by qPCR and normalised to GAPDH. Bars are mean arbitrary units ± SEM, N = 10. Letters denote significant differences (P ≤0.05) between sampling times.

3.4 Constitutive expression of IFNc, IFNd and IFNh

To establish the baseline expression of IFN subgroups tissue samples were taken from the gills, gut head kidney and spleen of healthy meagre. The constitutive expression profile of IFNc differed from the profiles of IFNd and IFNh, as seen in **Figure 5A-C**. Constitutive expression of IFNc was highest in the spleen, followed by the mid-gut and head kidney and was lowest expressed in the gills. In contrast, constitutive expression of IFNd and IFNh was highest in gills and spleen and lowest in the head kidney and mid-gut. When IFNc, d and h expression is compared within the same tissue **(Figure 5D)**, it is clear that IFNh is significantly lower than the other isoforms in each of the tissues studied, while IFNc is the highest expressed in the mid-gut, head kidney and spleen, and IFNd is highest in the gills.

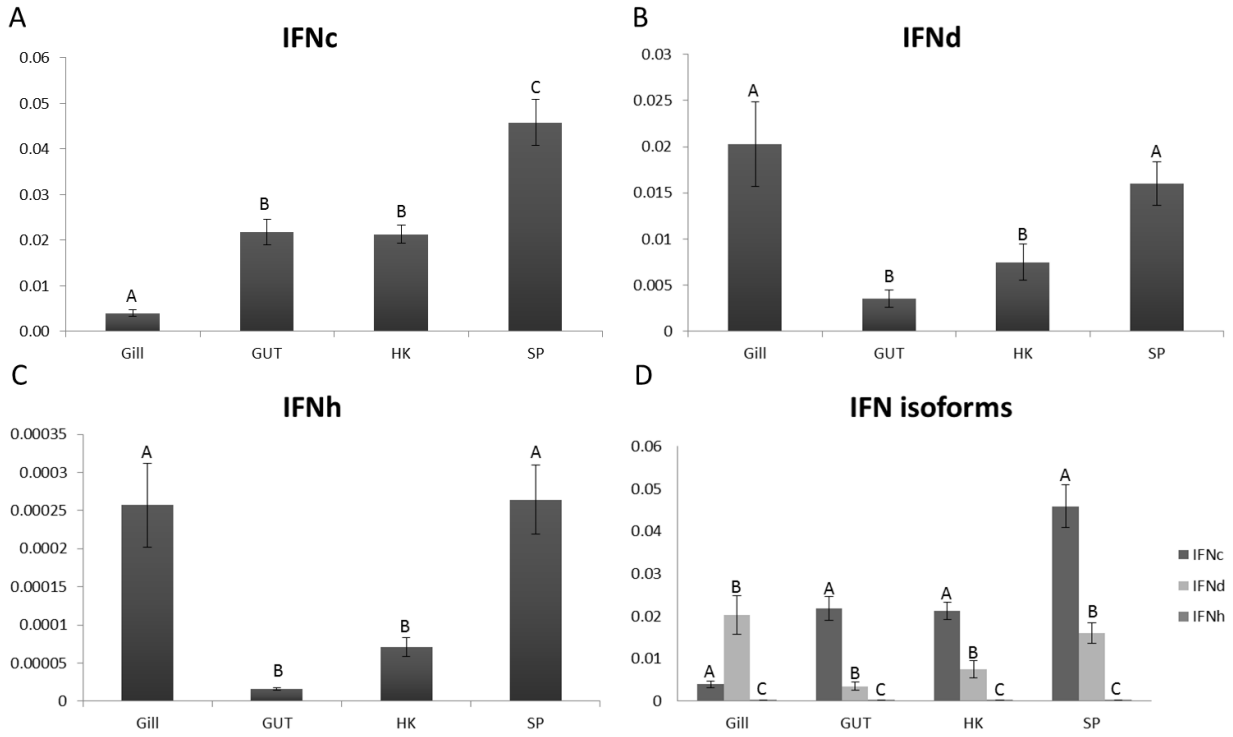

Figure 5. Constitutive expression of meagre IFN isoforms in four immune tissues. Total RNA was extracted from the tissues and IFN transcripts detected by qPCR and normalised to GAPDH. A, B and C show the constitutive expression of IFNc, IFNd and IFNh respectively, while D shows how each subgroup is expressed relative to each other within a tissue. HK = head kidney, SP = spleen. Bars are mean arbitrary units ± SEM, N = 10. Letters denote significant differences (P ≤0.05) between tissues in 5A-C and between subgroups within a tissue in 5D.

