Abstract

TNFα is a key cytokine involved in systemic inflammation and regulation of immune cells and is important during development. In the present study, 2 isoforms of TNFα were discovered in meagre, an emerging species in aquaculture. Phylogenetic analysis suggests these isoforms represent the type I and type II TNFα classes previously described in other teleost species. This study is the first to compare how these 2 types of TNFα behave in meagre and aims to provide insights into their expression in teleost fish by interrogating expression in whole tissues and isolated cell populations in four immunologically important sites (gills, intestine, head kidney and spleen) following PAMP stimulation, as well as monitoring gene expression during meagre development. Differential expression was seen in head kidney and gills, where TNFα1 was more highly expressed. Both isoforms increased in head kidney of meagre following injection with LPS, but this was not seen in other tissues or after injection with other PAMPs. However, in vitro studies hinted at a possible mucosal bias for TNFα1, which was more highly induced in gill and intestinal cell suspensions by PAMPs. In contrast TNFα2 was more highly induced in cells from systemic tissues. Through early development expression of both types of TNFα decreased as the meagre matured, with the exception of a transient increase shortly after the move to a dry feed diet. However, during the later stages of development expression of both isoforms increased in the gills. This data demonstrates a degree of differential expression of TNFα1 and TNFα2 in meagre with regard to expression regulation, and highlights the importance of TNFα during early development of teleost fish.

1. Introduction

Meagre (*Argyrosomus regius* - perciforms) is an emerging aquaculture species found in the Mediterranean sea, Black sea and eastern Atlantic coast, and is considered a prime candidate for aquaculture due to its large size, fast growth, low feed conversion ratio and high processing yield (Monfort, 2010). In recent years there have been major breakthroughs in production, due to extensive research, and meagre can now be produced in captivity, allowing for commercial production in Spain, France, Portugal, Italy, Greece and Croatia (FAO, 2012). However, several bottle necks in current and future production have been identified, of which disease control is considered critical, due to a number of emerging diseases and parasites in meagre populations (Toksen et al., 2007; Merella et al., 2009; Ternengo et al., 2010). As such, it is vital that a better understanding of the meagre immune response is obtained in order to effectively combat current and emerging pathogenic threats.

Tumor necrosis factor – α (TNFα) is a crucial cytokine, considered to be a central player in both anti and pro inflammatory responses, with roles in numerous cellular processes including cell survival, developmental regulation and pathogenic resistance (Chu, 2013). The human TNFα pro-protein consists of 233 amino acid residues, has a molecular mass of 26kDa, and contains a transmembrane domain with a downstream TNFα-converting enzyme (TACE) cleavage site, between residues Ala^{76} and Val^{77}, which facilitates the release of the 17kDa monomer (Sherry and Cerami, 1988; Horiuchi, 2010). However, both the soluble TNFα molecule and the membrane bound molecule must form a homotrimer to be biologically active (Horiuchi, 2010). TNFα is produced by macrophages, neutrophils, lymphocytes and other immune cells as part of a cytokine communication network during infection (Grivennikov, 2005), and plays an important role in both systemic and mucosal immunity (Munang'andu et al., 2015). Indeed, inhibition of TNFα results in increased susceptibility to disease and a reduced capability to resolve infection (Johnston and Conly,

2006). Furthermore, TNFα is a critical component for initiating the acute phase response, were it induces downstream effectors in a number of tissues, such as the production of transferrins and serum amyloids in the liver (Baumann and Gauldie, 1994). Despite TNFα being regarded as a component of the immune system it is also important for development. For example TNFα can affect osteoclast development and resorption rates (Van der Pluijm et al., 1991), is vital for selective apoptosis during embryogenesis, and plays a critical role in the development of the lung as well as lymphoid organs (Mitchell, 1998; Drayton, 2006).

TNFα has been identified in a number of teleost species, as in the cyprinids common carp (Saeij et al., 2003) and zebrafish (Savan et al., 2005), the salmonids rainbow trout and Atlantic salmon (Hong et al., 2013) and the perciforms seabream (Roca et al., 2008), grouper (Lam et al., 2011) and Japanese flounder (Hirono et al., 2000). There are multiple accounts of the biological function of the recombinant protein in various teleost species. These range from enhancing chemotactic responses, phagocytosis and nitric oxide production in gold-fish macrophages (Grayfer et al., 2008) to inducing expression of IL-1β, IL-8, TNFα and COX-2 in rainbow trout head kidney cells (Zou et al., 2003), and suggest teleost TNFα has similar functions to the mammalian counterparts. Interestingly, a number of teleost species appear to have multiple isoforms of TNFα (Falcoa et al., 2012; Kajungrio et al., 2015), mainly due to the whole genome duplications that have occurred in this lineage. Differential isoform expression is often apparent, as seen in rainbow trout head kidney cells when using immune stimulants or during infection with *Yersinia ruckeri* (Hong et al., 2013). Studies in perciforms have also shown differential expression of TNFα isoforms, as occurs in peripheral blood leukocytes after stimulation with Pathogen Associated Molecular Patterns (PAMPs)/ mitogens in tuna or in head kidney after injection with a recombinant chemokine (CCL4) in orange spotted grouper (Kadowakia et al., 2009; Hsua et al., 2013).Studies in cyprinids have

also demonstrated the receptor-independent activity of TNFα in common carp (*Cyprinus carpio*) by subjecting the bloodstream form of *Trypanoplama borreli* to recombinant common carp TNFα, resulting in the lysis of the parasites (Forlenza et al., 2009) and other studies in common carp have also demonstrated 'behavioural fever' linked to the regulation of TNFα (Rakus et al., 2017). Nevertheless, relatively few studies have directly compared the expression profiles of the different isoforms of TNFα, especially with respect to mucosal vs systemic expression and their appearance during development. As part of our studies into the immune system of meagre we have discovered two TNFα isoforms and in this paper we begin to characterise their expression patterns in head kidney, spleen, gut and gill cells, and during development.

2. Materials and Methods

2.1. Fish

Healthy meagre were provided by the Institute for Agri-Food Research and Technology (IRTA), San Carlos, Spain. Larvae were reared from fertilised eggs, produced from IRTA broodstock, in 1.5 m^3 tanks using a mesocosm system, with a water temperature of 20^{o}C. At two days post hatch (dph) enriched rotifers were introduced to the tanks, until 8 dph. From 9 dph to 31 dph enriched *Artemia metanauplii* were provided as a food source, and from 21 dph until the end of the trial a formulated artificial diet was provided as feed. All fish were overdosed with tricaine methanesulfonate (MS-222) anaesthetic (Sigma-Aldrich) and killed prior to sample collection.

2.2. Molecular cloning of meagre TNFα isoforms

Total RNA was extracted from meagre head kidney, gills (mixture of all 4 gill arches), gut and spleen with TRI reagent (Sigma-Aldrich) and pooled. RNA subsequently underwent reverse transcription using the SuperScript III (ThermoFisher) protocol with Oligo $(dt)_{26}$ as a primer. Partial sequences for each isoform were obtained using consensus primers (Supplementary Table 1), designed to conserved regions in closely related species. These primers were then used in a standard PCR assay, using MyTaq DNA polymerase (Bioline) to obtain a partial sequence, which was then ligated into pGEM-T easy vector (Promega). RapidTrans TAM1 competent cells (Active motif, 11096) were next transformed with the pGEM-T easy vector and plated onto MacConkey agar plates (Sigma-Aldrich) and incubated at 37^{o}C overnight. Positive colonies were selected and plasmid DNA extracted using a QIAprep Spin Miniprep Kit (Qiagen). Extracted DNA was then sequenced by Eurofins Genomics. 5' and 3' RACE PCR was then performed, using primers in Supplementary Table 1, as described by Hong et al. (2013), to obtain full length cDNA for both isoforms. Finally full length sequence assembly was confirmed using PFU DNA polymerase (Promega) and primers designed to the 5' and 3' ends (Supplementary Table 1), allowing for full length coding region synthesis.

