Gene expression analysis of the innate immune system during early rearing and weaning of meagre (*Argyrosomus regius*).

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Abstract

The present study is the first report of some representative innate immune genes in meagre (*Argyrosomus regius*) larvae. This study has specifically focused on the growth period from hatching to the juvenile stage, a critical time in marine fish development when reliance on innate immune mechanisms are required for survival. We report molecular cloning of partial open reading frames and expression patterns for some innate immune genes (*c3, cox2, met, lyzc, mxp, myd88, nod2, nod3*). In addition, phylogenetic analyses of some of the sequences obtained was performed where confusion among closely allied isoforms may have existed. These results show the *met* isoform from meagre is *met* II, an isoform more similar to a homolog described in *Larimichthys crocea;* lysozyme (*lyzc*) corresponds to the c-type and NOD isoforms (*nod2, nod3*) separate into different clades confirming their distinctness within a common evolutionary history. Gene expression profiles of innate genes were investigated, for nine developmental stages, from 8 days post-hatching (dph) to 120 dph. Present results demonstrated that *c3, cox2, met II, lyzc, mxp, myd88, nod2,* and *nod3* were expressed in all stages of larval development and displayed distinct expression profiles in separate tissues (kidney, spleen gut and gill). Moreover, expression patterns suggested theses innate immune genes may be influenced by feeding practices, *i.e.* switching from live prey (rotifer and *Artemia*) and weaning onto an inert commercial diet. In addition to evaluating changes in gene expression during early development, this study evaluated the modulation of gene expression by means of *in vivo* trials in juveniles that were stimulated with PAMPs (LPS, poly I:C, β-glucan). These results revealed significant changes in mRNA levels of target genes in the kidney, spleen, gut and gills. However, expression profiles differed in magnitude depending on the stimulant and/or tissue. These results are discussed in terms of their relevance and potential application in aquaculture practices.

Declarations of interest: none

Keywords: innate immunity, *Argyrosomus regius*, meagre, PAMP, ontogeny.

1. Introduction

In recent decades, the development of the aquaculture industry has been achieved by important advances in production techniques, as well as the introduction of new species [1]. To achieve greater sustainability for the industry, more enhanced production is needed to boost efficiency; thus, proper health management is a key issue in actual fish farming operations. One research area that could improve health management is vaccine development, among other preventive measures (*i.e*, functional feeds and/or therapeutic agents). However, in addition to the significant research and financial investments required for vaccine development for each particular infectious agent, there is a requirement for proper understanding of the immune functions and timing of their development in order to synchronize rearing practices (*i.e*. vaccination) to the stage of fish development.

Meagre (*Argyrosomus regius*) is an emerging species in aquaculture and is currently receiving a lot of attention within European region. Meagre can be found throughout the Mediterranean and Black Seas, as well as in the East Atlantic coasts [2]. As is common in production of a new species in aquaculture, the artificial rearing of meagre larvae needs to be optimized to improve survival rate in an effort to reduce larval rearing costs [3]. The successful culture of larvae from marine species not only requires optimized rearing and feeding protocols based on the species' nutritional requirements and digestive capabilities, but it also relies on an understanding of the function and timing of maturation of the immune system to aid management of the occurrence of infectious disease, which cause high losses in aquaculture, especially during early development [4].

Fish larvae are not capable of eliciting a specific immune response during early development since immune memory and a fully competent adaptive response develops later during the juvenile stage. Therefore, larvae are dependent on innate defenses against pathogens or opportunistic agents during their early life stages. While the epithelial layers of gills skin and digestive tract provide an initial physical barrier, if breached by a pathogen the innate immune system, composed of various effector molecules, provides the first line of defense. The innate immune system includes membrane-bound pathogen recognition receptors (PRRs), such as NOD-like receptors, and cytoplasmic proteins that bind pathogen-associated molecular patterns (PAMPs) expressed on the surfaces of invading microbes [5]. Innate immunity exerts its effect by employing a variety of cells and effector proteins able to destroy or inhibit the growth of infectious micro-organisms [6]. The complement system is one of the first lines of immune defense and is a regulator of acquired

immunity. The complement system is activated through three different, but partially overlapping routes: the classical, alternative and lectin binding pathways [7]. The classical pathway is triggered by antibody binding to the cell surface that in turn binds a cascade of complement factors; the alternative pathway is independent of antibody binding and is activated directly by foreign microorganisms; and the lectin pathway, which is activated by the binding of a protein complex consisting of mannose/mannan-binding lectin to oligosaccharide ligands on bacterial cells [8]. Lysozyme is expressed in a wide variety of tissues of the innate immune system of vertebrates and it is an important defense molecule that possesses lytic activity against microbial cell walls preventing invasion [9]. The role of Mx protein (mxp) in resistance to negative-strand RNA viruses has been well established, despite the precise mechanism of viral inactivation by Mxp not being fully understood [10]. Mx proteins are highly conserved in vertebrates, and are able to inhibit the virus life cycle in different phases, whether they are localized in the cytoplasm or in the nucleus of cells [11]. Cyclooxygenase (cox2) is responsible for synthesis of prostaglandins to maintain homeostatic functions and it also plays an important role in the inflammatory response, among other relevant functions [12]. Metallothionein is an important protein contributing to resistance to heavy metal contamination in aquatic organisms, but also has important immune functions [13]. Metallothionein plays a relevant role in the detoxification of heavy metal ions and plays a role in buffering action against heavy metals by binding essential metals such as Cu and Zn, which has an inhibitory effect on systemic pathogens and provides protection against oxidative stress [14]. For these effector molecules to carry-out their function in a coordinated manner there is a need for intra- and inter-cellular signaling. For this, the innate immune response relies on signaling by members of the toll-like receptor (TLR) family that are highly conserved in vertebrates, and associated adaptor molecules, such as Myd88 (myeloid differentiation primary response gene). MyD88 has been implicated in the downstream signaling of TLRs with the possible exception of TLR3 [15], and thus, it is implicated in defense against a variety of pathogens. MyD88 contains a Toll/Interleukin receptor (TIR) domain and is required for activation of mitogen–activated protein (MAP) kinase family members as well as nuclear factor kB (NF-kB) translocation, which in turn activates transcription of pro-inflammatory cytokines such as interleukin 1 beta (IL1B) [16]. Innate immunity additionally has the potential to respond to endogenous molecules [17] that are released by host cells as a result of necrosis, pathogen infection, and certain pathological conditions, which are directly or indirectly recognized by NOD-like receptors. The NOD-like receptors (NLRs) function as

cytoplasmic sensors of pathogen presence. NOD2 detects muramyl dipeptide (MDP) found in the peptidoglycan of the cell wall of both gram-positive and negative bacteria [17]. NOD2 in mammals is highly expressed in epithelial cells or macrophages associated with the intestine. Studies in zebrafish have detected NOD3 (*nod3*) which has been shown to be an orthologue of mammalian NOD3 (NLR3) and found to have similar NACHT domains and an equal number of Leucine-Rich-Repeat (LRR) domains, likely required for binding to pathogen associated molecular patterns [18], but its specific ligand remains to be identified.

The aim of this study is to improve understanding of the innate immune response of meagre during larval rearing when the adaptive immune response remains incomplete. To this end this study will examine i) the ontogenic changes in innate immune-relate gene expression of *c3*, *cox2*, *metII*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* during larval and juvenile development in meagre, and ii) how their expression is modulated by different PAMPs *in vivo* using adult fish*;* and iii) examine the modulation using PAMPs in isolated cells from specific organs *in vitro*. This information will be of value for better understanding early ontogeny of the immune system in this fish species, as well as serve as a basis for proper health maintenance in hatchery and nursery management practices.

2. Materials and methods

2.1 *Larval rearing and sample collection*

Larvae used in this study were obtained from a meagre broodstock held at IRTA-SCR (San Carlos de la Rapita, Spain). The broodstock was maintained under controlled simulated natural water temperature and photoperiod using a recirculation system (IRTAmar®). During the natural reproductive period (April-June), mature fish were selected based on oocyte size (< 550 µm) and spermiating condition. Pairs of mature fish (21.2 ± 3.7 kg females and 16.1 ± 2.6 kg males) were hormonally induced (15 µg/kg of des-Gly10, [D-Ala6]-gonadotropin-releasing hormone ethylamide (Sigma, Spain)) to spawn in 10,000 L tanks. The resulting fertilized eggs were collected using a passive egg collector placed in the outflow of the tank. Batches of 50,000 eggs were incubated at 18-19 ºC in 35 L mesh - bottomed (300 µm mesh) incubators with aeration and gentle air-lift water exchange that were placed within 2,000 L tanks. Hatching rate was determined by estimating the number of larvae (three 100 ml sub-samples) obtained from the stocked eggs.