3.5 In vivo expression of IFNc, IFNd and IFNh in response to PAMPs

To understand how these IFN isoforms are regulated in response to PAMPs *in vivo*, fish were challenged by injection with poly I:C, LPS and β-glucan for 24 h **(Figure 6).** Stimulation with poly I:C had a profound influence on IFN isoform regulation and resulted in a significant increase in the expression of IFNc and IFNd in all four tissues. IFNh expression was also increased following poly I:C stimulation in the mid-gut. Whilst LPS had no effect on IFN expression *in vivo*, β-glucan stimulation was shown to significantly down regulate IFNc in all the tissues monitored but increased expression of IFNh in the head kidney.

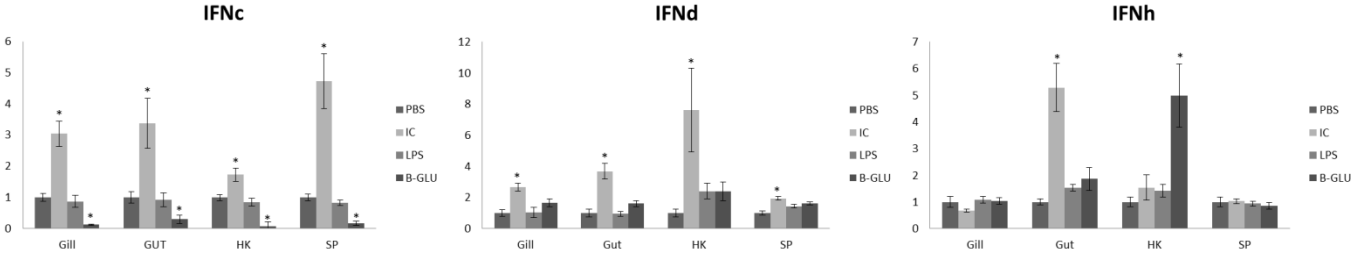

Figure 6. Effect of *in vivo* administration of immune stimulants on IFN subgroups. Meagre were injected with PBS, poly I:C, LPS or β-glucan and tissues collected 24 h later. Subsequently, total RNA was extracted from the tissues and IFN transcripts detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the same tissue in the control (PBS) fish. HK = head kidney, SP = spleen. Bars are mean fold change ± SEM, N = 10. Asterisks denote significant differences ($P \leq 0.05$) compared to the PBS control fish.