2.3. Sequence analysis of meagre TNFα isoforms

Amino acid sequence of each isoform was predicted from cDNA sequence using the ExPASy translate tool (http://web.expasy.org/translate) and then analysed using NCBI BLAST software (http://www.ncbi.nlm.nih.gov). Similarity and identity of the protein sequences were determined by MatGAT 2.0 software (Campanella et al., 2003). Phylogenetic analysis was conducted with MEGA 6 software using the neighbour-Joining test and Jones-Taylor-Thornton (JTT) model, and 10,000 bootstrap repetitions (Kumar et al., 2004). Isoelectric point and molecular weight were determined by Compute pI/Mw tool (http://www.expasy.ch/). The transmembrane domain was predicted using TMHMM tool

(http://www.cbs.dtu.dk/services/TMHMM/) and TNF signatures predicted by ExPASy Prosite (http://prosite.expasy.org).

2.4. Tissue distribution of meagre TNFα isoforms

Total RNA was extracted from the head kidney, gills, gut and spleen of healthy meagre of 30g, for TNFα isoform transcript analysis, quantified by qPCR as described in 2.8. The relative expression of each isoform was normalised to GAPDH expression and calculated as arbitrary units.

2.5. TNFα isoform expression ~~after in vivo~~ following intraperitoneal injection of PAMPs ~~stimulation~~

Fish were injected intraperitoneally with 100 µl PBS (Sigma-Aldrich), or PBS containing one of the following PAMPs: poly I:C (100 µg, Sigma-Aldrich), LPS (400 µg, Sigma-Aldrich) or β-glucan (100 µg, Sigma Aldrich). After 24 h the head kidney, gills, gut and spleen were collected and stored in RNA later (Sigma-Aldrich). Total RNA was subsequently extracted and qPCR performed as described in sections 2.2 and 2.8. Relative expression of each isoform was normalised to GAPDH expression and calculated as arbitrary units, then converted to a fold change relative to the PBS control.

2.6. TNFα isoform expression after in vitro stimulation with PAMPs

Tissues (head kidney, gills, gut and spleen) were collected from recently killed meagre, and passed through a 70 µm nylon mesh (Greiner) with 10 ml of L15 media (ThermoFisher)

containing penicillin/streptomycin (ThermoFisher) diluted 1:1,000 and 2% foetal calf serum (Sigma-Aldrich). The resulting cell suspension was collected and centrifuged at 400g for 10 min. The supernatant was removed and replaced with 10ml of the above media. Suspensions were again centrifuged and supernatants removed and replaced with 30ml of media. Cells were then transferred to 12 well plates (Greiner) in 5 ml aliquots and stimulated by addition of 250 µl of PBS containing LPS (50 µg/ml final concentration), poly I:C (100 µg/ml final concentration), or β-glucan (50 µg/ml final concentration). 250 µl of PBS was added to wells of control cultures. After 4, 12 and 24 h the cells were collected and centrifuged at 400 g for 10 min, the supernatant discarded, and the pellet suspended in RNA later. Total RNA was extracted and qPCR performed as described in sections 2.2 and 2.8. Relative expression of each isoform was normalised to GAPDH expression and calculated as arbitrary units, then converted to a fold change relative to the PBS control, for each time point.

2.7. TNFα isoform expression during ontogeny

Random samples (n=10) of whole larvae were collected 8, 15, 29, 40, 47 and 60 dph and stored in RNA later (Sigma-Aldrich) in order to allow the comparison of TNFα expression during key stages of meagre juvenile development. The fish ranged in size from of 0.1g to 40g during this period. The sampled fish were then individually homogenised in Tri Reagent (Sigma-Aldrich) and total RNA extracted and qPCR performed as described in sections 2.2 and 2.8. Relative expression of each isoform was normalised to GAPDH expression and calculated as arbitrary units. Once fish were larger, individual tissues (head kidney, gills, gut, spleen) were collected, at 85, 96 and 128 dph, and stored in RNA later. Total RNA was extracted and qPCR performed as described in sections 2.2 and 2.8. Relative expression of each isoform was normalised to GAPDH expression and calculated as arbitrary units.

144

145 2.8. Real time quantitative PCR

146 Total RNA from each tissue was extracted as above (section 2.2), then digested with TURBO
147 DNase (ThermoFisher) and subsequently underwent reverse transcription (section 2.2). The
148 transcripts for each gene of interest were quantified by real time quantitative PCR (qPCR) as
149 described by Wang et al., (2011). A reference containing a serial dilution of equal molar
150 quantities of each gene of interest (PCR products) was used to allow quantification.

151

152 2.9. Data transformation and statistics

153 Data from the baseline expression and PAMP stimulation (~~*in vivo*~~ intraperitoneal injection
154 stimulated and *in vitro* stimulated) was analysed by one way ANOVA and Tukey post hoc
155 test, with a sample size of n=10, n=10 and n=6 respectively ($p \leq 0.05$ was considered
156 significant). For baseline expression the two isoforms were compared to each other in each
157 tissue separately. For the PAMP stimulation data (both ~~*in vivo*~~ injection and *in vitro*
158 exposure) the different stimulants (poly I:C, LPS, β-glucan) were compared to the PBS
159 treated fish within the same tissue. A general liner model was used to analyse the ontogeny
160 data set, with each time point having a sample size of n=10. The analysis was conducted
161 using Statistical Product and Service Solutions (SPSS) software.

162

163 **3. Results**

164 3.1. Molecular cloning of Meagre TNFα1 and TNFα2

The meagre TNFα1 transcript (GenBank: MF186589) contains a 759bp open reading frame (ORF) and the transcript of TNFα2 (GenBank: MF186590) contains a 757bp ORF (Figure 1). The putative proteins for TNFα1 and TNFα2 consist of 251 amino acids (aa) and 237 aa, with an isoelectric point of 5.22 and 5.25 and molecular mass of 27.87 kDa and 26.09 kDa respectively. No signal peptide was found for either protein; however both encode a transmembrane region. Using the TMHMM transmembrane prediction software, TNFα1 aa 1-34 were predicted to be intracellular, aa 35-57 transmembrane and aa 58 – 251 extracellular. For TNFα2 aa 1-28 were predicted to be intracellular, 29-51 to be transmembrane and 52-237 extracellular. Both proteins contain TNF signatures, predicted by ExPASy prosite, which are essential for translocation across the endoplasmic reticulum membrane (Ishisaka et al., 1999; Horiuchi et al., 2010). The TNF signature for TNFα1 begins at the Ala^{97} and terminates at Leu^{251}, whilst for TNFα2 it begins at Ala^{81} and ends at Leu^{237}.

3.2. Amino acid sequence and phylogenetic analysis of meagre TNFα1 and TNFα2

TNFα putative proteins showed high homology with a number of perciform TNFα proteins (Figure 2). TNFα1 showed both high similarity (90.5 - 60.2%) and high Identity (81.4 - 38.8%) with other teleost TNFα1 molecules and reasonable similarity (45.8%) and identity (29%) to human TNFα. Similarly TNFα2 had high similarity (93.7 - 61.2%) and identity (91.1 - 36.5%) to other teleost TNFα2 proteins and reasonable similarity (48.5%) and identity (26.2%) to human TNFα. The multiple alignment (Figure 3) shows the sequence for each isoform is generally conserved and TNFα specific domains can be seen in both molecules, such as a the transmembrane domain, 10 beta strands which form the ‘jelly roll’ structure associated with TNFα molecules (Jones et al., 1989), the TNF family signature and 2 conserved cysteine residues which form a disulphide bond (Narachi et al., 1987). The conserved TACE cut site comprising of TL residues can also be seen in both meagre TNFα1 and TNFα2. To determine the relationship of the meagre TNFα isoforms and their homologs

a phylogenetic tree was constructed based on the aa sequences. As shown in Figure 4, both putative meagre proteins group within the TNFα clade, and cluster with the respective perciform type I and type II TNF subgroups, being highly associated to the large yellow croaker molecules in both cases.

3.3. Tissue distribution patterns of Meagre TNFα1 and TNFα2

Baseline expression of the two TNFα isoforms was established for head kidney, spleen, gut and gills in healthy, untreated fish by qPCR. As shown in Figure 5, both TNFα isoforms are lowly expressed in the gut and show highest expression in the spleen. However, a differential expression between the isoforms was apparent in the head kidney and the gills, where in both cases TNFα1 is expressed at higher levels than TNFα2 (eg. in head kidney TNFα1 is ~x3 that of TNFα2).