Larvae were then transferred to two 1.5 m^3 tanks and reared using a mesocosm system. Water temperature was maintained at 20 ºC. From two days post-hatching (dph), larvae were fed enriched rotifers (*Brachionus sp.*) until 11 dph. Freshly enriched *Artemia* metanauplii were introduced from 9 dph until 31 dph. The experimental tanks were fed with a dry commercial feed for fish larvae containing 60 % protein, 15 % lipids, 9 % ash, 0.5% fibre, 0.5% phosphorus, (Gemma Wean 0.2, Trouw France S.A, France). The diet was incorporated from 21 dph until the end of the experiment. Random samples of larvae and juvenile meagre were taken at 8, 15, 29, 40, 43, 60, 85, 96, and 120 dph. Each sample consisted of ten animals that were placed in an Eppendorf tube on ice, containing RNAlater™ (Ambion, Austin, Texas), then preserved at -80 ºC until RNA extraction. Time points for analysis were chosen to coincide with the specific periods of change in rearing practices; primarily changes in diet, but also rapid changes in organogenesis, all of which increase stress and enhance susceptibly to diseases. Before immersion in RNAlater™ (Ambion, Austin, Texas) the fish were euthanized using a high concentration of MS222 (1 g/l) (Aldrich, E10521). Larger larvae (post-29 dph) had an excess of tissue, mainly muscular tissue that was trimmed to reduce signal dilution from non-target tissues. The anterior section from the gills forward and the posterior part from the anus to the tail were removed (Fig. 1), whereas younger larvae were processed entire. At days 85, 96, and 120 individual tissues (gill, kidney, spleen, and intestine) were excised aseptically.

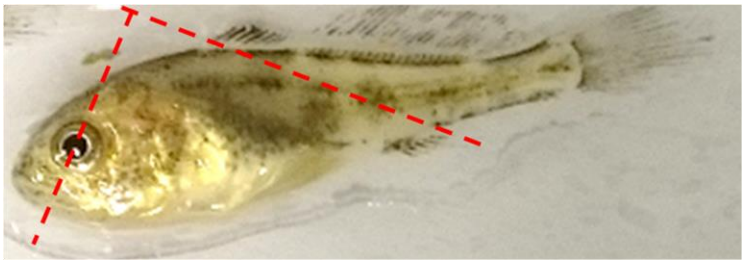


Fig. 1. Prior to the extraction of RNA from the samples, the rostrum and caudal portions were removed by cutting with scalpel as illustrated with dashed lines. Only the remaining ventral part was used for further analysis.

2.2 *Total RNA extraction and reverse transcription*

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. RNA concentration and purity was determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at 260 and 280 nm. The quality of extracted RNA was verified with visualization of the 28S and 18S ribosomal RNA bands by agarose gel electrophoresis. Prior to reverse transcription

total RNA was treated with the DNase 1 AMPD1 kit, (Sigma–Aldrich), according to manufacturer's instructions to remove possible contaminating genomic DNA. Total RNA then underwent reverse transcription by adding 2 µg total RNA using the ThermoScript ™ Reverse Transcriptase (Invitrogen) with oligo-dT (0.5 µg/µl) and random hexamer primers (50 ng/µl) 10X RT buffer [200 mM Tris-HCl (pH8.4), 500 mM KCl] 1.5 mM $MgCl_2$, 800 mM dNTP mix, RNase inhibitor, SuperScript ^{TM} II RT, followed by RNAse H (Invitrogen) treatment. Once reverse transcription reactions were prepared they were placed in a Mastercycle® nexus GSX1 (Eppendorf AG, Hamburg, Germany) to complete first.strand cDNA synthesis. All the samples were diluted 1:20 in molecular biology grade water (Sigma-Aldrich) and stored at -20 ºC. Negative controls (no RT enzyme) were included to confirm absence of genomic DNA contamination.

2.3 *Gene isolation*

Nucleotide sequences of target genes from the Genebank (www.nbci.gov) were chosen from different marine teleost species, such as large yellow croaker (*Larimichthys crocea*), mandarin fish (*Synchiropus splendidus*), Humphead snapper (*Lutjanus sanguineus*), European seabass (*Dicentrarchus labrax*), orange spotted grouper (*Epinephelus coioide),* turbot (*Scophthalmus maximus),* Asian sea bass (*Lates calcarifer*) and gilthead sea bream (*Sparus aurata***)**. The sequences were aligned using CLUSTAL W (BioEdit package; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) for designing degenerate/consensus primers for amplification from cDNA produced from meagre tissues as described previously (Campoverde et al., 2017). The fragments amplified were separated by gel electrophoresis and resulting bands of the expected length were excised, purified using the QIAQuick PCR purification kit (Qiagen) and sequenced by Sistemas Genomicos (Valencia, Spain). The transcript sequences obtained were then used as templates for designing primers for quantitative PCR assays.

2.4 *Phylogenetic analysis*

Some of the genes chosen for analysis are known to be members of larger more diverse and previously studied families of genes. As such, there exists in genetic databases other homologues for comparison to establish the member of such a gene family that has been identified for study. To clarify this, evolutionary analyses were conducted in MEGA5 [19] after alignment of all selected homologs using CLUSTAL W. Nucleotide substitution models were chosen for each analysis using the utility included in MEGA 5. The evolutionary reconstruction of the NOD isoforms was performed using the Kimura 2-

parameter nucleotide substitution model [20] and Neighbor-Joining method [21]. The evolutionary history for metallothionein and lysozyme were inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [22]. For these analyses, the initial trees for the heuristic search were obtained automatically as follows: when the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise Neighbor-Joining method with MCL distance matrix was used. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 200,0000)]. Statistical robustness of data for all analyses was analyzed using the bootstrap method [23] with bootstrap confidence values from 1,000 replicates shown at branch nodes (values < 40 not shown). During analyses all ambiguous positions were removed for each sequence pair. The analysis of NOD isoforms (*nod2* and *nod3*) involved 129 positions from 25 sequences. For metallothionein *(metII*), a total of 22 sequences were included in the final dataset each with 71 positions. The analysis of lysozyme (l*yzc*) included 25 sequences with 148 positions in each.

2.5 *PAMP stimulation*

To investigate the effect of PAMP stimulation on the expression of the selected genes, 38 healthy juvenile individuals (30 - 40 g each) were held in a recirculating water tank (100 l) at 20 ˚C. Fish were fed with a commercial diet, 58% protein, 17% lipids, 10 % ash, 0.6% fibre, 1,3 % phosphorous (Gemma Wean 0.3, Trouw France S.A, France) twice a day (09:00 and 16: 00) for two weeks to acclimate them to the indoor culture environment. For primary cell culture and collection of samples, fish were euthanized with an overdose of anesthesia (50 mg/l MS-222).

2.5.1 *In vivo stimulation*

Fish were injected (n = 8) intraperitoneally with 100 µl PBS containing either 100 µg poly I: C (3.3 mg/kg) (Sigma, UK. P1530), 400 µg LPS (13.3 mg/kg) (Sigma, UK. L3129) or 100 µg β-glucan (3.3 mg/kg) (Sigma, UK. 89862). The control animals were injected with PBS only. After 24 h, the individuals were dissected and tissues (head kidney, spleen, gut and gill) sampled. Total RNA was isolated and cDNA was prepared as described above. The mRNA expression level of *metII*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* were determined by qPCR. Relative expression was normalized to GAPDH expression and calculated as arbitrary units and converted to a proportion relative to the PBS control samples.

2.5.2 In vitro stimulation

Tissues (kidney, spleen, gills, and gut) from apparently healthy fish (n = 6) were collected from euthanized fish for primary cell culture. Tissue from gills and gut were pre-treated with collagenase in HBSS with FBS (0.37mg/ul – Sigma Aldrich, Ref# C0130-100MG) for 30 min at 37ºC to facilitate dissociation of cells from intercellular connective tissue. Afterwards these tissues, together with the spleen and kidney samples, were passed through a 100 µm nylon mesh cell strainer (SefarNytal PA-13xxx/100, Spain) in Leibovitz L15 medium (Gibco) containing penicillin/streptomycin (Gibco, #15140-122) at 1:1000 and 2% foetal calf serum (Gibco, #10270-098). The resulting cell suspension was collected and centrifuged at 400 x g for 10 min. The supernatant was removed and replaced with 10 ml of previously described L15 media. The cell suspension was again centrifuged and supernatants removed and replaced with 30 ml of media. Cells were distributed to 12 well microtiter plates in 5 ml aliquots. Wells were stimulated using LPS (Sigma, #L3129-100MG) at 50 µg/ml, poly (I:C) (Sigma, #P1530-25MG) at 100 µg/ml, and B-glucan (Sigma, #89862-1G-F) at 50 µg/ml in triplicate. Control samples included 250 µl of PBS. Four, 12 and 24 h after stimulation the cells were harvested and centrifuged at 400 x g for 10 min, the supernatant discarded, and the pellet suspended in RNAlater. Total RNA was isolated and cDNA was prepared as described above.

2.6 *Real-time qPCR*

The qPCR reactions for innate immune gene expression were carried out using a LightCycler® 480 Real-Time (Applied Biosystems, Roche). A master mix was prepared containing: 5 µl SYBR Green Supermix (Life Technologies), 1µl of molecular biology grade water (Sigma) containing 10 µM of forward and reverse gene-specific primers and 4 µl of sample cDNA in a final volume of 10 µl. The real-time qPCR cycling was carried out as follows: 10 min at 95 ºC, 40 cycles of 95 ºC for 25 s, followed by an annealing step of approximately 59 ºC for 25 s (annealing temperatures were adjusted for each specific primer pair), followed by 72 ºC for 15 s, with a final dissociation stage of 0.5 ºC increments from 75 ºC to 95 ºC. Each sample on the qPCR plate had two methodological replicates. Primer sequences used and amplicon lengths for each assay are shown in Table 1. The specificity of the primers was checked by confirming that only one melt peak was produced, and also by running a subsample on an agarose gel (2%) to confirm the presence of a single band of the expected size. The efficiency of amplification (E, %) of each primer pair was assessed from five serial ten-fold dilutions of cDNA from individual tissues, then

calculated following the equation: E (%) = $10^{(-1/slope)} - 1$, where the slope is that calculated from the regression line of the standard curve. Efficiencies of the gene expression assays ranged from 96.2% to 100.9% (100.27 ± 1.58, mean ± SD) (Table 1). The absence of primer-dimer formation in the NTC (non-template control) was also confirmed. Relative expression of genes from larvae, and each tissue from juveniles (ontogeny study), was normalized using the three endogenous controls glyceraldehyde phosphate dehydrogenase (*gpdh*), beta-actin (*β-act*), and hypoxanthine-guanine phosphoribosyltransferase (*hprt*) as determined using geNORM (http://www.primerdesign.co.uk/products/9461-genormplus-kits/), while the relative quantification at 120 dph, showing the lowest expression, was used as the calibrator.