3.6 In vitro expression of IFNc, IFNd and IFNh in response to PAMPs

To further interrogate the differential modulation of the IFN subgroups in meagre, cells were isolated from the gills, gut, head kidney and spleen and stimulated *in vitro* with the various PAMPs for 4, 12 and 24 h. As shown in **Figure 7**, poly I:C stimulation increased the expression of IFNc and IFNh in gill cells after 4 h, although this expression had returned to baseline levels by 12 h. IFNd expression, however, remained at baseline levels until 24 h when a significant increase was observed. In mid-gut cells no increase in expression was observed until 24 h post-stimulation, when poly I:C stimulation resulted in an increased expression of all three subgroups, with IFNd showing the highest fold increase in expression at this time. Head kidney cells also had increased IFN expression in response to poly I:C, with all three isoforms upregulated at 4 and 12 h post stimulation but only IFNd remaining elevated at 24 h. Splenic cells also responded strongly to poly I:C stimulation, where IFNc was upregulated at 12 and 24 h post stimulation, whilst both IFNd and IFNh were upregulated at all time points. A stimulatory effect of β-glucan was also seen with splenocytes, with a small upregulation of IFNd at 24 h. As with the *in vivo* trial, LPS had no effects. Overall, IFNc was the least upregulated of all the isoforms following poly I:C stimulation *in vitro*, and IFNh was the most upregulated of the IFN isoforms in the spleen and head kidney. Whilst some kinetic differences were apparent between the different isoforms, it was curious that with gut cells they all had a delayed upregulation.

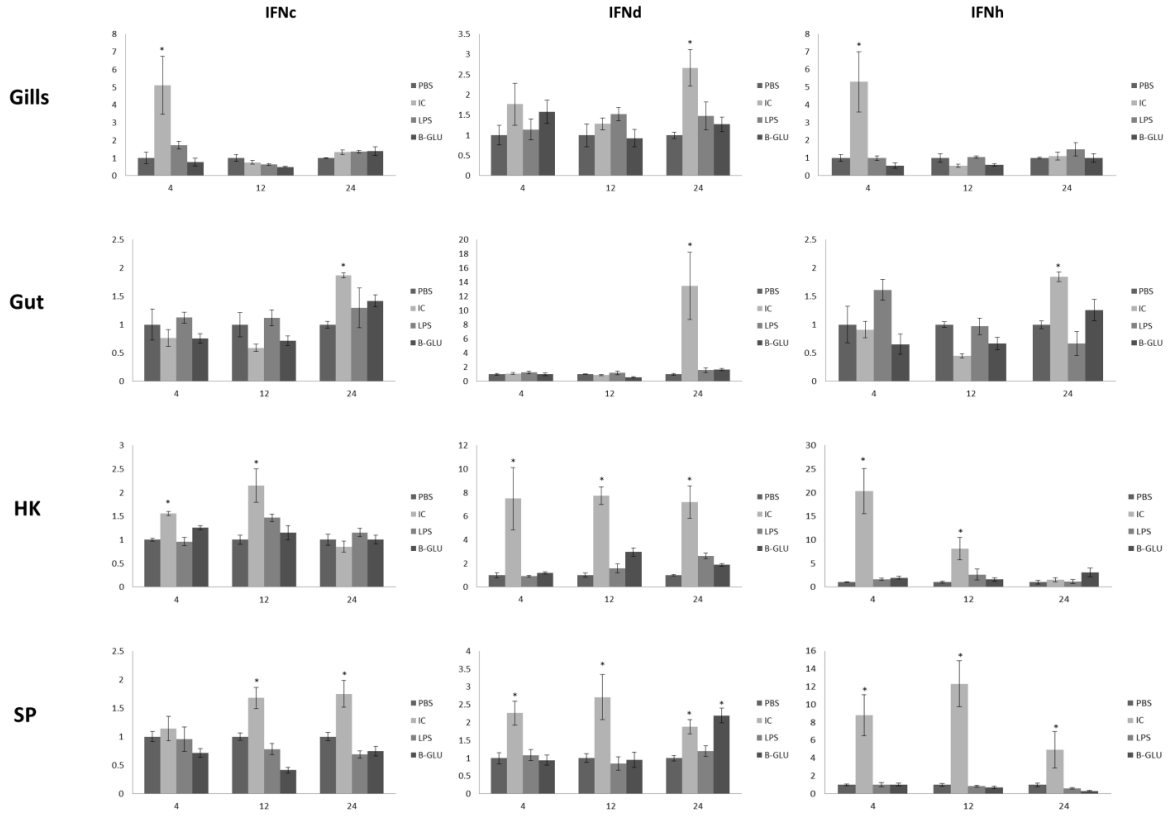

Figure 7. The effects of immune stimulants on IFN subgroup expression in primary cell cultures. Gill, mid-gut, head kidney (HK) and spleen (SP) cells were incubated with PBS, poly I:C, LPS or β-glucan for 4, 12 and 24 h. Subsequently total RNA was extracted from the cells and IFN transcripts detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the control (PBS) cells at the same time point. Bars are mean fold change ± SEM, N = 6. Asterisks denote significant differences (P ≤0.05) compared to the PBS control cells.