3.4. Meagre TNFα1 and TNFα2 expression in response to intraperitoneal injection of PAMPs~~immune stimulants in vivo~~

To investigate whether the expression of the TNFα isoforms could be modulated *in vivo*, fish were injection challenged with various PAMPs for 24 h. As shown in Figure 6, when compared to the control (PBS injected) fish, a significant increase in expression was seen with both isoforms after challenge with LPS in head kidney, but not in other tissues. Curiously a significant decrease in expression in the spleen and the gut was seen after challenge with poly I:C also for both isoforms. A differential effect was only seen in the case of TNFα1, which was significantly downregulated in the gills after challenge with LPS and β-glucan while TNFα2 expression was unchanged.

3.5. Meagre TNFα1 and TNFα2 expression in response to PAMP~~immune~~ stimula~~n~~tions~~s~~ in vitro

To further investigate the potential differential modulation of each TNFα isoform in meagre, cells were isolated from the above four tissues and challenged *in vitro* with the various PAMPs for 4, 12 and 24 h. As shown in Figure 7a, a differential expression profile of the two isoforms was apparent in the head kidney cells. TNFα1 showed almost no change in expression compared to the baseline, with only a small increase seen when stimulated with LPS after 4 h and a small decrease after 24 h. By contrast, there was a relatively large increase in expression of TNFα2 for all stimulants after 4 h, which remained at 8 h and had returned to control levels (or lower – LPS) by 24 h. Similarly with splenocytes (Figure 7b) a differential expression profile was seen, with both isoforms being down regulated after 12 and 24 h stimulation with the PAMPs, but with TNFα2 only being significantly increased by all of the stimulants at 4 h. Gut cells (Figure 7c) also showed down-regulation of both isoforms after stimulation with poly I:C for 4 h, and poly I:C or β-glucan for 12 h. However, at 24 h upregulation occurred in cells stimulated with LPS and β-glucan. Despite the profiles for each isoform being similar the level of expression differed greatly, with TNFα1 being upregulated more highly than TNFα2. Lastly, with gill cells (Figure 7d) there was also a clear difference in expression profile between the two isoforms, with increased expression of TNFα1 seen in response to all three PAMPs at 4h, and remaining elevated at 12 h and 24 h with LPS stimulation. This contrasted with TNFα2 expression, which was downregulated after 4 h stimulation with LPS, and with all stimulants after 12 h.

3.6. Meagre TNFα1 and TNFα2 expression during early ontogeny

Immune genes may have a role in development in addition to their immune function. For this reason TNFα expression was monitored in whole fish for the first 60 days post hatch (Figure 8). Both isoforms followed the same trend in expression, namely a decrease from day 8 to day 60, where expression was 8 times higher at day 8 compared with day 60 for TNFα1 and 3

237 times higher for TNFα2. Both isoforms deviated from this trend at day 29, when an increase
238 in gene expression was seen compared to the previous time point (day 15).

239 3.7. Meagre TNFα1 and TNFα2 expression during late ontogeny

240 TNFα gene expression appeared quite low at day 60, but as whole fish had been used this
241 might mask more intricate expression profiles in individual tissues. Therefore, later time
242 points (days 85, 96 and 128) were sampled where individual tissues could be collected (head
243 kidney, spleen, gut, gills). As seen in Figure 9, the head kidney and gut expression level of
244 both isoforms seemed to have plateaued, with no changes apparent over time. In the spleen
245 isoform expression level was seen to continue to decrease over these later ontogeny time
246 points. However, in gills an increase in expression of both TNFα isoforms occurred as time
247 progressed, such that by day 128 spleen and gill levels were relatively comparable, as seen in
248 Figure 5.

249 **4. Discussion**

250 In mammals TNFα acts a as a critical link for many inflammatory and developmental
251 processes. It appears to have evolved early in jawed vertebrates, and is present in
252 cartilaginous as well as bony fish (Secombes et al., 2015). It was duplicated at some point
253 during vertebrate evolution to generate TNFβ (LTα) and LTβ that have acquired unique
254 functions and cell specific expression patterns (Secombes et al., 2016). The exact timing of
255 these duplications is not certain, but clear homologues are present in all tetrapods, although
256 these genes appear to have been lost in some lineages (eg. in birds), with some novel
257 sequences in fish still to be verified as to whether they are due to lineage specific duplications
258 of TNFα (eg. TNF-N in teleosts, Savan et al., 2005), or are more ancient duplications related
259 to the above. However, it is clear that in teleosts there has been duplication of the TNFα locus
260 as a consequence of whole genome duplication (WGD) events (Ravi et al., 2008, Flajnik et

al., 2010), generating further TNFα genes in this group of bony fish. One WGD happened at the base of the teleost fish, when the locus was duplicated and 2 types of TNFα were generated, termed TNFα1 and TNFα2 (Lepen et al., 2015; Falco et al., 2012). These molecules have subsequently undergone a degree of sub-functionalisation and neo-functionalisation (He and Zhang, 2005). Further duplication of TNFα genes has occurred in more recent times mainly through additional WGD events in particular lineages, as seen in salmonids and cyprinids (Savan and Sakai, 2004; Hong et al., 2013). For example, in rainbow trout two type I and two type II genes exist. In perciforms, though, only a single type I and type II TNFα have been described to date.

The molecules identified in this study in meagre contain all the key features of TNFα molecules; the TNF signature associated with TNF family molecules (Savan et al., 2005), the TL duplet that acts as a TACE cleavage site (Garcia-Castill et al., 2002), a transmembrane region and conserved cysteines (Figures 1 and 2). This coupled with the high similarity/identity and phylogenetic analysis with other teleost and tetrapod TNFα molecules confirms that the molecules reported in this paper are both TNFα homologues. However, differences in sequence and position in the phylogenetic analysis indicate these two meagre TNFα molecules do differ significantly, being equivalent to the TNFα1 and TNFα2 genes. That they may have undergone sub-functionalisation is immediately apparent from the baseline expression analysis, where they show different transcript level in fish specific tissues, namely the head kidney and the gills (Figure 5), which may hint at different roles within the meagre immune system.

We next examined whether the expression of these genes could be modulated by PAMPs, and if this revealed further evidence of sub-functionalisation. An initial ~~*in vivo*~~ *intraperitoneal injection* study was performed (Figure 6), with three PAMPs injected individually and responses studied 24 h later. This showed that both types of TNFα followed a very similar

profile, where in response to LPS stimulation a doubling of the TNFα transcript levels occurred in head kidney cells, likely due to the presence of macrophages (Norte dos Santos et al., 2014), T-cells (Roggia et al., 2001) or TNFα producing dendritic cells (Serbina et al., 2003). However, despite the similar fold change seen, it is important to remember that basal expression of the 2 types of TNFα differ, and hence the level of TNFα1 remained higher relative to TNFα2. The shared gene expression profile of the two types of TNFα has also been observed in head kidney and intestine of common carp when injected with *Aeromonas salmonicida* (Falco et al., 2012) and in head kidney of orange-spotted grouper injected with CCL4 (Hsu et al., 2013). Curiously injection of meagre with poly I:C resulted in significant down-regulation of TNFα1/2 in spleen and gut, perhaps related to the induction of anti-viral immune pathways. However, a difference between these two TNF isoforms was apparent in gills, where LPS and β-glucan induced a significant down-regulation of TNFα1 but not TNFα2.