For analyzing the relative expression *in vivo* and *in vitro* after PAMP stimulation, data was normalized using just GAPDH expression values, as this endogenous control gene showed less variability under the conditions tested. After normalization to this endogenous control as arbitrary units, results were converted to a proportion relative to the control group (PBS injected fish).

Table 1. Primers used for gene expression analysis by qPCR, including the amplicon size and primer sequences. Abbreviations: *gpdh*, glyceraldehyde 3-phosphate dehydrogenase; *β-act*, beta-actin; *hprt*, hypoxantine-guanine phosphoribosyltransferase; *metII*, methallothionein*; c3*, complement*; cox2*, cyclooxygenase; *lyzc*, lysozymes; *mxp*, mx protein; *myd88*, myeloid differentiation primary response gene 88; *nod2*, nucleotide-binding oligomerization domain-containing protein 2; *nod3*, nucleotide-binding oligomerization domain-containing protein 3.

	Genes	E (%)	Forward primer (5' → 3´)	Reverse primer (5' → 3´)	Size (bp)
Reference genes	*gpdh*	100.0	CCAGTACGTGGTGGAGTCCACTG	AGCGTCAGCGGTGGGTGCAGAG	109
	β-act	100.0	TGGGGGAGCAATGATCTTGATCTTCA	AGCCCTCTTTCCTCGGTATGGAGTC	212
	hprt	100.9	CATGGACTCATCTTGGACAGGACAGA	GCCTTGATGTAGTCCAGCAGGTC	137
Immune genes	*metII*	102.2	GATCCTGCAATTGCAAAGACTGTTC	CCGGATGGGCAGCATGGGCAG	70
	c3	100.1	AACCCATACGCTGTTGCCATGACG	CACGTCCTTTAGGTACTGGGCCAG	120
	cox2	100.5	GGAAGTTGGTGTTGACATGCACTAC	CAATCAGGATGAGCCGTGTGGTC	211
	lyzc	100.0	GATGGATCCACTGACTACGGCATC	AAGCTGGCTGCACTGGATGTGGC	100
	mxp	96.2	AGTCAGTGGTTGACATTGTTCATAATG	AACAGTGGCATGACCGTCATTGTAG	187
	myd88	102.2	GCTACTGCCAGAGTGACTTCGAGT	TCCATACACACGAACCCGGGAGG	120
	nod2	100.1	CTCAATACTGTGCTGATGTCCATGG	CAAGTGTAACCTTTGGAGTAAGGTAG	145
	nod3	100.8	CAGCTTGGTGGAACTTGTTCATCAC	TAACATCAGTCAGGATCTCAGTGTTG	130

306 2.7 *Statistical analysis*

307 All data sets were checked for homogeneity of variances by subjecting them to a Levene's
308 test using univariate analysis in a general linear model, based on a Tukey HSD post-hoc test,
309 with a sample size of n =10 (larva) and n = 8 (juvenile) to determine differences between time
310 points ($P \leq 0.05$). The Kruskal-Wallis test was used for analyzing the expression for the *in*
311 *vivo* (n = 8) samples after PAMP stimulation, while the ANOVA test was used for analyzing
312 expression for the *in vitro* (n = 6) based on a Tukey HSD post-hoc test, using the statistical
313 software package SPSS 20.0 (SPSS Inc., US).

314 **3. Results**

315 3.1 *Gene Isolation*

316 Specific gene products were obtained using degenerate or consensus-primed PCR assays. All
317 amplified fragments were sequenced bi-directionally and analyzed using the BLAST utility
318 via the National Center for Biotechnology Information (NCBI) database
319 (http://www.ncbi.nlm.nig.gov/) to confirm their identity as proper orthologues of the intended
320 target gene. Sequences for transcripts from *met*, *c3, cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3*, are
321 reported for the first time in this species. These sequences have been uploaded to GenBank
322 under the following accession numbers: *met* = MF281966, *c3* = MF281960, *cox2* =
323 MF281967, *lyzc* = MF281968, *mxp* = MF281965, *myd88* = MF281964, *nod2* = MF281970,
324 *nod3* = MF281969. Sequences were also obtained for GAPDH, β-actin and HPRT as
325 endogenous control genes for gene expression assays and have been entered into the GenBank
326 database under the following accession numbers MF281962, MF186587 and MF186588,
327 respectively.

328 3.2 *Phylogenetic analysis*

329 Some of the genes of interest for this study are known to have additional isoforms. For this
330 reason, phylogenetic analysis was performed to properly identify such genes. Homologs of
331 metallothionein, nod2, nod3, and lysozyme from meagre were further characterized as to class
332 or isoform to give clarity to resulting gene expression analyses. To achieve this, extant
333 sequences from GeneBank were aligned using CUSTAL W and the alignment edited
334 manually. For all analyses, the percentage of trees in which the associated taxa clustered
335 together (bootstrap values) is shown next to the branch nodes. Phylogenetic analysis of
336 representative homologs of *nod2* and *nod3* sequences from GenBank were compared to that
337 obtained from meagre resulting in an optimal tree with a sum branch length = 1.89832313.
338 The two different Nod isoforms, *nod2* and *nod3*, clearly separated into distinct and differing

clades. Analysis of metallothionein II confirmed its segregation from metallothionein I and generated a tree with the highest log likelihood of -157.7290. The tree resulting from the analysis of lysozyme had highest log likelihood value of -2526.8685 and demonstrated its membership in the clade with *lyzc*.