4. Discussion

The production of type I IFNs during viral infection is a crucial component of the antiviral response in jawed vertebrates. Multiple IFN genes are commonly present, usually as a result of tandem gene duplication within the IFN locus/loci. This gives the potential for sub-functionalisation and neo-functionalisation (He and Zhang, 2005), whereby each IFN subgroup may acquire a unique role in the antiviral response. Multiple subgroups are present in mammals (eg α, β, κ, ε, ω/τ, δ/ζ), and similarly in amphibians and teleost fish large numbers of IFN genes are present that fall into a number of subgroups (Zou et al., 2014; Gan et al., 2016; Sang et al., 2016). In teleost fish these are broadly classified into group 1 and group 2 molecules that are characterised by the possession of 2 or 4 conserved cysteines. These can be further divided into seven subgroups (a-f and h), although it is clear that they are not all present in every teleost lineage. Thus, to date salmonids have been shown to have 6 subgroups IFNa-f, cyprinids 3 subgroups IFNa, c and d, but until recently Acanthopterygians (perciformes, pleuronectiformes, etc) were thought to possess only 1 subgroup IFNd, although multiple copies could be present (Wan et al., 2012). However, it has been shown recently that Acanthopterygians can in fact possess 3 subgroups, IFNc, d and h, the latter likely

Acanthopterygian specific. Only one study has compared the expression profiles of all three subgroups, in Japanese flounder, but curiously could only detect induced expression of IFNh (Hu et al., 2017). Hence in this study we have attempted to help clarify the role of the three isoforms by analysis of their expression in the perciform fish, meagre, a newly emerging species for aquaculture.

Initially the three meagre genes were cloned and the sequence analysed to verify they were equivalent to IFNc, d and h. Meagre IFNc shows a high similarity/identity to IFNc in other perciform species, and had highest homology to rainbow trout IFNc of the 6 IFN subgroups analysed in this species. It clustered with IFNc's in phylogenetic tree analysis, and had the 4 conserved cysteines typical of group 2 IFNs, that form two disulphide bridges to ensure the correct folding of the protein (Chen et al., 2017; Robertson, 2006). However, it should be noted that the Acanthopterygian IFNc protein sequences, including meagre IFNc, actually have 6 cysteines present. Lastly meagre IFNc has a conserved CxW motif, as described by Zou et al. (2007) for trout IFNb/ c. Meagre IFNd showed very high similarity (98.9 – 91.4%) and identity (96.2 -83.9%) with IFNd in other Acanthopterygian species, the highest of all the meagre IFN subgroups. It also had the highest similarity/ identity with rainbow trout IFNd and grouped with IFNd from other species in phylogenetic tree analysis. As a group 1 IFN it has only 2 conserved cysteines present. Interestingly, there are several potential glycosylation sites in the Acanthopterygian IFNd sequences in contrast to salmonid IFNd, which could affect IFNd secondary structure and hence function of these molecules (Meng et al., 2007). Lastly, meagre IFNh has high similarity/ identity with large yellow croaker IFNh (93.2%/89.5), but when compared to Japanese flounder IFN4 and Nile tilapia IFNa3 there is a large drop in similarity/ identity indicating IFNh is highly variable within the Acanthopterygians. Meagre IFNh also has 2 conserved cysteines as with IFNd but possesses another 3, in common with large yellow croaker that in fact has an additional (6th) cysteine. Such features and analysis confirm that the genes cloned were indeed meagre IFNc, d and h.