In view of the above results we undertook a time course study of the effect of PAMP stimulation on *in vitro* cultured cells from head kidney, spleen, gut and gills. This revealed some further evidence of sub-functionalisation between the two genes, where in general larger increases in TNFα1 expression were induced in the gut and gill cells by the PAMPS whereas larger increases in TNFα2 were seen in head kidney cells and splenocytes. This difference may reflect differences in the cell composition present in the cultures, which are derived from systemic vs. mucosal tissues. For example, it has been shown in trout that some cell lines preferentially express type I TNFα (eg. RTGill, RTL), whilst macrophages (RTS-11 cells and primary cultures) express both forms (Hong et al., 2013). The increase in TNFα expression in cultured head kidney cells shows that the increases seen ~~*in vivo* in~~ following injection of the immune stimulants in this tissue likely come from resident ~~-~~cells ~~located there~~ rather than cells that have migrated to the tissue ~~in~~. However, there are two key differences in

the head kidney cell expression results *in vitro* compared to the ~~*in vivo*~~ injection experiment. The first is the early expression modulation seen *in vitro* with LPS that had waned by 24 h. This could be attributed to the direct contact of the cells in culture with the PAMP, whereas following injection ~~*in vivo*~~ there may be a lag due to the time needed to travel to the head kidney to stimulate the cells resulting in the apparently prolonged effect. The second is that the expression of TNFα2 was increased with all of the stimulants *in vitro*, whilst injection of ~~*in vivo*~~ poly I:C and β-glucan had no effect. This may hint at a suppression of TNFα2 *in vivo*. In the other tissues studied (spleen, intestine, gills) there was also no increase in TNFα expression after~~in the *in vivo* study~~ injection, and so perhaps these differences are due to the sampling being too late and that in fact earlier responses were occurring. This was seen in carp fed a β-glucan supplemented diet and then challenged by injection with *Aeromonas salmonicida*, where modulation of TNFα in head kidney occurred at 6 h post-challenge but had waned by 12 h (Falco et al., 2012). Similarly, in genetic lines of rainbow trout challenged by injection with *Flavobacterium psychrophilum* up regulation of TNFα1, TNFα2 (both type I TNFα's) and TNFα3 (type II TNFα) was seen at 6 h but not 24 h, although TNFα1 and TNFα3 were again upregulated at 48 h (Kutyrev et al., 2016). However, it should also be noted that whole cell suspensions were used in this experiment that contain a mixture of immune and non-immune cells, with some non-immune cells known to be capable of expressing TNF α (Redlich et al., 2002) and potentially contributing to the transcript levels seen after stimulation.

The present study also compared the expression profiles of the two types of TNFα during development for the first time. From an initial relatively high expression level early in meagre development, we found a decrease in the production of both isoforms over time. This hints at an important role for TNFα in development, likely due to its function in apoptosis of selected cells, in remodelling of the skeletal structure and in development of a crude

lymphoid system (Van der Pluijm et al., 1991; Mitchell et al., 1998; Drayton et al., 2006). There was one major deviation from this trend, which occurred shortly after the switch from a diet of *Artemia* to a dry pellet commercial feed, and may indicate this diet change affected the immune system, possibly through an effect on the gastro intestinal tract. It is well known that dietary components can cause enteritis within the gut, as seen when feeding soybean meal in salmonids (Krol et al., 2016). During late ontogeny it was possible to study individual tissues rather than whole larvae. In head kidney and gut there was no further change in expression at the times studied, indicating homeostatic levels had been reached in these tissues. However, this was not the case in the gills and the spleen. In spleen there was a continued decrease in expression of both isoforms, whilst in the gills a steady increase was observed. The latter may indicate that TNFα producing cells are accumulating at this site for defence against potential pathogens. ~~infection., possibly linked to development of specialised gill associated immune tissue (GIALT) (Salinas et al., 2011; Lazado and Caipang, 2014), concentrated or diffuse, such as the interbranchial lymphoid tissue recently identified in salmonid gills (Norte dos Santos et al., 2014).~~

In summary, this study reports the discovery of 2 types of TNFα in meagre, an emerging species for aquaculture. These 2 isoforms were expressed at different levels in key immunological tissues, and showed some differential expression patterns in response to stimulation with PAMPs, indicating a degree of sub-functionalisation is present. We also showed that both isoforms were expressed highly during early ontogeny and gradually decreased as development progressed. However, during late ontogeny there was an increase in expression of both isoforms in the gills hinting at the ~~development GIALT~~ importance of TNFα at this site~~time~~.

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Disclosures

The authors have no financial conflicts of interest.

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Highlights

- Two isoforms of TNFα have been identified in the perciform Meagre (Argyrosomus regius).
- Gene expression for both TNFα isoforms is modulated by PAMP stimulation in vivo and in vitro.
- In vitro studies suggest a possible mucosal bias for TNFα1 and a systemic bias for TNFα2.
- Both TNFα isoforms appear important during early development and following the first feed on a commercial diet.

Figure 1. Nucleotide and deduced amino acid (aa) sequence of meagre TNFα1 (left) and TNFα2 (right). The putative aa sequence is shown under the triplet codon. Start and stop codons are in bold. The transmembrane regions are underlined, TACE cut sites have been boxed and the TNF signature regions are highlighted in grey.

Figure 2. Amino acid (aa) similarity (white) and identity (grey) of meagre TNFα1 and TNFα2 with the known aa sequences of other vertebrates. Accession numbers are as follows: Meagre TNFα1 : MF186589, Croaker TNFα1 : KKF15586.1, Grouper TNFα1 : AEH59794.1, Tuna TNFα1 : BAG72141.1, Tilapia TNFα1 : NP_001266462.1, Trout TNFα1 : NP_001117846.1, Zebrafish TNFα1 : Q4W898, Meagre TNFα2 : MF186590, Croaker TNFα2 : XP_010744292.2, Grouper TNF α2 : AEH59795.1, Tuna TNFα2 : BAG72142.1, Tilapia TNFα2 : XP_013122429.1, Trout TNFα3 : CCH10518.1, Zebrafish TNF α2 : NP_001019618.1, Frog TNFα : NP_001107143.1, Human TNFα : CAA78745.1

Figure 3. Multiple alignment of known TNFα1 and TNFα2 molecules in bony fish species, with the human TNFα sequence. The multiple alignment was produced using CLUSTAL OMEGA. The transmembrane domain is indicated by a double underline, the TACE cut site (TL) is in bold with a single underline, the 10 human beta strands are boxed and the 2 conserved cysteines are indicated by grey shading. Accession numbers are as follows : : Meagre TNFα1 : MF186589, Croaker TNFα1 : KKF15586.1, Grouper TNFα1 : AEH59794.1, Tuna TNFα1 : BAG72141.1, Tilapia TNFα1 : NP_001266462.1, Trout TNFα1 : NP_001117846.1, Meagre TNFα2 : MF186590, Croaker TNFα2 : XP_010744292.2, Grouper TNF α2 : AEH59795.1, Tuna TNFα2 : BAG72142.1, Tilapia TNFα2 : XP_013122429.1, Trout TNFα3 : CCH10518.1, Human TNFα : CAA78745.1.

Figure 4. An unrooted phylogenetic tree of currently known TNFα sequences, constructed using amino acid multiple alignment software CLUSTAL W and the neighbour-joining method in MEGA 6. Node values represent the bootstrap percentage confidence following 10,000 runs. Groupings of families and TNF types are indicated on the right. Accession numbers follow immediately after of each species common name**.**

Figure 5. Distribution of meagre TNFα1 and TNFα2 in four key immune tissues. Total RNA was extracted from the tissues and TNFα1 and TNFα2 transcripts detected by qPCR and normalised to GAPDH. HK = head kidney, SP = spleen. Bars are means ± SEM, n = 10. Asterisks denote significant differences at $P \leq 0.05$ between isoforms within a tissue.

Figure 6. Effect of intraperitoneal injection of immune stimulants on TNFα1 (left) and TNFα2 (right) expression. 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 TNFα1 and TNFα2 transcripts detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the control (PBS) fish for the same tissue. HK = head kidney, SP = spleen. Bars are means ± SEM, n = 10. Asterisks denote significant differences at $P \leq 0.05$ compared to the PBS control fish.