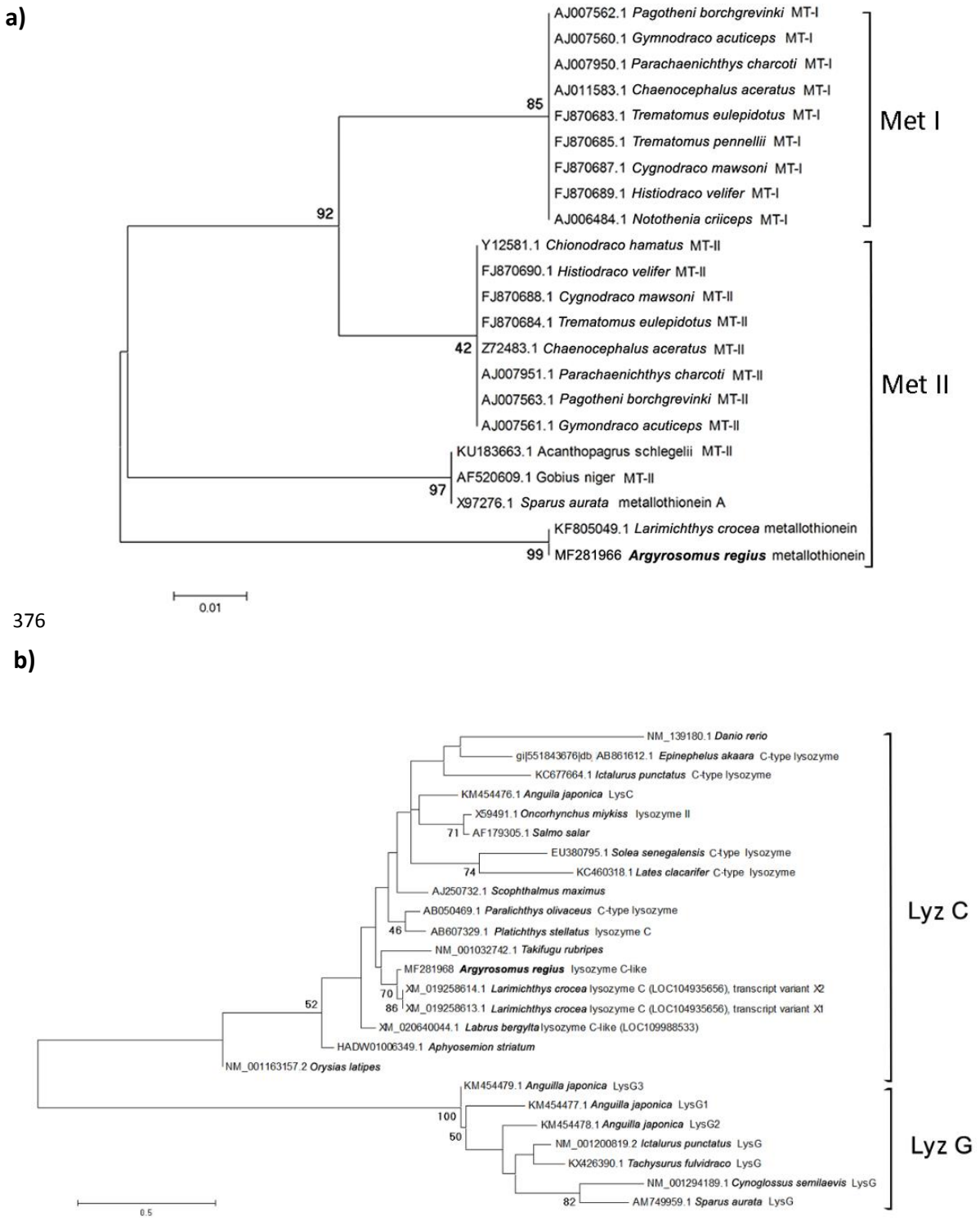
3.2 *Gene expression analyses*

3.2.1 *Gene expression during ontogeny*

In order to better understand the biological role of several significant innate immune genes in meagre, mRNA levels for *met II, c3, cox2, lyzc, mxp, myd88, nod2, nod3* were analyzed by quantitative real-time PCR from 8 until 120 dph (Fig. 2). The expression of the above-mentioned genes were variable throughout development and growth of larvae and fry. However, among genes studied three general patterns of expression were evident. In the first group, including *cox2,* there was a general decrease over time between 8 and 60 dph. A second group, composed of *mxp* and *myd88,* showed an expression pattern characterized by an initial low expression that increased over time with an important peak in expression at 60 dph. A third group of genes, consisting of *lyzc, nod2, nod3,* was also discernable, which showed low levels initially with peaks at 29 and 60 dph. The pattern of *met II* and *c3* expression was clearly at a high level at day 8, and then it decreased to a moderate level at day 29 and followed by a substantial increase at day 60. However, a different expression pattern was found for *mxp* and *myd88* transcripts. They were detected at day 8 and subsequently the expression level increased gradually until day 60. In contrast, the expression of *cox2* gradually decreased during the study period, possibly due to the expression signal being diluted by accumulation of non-expressing cells as the fish larva grew. A comparison of *nod2* and *nod3* show their expression profiles were similar. In fact, the levels of expression of both transcripts were higher at day 29 and then, decreased to moderate levels (40-43 dph), followed by an increase at day 60. The pattern of expression seen with *lyzc* expression was similar, but with greater differences in expression between 29 and 60 dph. Interestingly, changes in the expression of these genes seemed to correlate to changes in the diet during on-growing (Fig. 3).

As larvae increased in size during the latter part of the ontogeny study (85, 96, or 120 dph) the transcript expression was monitored in individual tissues (kidney, spleen, gill and gut). As shown in Figure 4, the eight genes exhibited different patterns of tissue specific expression. The expression of *cox2* showed a significant increase in gill, gut and kidney at day 120 compared to earlier time points (85-96 dph). Expression of *myd88* transcripts showed a significant increase in the kidney at 96 dph that was maintained high and stable until 120 dph.

However, *met II* and *mxp* transcripts showed significant increases in the gut during 85-96 dph. Finally, *nod3* transcript levels increased significantly ($P \leq 0.05$) in the spleen (85 dph) and gut (120 dph) in comparison with other preceding time points (Fig. 4).



c)

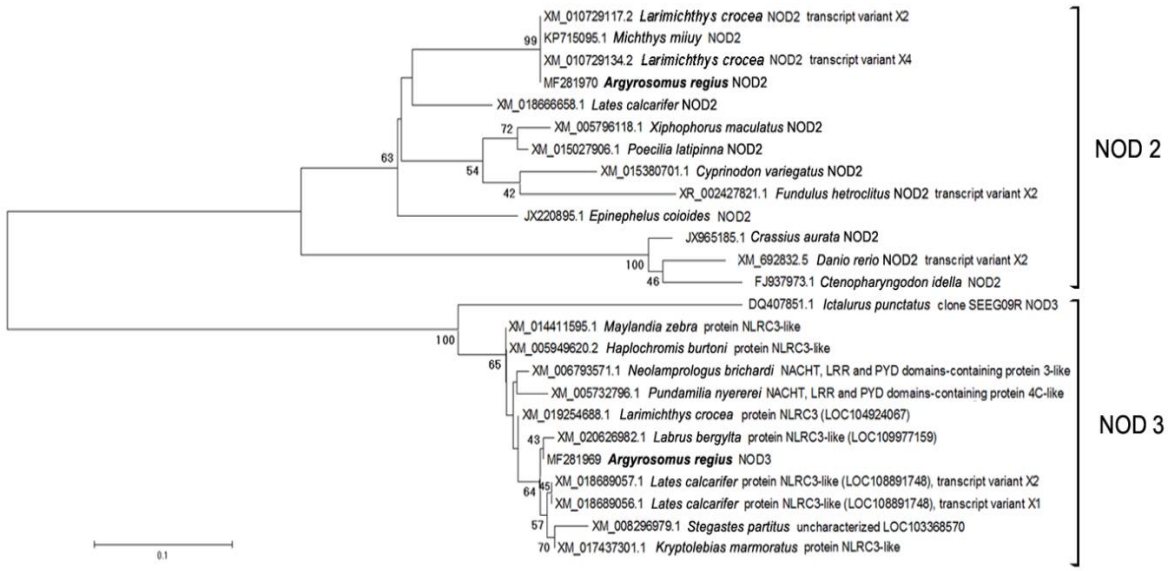
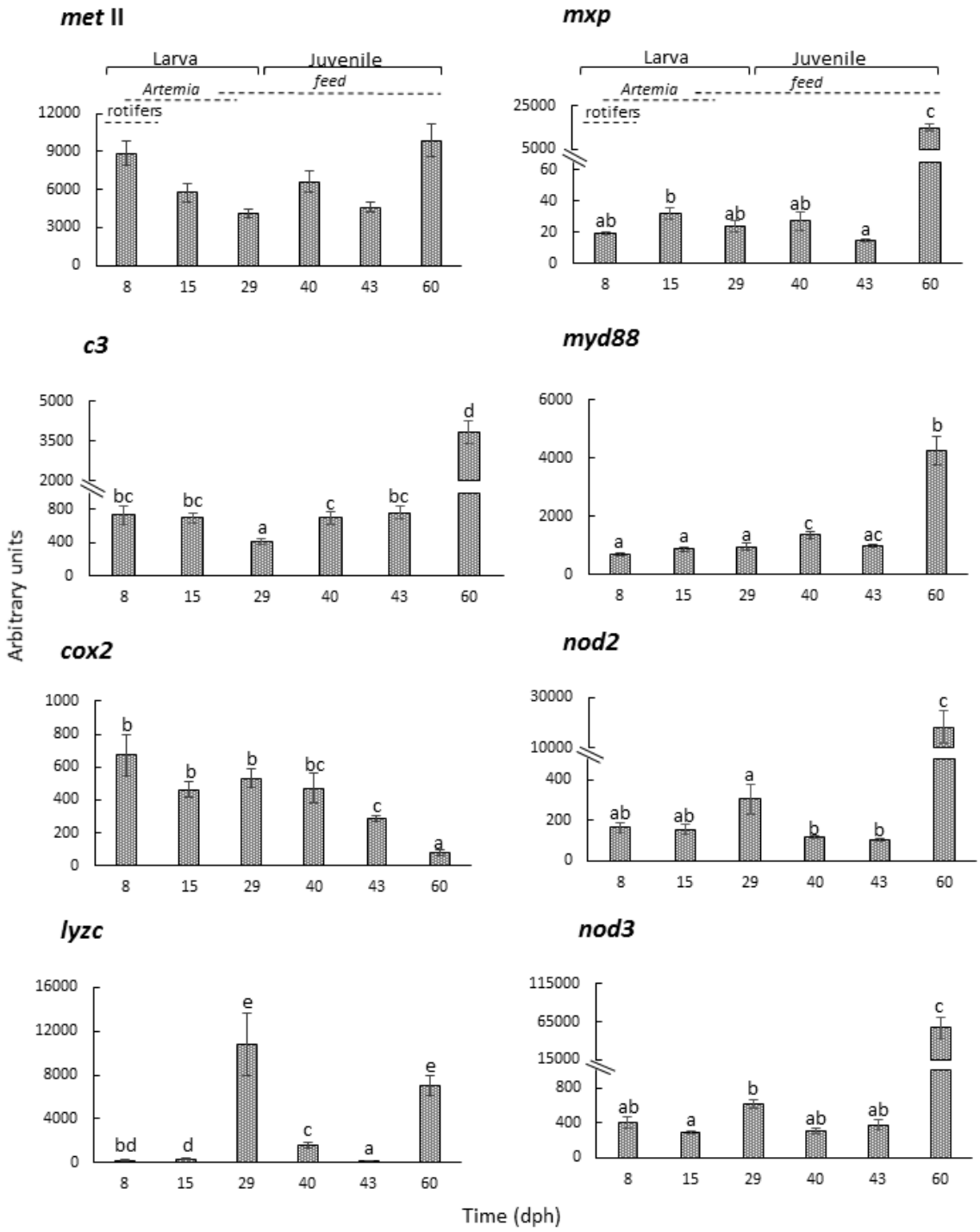


Fig. 2. The evolutionary history for (a) *met II*, (b) *lyzc* (c) *nod2*, *nod3* from meagre (shown in bold) was inferred using the maximum likelihood (a), (b) and neighbor-joining (c) method conducted in MEGA5. All coding positions were included and values for the bootstrap confidence values (1,000 replicates) are shown at branch nodes.



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393 **Fig. 3.** The temporal change in gene expression of larval and juvenile (n = 10) meagre for *met II*, *c3*, *cox2*, *lyzc*,
394 *mxp*, *myd88*, *nod2*, *nod3*. Transcripts were normalized using an arithmetic mean of three housekeeping genes:
395 GAPDH, HPRT, and β-actin. Different letters above the bars indicate significant differences among different
396 time points (Tukey´s test, $P \leq 0.05$) and the larval feeding schedule also are shown. Results are expressed as the
397 mean ± SEM.