During early ontogeny of meagre larvae, 8-60 dph, the expression profiles of the IFN subgroups were quite similar and constant with the exception of 29 dph where there was a transient yet dramatic increase in expression of all IFN subgroups. This time point is during the period when the larvae are being introduced to a standard inert pelleted feed, and perhaps is caused by changes in the intestine at this time, as diet can be linked to gastro intestinal irritation in young mammals (Pancaldi et al., 2008) and fish (Romarheim et al., 2006). The transient up-regulation of inflammatory cytokines (Milne et al., 2017) and antimicrobial peptides (Campoverde et al., 2017) has also been seen in meagre following introduction of a pelleted diet. Whilst IFNd and IFNh were expressed at similarly low levels during early ontogeny, IFNc was expressed at levels approximately 400 fold higher. This suggests IFNc plays an important role in larval anti-viral defence in meagre. IFNc is certainly a potent IFN in other species. For example, in juvenile Atlantic salmon (*Salmo salar*) injection with IFNc producing constructs dramatically upregulates key ISGs in the muscle, head kidney and spleen, and these fish have a significant increase in survival when exposed to infectious salmon anemia virus (ISAV) compared to control fish (Chang et al., 2014).

As the fish grew it was possible to look at the constitutive expression of the three isoforms in individual tissues. The expression profiles for IFNd and IFNh were identical, being highest in the gills and spleen and lowest in the mid-gut and head kidney. Whilst IFNc expression was also high in spleen, it differed in having lowest expression in the gills. The difference in constitutive expression profiles could relate to differences in expression regulation between the group 1 and group 2 IFNs,

and requires further investigation. The high constitutive expression in the spleen of all isoforms suggests cell types expressing IFN are prevalent at this site, likely due to the function of the spleen in filtering and destroying blood born antigens and protecting splenic cells from infection. When comparing constitutive expression levels between IFN subgroups it was clear that IFNh had the lowest expression level in the four tissues monitored in this study, and perhaps plays a minor role in homeostatic immunity and preventing initial infection. IFNc was again more highly expressed in three of the four tissues, with the exception of gills where IFNd was highest. Such data imply a degree of sub-functionalisation between the IFN subgroups in this species.

To study the potential differences in induction of IFN isoform expression, an *in vivo* stimulation experiment was performed using PAMPs. Injection of poly I:C resulted in up regulation of IFNc and IFNd in all tissues. However, IFNh expression only increased in response to poly I:C stimulation in the mid-gut, which is in contrast to the results with large yellow croaker where upregulation occurred in spleen and head kidney following poly I:C injection (Ding et al., 2016). Surprisingly, IFNh expression was also increased following β-glucan administration in the head kidney. IFNh was not the only IFN affected by β-glucan as IFNc expression was significantly downregulated in each tissue upon exposure to this PAMP, suggesting a negative regulation of this IFN subgroup by β-glucan. That IFNh was not induced in head kidney and spleen by poly I:C clearly shows there are some unique aspects of the regulation of this isoform. Indeed, in large yellow croaker, promoter analysis of IFNd and IFNh has shown that IRF7 is more important for IFNd expression (although it interacts with IRF3 to further enhance expression) whereas IRF3 is more important for IFNh expression (Ding et al., 2016). In addition, it was found that in cells stimulated with rIFNd both unphosphorylated and phosphorylated IRF3 and IRF7 are increased, but this is not seen when rIFNh is used. So in the latter case STAT1/ STAT2 complexes presumably activate downstream ISG without the involvement of IRF3/ 7.