Figure 7. The effects of immune stimulants on TNFα1 and TNFα2 expression in primary cell cultures. Effects of immune stimulants on cells isolated from the (**a**) head kidney, (**b**) spleen, (**c**) gut, (**d**) gills. Cells from each tissue were incubated

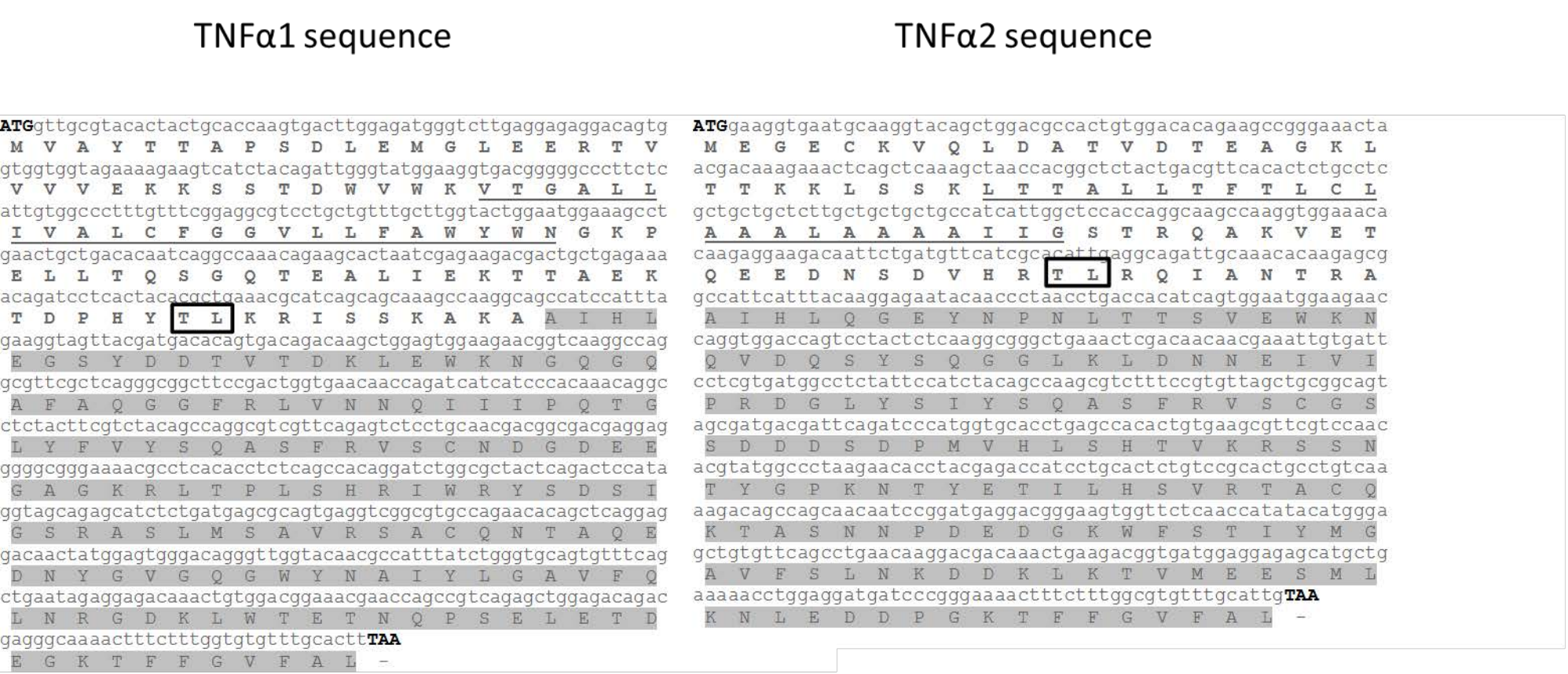
with PBS, poly I:C, LPS or β-glucan for 4, 12 and 24 h. Subsequently, total RNA was extracted from the cells and TNFα1 and TNFα2 transcripts detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the control (PBS) cells at the same time point. Bars are means ± SEM, n = 7. Asterisks denote significant differences at $P \leq 0.05$ compared to the PBS control cells.

Figure 8. The expression of meagre TNFα1 and TNFα2 during development in juvenile fish. The dashed line indicates the switch from feeding exclusively on *Artemia* to introducing a standard commercial dry pellet feed. Whole fish were homogenised and total RNA extracted at 8, 15, 29, 40, 47 and 60 days post hatch. Subsequently TNFα1 and TNFα2 transcripts were detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the day 60 samples. Bars are means ± SEM, n = 10. Letters denote significant differences between sampling times ($P \leq 0.05$).

Figure 9. The expression of meagre TNFα1 and TNFα2 in specific tissues during the later stages of development. Tissues were taken 85, 96 and 128 days post hatch and total RNA extracted. Subsequently TNFα1 and TNFα2 transcripts were detected by qPCR and normalised to GAPDH, then expressed as a fold change compared to the day 85 samples for each tissue. HK = head kidney, SP = spleen. Bars are means ± SEM, n = 10. Letters denote significant differences at $P \leq 0.05$ within a tissue compared to day 128 samples.

	5	6	7	8	9
6.1	70.6	38.8	41.0	26.7	29.0
3.0	70.0	38.2	41.1	25.5	28.1
2.7	70.9	40.5	40.7	24.2	30.9
	64.7	38.6	38.6	27.5	30.2
7.3		38.3	41.3	24.2	28.4
8.3	58.3		34.5	28.5	26.6
5.5	55.1	57.4		26.1	25.9
0.2	47.2	48.3	47.5		30.0
9.0	47.4	48.8	45.9	53.6	

TNFα2	1	2	3	4	5	6	7	8	9
1. Meagre		91.1	66.5	68.3	63.0	45.6	36.5	28.0	1
2. Croaker	93.7		66.9	67.1	63.0	45.3	36.9	28.5	1
3. Grouper	80.0	79.2		70.7	66.8	47.8	37.9	28.0	1
4. Tuna	79.2	78.4	82.9		64.3	51.4	37.0	26.2	1
5. Tilapia	77.0	75.3	77.5	75.1		47.8	37.6	28.1	1
6. Trout	67.5	67.1	67.1	67.3	66.7		42.2	28.5	1
7. Zebrafish	61.2	61.6	62.1	58.8	56.9	64.6		25.7	1
8. Frog	46.4	48.1	45.8	47.8	47.3	48.3	51.3		3
9. Human	48.5	50.2	48.3	45.7	49.8	48.8	50.9	53.6	


TNFα1 sequence
TNFα2 sequence
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M V A Y T T A P S D L E M G L E E R T V
gtggtggtagaaaagaagtcatctacagattgggtatggaaggtgacgggggcccttctc
V V V E K K S S T D W V W K V T G A L L
attgtggccctttgtttcggaggcgtcctgctgtttgcttggtactggaatggaaagcct
I V A L C F G G V L L F A W Y W N G K P
gaactgctgacacaatcaggccaaacagaagcactaatcgagaagacgactgctgagaaa
E L L T Q S G Q T E A L I E K T T A E K
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T D P H Y T L K R I S S K A K A A I H L
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E G S Y D D T V T D K L E W K N G Q G Q
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K N L E D D P G K T F F G V F A L –