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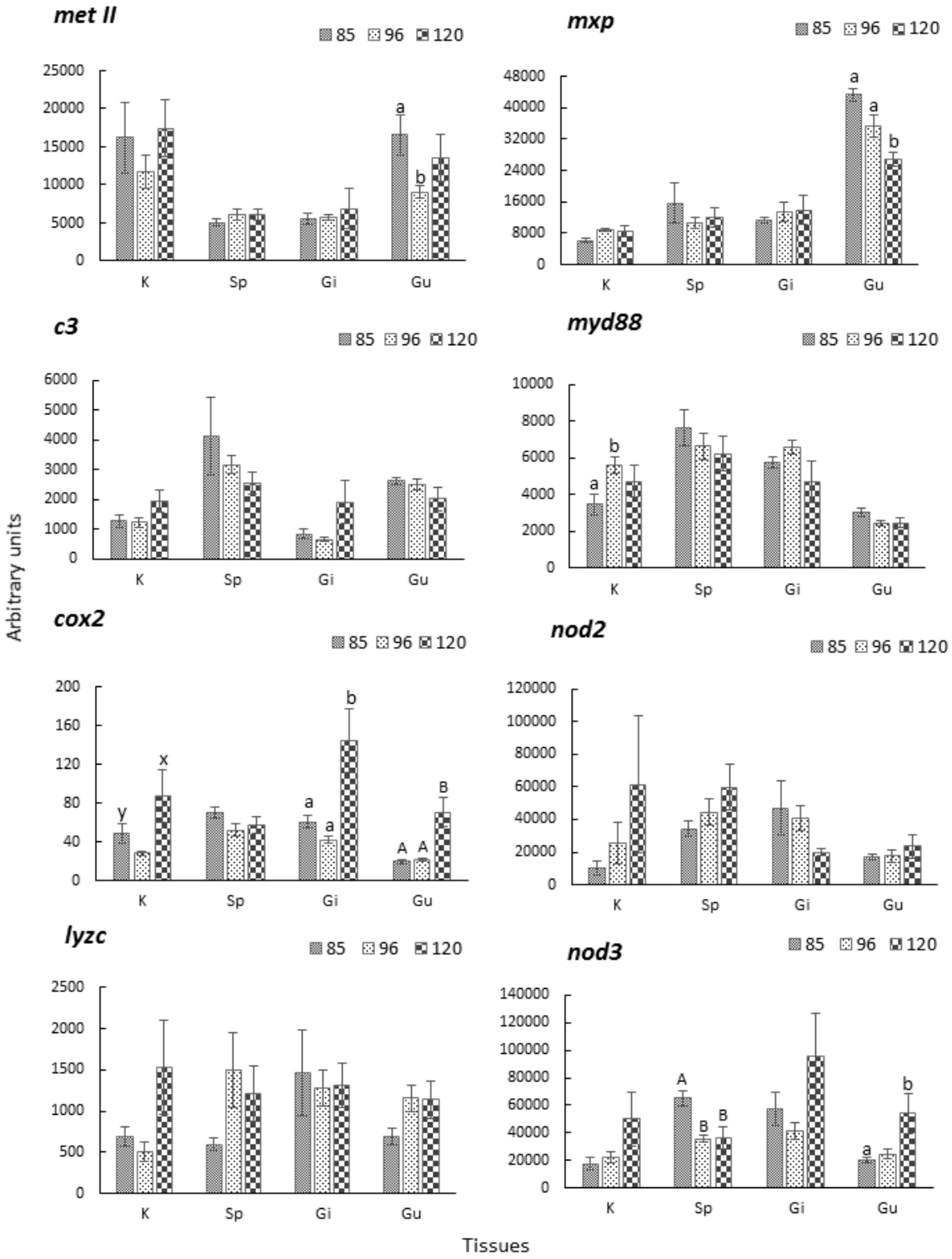

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400

401 **Fig. 4.** The temporal change in gene expression of *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* in the kidney,
402 spleen, gill, and gut (n = 8) from 85-120 dph in meagre. Transcripts were normalized using an arithmetic mean
403 of three housekeeping genes: *gpdh*, *hprt*, and *β-act*. Different letters above the bars indicate significant
404 differences among different time points (Tukey´s test, $P \leq 0.05$). Results are expressed as the mean ± SEM. K =
405 kidney, Sp = spleen, Gu = gut, Gi = gill.

406

3.2.*2 Response of meagre innate immune genes to in vivo PAMP stimulation*

To better understand how different pathogenic stimuli influence changes in the expression of these genes, an *in vivo* challenge with various PAMPs was performed, with samples collected for analysis after 24 h. Post stimulation, *c3* mRNA levels were most highly up-regulated in the kidney, spleen, gut and gills by β-glucan. However, LPS seemed to be a more potent stimulator of expression in the gut and gills. Lysozyme was significantly up-regulated in the kidney, spleen, gut and gill by LPS, but, curiously, there was significant down-regulation in mucosal tissues (gut and gill) when poly I:C was used, while there was a significant up-regulation in systemic tissues (kidney and spleen) by β-glucan stimulation. The expression of *met II* was potently induced in the spleen, gut, and gills by LPS stimulation and by poly I:C and β-glucan stimulation in the spleen and gut. The expression of *myd88* was significantly up-regulated in the gut by all the PAMP stimulants. The abundance of *mxp* transcripts were increased in all the tissues tested when stimulated by poly I:C and in the gut there was also a significant up-regulation ($P \leq 0.05$) when stimulated by LPS and β-glucan. The expression level of *cox2* was significantly up-regulated in kidney and spleen by LPS. The expression of *nod2* transcripts showed the highest up-regulation in spleen and gut when stimulated by LPS and β-glucan. Different expression profiles were observed for *nod3*; there was significant up-regulation ($P \leq 0.05$) in the kidney and spleen by β-glucan, while the other PAMP stimulants had little significant effect on mRNA levels, as seen in Figure 5.

3.2.*3 Response of meagre innate immune genes to in vitro PAMP stimulation*

Only the lymphoid organs of kidney and spleen were analyzed for the *in vitro* part of this study. The kidney is the first major lymphoid organ to develop during early ontogeny and the spleen plays an important role in hematopoietic and immune functions. Cells were isolated from these lymphoid organs (kidney and spleen) and challenged with 3 PAMPs for 4, 12 and 24 h. The expression of *met* II was significantly upregulated (Fig. 6) in splenocytes for all stimulants after 24 h. The expression of *mxp* was up-regulated significantly in the spleen and kidney cells after 4 and 12 h stimulation with poly I:C, but in the kidney cells the only significant increase by all of the stimulants was at 24 h. Similarly, with kidney cells a differential expression profile was seen with increased expression of *c3* in response to all three PAMPS at 24 h, but splenocytes showed an up-regulation after 24 h stimulation with LPS and β-glucan. No change in expression of *myd88* was found in the stimulated kidney and spleen cells at 4 h, but at 12 h there was up-regulation using poly I:C. However, at 24 h up-regulation occurred in spleen cells with LPS. The mRNA expression of *cox2* in kidney cells was up-regulated at 4 h by all 3 stimulants. In splenocytes, significant expression levels were only detected at 12 h after the stimulation by poly I;C and β-glucan. A significant up-regulation of *lyzc* expression was seen in kidney cells at 4 h after stimulation by LPS and β-glucan and at 24 h after stimulation using poly I:C. In splenocytes, up-regulation was seen at 12h after stimulation by poly I:C and β-glucan at 24h after stimulation by LPS and β-glucan. For *nod2*, transcription levels in kidney cells was significantly up-

regulated at 24 h post-stimulation with LPS and β-glucan. This contrasted with *nod3* expression, which was up-regulated in kidney cells after 4h stimulation with LPS and β-glucan and had returned to control level by the other later time points. For splenocytes, *nod3* expression was significantly up-regulated at 24 h post-stimulation with LPS and β-glucan.