To gain further insights into factors controlling IFNh regulation in meagre, *in vitro* stimulation of isolated cells was studied using the same PAMPs. Unlike the situation *in vivo*, IFNh was induced by poly I:C in all the cell suspensions, although the kinetics varied by cell source. An early induction was seen in gills/ head kidney/ spleen, which was sustained in head kidney and spleen. However, in gut cells the induction was only seen 24 h post-stimulation. Similarly in large yellow croaker IFNh expression was upregulated by poly I:C stimulation in spleen and head kidney and had returned to baseline expression in both tissues by 24 h (Ding et al., 2017). IFNd expression showed a similar profile to IFNh, in being upregulated early in head kidney and spleen, and was sustained in both cases. It also had a delayed upregulation in gut cells. However, it differed to IFNh in showing a late induction in gill cells, giving a clear mucosal-derived vs. systemic-derived difference in response kinetics of the cells. Lastly, IFNc was also upregulated in each cell suspension following poly I:C stimulation but the speed and duration again varied. Early upregulation was seen in gill and head kidney cells similar to IFNh, but not in splenocytes. This was sustained in head kidney cells. In spleen cells induction was later and remained elevated to 24 h, also seen with IFNh. Lastly, as with the other isoforms, delayed upregulation was seen in gut cells. The most noticeable difference to the *in vivo* results was the fast and sustained induction of IFNh. Some of the differences to the in vivo results may be explained by a more chronic exposure to poly I:C in the cell suspensions whereas *in vivo* it will likely be cleared (eg. by splenic macrophages) after a relatively short time and this may result in a faster return to base line expression *in vivo* than *in vitro*. Interestingly β-glucan again had an effect on expression of one IFN subgroup, in this case IFNd in splenocytes 24h post-stimulation.

That this was not observed *in vivo* is potentially due to the disruption of negative feedback loops or the chronic stimulation *in vitro*.

In summary, this study is the first in-depth expression analysis of the 3 IFN subgroups (c, d and h) in the perciform fish, meagre. It shows that IFNc is highly expressed in developing meagre, highlighting its potential importance in juvenile fish. Analysis of constitutive expression of meagre IFNs in individual tissues also found that IFNc was relatively highly expressed, with the exception of gills. Comparison between tissues for individual isoforms showed that spleen had high transcript levels of all three IFNs, and that IFNd/ IFNh were also high in gills. Poly I:C stimulation *in vivo* and *in vitro* resulted in the upregulation of the IFN subgroups, although differences in kinetics and the cell source used were apparent. These results demonstrate a complex pattern of regulation between the different IFN isoforms present in meagre and highlights potential sub-functionalisation of these IFN subgroups during perciform anti-viral responses.

Acknowledgments

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.

References

Campanella J., Bitinicks L. and Smalley.J. 2003. MATGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics. 4: 29.

Campoverde C., Milne D., Estevez A., Duncan N., Secombes C.J. and Andree K. 2017. Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*). Fish Shellfish Immunol. 69: 200-210.

Casani D., Randelli E., Costantini S., Facchiano A., Zou J., Martin S., Secombes C.J., Scapigliati G. and Buonocore F. 2009. Molecular characterisation and structural analysis of an interferon homologue in sea bass (*Dicentrarchus labrax L*.). Mol Immunol. 46(5): 943-952.

Chang C., Robertsen C., Sun B. and Robertsen B. 2014. Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. Vaccine. 32(36): 4695-4702.

Chang M., Nie P., Collet B., Secombes C.J. and Zou J. 2009. Identification of an additional two-cysteine containing type I interferon in rainbow trout *Oncorhynchus mykiss* provides evidence of a major gene duplication event within this gene family in teleosts. Immunogenetics. 61(4): 315-325

Chen K., Liu J. and Cao X. 2017. Regulation of type I interferon signaling in immunity and inflammation: A comprehensive review. J Autoimmun. 83: 1-11.

Ding Y., Ao J., Huang X. and Chen X. 2016. Identification of two subgroups of type I IFNs in Perciforme fish large yellow croaker *Larimichthys crocea* provides novel insights into function and regulation of fish type I IFNs. Front Immunol. 7: 343. doi: 10.3389/fimmu.2016.00343.