Meagre_TNFa1 MVAYT-TAPSD--LEMGLEERTV----------VVVEKKSSTDWVWKVTGALLIVALCF
Croaker_TNFa1 MVAYT-TAPSD--LEMGLEERTV----------VVVEKKSSTDWIWKVTGALLVVALCF
Grouper_TNFa1 MVAYT-TAPGD--VEMGPEERTV----------VLVEKKSSAVQIWKVSVALLTVALCI
Tuna_TNFa1 MVAYT-TAPAD--VETGLEERTV----------VLVEKKSSTGWIWKVSGTLLIILLCL
Tilapia_TNFa1 MVAYT-TTPVD--VEAGPEAKTV----------VLVEKKSPAEWIWKVCAVLVVVALCL
Trout_TNFa1 MEGYA-MTPED--MERGLENSLVDSGPVYKTTVTAVAERKASRGWLWRLCGVLLIAALCA
Meagre_TNFa2 MEGEC-KVQLDATVDTEAGKLTT----------K--KLSS------KLTTALLTFTLCL
Croaker_TNFa2 MEGEC-KVQLDATVDTEAGKQTT----------Q--RFSS------KLTTALLTFTLCL
Tuna_TNFa2 MEGEC-KVALDAAVHIGARKHTT----------QSVKPSS------KLTTAVLAFTFCF
Grouper_TNFa2 MEGEC-KVMLDAAVDADARKQTT----------PVRPGS------KLTTGLLVFTLCL
Tilapia_TNFa2 MEGEC-MVVLDTTINKDTEKQATQR---------TQREPRS------KLTMVLLAFTLCL
Trout_TNFa3 MEGDCSRVTVD--LENGPVSPTV----------TLVREKSTQR--WRLCGALLAMALCV
Human_TNFa MSTES--MIRD--VELAEEALPK----------KTGGPQGSRR---CLFLSLFSFLIVA
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GGVLVFA-WYWTGKPELLTQSGQTEALIEKTTAEKTDPHYTLKRISS--KAKAAIHLEGS
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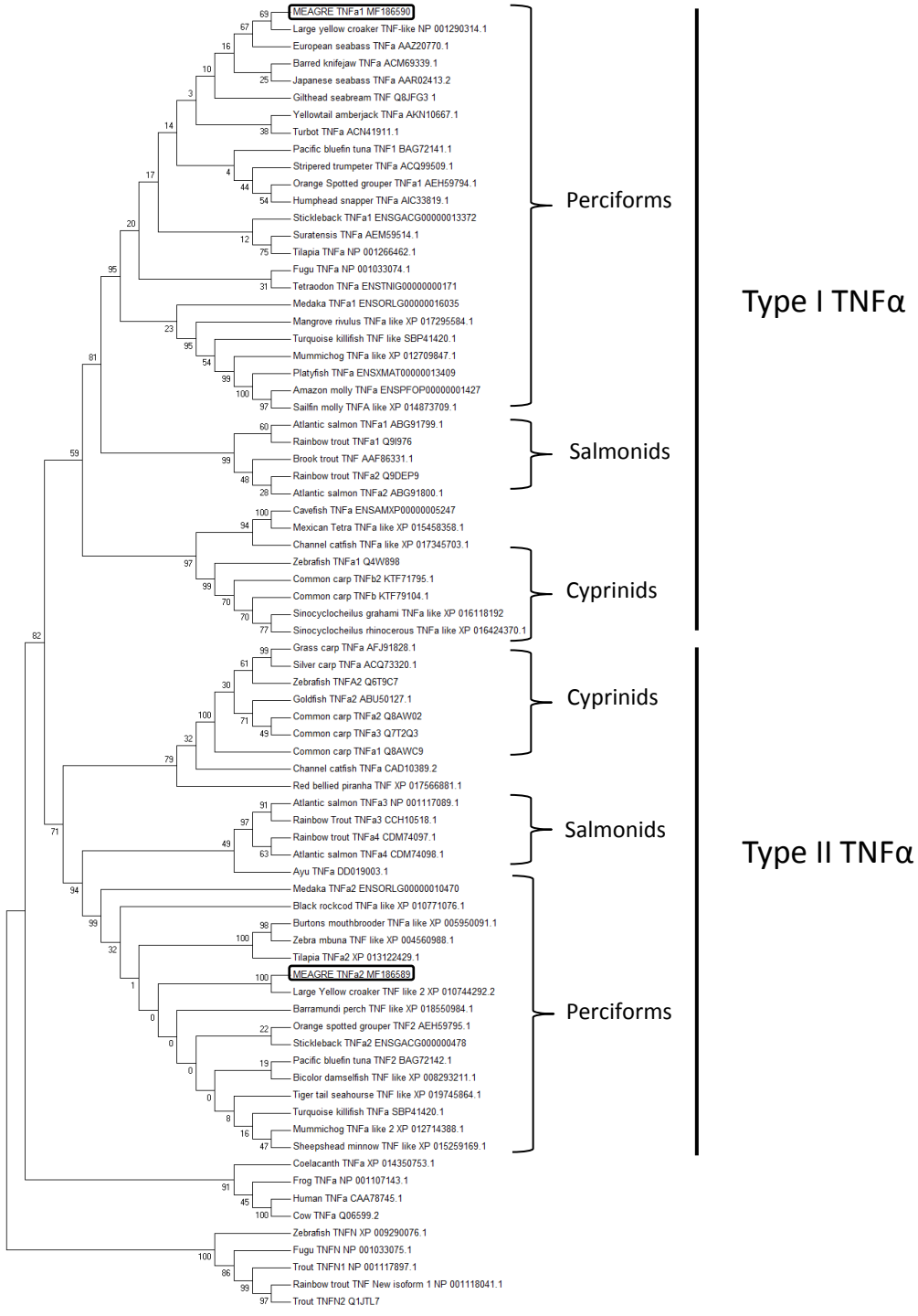
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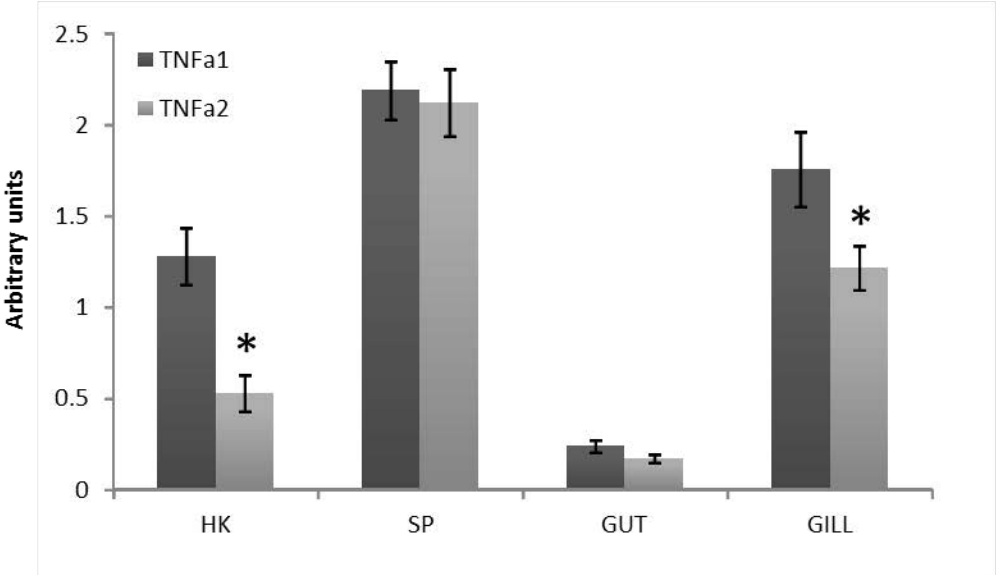
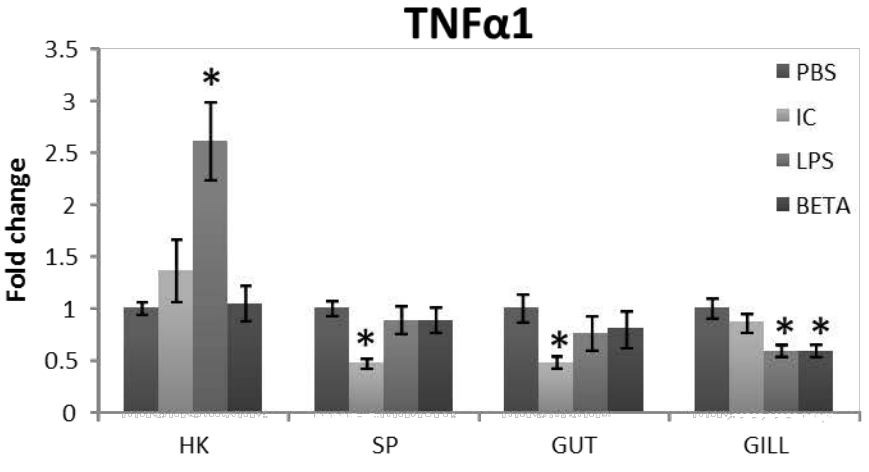
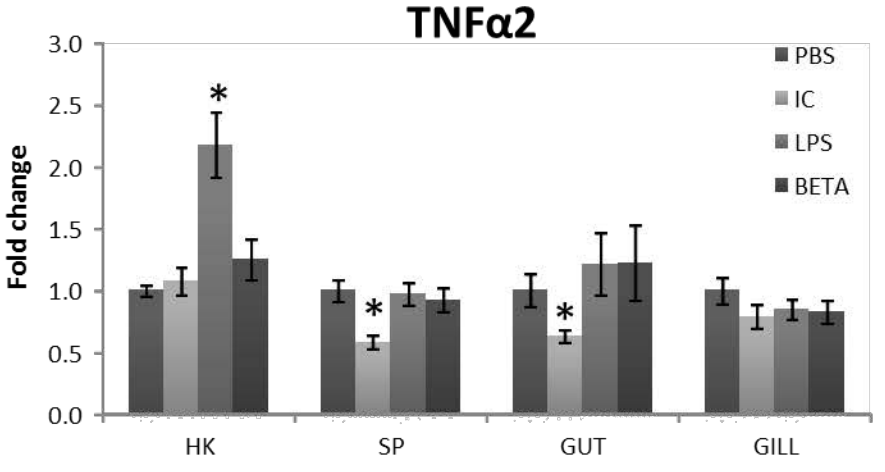
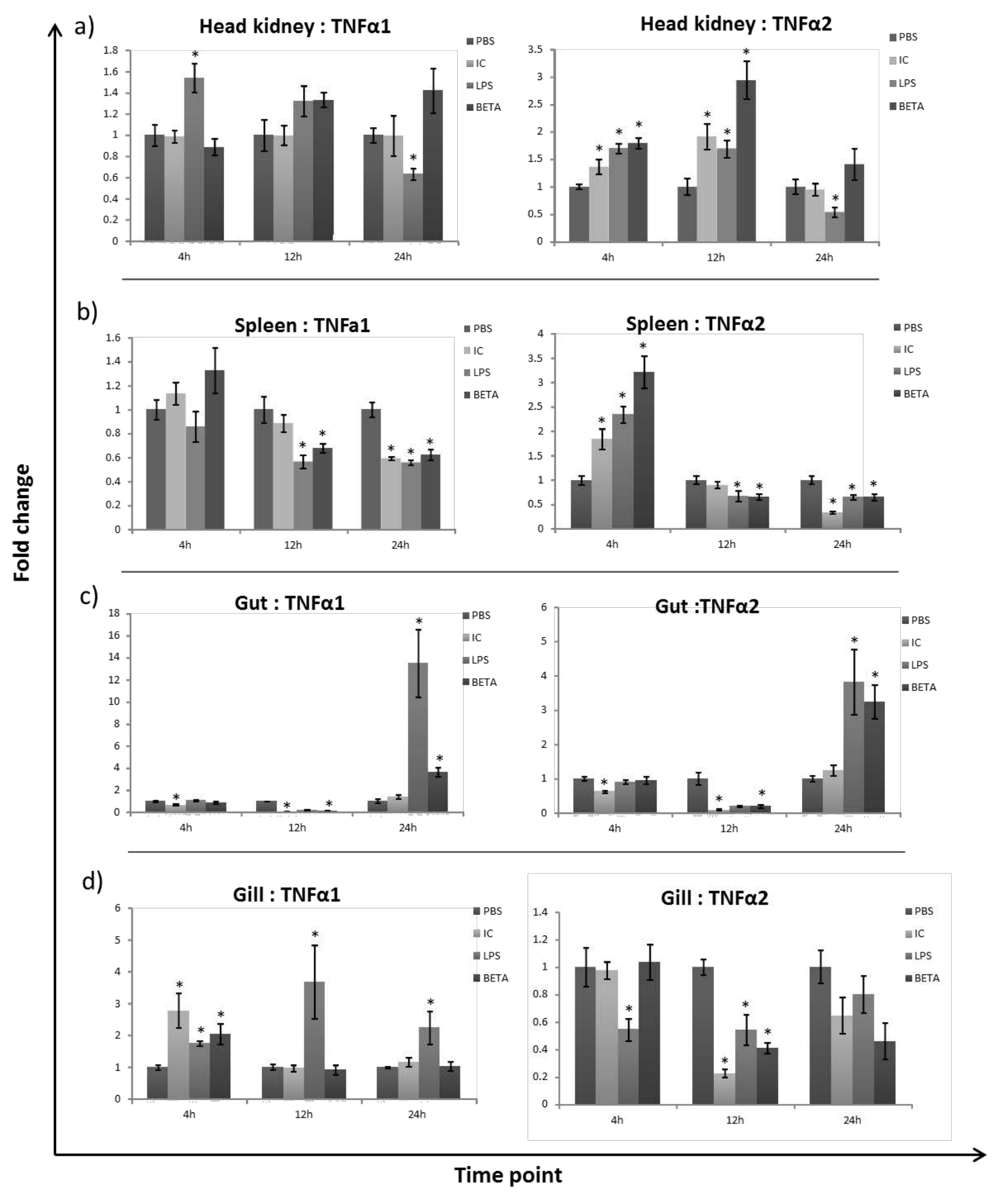
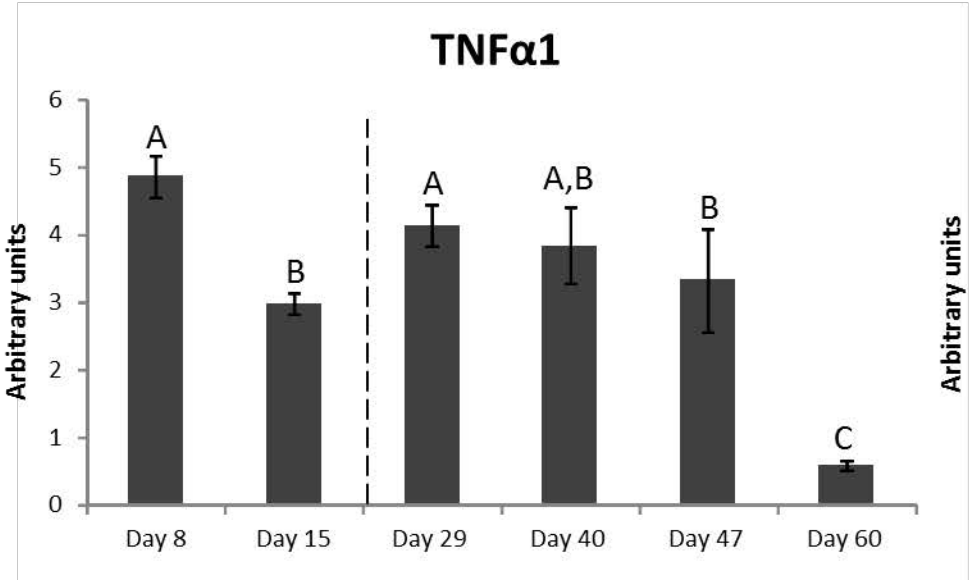
Large yellow croaker TNF-like NP 001290314.1
European seabass TNFa AAZ20770.1
Barred knifejaw TNFa ACM69339.1
Japanese seabass TNFa AAR02413.2
Gilthead seabream TNF Q8JFG3.1
Yellowtail amberjack TNFa AKN10667.1
Turbot TNFa ACN41911.1
Pacific bluefin tuna TNF1 BAG72141.1
Stripered trumpeter TNFa ACQ99509.1
Orange Spotted grouper TNFa1 AEH59794.1
Humphead snapper TNFa AIC33819.1
Stickleback TNFa1 ENSGACG00000013372
Suratensis TNFa AEM59514.1
Tilapia TNFa NP 001266462.1
Fugu TNFa NP 001033074.1
Tetraodon TNFa ENSTNIG00000000171
Medaka TNFa1 ENSORLG00000016035