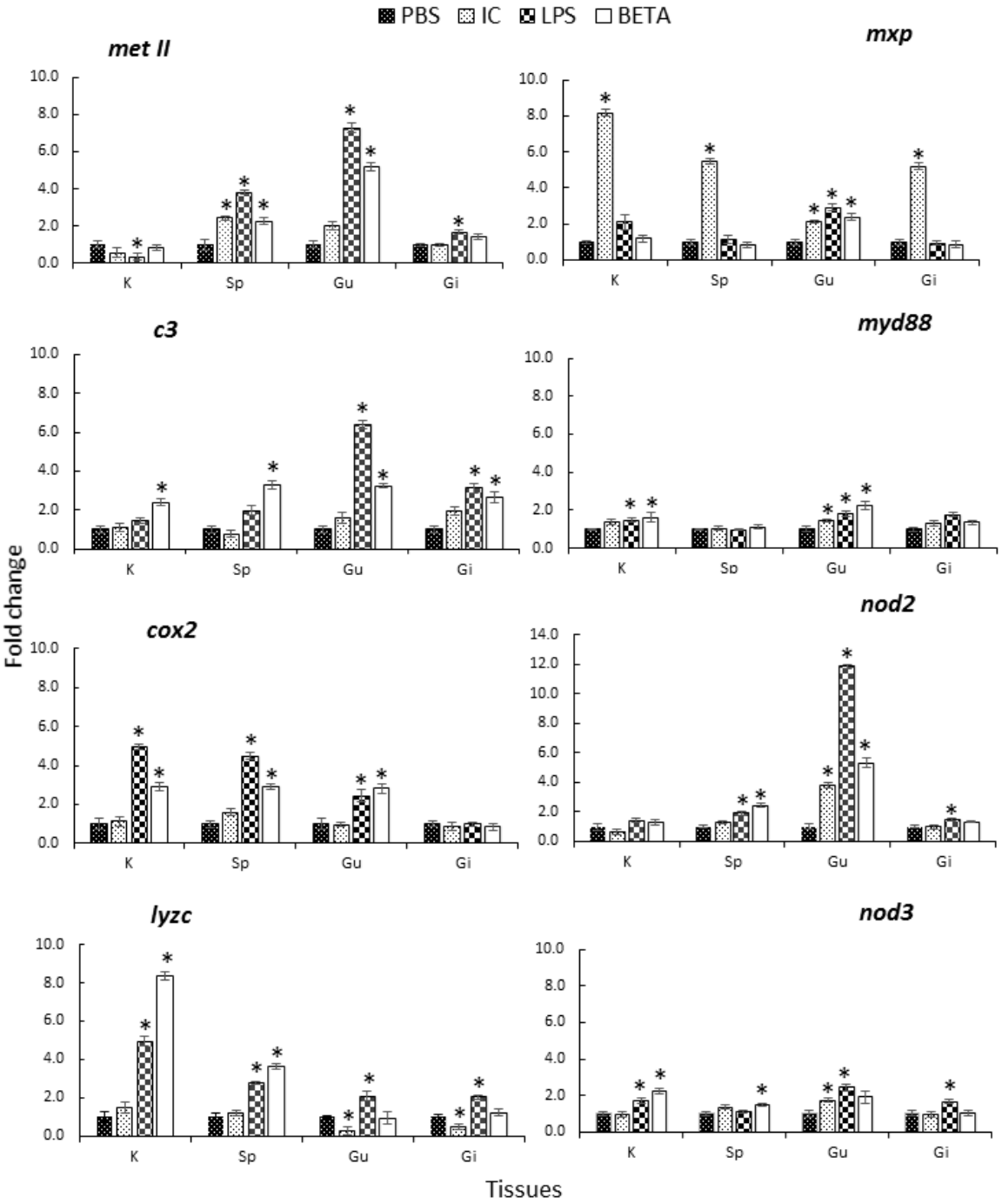
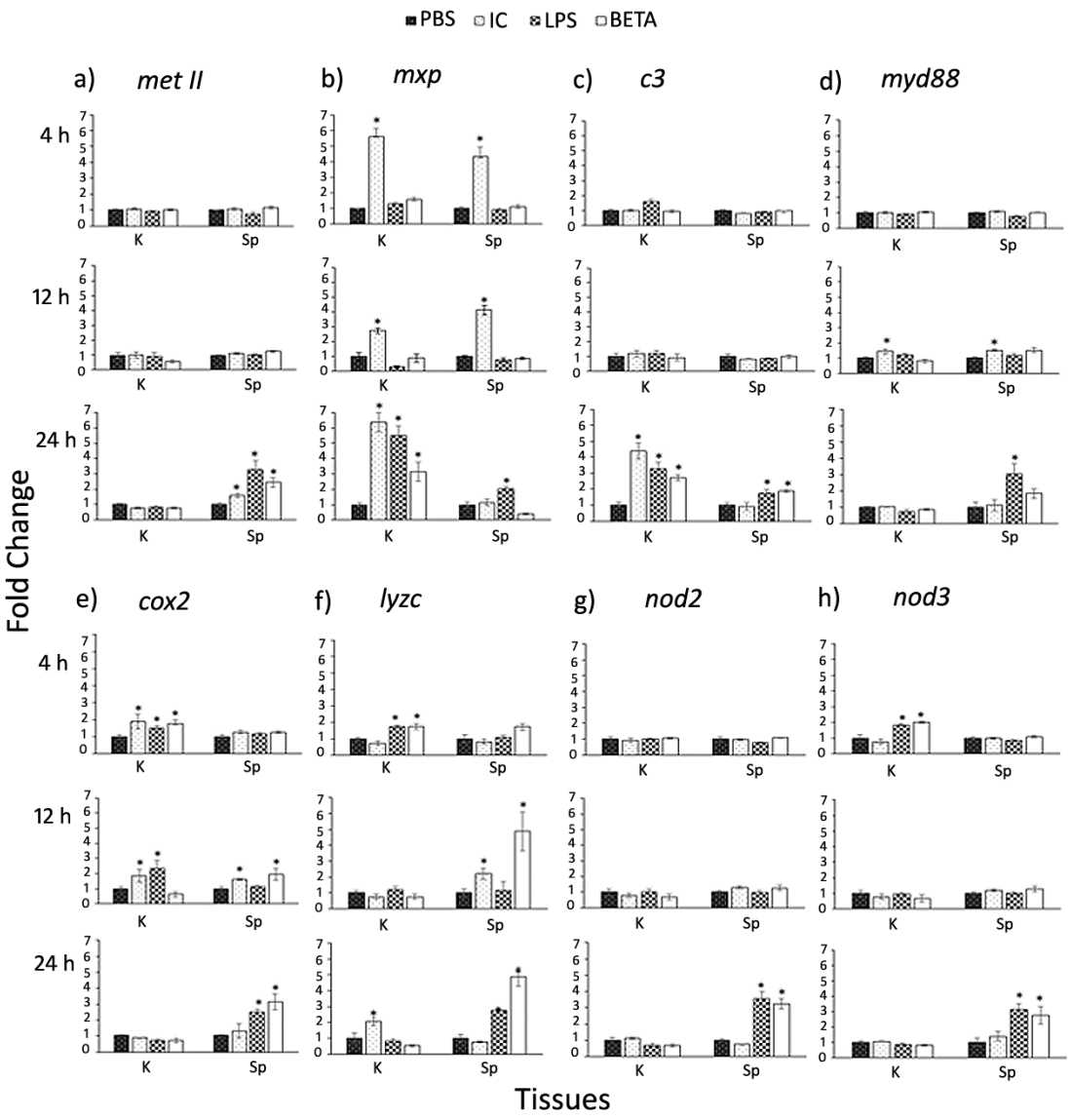


Fig. 5. The *in vivo* expression of *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* in meagre kidney, spleen, gill and gut following PAMP stimulation. Fish (n = 8) were injected intraperitoneally with PBS, poly I:C, LPS and β-glucan and sampled at 24 h post-injection. The gene for GAPDH, was employed as an internal reference. Asterisks (*) mark significant between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. Abbreviations: K = kidney, Sp = spleen, Gu = gut, Gi = gill.



453

454 **Fig. 6.** The expression patterns *in vitro* of a) *met II*, b) *mxp*, *c) c3*, d) *myd88*, *e) cox2*, f) *lyzc*, g) *nod2*, h) *nod3*
455 mRNA after PAMP stimulation of cell suspensions isolated from meagre (*A. regius*) kidney and spleen tissues,
456 collected from healthy fish (n = 6). The cells were stimulated with poly I:C, LPS and β-glucan and sampled at
457 different time points post-stimulation (4, 12 and 24 h), for RNA extraction and qPCR. All samples were
458 compared to PBS treated controls. Asterisks (*) mark significant between stimulated and PBS control groups
459 (Tukey's test, $P \leq 0.05$). Data are means ± SEM. Abbreviations: K = kidney, Sp = spleen.

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4. Discussion

4.1 Ontogeny of Innate Gene Expression

The present study is the first report of some representative innate immune genes in meagre and the characterization of their expression patterns in larvae and early juveniles, which is a critical time in fish development, when reliance on innate immune mechanisms is required for combatting infectious disease. Although previous studies have focused on identifying immune genes and/or characterizing their response to certain pathogens, there are few comparative studies reporting their temporal appearance during development. Further we have characterized the expression of these gens under specific immune stimulation using four different PAMPs. This immune stimulation study was performed in whole animals with all the consequent communication among various tissues, and in primary cell isolations from those same organs to see how organ-specific responses might differ from the response obtained from the intact animal.

Phylogenetic analysis was used to confirm the specific identity of genes where closely allied isoforms are known that could confound gene expression analyses. The phylogenetic analysis of *met* revealed that the isoform identified from meagre was *met II*, and it is an isoform more similar to a homolog described in *Larimichthys crocea* as expected, since both species are quite close, from a taxonomical point of view. Metallothionein has been shown to be a potential biomarker for metal contamination, but it also has an important role in Zn regulation during development [24]. Zinc is one of the major trace elements indispensable for functioning of, among other things, zinc-finger transcription factors. In this study, *met II* showed a higher expression level from 8 dph (4.49 ± 0.39 mm SL), but slowly decreased during development in meagre. In specific tissues during the early juvenile period, the abundance of *met II* transcripts increased, particularly in the kidney and gut. In these tissues, metallothionein is especially important in development due to cellular signals that are required during cell growth and differentiation, which require nuclear localization of this intracellular zinc regulator [25] and maintaining Zn homeostasis at the cytoplasmic level for regulation of several crucial processes implicated in the innate immune [26]. For example, Zn ions are important for the production of pro-inflammatory cytokines such as interleukins IL-1β, IL-6 and tumor necrosis factor α (TNF-α), and Zn deficiency leads to reduced chemotaxis by polymorphonuclear cells [26], and impaired formation, activation, and maturation of lymphocytes, disturbed intercellular communication via cytokines, and weakened innate host defense via phagocytosis and oxidative burst [27].