FAO, 2012. FAO Fisheries Department, Fishery Information, Data and Statistics Unit. FISHSTAT Plus: Universal software for fishery statistical time series. Version 2.3. 2000. Data sets: Aquaculture production: quantities and values 1950-2010.

Flajnik M. and Kasahara M. 2010. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet. 11: 47–59.

Gan Z, Chen S.N., Huang B., Hou J. and Nie P. 2016. Intronless and intron-containing type I IFN genes coexist in amphibian *Xenopus tropicalis*: Insights into the origin and evolution of type I IFNs in vertebrates. Dev Comp Immunol. 67: 166-176.

He X. and Zhang J. 2005. Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. Genetics. 169: 1157–1164.

Hong S., Li R., Xu Q., Secombes C. and Wang T. 2013. Two types of TNF-α exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF-α3 in rainbow trout *Oncorhynchus mykiss*. J Immunol. 191(12): 5959-5972.

Hu Y., Yoshikawa T., Chung S., Hirono, I. and Kondo H. 2017. Identification of 2 novel type I IFN genes in Japanese flounder *Paralichthys olivaceus*. Fish Shellfish Immunol. 67: 7-10.

Imperiali B. and O'Conner S. 1999. Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. Curr Opin Chem Biol. 3(6): 643-649.

Ivashkiv L. and Donlin L. 2014. Regulation of type I interferon responses. Nat Rev Immunol. 14: 36-49.

Kumar S., Tamura K. and Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5: 150-163.

Kontsek P. 1994. Human type I interferons: structure and function. Acta Virol. 38(6): 345-60.

Love A. 2007. Functional homology and homology of function: biological concepts and philosophical consequences. Biology & Philosophy. 22(5): 691-708.

Martin S.A.M., Taggart J.B., Seear P., Bron J.E., Talbot R., Teale A.J., Sweeny G.E., Hoyheim B., Houliham D.F., Tocher D.R., Zou J. and Secombes C.J. 2007. Interferon type I and type II responses in an Atlantic salmon (*Salmo salar*) SHK-1 cell line by the salmon TRAITS/SGP microarray. Physiol Genomics. 32(1): 33-44.

Meng J., Parroche P., Gloenbock D. and McKnight C. 2007. The differential impact of disulphide bonds and N-linked glycosylation on the stability and function of CD14. J Biol Chem. 283: 3379-3384.

Merella, P., Cherchi, S., Garippa, G., Fioravanti, M., Gustinelli, A. and Salati, F. 2009. Outbreak of *Sciaenacotyle panceri* (Monogenean) on cage-reared meagre *Argyrosomus regius* (Osteichthyes) from the western Mediterranean Sea. Dis Aquatic Organisms 86: 169-73.

McNab F., Mayer-Barber K., Sher A., Wack A. and O'Garra A. 2015. Type I interferons in infectious disease. Nat Rev Immunol. 15: 87-103

Milne D., Campoverde C., Andree K., Zou J. and Secombes C.J. 2017. Two types of TNFα in meagre (*Argyrosomus regius*): Discovery, distribution and expression modulation. Mol Immunol. 92: 136-145. doi: 10.1016/j.molimm.2017.10.007

Monfort, M. 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. 80: 28.

Pancaldi M., Mariotti I. and Balli F. 2008. Intestinal inflammation in nursing infants: different causes and a single treatment ... but of protected origin. Acta Biomed. 79(2) : 144-50.

Pereiro P., Costa M., Diaz-Rosales P., Dios S., Figueras A. and Novoa B. 2014. The first characterization of two type I interferons in turbot (*Scophthalmus maximus*) reveals their differential role, expression pattern and gene induction. Dev Comp Immunol. 45(2): 233-244.