Mangrove rivulus TNFa like XP 017295584.1
Turquoise killifish TNF like SBP41420.1
Mummichog TNFa like XP 012709847.1
Platyfish TNFa ENSXMAT00000013409
Amazon molly TNFa ENSPFOP00000001427
Sailfin molly TNFA like XP 014873709.1
Perciforms
Atlantic salmon TNFa1 ABG91799.1
Rainbow trout TNFa1 Q9I976
Brook trout TNF AAF86331.1
Rainbow trout TNFa2 Q9DEP9
Atlantic salmon TNFa2 ABG91800.1
Salmonids
Cavefish TNFa ENSAMXP00000005247
Mexican Tetra TNFa like XP 015458358.1
Channel catfish TNFa like XP 017345703.1
Zebrafish TNFa1 Q4W898
Common carp TNFb2 KTF71795.1
Common carp TNFb KTF79104.1
Sinocyclocheilus grahami TNFa like XP 016118192
Sinocyclocheilus rhinocerous TNFa like XP 016424370.1
Cyprinids
Type I TNFα
Grass carp TNFa AFJ91828.1
Silver carp TNFa ACQ73320.1
Zebrafish TNFA2 Q6T9C7
Goldfish TNFa2 ABU50127.1
Common carp TNFa2 Q8AW02
Common carp TNFa3 Q7T2Q3
Common carp TNFa1 Q8AWC9
Cyprinids
Channel catfish TNFa CAD10389.2
Red bellied piranha TNF XP 017566881.1
Atlantic salmon TNFa3 NP 001117089.1
Rainbow Trout TNFa3 CCH10518.1
Rainbow trout TNFa4 CDM74097.1
Atlantic salmon TNFa4 CDM74098.1
Salmonids
Ayu TNFa DD019003.1
Medaka TNFa2 ENSORLG00000010470
Black rockcod TNFa like XP 010771076.1
Burtons mouthbrooder TNFa like XP 005950091.1
Zebra mbuna TNF like XP 004560988.1
Tilapia TNFa2 XP 013122429.1
MEAGRE TNFa2 MF186589
Large Yellow croaker TNF like 2 XP 010744292.2
Barramundi perch TNF like XP 018550984.1
Orange spotted grouper TNF2 AEH59795.1
Stickleback TNFa2 ENSGACG000000478
Pacific bluefin tuna TNF2 BAG72142.1
Bicolor damselfish TNF like XP 008293211.1
Tiger tail seahourse TNF like XP 019745864.1
Turquoise killifish TNFa SBP41420.1
Mummichog TNFa like 2 XP 012714388.1
Sheepshead minnow TNF like XP 015259169.1
Perciforms
Type II TNFα
Coelacanth TNFa XP 014350753.1
Frog TNFa NP 001107143.1
Human TNFa CAA78745.1
Cow TNFa Q06599.2
Zebrafish TNFN XP 009290076.1
Fugu TNFN NP 001033075.1
Trout TNFN1 NP 001117897.1
Rainbow trout TNF New isoform 1 NP 001118041.1
Trout TNFN2 Q1JTL7