The complement system is known as an integral aspect of innate immunity, of which *c3* is a central component. This system also links with the adaptive immune response and has been shown to be important for C3-trophic driven morphogenesis of developing embryos/larvae [28]. In this study, the expression of *c3* was detected from the initial time point of the study (8 dph), but transcript levels of *c3* reached their lowest levels at 29 dph (14.41 ± 1.62 mm SL), coinciding with the end of the weaning period (the transition from artemia to an artificial diet) where as a result, larvae may be immunosuppressed in comparison to other life development periods and possibly more sensitive to potential pathogenic organism. As larvae matured, the expression level of *c3* in tissues increased in the spleen and gut tissues, probably acting to facilitate hematopoietic development [29], stem-cell differentiation [30] and defense against allochthonous bacteria ingested with feed, some of which may be potential pathogens [28]. These previous findings support the idea that complement and its recognized role in immune defense, may also play a role in the formation and generation of different organs during development [31].

Cyclooxygenase, is the enzyme responsible for the initial rate-limiting conversion of arachidonic acid to prostaglandins (PGE2 and PGH_2). Mammals contain two *cox* genes, *cox1* and *cox2*. Homologs of these genes have been found in many fish species, including zebrafish (*Danio rerio*) [32]. Although, it is known that both isoforms are similar, *cox1* is generally assumed to be constitutively expressed performing homeostatic and maintenance functions, while *cox2* is induced by inflammation, cytokines, endotoxin and other pathophysiological processes [33]. In the present study, *cox2* displayed a decrease in expression between 8 dph and 60 dph (during which period whole larvae were analyzed), that may be due to the expression signal being diluted as the larvae grew, since the abundance of *cox2*-postive cells likely did not increase in the same proportion as all other somatic cells. Although Ishikawa *et al*. [34] observed *cox2* expression in zebrafish, it exhibited a more restricted pattern of expression and no evidence of an association to morphogenic development. In Atlantic salmon (*Salmo salar*) [33] and mummichug (*Fundulus heteroclitus*) [35], *cox2* was observed to have the highest level of expression in the gills, and it was suggested that there was a possible higher requirement for osmoregulation processes and/or stress responses. In the present study, high *cox2* expression levels were seen in the kidney, gills and gut in early juveniles aged 120 dph, although the relation of this expression to PGE production in these tissues was not evaluated. High *cox2* expression with a potential increase in production of PGs [12][36] in some tissues such as gills possibly aid the process of osmoregulation [12] and ion transport in euryhaline species [35], and in the gut to maintain the integrity of the mucosal barrier [37].

Significantly, expression of *cox2* is associated with several cell types, e.g. monocytes and macrophages [38] that likely aid in surveillance of mucosal barriers

In fish, there exist two variants of lysozyme: c and g- types. The lysozyme isoform identified in meagre was shown to be the c-type, which has been reported previously in many teleost species [39–41]. Lysozyme possesses lytic activity against both gram-positive and gram-negative bacteria. For this reason, it is an important defense molecule of the immune system, widely present at host /environment interfaces including gills, and gastrointestinal tract, but it is also present in lymphoid tissues, serum, mucus, and other body fluids [29]. Aside from its antibacterial function, it promotes phagocytosis by directly activating polymorphic nuclear leucocytes and macrophages, or indirectly, by an opsonizing foreign bodies. At 29 dph, transcript levels of *lyzc* were found to be significantly up-regulated around the time of weaning (transfer to *Artemia* and dry feed). Possibly, this increased expression during the transition of diet may reflect influence from certain nutrients or exposure to new cohorts of bacterial species in different feed sources that are stimulating expression of this immune effector. It may also be that specific components supplemented in the feed modulate lysozyme expression [42]. Lysozyme gene expression in teleost has been assessed in tissues from healthy fish, but the levels and pattern of expression varied for individual species, the isoform under study, and tissues examined [40,43]. In the current study, analysis of *lyzc* in tissues showed an increase in the kidney, spleen and mucosal tissues (gut and gill) in juveniles aged 60-85 dph, whereas its expression was even higher at later time points (120 dph). This observation could be correlated with the structure of the organs and increase in mucus/goblets cells in mucosal tissues during this period in this species [44]. Further, some reports have found the c type lysozyme in rainbow trout, (*Oncorhynchus mykiss)* [45] was specifically expressed in the liver and kidney [46], whereas in Japanese flounder, *Paralichthys olivaceus*, the c-type lysozyme gene was expressed in head kidney, spleen, brain and ovary, while in brill (*Scophthalmus rhombus*) expression was observed in liver and stomach [39]. Therefore, differences in the *lyzc*-positive tissue distribution and levels of expression of *lyzc* among fish species might suggest some variation in function, and potentially multiple roles.

The induction of the gene *mxp* in mammals is specifically induced by type I-IFN [47]. The antiviral effect of type I IFN is exerted through its binding to the IFN-α/β receptor, which triggers a signal transduction through the Jak-Stat pathway resulting in expression of *mxp* and other antiviral proteins. In the present study, during early development the peak in expression of this gene was observed at 60 dph and it was also expressed in all tissues examined during later stages of development, with a prominent increase in the gut, coincident with an

abundance of granulocytes in this tissue at later developmental periods [44]. The gut epithelium is a continuation of the interface between the external environment and internal host environment, therefore it requires defense mechanisms to be operating continuously to act rapidly for limiting infection and maintaining gut homeostasis. Mxp is among those proteins that responds quickly to a viral attack, but as the immune system matures might be maintained at a constitutively higher level in the gut relative to other tissues (Fig. 4).

Myeloid differentiation factor 88 (Myd88) is an important adaptor protein in the Toll-like receptor (TLR) signaling pathway and is used by all TLRs except for TLR3. Orthologues of *myd88* have been found in multiple fish species [15,48,49], demonstrating among fish the wide evolutionarily distribution of this intracellular immune mediator due to its crucial role in host immunologic surveillance where it functions as an accessory protein to ligand receptors. The gene for Myd88 was found expressed continuously in all stages of larval development with a sharp increase at 60 dph. In more developed juveniles where individual tissues were examined, *myd88* transcripts were detected in all tissues, while high levels of transcripts were observed in the gill, spleen and kidney at 85, 96 and 120 dph.

The phylogenetic analyses of NOD isoforms from meagre demonstrated the two isoforms identified reside hierarchically in different clades, consistent with their presumed separate functions. The genes *nod2* and *nod3* belong to the subfamily of NOD-like receptors (NLRs) characterized by CARD-containing effector-binding domains. Nod2 is a member of the cytoplasmic pattern recognition receptors (PRRs) family that recognizes muramyl dipeptides derived from peptidoglycan, present both in gram-positive and negative bacteria. Upon ligand recognition, Nod2 induces the activation of the NF-kB and MAPK pathways. Activation of NFkB and Mapk induce transcription and production of inflammatory cytokines, chemokines and antimicrobial peptides which mediate the antimicrobial response. Nod3 (NLRC3) belonging to the NLR-C subfamily, plays a role in the innate immune response against bacteria and virus. Studies have also suggested that it has a role in modulating T cells and inhibiting inflammatory mechanisms, although studies in species such as catfish have shown *nod3* is present in many tissues [50]. Little is known about the precise mechanism, activation and signaling cascades of members of the NLRC subfamily. During the first 60 days of this study, *nod2* and *nod3* genes showed similar expression patterns during larval development, with the transcripts exhibiting abrupt up-regulation at 29 dph and 60 dph, which could suggest a coordinated activity between the two peptides these genes encode. In contrast, differences were observed in the individual tissues examined. Expression of *nod2* increased in the kidney and spleen while *nod3* transcripts were highly expressed in gill and gut at 120 dph. Similar

599 results for *nod2* expression in the kidney were obtained from grass carp (*Ctenopharyngodon*
600 *idella*) [51], and grouper (*Epinephelus coioides*) [52], while in rainbow trout (*Oncorhynchus*
601 *mykiss*), *nod2* isoforms (*nod2a* and *nod2b*) were detected highly expressed in muscle [53].
602
603 *4.2 In Vivo Stimulation*
604
605 While studies of immune gene expression are becoming more common with fish, the totality
606 of data is somewhat limited and comparative studies on molecular effectors of the innate
607 immune system often rely on the greater abundance of literature from mammalian studies as
608 a starting point. Herein is presented a study of the expression of key innate immune genes as
609 modulated by PAMPs. An *in vivo* study was performed with three different PAMPs injected
610 intraperitoneally and measured the immune gene expression responses to each PAMP 24 h
611 after their administration.
612 In our study, we found an increase in expression of *met II* in the spleen, after stimulation by
613 the three different PAMPs, while in gut the expression of *met II* was only up-regulated by
614 LPS and β-glucan. In contrast, it was only slightly stimulated in the gills by LPS. These results
615 suggest that the spleen is somewhat more responsive with regard to *met II* activation where
616 recruitment of lymphocyte lineages are an important response for preventing systemic
617 infections. The response in the gut was much more pronounced using LPS and β-glucan as
618 stimulants, demonstrating that this tissue is functioning as a first line of defense with a much
619 faster, stronger response against invading pathogens. Interestingly, there was a slight, but
620 statistically significant, decrease in expression in the kidney under stimulation by LPS. The
621 induction of *met II* is mediated by immune cells and inflammatory signals. Additional gene
622 products may be working to suppress the expression in kidney tissue where filtration of excess
623 ions and their removal from the blood occurs.
624 In this study, *c3* transcripts were up-regulated in all the tissues exposed to β-glucan.
625 Interestingly, Campoverde et al. [54] detected a similar response in piscidin (*pisc*), after β-
626 glucan stimulation, which could suggest a common up-stream response mechanism when
627 meagre are exposed to β-glucan, such as a shared regulation by pro-inflammatory cytokines
628 or toll-like receptors.
629 With regard to *cox2* expression, significant differences were observed in the kidney, spleen
630 and gut after injection with LPS and β-glucan, but there were no changes in gill tissue. This
631 makes sense physiologically, as there is a need to maintain respiratory functions and avoid
632 unnecessary damage to respiratory epithelia from an excessive inflammatory response.