Ravi V. and Venkatesh B. 2008. Rapidly evolving fish genomes and teleost diversity. Curr Opin Genet Dev. 18: 544–550.

Robertsen B. 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20(2): 172 – 91.

Rodriguez I., Chamorro R., Novoa B. and Figueras A. 2009. β-Glucan administration enhances disease resistance and some innate immune responses in zebrafish (*Danio rerio*). Fish Shellfish Immunol. 27(2): 369-373.

Romarheim O., Skrede A., Gao Y., Krogdahl A., Denstadli V., Lilleeng E. and Storebakken T. 2006. Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 256(1-4): 354 – 364.

Sakurai T., Ohno N. and Yadomae T. 1996. Effects of fungal beta-glucan and interferon-gamma on the secretory functions of murine alveolar macrophages. J Leukoc Biol. 60(1): 118-124.

Sang Y., Liu Q., Lee J., Ma W., McVey D.S. and Blecha F. 2016. Expansion of amphibian intronless interferons revises the paradigm for interferon evolution and functional diversity. Scientific Reports 6: 29072.

Secombes C.J. and Zou J. 2017. Evolution of interferons and interferon receptors. Front Immunol. 8: 209. doi: 10.3389/fimmu.2017.00209.

Sun F., Peatman E., Li C., Liu S., Jiang Y., Zhou Z. and Liu Z. 2012. Transcriptomic signatures of attachment, NF-kappaB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. Dev Comp Immunol. 38(1): 169-180.

Svingerud T., Solstad T., Sun B., Nyrud M., Kileng O., Greiner-Tollersrud L. and Robertsen B. 2012. Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: Evidence for high IFNb/IFNc–producing cells in fish lymphoid tissues. J Immunol. 189(12): 5912-5923.

Ternengo S., Agostini S., Quilichini Y., Euzet L. and Marchand B. 2010. Intensive infestations of *Sciaenocotyle pancerii* (Monogenea, Microcotylidae) on *Argyrosomus regius* (Asso) under fish-farming conditions. J Fish Diseases. 33: 89–92.

Toksen E., Buchmann K. and Bresciani J. 2007. Occurrence of *Benedenia sciaenae* van Beneden, 1856 (Monogenea: Capsalidae) in cultured meagre (*Argyrosomus regius* Asso, 1801) (Teleost: Sciaenidae) from western Turkey. Bull Eur Ass Fish Pathol. 27(6): 250.

Wan Q., Wicramaarachchi W., Whang I., Lim B., Oh M., Jung S., Kim H., Yeo S. and Lee J. 2012. Molecular cloning and functional characterization of two duplicated two-cysteine containing type I interferon genes in rock bream *Oplegnathus fasciatus*. Fish Shellfish Immunol. 33(4): 886-898.

Wang T., Diaz-Rosales P., Costa M.M., Campbell S., Snow M., Collet B., Martin S., Secombes C.J. 2011. Functional characterization of a nonmammalian IL-21: rainbow trout *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22. J Immunol. 186: 708–721.

Xiao Z., Trincado C. and Murtaugh M. 2004. β-Glucan enhancement of T cell IFNγ response in swine. Vet Immunol Immunopathol. 102(3): 315-320.

Zou J., Tafalla C., Truckle J. and Secombes C.J. 2007. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. J Immunol. 179(6): 3859-3871.

Zou J. and Secombes C. 2011. Teleost fish interferons and their role in immunity. Dev Comp Immunol. 35(12): 1376-1387.

Zou J., Gorgoglione B., Taylor N., Summathed T., Lee P., Panigrahi A., Genet C., Chen Y., Chen T., Hassan M., Mughal S., Boudinot P. and Secombes C.J. 2014. Salmonids have an extraordinary complex type I IFN system: Characterization of the IFN locus in rainbow trout *Oncorhynchus mykiss* reveals two novel IFN subgroups. J Immunol. 193(5): 2273-2286.