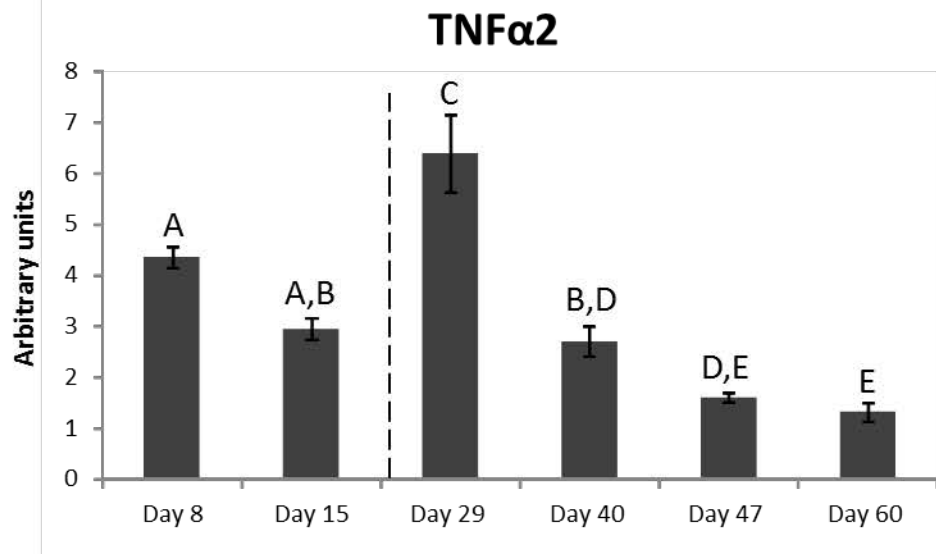

2.5
2
1.5
1
0.5
0
Arbitrary units
TNFa1
TNFa2
*
*
HK
SP
GUT
GILL

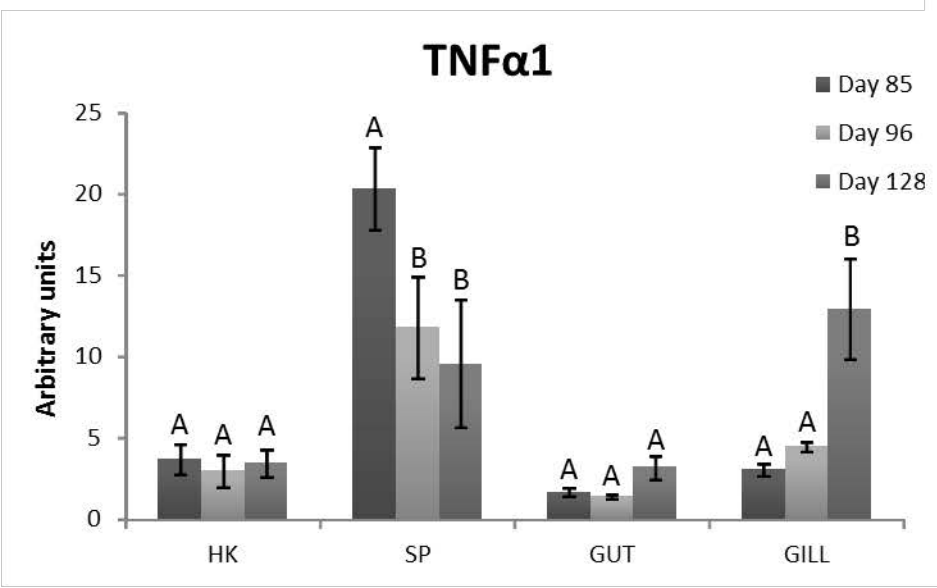

TNFα1
Fold change
3.5
3
2.5
2
1.5
1
0.5
0
PBS
IC
LPS
BETA
HK
SP
GUT
GILL

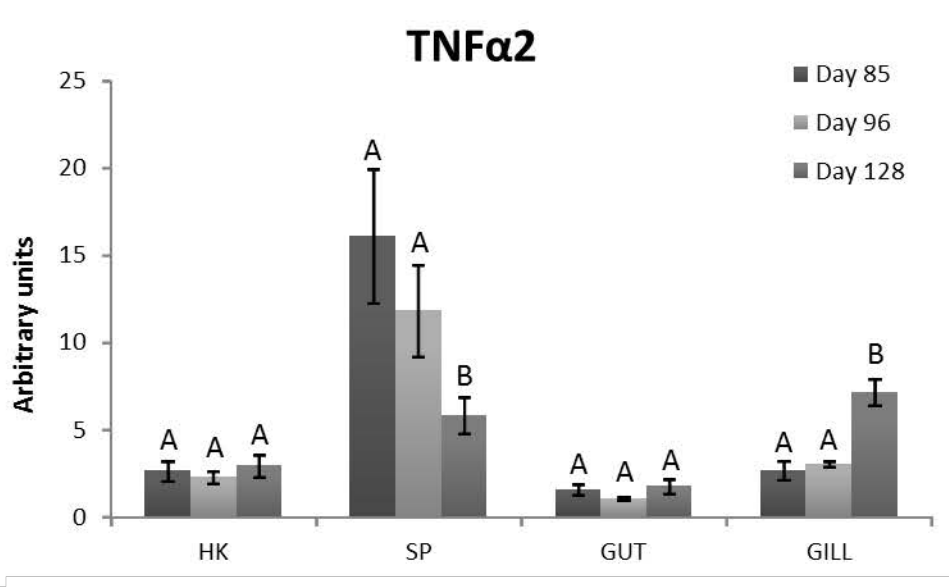

TNFα2
Fold change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PBS
IC
LPS
BETA
HK
SP
GUT
GILL


a)
Head kidney : TNFα1
Head kidney : TNFα2
b)
Spleen : TNFa1
Spleen : TNFα2
c)
Gut : TNFα1
Gut :TNFα2
d)
Gill : TNFα1
Gill : TNFα2
PBS
IC
LPS
BETA
4h
12h
24h
Fold change
Time point


TNFα1
Arbitrary units
0
1
2
3
4
5
6
A
B
A
A,B
B
C
Day 8
Day 15
Day 29
Day 40
Day 47
Day 60


TNFα2
Arbitrary units
0
1
2
3
4
5
6
7
8
A
A,B
C
B,D
D,E
E
Day 8
Day 15
Day 29
Day 40
Day 47
Day 60


TNFα1
Day 85
Day 96
Day 128
Arbitrary units
25
20
15
10
5
0
A
A
A
A
B
B
A
A
A
A
A
A
A
A
B
HK
SP
GUT
GILL


TNFα2
Day 85
Day 96
Day 128
Arbitrary units
25
20
15
10
5
0
A
A
B
A
A
A
A
A
A
A
A
B
HK
SP
GUT
GILL