Therefore, in gills more stimulation might be needed to elicit significant up-regulation of *cox2* and the concomitant increase in prostaglandin synthesis. These results demonstrate that *cox2* can be induced by PAMPs binding to PRRs in the kidney, spleen, and gut; all tissues that need a thorough humoral and cellular response to clear pending systemic infections.

Multiple genes encoding the c-type lysozyme have been identified in different teleost species, and in some species different isoforms have been shown to perform distinct functions, or have tissue-specific expression patterns [40]. In the case of meagre, *lyzc* transcripts were found to increase significantly in the kidney and spleen after injection with LPS and β-glucan, while *lyzc* expression in the gut and gills was up-regulated only by LPS stimulation. However, poly I:C stimulation down-regulated *lyzc* expression in gills and gut. The higher levels of expression detected in the gill after injection with LPS and β-glucan are coherent with the idea of epithelia cells forming the primary barrier against a multitude of potential pathogens requiring a quick and rapid response to obviate any ingression of invaders [9]. These data from meagre are consistent with other studies from teleosts, but contrasting results have been obtained; studies of c-type lysozyme in brill showed significant changes in mRNA levels in stomach and liver after a bacterial challenge [40] possibly indicating this isoform might provide some direct protection in the digestive system in brill, whereas for g-type lysozyme similar tissue expression profiles were observed.

A relatively high *mxp* expression was observed in all tissues stimulated by poly I:C, but *mxp* expression was also stimulated to a lesser degree in the gut by LPS and β-glucan. High expression of *mxp* transcripts in mucosal tissues (gill and gut) was expected as these are the main entrance route for viral infections in aquatic organisms, while systemic tissues like the kidney and spleen have a high abundance of lymphocytes that respond to viral attack and produce Mx protein.

The expression of *myd88* was found to be up-regulated in the gut by all PAMPs tested. The expression of *myd88* is ubiquitous as it functions as a mediator for intracellular signaling in immune cells. Increased expression in the gut may facilitate and improve the integrity of the intestinal barrier following PAMP recognition [55]. Toll-like receptors (TLRs) recruit adaptor molecules, like Myd88, for signal transduction to activate nuclear factor-kappa B (NF-kB).

In the case of *nod2,* transcripts were up-regulated in the spleen following injection of LPS and β-glucan, whereas in the gut an up-regulation was observed following stimulation with each PAMP. In gills, there was a weak response when fish were injected with LPS, which might again be explained as a protective measure to avoid an unregulated pro-inflammatory response leading to tissue trauma. The aquatic habitat is a rich source of bacterial LPS and the immune

responses activated by the presence of LPS would need to be under tight control to differentiate presence of LPS liberated from natural bacterial cell death from an active invasion.

Gene expression levels for *nod3* were generally weak in all tissues; although there was some significant up-regulation, in the kidney by LPS and β-glucan, in spleen by β-glucan, in gut by poly I:C and LPS, and in gill tissue by LPS.

This in vivo trial using poly I:C, LPS, and beta-glucan has demonstrated that gene expression was effectively modulated in response to these PAMPs for all the studied genes, while the induction of gene expression by each stimulant demonstrated tissue-specific differences.

4.3 In Vitro Stimulation

Due to some difficulties in obtaining cell suspensions of sufficient quality from gills and intestine, we analyzed *in vitro* results only for the spleen and kidney. Exposure of kidney and spleen cells to different PAMPs elicited differential responses as compared to control cell suspensions treated with only PBS, demonstrating that these cells have the ability to recognize molecular patterns mimicking diverse pathogen types.

Early modulation of expression was seen for *mxp, cox2, lyzc, nod3* in kidney cells. An important component of the fish antiviral response is the IFN-inducible protein *mxp*. In Atlantic salmon and Japanese flounder *mxp* was found to significantly protect fish cells against viral infection and induce the gene expression levels of type *1 IFN* and *mxp* [56,57]. In the present study kidney and spleen cells responded strongly to poly I:C stimulation with an increase in mRNA transcripts of *mxp* as early as 4 h, and up to 12 h following exposure. At the same time expression *myd88* reached maximum levels following exposure to poly I : C in both systemic tissues, then recovered its basal levels at 24 h. These results suggest stimulation of kidney and spleen cells with poly I:C elicits a significantly increased expression of *mxp* and *myd88* where they may function in synergy against viral infections [58].

The antimicrobial activity of c-type lysozyme is due to its hydrolytic activities, inhibition of viral genomic RNA or RNA transcripts, and degradation of viral polysaccharides. In orange-spotted grouper, *E. coioides* and Japanese flounder, *Paralichthys olivaceus* the expression of c-type lysozyme transcripts was most abundant in head kidney [59,60] and in grouper cells when they were exposed to Singapore grouper iridovirus (SGIV). In the present study, expression of lysozyme was up-regulated in kidney cells at 4 h post exposure to LPS and β-glucan. However, this level increased significantly in spleen at 12 h post exposure to poly I:C

and β-glucan demonstrating lysozyme is more active as an immediate response element when bacteria (LPS) is the source of stimulation.

The complement system, present in the blood plasma, plays a central role in recognizing foreign antigens and subsequent microbial killing, and phagocytosis. In the present study, when kidney cells were exposed to PAMPS, *c3* gene expression increased at 24 h exposure, but was more highly expressed in spleen cells stimulated with LPS and B-glucan. The difference in expression between these tissues may be due in part to the types of cells present in each organ since kidney tissue has significant osmoregulatory roles as well as functioning in hematopoiesis.

Prostaglandin synthesis via cox2 (a.k.a. prostaglandin synthase) activity, among other things, functions as a mediator of inflammation. It has been reported that the expression of *cox2* is not constitutive in rainbow trout and sea bass, but can be induced by factors such as LPS [61,62]. In this study, there was an immediate and early up-regulation of expression of *cox2* in stimulated kidney cells using all three PAMPS, whereas there was a later, but more prolonged increase of expression, in spleen cells stimulated with LPS and beta-glucan. By 24 h post exposure, the levels of expression in kidney cells were not significantly different from the control PBS-treated cells. The significance of the response in kidney cells may be related to the need for production of O_2 radicals generated as by-products of prostaglandin synthesis that would negatively impact bacterial agents potentially found in the blood during an episode of bacteremia.

The *met II* gene, has been used as a biomarker of heavy metals exposure in aquatic animals [63] and can also be implicated in protection from cellular and oxidative stress. Met II has been detected mainly in liver and kidney in mandarin fish and catfish [64,65] after exposure to cadmium and selenium, respectively, however this study found that spleen cells from meagre responded late to all 3 PAMPS at 24 h post-exposure; in contrast to kidney cells which did not show any significant stimulation by the three PAMPs used. Chelation of metals such as iron and zinc may provide a significant edge in combatting pathogens that require such trace metals for regulation of gene expression (eg. - zinc-finger transcription factor proteins) or metabolic processes requiring biologically available forms of iron. The response from the spleen cells seen with this perspective has sense. That there is no response seen from kidney cells is an area that could be profitable for future study.

NLRs are important intracellular cytosolic sensors for the initiation of innate immune responses against infectious agents [17]. Recently, genes grouped in the NLR family have been characterized in Japanese flounder [66] and seabass [67]. High levels of nod2 mRNA

have been found in the spleen and different immune cell populations and in particular neutrophils [68]. Nod3 plays a role in modulating T cell responsiveness and inhibits inflammatory mechanisms [67]. The current work with meagre has shown that only the expression of *nod3* in kidney cells increased at 4 h following an exposure to LPS and β-glucan. However both kidney and spleen cells respond strongly at 24 h to LPS and β-glucan with an increase of mRNA transcripts of *nod2* and *nod3*. The difference in the timing of expression of *nod2* and *nod3* suggests different regulatory pathways may be controlling each in the immediate early response. In no instance was poly I:C seen to function as a stimulant for either NLR. This is in agreement with previous findings, considering that muramyl dipeptides are previously described as a ligand for nod2 [17].

The findings from both the in vivo and in vitro experimental data confirm the genes studied are responsive to pathogen associated molecular patterns and the host immune response is tissue specific. The timing of expression during on-growing of larval and juvenile meagre, can depend on the state of maturity of the organs and their requisite cellular composition. In conclusion, the current study is the first to identify the genes for *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2* and *nod3*, in meagre and examine how these immune genes are expressed during larval and juvenile development, and the effects of PAMP stimulation on the modulation of expression *in vivo*. Interestingly, the data from this study suggests that the transition from live feed to a commercial dry feed might be a time when the innate immune response is in flux, and that it may be the change in diet and the nutritional and/or compositional changes of the feed that are inducing the significant alterations in expression seen at these times. This study, by using poly I:C, LPS and β-glucan as proxies for actual viral, bacterial and fungal pathogens pathogens, also provides valuable insight into how the meagre innate immune response may respond in comparison to other species. However, further studies focused on larval nutrition, specifically identifying changes in the components in the diet that can beneficially modulate gene expression of the immune system, will enhance larval rearing protocols by reducing mortalities.

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